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Chemistry, Department of Adult and Elderly Nursing, University of Florida,
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
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Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: bpgoffice@wjgnet.com
<http://www.wjgnet.com>

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Increased susceptibility of aging gastric mucosa to injury: The mechanisms and clinical implications

Andrzej S Tarnawski, Amrita Ahluwalia, Michael K Jones

Andrzej S Tarnawski, Amrita Ahluwalia, Michael K Jones, University of California Irvine and the Veterans Administration Long Beach Healthcare System, 5901 E. 7th Street, Long Beach, CA 90822-5201, United States

Author contributions: Tarnawski AS, Ahluwalia A and Jones M contributed to this paper; Tarnawski AS designed the overall concept and outline of the manuscript; Ahluwalia A and Jones MK contributed to the discussion and design of the manuscript; Tarnawski AS, Ahluwalia A and Jones MK contributed to the writing, editing and revision of the manuscript, illustrations, and review of literature (55%, 30% and 15%, respectively).

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Correspondence to: Andrzej S Tarnawski, MD, PhD, DSc, University of California Irvine and the Veterans Administration Long Beach Healthcare System, 5901 E. 7th Street (09/151), Long Beach, CA 90822-5201,

United States. atarnawski@yahoo.com

Telephone: +1-562-8265437 Fax: +1-714-8464496

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Abstract

This review updates the current views on aging gastric mucosa and the mechanisms of its increased susceptibility to injury. Experimental and clinical studies indicate that gastric mucosa of aging individuals—"aging gastropathy"—has prominent structural and functional abnormalities *vs* young gastric mucosa. Some of these abnormalities include a partial atrophy of gastric glands, impaired mucosal defense (reduced bicarbonate and prostaglandin generation, decreased sensory innervation), increased susceptibility to injury by a variety of damaging agents such as ethanol, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), impaired healing of injury and reduced therapeutic efficacy of ulcer-healing drugs. Detailed analysis of the above changes indicates that the following events occur in aging gastric mucosa: reduced mucosal blood flow and impaired oxygen delivery cause hypoxia, which

leads to activation of the early growth response-1 (egr-1) transcription factor. Activation of egr-1, in turn, upregulates the dual specificity phosphatase, phosphatase and tensin homologue deleted on chromosome ten (PTEN) resulting in activation of pro-apoptotic caspase-3 and caspase-9 and reduced expression of the anti-apoptosis protein, survivin. The imbalance between pro- and anti-apoptosis mediators results in increased apoptosis and increased susceptibility to injury. This paradigm has human relevance since increased expression of PTEN and reduced expression of survivin were demonstrated in gastric mucosa of aging individuals. Other potential mechanisms operating in aging gastric mucosa include reduced telomerase activity, increase in replicative cellular senescence, and reduced expression of vascular endothelial growth factor and importin- α nuclear transport protein essential for transport of transcription factors to nucleus. Aging gastropathy is an important and clinically relevant issue because of: (1) an aging world population due to prolonged life span; (2) older patients have much greater risk of gastroduodenal ulcers and gastrointestinal complications (*e.g.*, NSAIDs-induced gastric injury) than younger patients; and (3) increased susceptibility of aging gastric mucosa to injury can be potentially reduced or reversed pharmacologically.

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Key words: Aging gastric mucosa; Injury; Phosphatase and tensin homologue deleted on chromosome ten-PTEN; Survivin; Apoptosis; Hypoxia

Core tip: This review focuses on aging gastric mucosa and its increased susceptibility to injury. The following events occur in aging gastric mucosa: reduced mucosal blood flow and hypoxia, upregulates PTEN that activates pro-apoptotic caspases and reduces anti-apoptosis protein, survivin. The imbalance between pro- and

anti-apoptosis mediators results in increased apoptosis and increased susceptibility to injury. Aging gastropathy is an important and clinically relevant issue because of: (1) an aging world population; (2) older patients have much greater risk of gastroduodenal ulcers and gastrointestinal complications (*e.g.*, non-steroidal anti-inflammatory drugs-induced gastric injury) than younger patients; and (3) increased injury of aging gastric mucosa can be reversed pharmacologically.

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BIOGRAPHY

Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, University of California Irvine, Editor-in-Chief, *World Journal of Gastroenterology* (Figure 1), Graduated (MD) from the University Medical School, Krakow, Poland, where he also received PhD (pathology) and DSc (gastroenterology) and served as Assistant and Associate Professor and V-Chair, Department of Gastroenterology. Following gastrointestinal fellowship at the University of Missouri, Columbia, MO, United States, he was appointed as Associate Professor (1982-1986) and full Professor (1986-present) at the University of California, Irvine, United States. He served as: Associate Chair, American Gastroenterological Association/EGD 1997-1999 and 2008-2010; Scientific Director, Shimoda Symposia on Mucosal Defense in Japan (8x), Chairman of Research Fora at DDW/AGA annual meetings (12 times; 1996-2011), Chair, Pasteur Institute Euroconference 2005 and as Chair and or Co-chair of 68 International Symposia.

Publications, presentations and grants: 347 full, peer reviewed publications [*Lancet*, *Nature Med*, *JCI*, *Gastroenterology* (over 30 papers), *Hepatology*, *Gut*, *FASEB J*, *Am J Pathol*, *Am J Physiol*, *Am J Gastroenterol* and others]; 20 book chapters; 507 presentations at international and United States meetings; 20 peer reviewed funded grants (NIH, VA Merit Review 1984-present), 4 United States patents. Clinical and Research interest: endoscopic, histologic, functional assessment of injury and protection of gastrointestinal mucosa; cellular and molecular mechanisms of gastroduodenal and esophageal ulcer healing-role of growth factors, signaling pathways, angiogenesis, non-steroidal anti-inflammatory drugs (NSAIDs), prostaglandins and *Helicobacter pylori* (*H. pylori*) toxins; injury and protection of portal hypertensive gastric mucosa and aging gastric mucosa; confocal endomicroscopy and molecular imaging; gene therapy. Received numerous prestigious academic honors including Glaxo International Research Award, Athalie-Clarke, Merenti-



Figure 1 Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, University of California, Irvine; and VA Long Beach Health Care System, 5901 E. Seventh Str., Long Beach, CA 90822, United States; Honorary Professor, Chinese University of Hong Kong; Editor-in-Chief, *World Journal of Gastroenterology*.

bus Medal, Peregrinator of Science awards and others. Memberships: American Gastroenterological Association (Fellow), American College of Gastroenterology (Fellow), British Society of Gastroenterology, Japanese Society of Gastroenterology (Honorary), Hungarian Society of Gastroenterology (Honorary), American Society for Investigative Pathology, Association of American Physicians (by election) and others. Editorial Boards-9 scientific journals. Sixteen of his former GI Clinical Fellows and/or Residents or Research postdoctoral fellows hold academic positions in United States Medical Schools (4 being Chairs of Departments). Twenty of his former international trainees and/or associates hold academic positions abroad (France, Germany, Hungary, Japan, Poland, Sweden, Switzerland).

INTRODUCTION

Experimental and clinical studies indicate that the gastric mucosa of aging individuals (which we refer to herein as aging gastric mucosa and/or “aging gastropathy”-the term that we proposed earlier^[1]) has prominent structural and functional abnormalities *vs* young gastric mucosa^[1-3] that impair gastric mucosal defense.

Gastric mucosal defense and its impairment in aging

Mucosal defense in normal stomach, its particular components, and the mechanism of gastric mucosal injury have been reviewed in detail in previous papers^[4-6]. Under normal conditions, gastric mucosal integrity is maintained by defense mechanisms (Figure 2), which include pre-epithelial, epithelial and post-epithelial components^[4,5]. The pre-epithelial component: mucus-bicarbonate-phospholipid “barrier”-constitutes the first line of gastric mucosal defense^[4]. The epithelial component consists of a continuous layer of surface epithelial cells interconnected by tight junctions and forming the epithelial “barrier”. These epithelial cells generate and secrete bicarbonate, mucus, phospholipids, trefoil peptides, prostaglandins (PGs) and heat shock proteins^[4]. The integrity of the epithelial cell layer is maintained by continuous cell renewal that is accomplished by proliferation of progenitor cells regulated by growth factors, prostaglandin E₂ and survivin-an anti-apoptosis and mitosis-promoting protein^[4]. The post-ep-

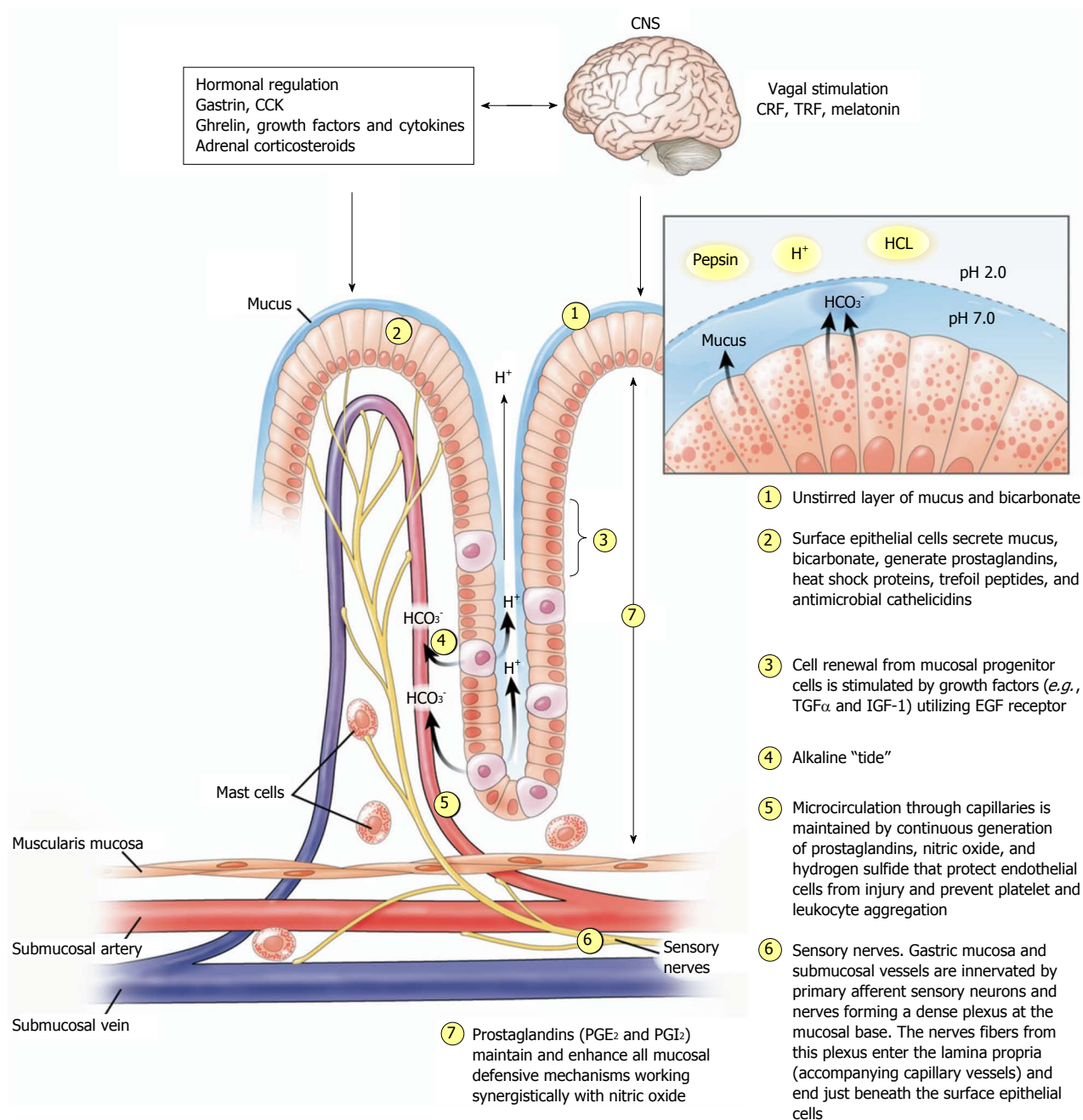


Figure 2 Gastric mucosal defense. Schematic representation of gastric mucosal defense mechanisms. Reproduced with permission from Laine, Takeuchi and Tarnawski^[4]. (1) "Unstirred" layer of mucus/bicarbonate/phospholipids above surface epithelial cells constitutes the first line of defense. It maintains a pH of approximation 7.0 (close to the physiological cell pH) at the surface epithelial cells, while pH in the lumen is about 1.0-3.0; (2) the surface epithelial cells secrete mucus, bicarbonate and synthesize prostaglandins and heat shock proteins; (3) mucosal cell renewal from mucosal progenitor cells is driven by growth factors (transforming growth factor α and insulin like growth factor-1 α) utilizing the epidermal growth factor receptors). Expression of survivin in epithelial progenitor cells prevents apoptosis and is the key for "immortality" of these cells under normal conditions; (4) "Alkaline tide"-parietal cells secreting HCl into the gastric gland lumen concurrently secrete bicarbonate into the lumen of adjacent capillary blood vessels. Bicarbonate is transported to the surface and contributes to the first line of defense; (5) mucosal microcirculation through the capillary microvessels is essential for delivery of oxygen and nutrients. Endothelial cells of microvessels generate prostaglandins, mainly PGI₂ (prostacyclin) and nitric oxide, which exert vascular and mucosal protective actions; (6) sensory nerve stimulation by H⁺-ion or other irritants causes release of neurotransmitters such as calcitonin gene related peptide (CGRP) and substance P in nerve terminals, which induce vasodilatation and enhance mucosal blood flow; and (7) continuous generation of prostaglandin E₂ (PGE₂) and prostacyclin (PGI₂) by the gastric mucosal cells is crucial for the maintenance of mucosal integrity. Almost all of the above (1-6) mucosal defense mechanisms are stimulated or facilitated by endogenous or exogenous prostaglandins. CRF: Corticotrophin-releasing factor; TRF: Thyrotropin-releasing factor; CCK: Cholecystokinin.

ithelial component of mucosal defense includes continuous blood flow through mucosal microvessels lined with endothelial cells forming an endothelial "barrier", sensory nerves releasing calcitonin gene-related peptide (CGRP) and hence regulating mucosal blood flow; and, the gener-

ation of PGs and nitric oxide^[4,5]. The structural elements of normal gastric mucosal defense were reviewed and discussed in detail in our previous paper^[4] and are presented in Figure 3. Importantly, gastric mucosal defense is also regulated by the central nervous system through

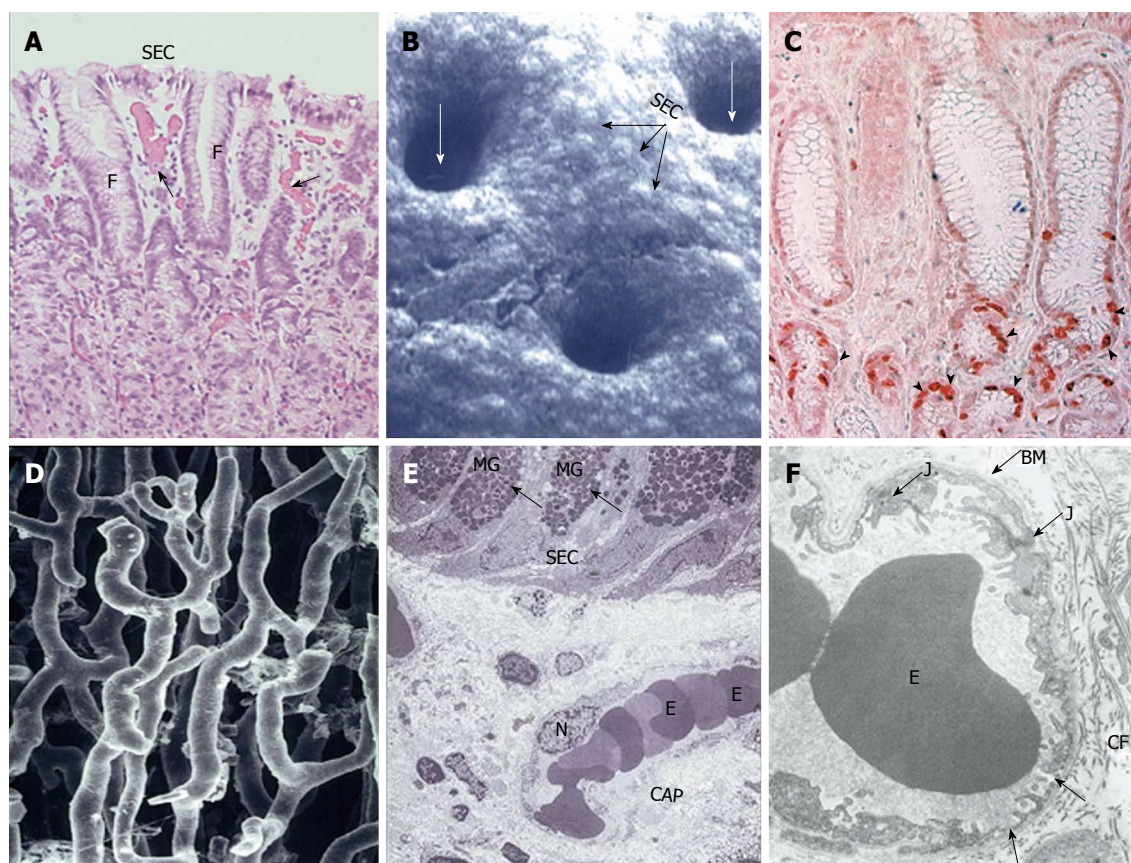


Figure 3 Structural components of gastric mucosal defense: surface epithelial cells, progenitor cells and blood microvessels. Reproduced with permission from Laine, Takeuchi and Tarnawski^[4]. A: Histology of upper part of human gastric mucosa visualizing surface epithelial cells (SEC), foveoli (F), and upper gland area. (Hand E staining; original magnification, $\times 50$). Blood microvessels with erythrocytes in the lumen are present in the lamina propria (arrows); B: Scanning electron micrograph of human gastric mucosal luminal surface. The unstirred mucus gel layer is not seen because of dissolution during fixation. Individual SEC are clearly visible as are lumina of the gastric pits (white arrows). Reproduced with permission from Tarnawski *et al.*^[7]; C: Immunostaining of human gastric mucosa with survivin (anti-apoptosis protein) antibody. Survivin is strongly expressed (brown-red staining) in the epithelial progenitor cells located in the foveolar/neck area (arrowheads). Reproduced with permission from Tarnawski *et al.*^[1]; D: Vascular cast study of capillary blood vessels in the gastric mucosa using Mercor resin. The remaining components of the mucosa were dissolved with concentrated NaOH. Reproduced with permission from Ichikawa, Tarnawski *et al.*^[8]; E: Transmission electron micrograph of normal human gastric mucosa. SEC contain dark mucus granules (MG, arrows). Below the surface epithelial cells, a capillary blood vessel (CAP) with erythrocytes (E) in the lumen is present in the lamina propria. N, nucleus of endothelial cell lining capillary vessel (original magnification, $\times 2000$). Reproduced with permission from Tarnawski *et al.*^[9]; F: Transmission electron micrograph of a portion of human gastric capillary blood vessel. The structure of the capillary wall and endothelial cell cytoplasm is normal with a characteristic fenestrations (arrows) allowing transport. BM: Basement membrane; E: Erythrocytes in the capillary lumen; J: Junction between two neighboring endothelial cells; CF: Collagen fibers. Original magnification, $\times 17400$. Reproduced with permission from Tarnawski *et al.*^[9].

vagal innervation, the release of corticotrophin-releasing factor, thyrotrophin-releasing factor, melatonin and others; by hormones including gastrin, cholecystokinin, adrenal corticosteroids; and by growth factors and cytokines^[4].

Gastric mucosal injury occurs when injurious factors “overwhelm” a normal, intact mucosal defense or when the mucosal defense is impaired^[4,5]. The mechanisms of mucosal injury and its repair were described in detail in our previous publications^[5,6].

Impaired gastric mucosal defense in aging individuals

Previous studies showed that aging gastric mucosa has impaired mucosal defense including reduced mucus and bicarbonate secretion, decreased prostaglandin generation, reduced nitric oxide synthase (NOS) activity; and, impaired sensory nerve responses to luminal acid^[10-16]. Lee and Feldman demonstrated in Fisher 344 rats *in vivo* that gastric mucosal prostaglandin synthesis

is significantly reduced in aging *vs* young rats; and, that aging rats are more susceptible to aspirin-induced acute gastric mucosal injury^[10]. Gronbech and Lacy examined in young and aged Fisher 344 rats damage of gastric mucosa by exposure to either 80% ethanol for 30-45 s or 1 mol/L NaCl for 10 min followed by saline in a chambered stomach model^[11]. They found that the mucosal lesions were significantly more extensive, and epithelial restitution was significantly reduced and delayed in aging *vs* young rats after both types of injury^[11]. In separate experiments, they monitored changes in gastric mucosal blood flow using a laser-Doppler flow-meter and demonstrated that young rats had a marked increase in gastric mucosal blood flow in response to 1 mol/L NaCl, luminal acid challenge, and capsaicin treatment; and, that these responses were abolished in aging rats^[11]. Moreover, aging rats had a lower density of CGRP (+) positive nerve fibers around gastric submucosal blood

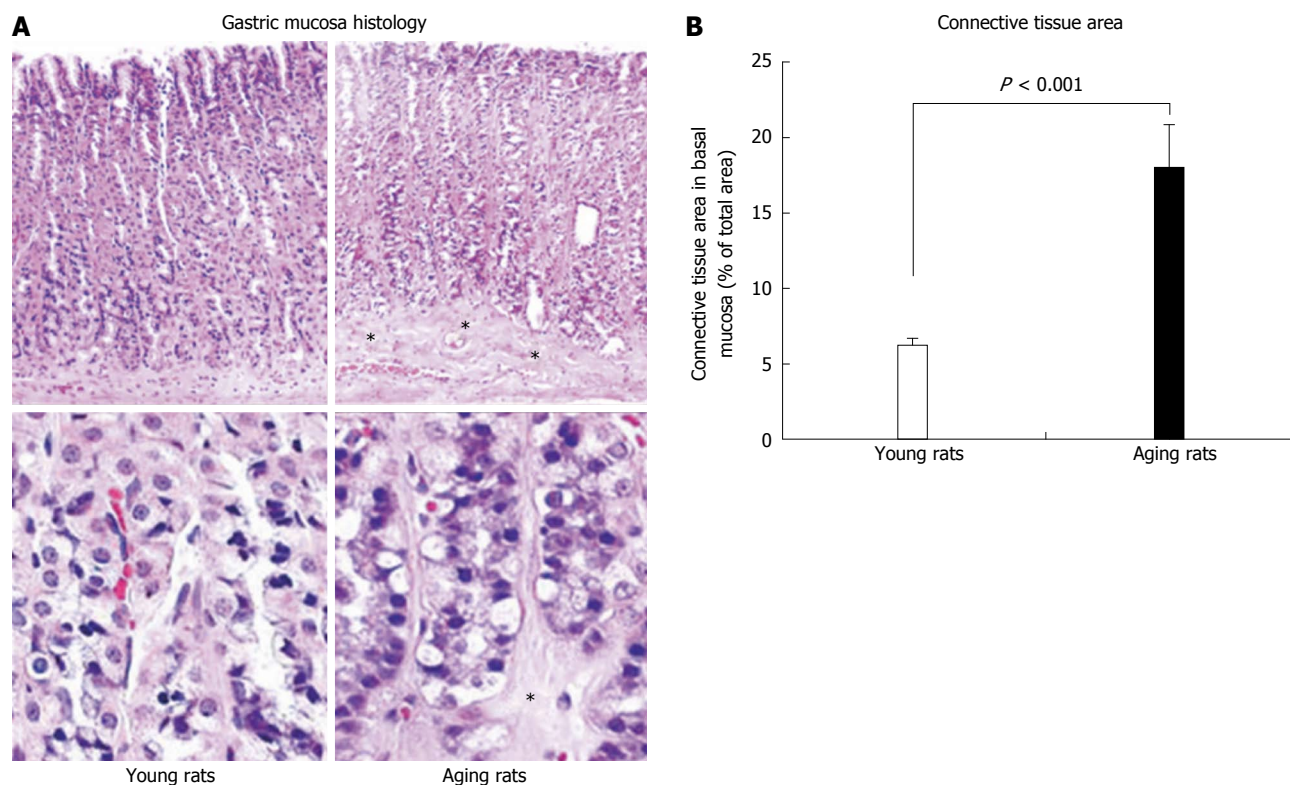


Figure 4 Photomicrographs of gastric mucosa in young and aging rats. In gastric mucosa of aging rats there is partial atrophy of gastric glands in the basal mucosa and their replacement with connective tissue (*). A: Hematoxylin and eosin staining at low magnification (x 100) is shown in the upper panels and higher magnification (x 500) is shown in the lower panels; B: Quantification of connective tissue in the lower one third of the gastric mucosa shows a significant increase in connective tissue replacing glandular cells in aging rats. Quantification of the number of inflammatory cells in gastric mucosa shows no inflammation (only a minimal number of inflammatory cells) and no difference between young and aging rats indicating that atrophic changes are not accompanied by an inflammation. Reproduced with permission from Tarnawski *et al*^[1].

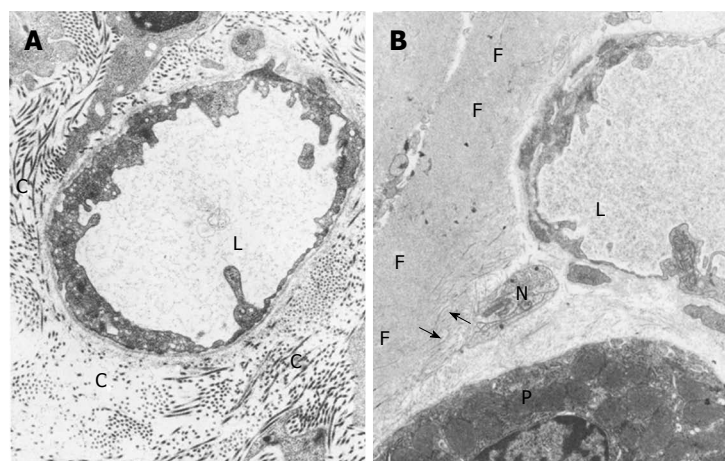


Figure 5 Transmission electron microscopy. A: Transmission electron micrograph of perivascular connective tissue from a 3-month-old control rat from the basal portion of the oxyntic mucosa. The connective tissue shows numerous collagen fibers (C); L, Microvessel lumen. Magnification x 19000; B: Transmission electron micrograph of perivascular connective tissue from an old rat. In the basal portion of the oxyntic mucosa, collagen fibers are mostly absent and replaced by rudimentary collagen fibers (arrows) and deposits of amorphous fibrillar material (F). P: Parietal cells; L: Blood microvessel lumen; N: Nerve bundle. Magnification x 19000. Reproduced with permission from Hollander, Tarnawski *et al*^[2].

vessels and decreased mucosal release of prostaglandin E2 compared to young rats^[11]. These data demonstrated impaired gastric mucosal defense and reduced gastric epithelial restitution in aging rats, which were related to the lack of hyperemic response to mucosal injury likely

due to reduced CGRP (+) nerve fibers and decreased prostaglandin generation in aging gastric mucosa^[11]. Other studies demonstrated aging-related changes in gastric mucosal glycoprotein synthesis, reduced gastric mucosal bicarbonate secretion and reduced gastric mucosal blood

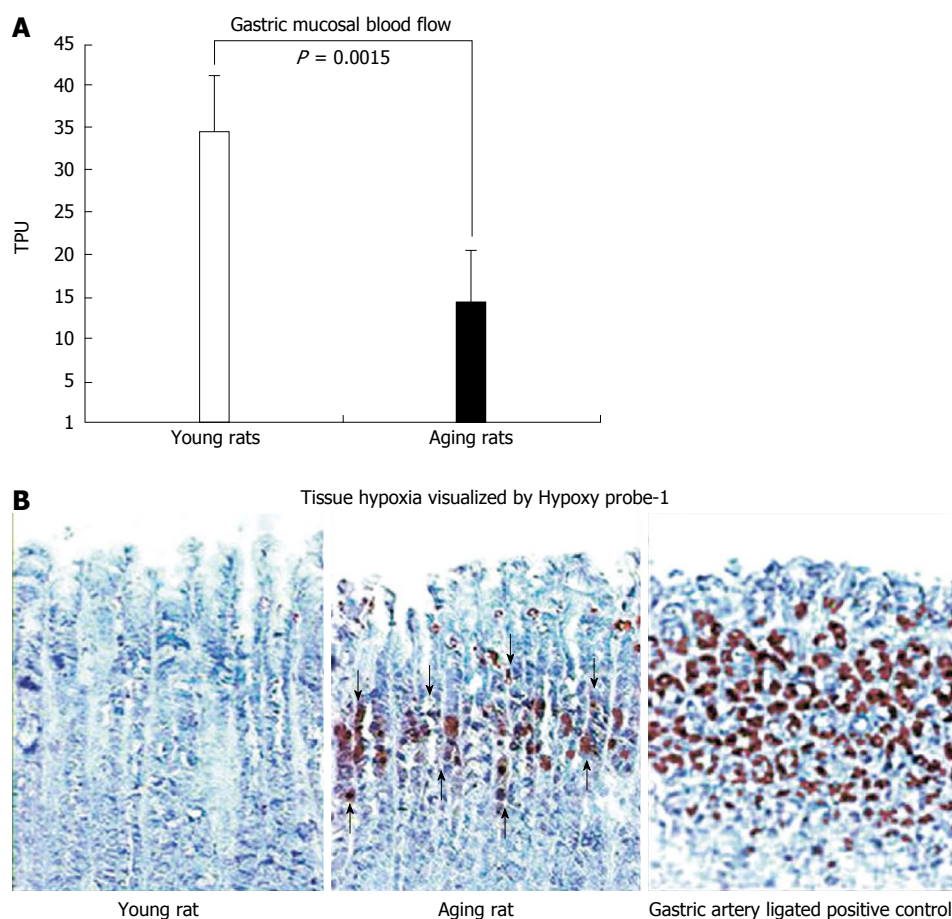


Figure 6 Gastric mucosal blood flow measured with BLF21 Laser-Doppler flow meter and mucosal hypoxia visualized by Hypoxy probe-1. A: In gastric mucosa of aging rats at baseline, mucosal blood flow, expressed in perfusion units, is significantly reduced by approximation 60% (vs young rats; $P = 0.0015$). Such a dramatic reduction in blood flow likely leads to chronic hypoxia; B: Photomicrographs of rat gastric mucosa. Gastric mucosal hypoxia is visualized by immunohistochemical staining utilizing the small molecular marker, pimonidazole HCl (Hypoxy probe-1), which binds selectively to oxygen starved cells^[30]. In young rats, Hypoxy probe-1 staining is negative in both connective tissue and epithelial cells of the gastric mucosa demonstrating the absence of hypoxia. In aging rats, positive staining is strongly expressed (brown staining) in the upper and mid-mucosa, mainly in the progenitor and parietal cell zone (arrows), reflecting severe hypoxia in these cells. As a positive control we used gastric mucosa of young rats that had all major gastric arteries ligated for 1 h. A strong accumulation Hypoxy probe-1 is present in the majority of epithelial cells (brown staining) reflecting profound cell hypoxia. Reproduced with permission from Tarnawski *et al*^[1].

flow in aging *vs* young rats^[12-14]. Importantly human studies confirmed clinical relevance of these experimental findings. Cryer *et al*^[17] and Goto *et al*^[18] demonstrated in humans an age-associated decrease in gastric mucosal prostaglandin concentration *vs* young individuals. In another human study, Feldman and Cryer^[19] showed that aging is associated with a significant reduction in gastric bicarbonate, sodium ion and non-parietal fluid secretion. Since mucosal defense is significantly reduced in aging gastric mucosa, not surprisingly one can anticipate increased susceptibility of aging gastric mucosa to injury.

Increased susceptibility of aging gastric mucosa to injury

Experimental studies showed that gastric mucosa of aging rats has increased susceptibility to injury by a variety of damaging agents such as ethanol, aspirin and other NSAIDs, hypertonic saline, bile acids, cold restraint-induced stress and other factors^[10,11,20-25]. Human studies fully confirmed these experimental findings and demonstrated that patients over 65 years of age have significant-

ly increased gastric mucosal injury by aspirin and other NSAIDs^[23,26-29]. Older patients taking low-dose aspirin or NSAIDs also have a much greater absolute risk of gastrointestinal (GI) complications than younger patients. Patrono *et al*^[28] reported that the risk of ulcer complications in subjects under 50 years of age was below 0.5% while the risk was nearly 4% in subjects aged 70-79 years and approximately 6% in subjects over 80 years of age. Even though a 2-fold increase in risk with low-dose aspirin is consistent across the different age groups, the incidence of complications and the absolute increase in complications with aspirin *vs* controls is dramatically higher in the older population due to their higher baseline risk^[28]. Furthermore, the concurrent use of other medications (*e.g.*, NSAIDs) that increase the risk of bleeding in low-dose aspirin users also increases with age^[26-29].

Structural abnormalities of aging gastric mucosa

In a previous study we analyzed structural changes in gastric mucosa of aging (*vs* young) rats by quantitative histology^[1]. That study demonstrated a partial atrophy of

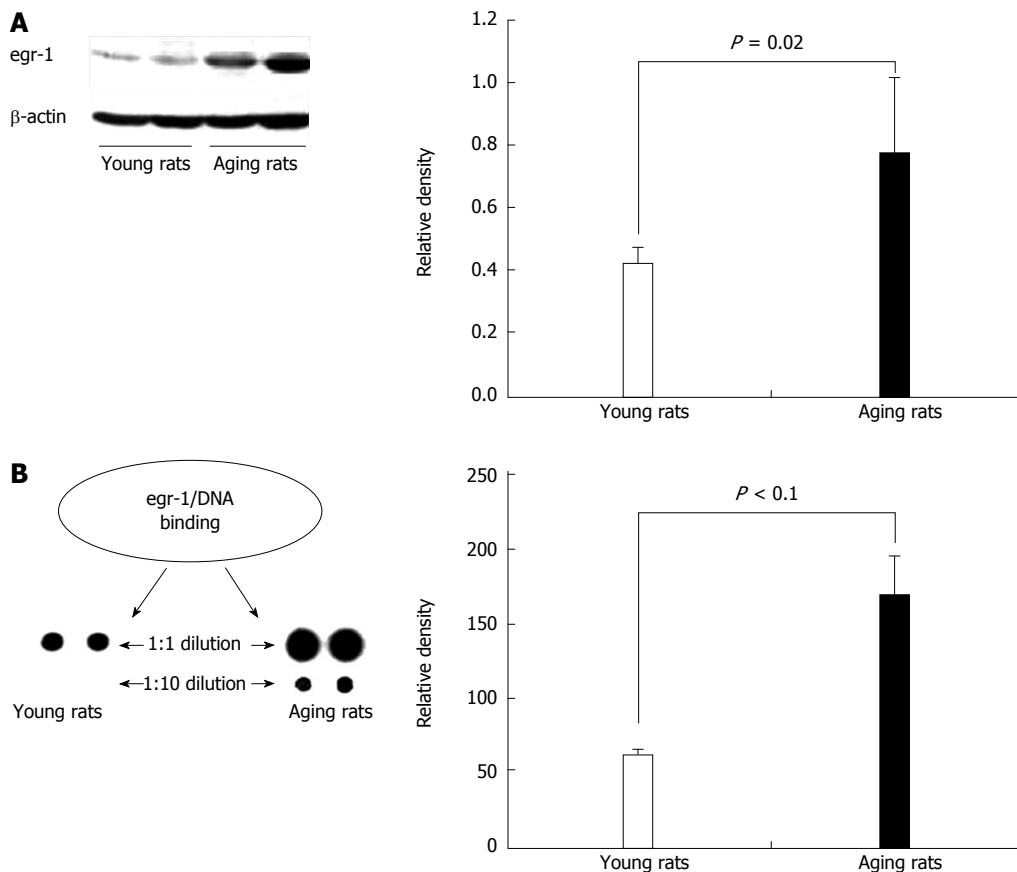


Figure 7 Increased expression of early growth response-1 and increased early growth response-1 transcriptional activity in gastric mucosa of aging vs young rats. A: Representative Western blotting demonstrate increased early growth response-1 (egr-1) protein expression in gastric mucosa of aging (vs young) rats; B: Assessment of egr-1 transcriptional activity in gastric mucosa of young and aging rats was performed using the TransSignal™ TF-TF Interaction Array (Panomics, Redwood City, CA). The egr-1 *cis*-element is spotted in duplicate: in the first row DNA was spotted without dilution; in the second row DNA was diluted ten times (1:10). In gastric mucosa of aging rats there is a significant, 2.7-fold increase (vs that of young rats; $P < 0.02$) in binding of egr-1 protein to its GC-rich *cis* elements that are highly expressed in the PTEN gene promoter. Reproduced with permission from Tarnawski *et al.*^[1]

gastric glands and their replacement with increased connective tissue in the basal one third of the mucosa (Figure 4A). Quantification of connective tissue in the lower one third of the gastric mucosa shows a significant approximately 3 fold increase in connective tissue replacing glandular cells in aging rats (Figure 4B). These findings were independently confirmed later by another group^[3].

In a separate study using transmission electron microscopy (TEM) (Figure 5) we demonstrated prominent histologic and ultrastructural alterations in gastric mucosa of aging rats including disorganized collagen fibrils in connective tissue immediately adjacent to capillary blood vessels (Figure 5B)^[2]. We postulated that these changes could interfere with nutrient and oxygen transport and hence lead to hypoxia as well as the accumulation of toxic metabolites^[2].

Mechanisms of aging gastropathy-novel insight

While previous studies showed reduced gastric mucosal blood flow in aging rats, those studies did not examine mucosal hypoxia directly. To fill this gap we examined gastric mucosal blood flow in young (3 mo of age) and aging (24 mo of age) rats using a laser Doppler flowmeter as well as determined mucosal oxygenation^[1]

using the specific Hypoxy-1 probe, which visualizes tissue and individual cell hypoxia^[30]. In addition, we also examined expression of early growth response-1 (egr-1), a transcription factor (which is activated by hypoxia) and expression of dual phosphatase and tensin homologue deleted on chromosome ten (PTEN). PTEN is a dual specificity phosphatase that inhibits the PI3K/Akt signaling pathway crucial for cell survival and therefore promotes apoptosis^[31-34]. Furthermore, the same study^[1] examined apoptosis in the gastric mucosa of aging vs young rats using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) method and also quantified expression and activation of the apoptosis-inducing executioner proteases: caspase-3 and caspase-9 described in our previous paper^[35], as well as the anti-apoptosis protein, survivin described in our previous studies^[36,37]. That experimental study^[1] showed that gastric mucosa of aging rats exhibits: (1) Significantly reduced mucosal blood flow (by approximately 60%) compared with gastric mucosa of young rats (Figure 6A) resulting in marked hypoxia (reflected by the accumulation of Hypoxy-1 probe) of the upper and middle gastric mucosa, mainly in parietal and progenitor cells (Figure 6B). It should be noted that a recent human

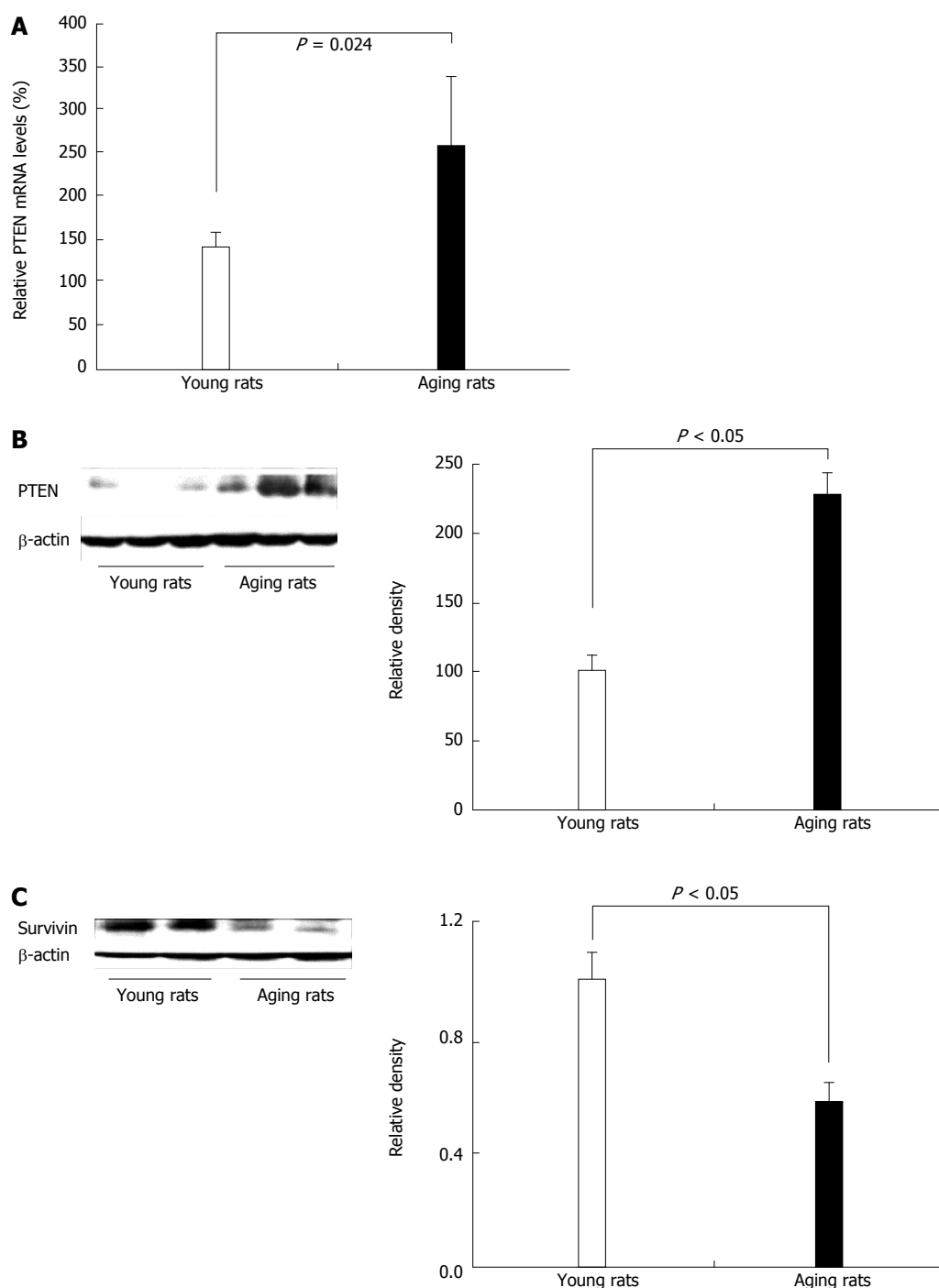


Figure 8 Increased expression of dual phosphatase PTEN and reduced expression of survivin in gastric mucosa of aging vs young rats. A: Real time PCR; B: Representative Western blotting showing a significant increase in phosphatase and tensin homologue deleted on chromosome ten (PTEN) mRNA and protein expression, respectively in gastric mucosa of aging vs young rats; C: Representative Western blotting showing a significant decrease in survivin (anti-apoptosis protein). Reproduced with permission from Tarnawski *et al*^[1].

study fully confirmed these experimental findings and demonstrated abnormalities in gastric submucosal vessels and gastric submucosal arteriolar dysfunction in elderly patients, which may lead to reduced blood supply^[38]; (2) Increased expression of egr-1 protein, which is activated by hypoxia, and increased egr-1 transcriptional activity (Figure 7); (3) Increased expression of PTEN mRNA and protein, and reduced expression of survivin (Figure 8). This is mechanistically important since increased PTEN arrests cell growth and inhibits cell survival by reduc-

ing survivin and inducing apoptosis^[33,34]; (4) Significantly increased apoptosis demonstrated by TUNEL assay (Figure 9A and B); (5) Significantly increased expression of cleaved caspase-3 and caspase-9, which induce apoptosis (Figure 10); and (6) Significantly increased susceptibility to ethanol-induced injury compared with gastric mucosa of young rats (Figure 11A). The crucial mechanistic role of PTEN in the increased susceptibility of aging gastric mucosa to injury is evidenced by the finding that down-regulation of PTEN protein expression by local admin-

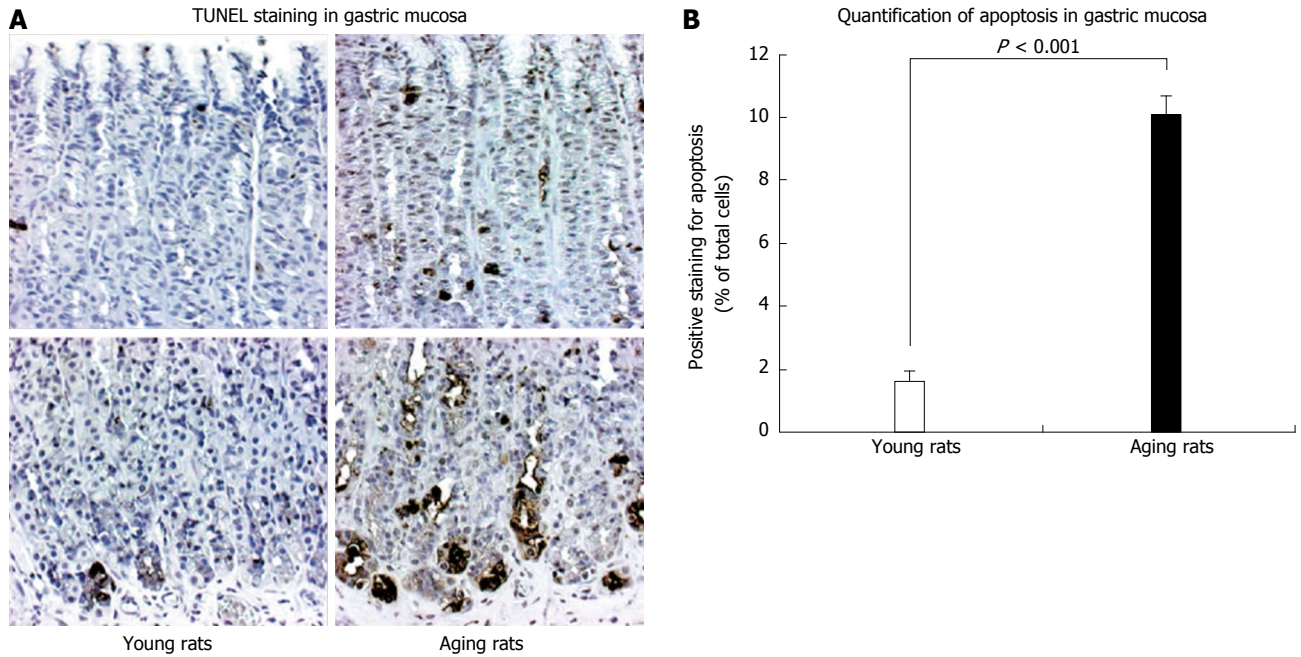


Figure 9 TUNEL staining for apoptosis in gastric mucosa of young and aging rats. A: The photomicrographs of gastric mucosa of young and aging rats at baseline (magnification $\times 100$). *In situ* cell death (apoptosis) detection by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was used to visualize apoptotic-positive cells (brown staining); B: Quantification of the number of positively labeled cells demonstrated that gastric mucosa of aging rats exhibits a significantly increased number of apoptotic cells vs mucosa of young rats. The increased apoptosis prominently involved epithelial cells at the basal mucosa explaining atrophy of the basal gastric glands shown in Figure 4. Reproduced with permission from Tarnawski *et al*^[1].

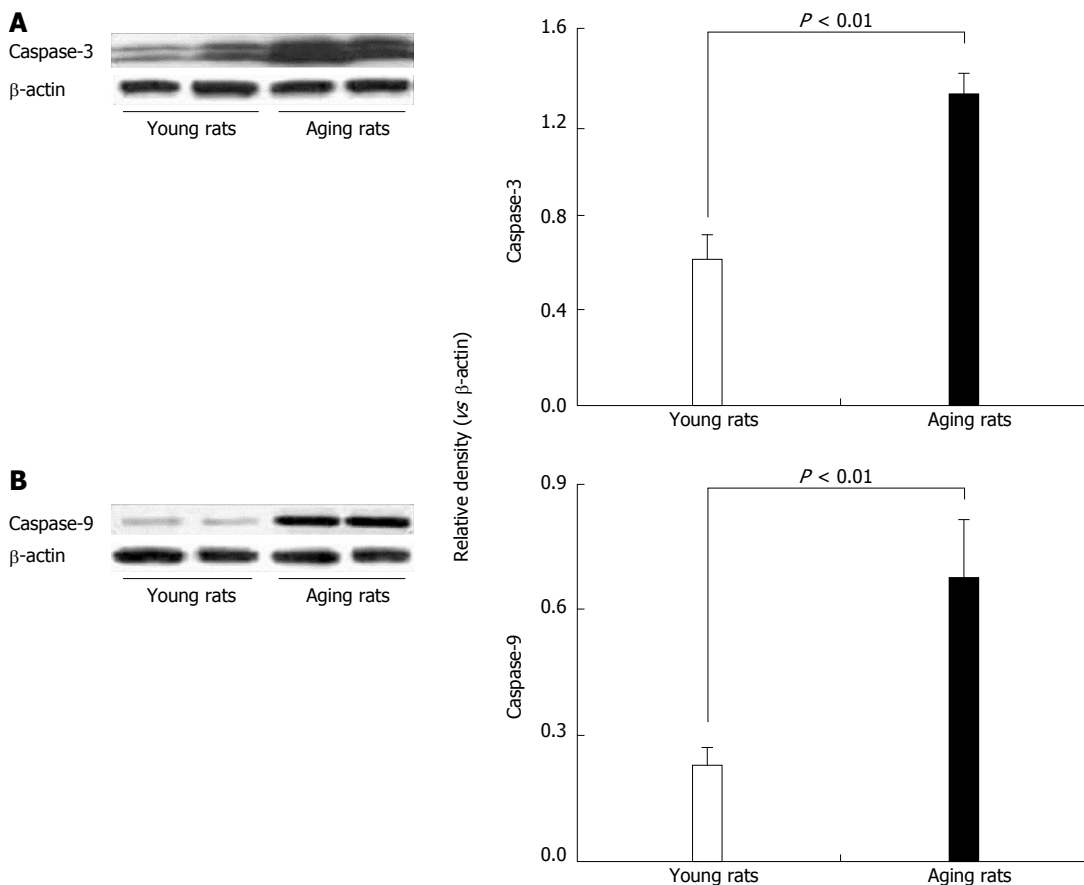


Figure 10 Expression of cleaved caspase-3 and caspase-9 protein levels by Western blotting in gastric mucosa of young and aging rats. In gastric mucosa of aging rats there is a significant increase in apoptotic cis-inducing (A) cleaved caspase-3 and (B) caspase-9 compared to gastric mucosa of young rats. Reproduced with permission from Tarnawski *et al*^[1].

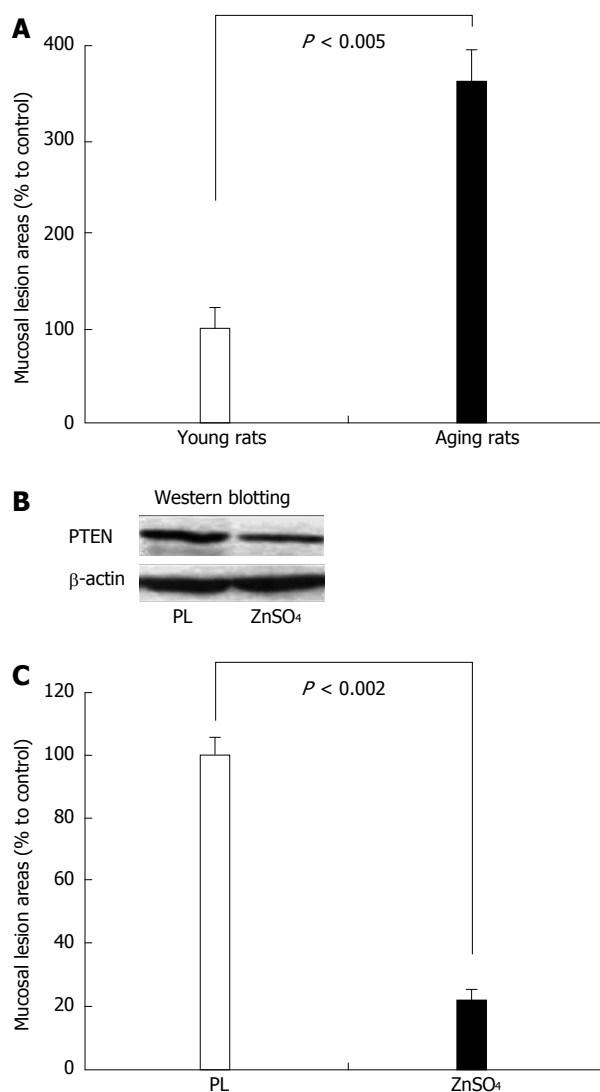


Figure 11 Extent of ethanol-induced gastric mucosal injury in young and aging rats. **A:** Three hours after intragastric administration of 8 mL/kg of 50% ethanol, gastric mucosal injury is significantly increased in aging rats vs young rats; **B:** Intragastric administration of ZnSO₄ for 4 h (2 mL 0.5% solution) down-regulates phosphatase and tensin homologue deleted on chromosome ten (PTEN) protein expression in gastric mucosa of aging rats vs placebo control (PL); **C:** Intragastric administration of ZnSO₄ to aging rats for 4 h completely reverses the increased susceptibility of gastric mucosa to ethanol-induced injury indicating a causal relationship between PTEN and mucosal injury. Reproduced with permission from Tarnawski *et al*^[1].

istration of ZnSO₄ completely reversed the increased susceptibility of gastric mucosa of aging rats to ethanol-induced injury^[1] (Figure 11B and C).

We also tested human relevance of these experimental findings. These studies^[1] demonstrated that gastric mucosa of aging humans has increased expression of PTEN; and, reduced expression of survivin, anti-apoptotic and mitosis-promoting protein (Figure 12)^[1], which is a major target for NSAIDs-induced gastric mucosal injury^[36,37].

Other mechanisms of impaired gastric mucosal defense in aging gastric mucosa

Other abnormalities including reduced telomerase activity^[39], cellular senescence^[40] and increased lipid peroxi-

dation also significantly contribute to impaired gastric mucosal defense and increased susceptibility to injury of aging gastric mucosa. Shortening of telomeres or loss of telomere function seen during aging, results in activation of DNA damage checkpoint responses^[39]. In aging gastric mucosa these events result in a biologically irreversible state of cell-growth arrest or cellular senescence^[40], which increases susceptibility to injury from damaging agents.

In addition, our recent study in rats identified in aging gastric mucosa reduced expression of vascular endothelial growth factor (VEGF)-which is a pro-angiogenic factor and protects gastric endothelial cells^[41]. Our subsequent study showed that reduced VEGF expression in aging gastric mucosa is mediated by the downregulation in gastric endothelial cells of importin- α , nuclear transport protein essential for transport of transcription factors to the nucleus^[42].

One of the potential factors and targets operating in aging may be Klotho-a membrane protein related to β glucuronidase. Mutation of this protein has been associated with human aging and circulating levels of Klotho protein decline with age^[43]. Klotho deficient mice have many features of human premature aging syndrome-progeria^[43,44]. Klotho overexpression in mice extended lifespan of mice by 19%-31% *vs* normal mice^[44]. A study examining Klotho expression in mice demonstrated that in normal mice Klotho is expressed in the stomach, mainly in the myenteric plexus and the loss of Klotho (in homozygous Klotho^{-/-} mice) causes depletion of interstitial cells of Cajal and their progenitors resulting in gastric motor dysfunction^[45]. Our preliminary data indicate that in contrast to Klotho deficient mice with premature aging syndrome, in normally aging rats Klotho expression in gastric mucosa in epithelial and vascular compartments is similar to that in young rats.

A summary of structural, functional and biochemical abnormalities of aging gastric mucosa is presented in Table 1.

DISCUSSION

Detailed analysis of the above changes indicates the following sequence of events taking place in aging gastric mucosa: (1) reduced mucosal blood flow and impaired oxygen delivery causes hypoxia, which leads to activation of the *egr-1* transcription factor; (2) Activated *egr-1* in turn upregulates PTEN, which induces cleavage-mediated activation of the pro-apoptotic proteases, caspase-3 and caspase-9^[1]. In addition, upregulated PTEN exerts a pro-apoptotic action by reducing expression of the anti-apoptosis protein, survivin; and (3) This imbalance between pro- and anti-apoptosis factors results in increased apoptosis and increased susceptibility to injury^[1].

We also tested human relevance of this concept and demonstrated increased expression of PTEN and reduced expression of survivin in human gastric mucosa of aging individuals^[1]. This clearly indicates the human relevance of our experimental findings and also can ex-

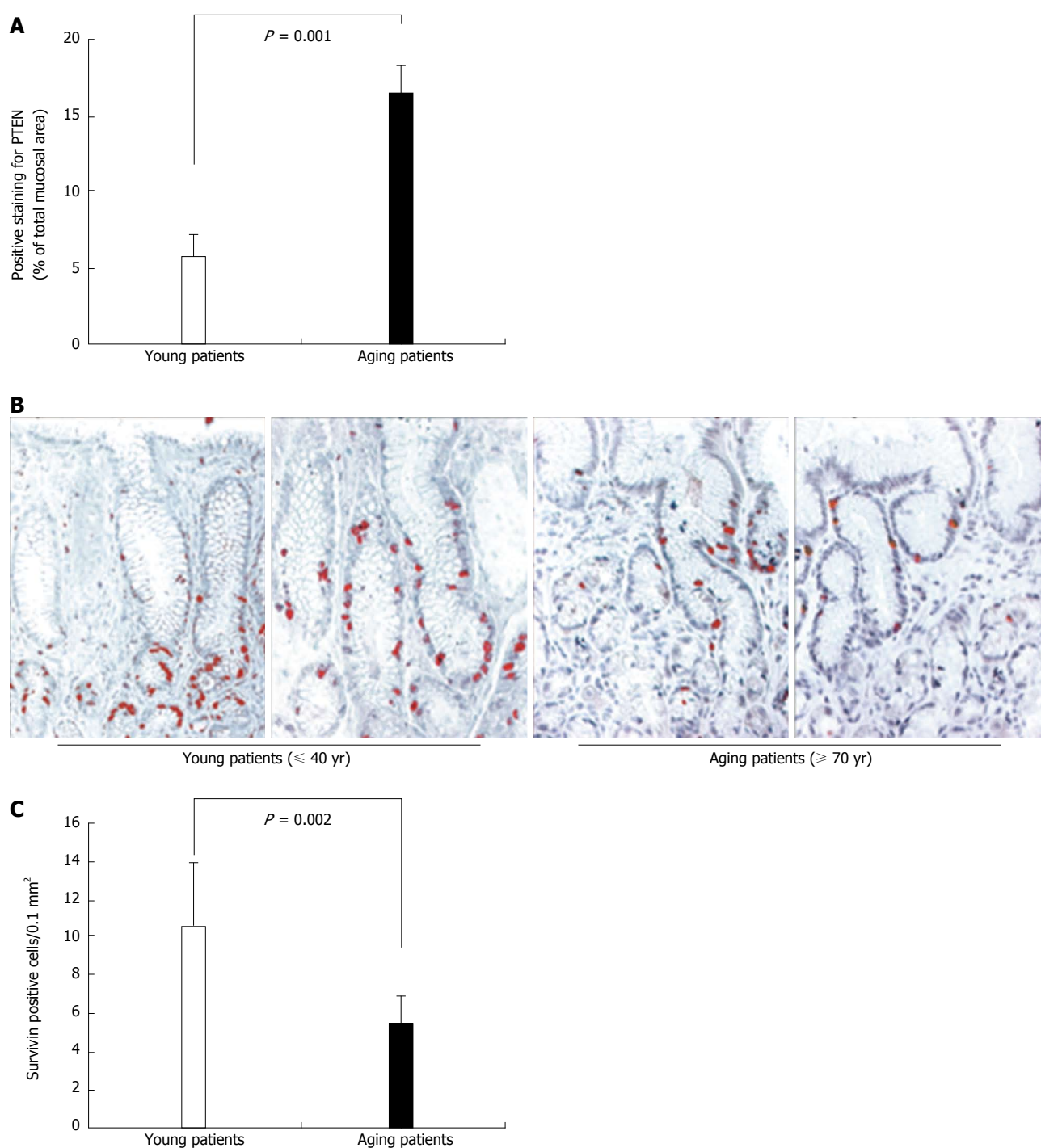


Figure 12 Human relevance: expression of phosphatase and tensin homologue deleted on chromosome ten and survivin in gastric mucosa of young and aging individuals. A: Quantification of phosphatase and tensin homologue deleted on chromosome ten (PTEN)-positive cells in gastric mucosal sections demonstrated a significantly increased number and significantly increased mucosal area of positively stained cells, mainly epithelial cells in aging (≥ 70 years of age) vs young patients. The threshold of positive staining was set at level 75 on scale 0-255. Reproduced with permission from Tarnawski *et al.*^[1]; B: Photomicrographs of representative sections of human gastric mucosa of young and aging patients immunostained for survivin^[1]. In gastric mucosa of young patients (40 years of age and younger), survivin expression is strong (brown-red staining) in the nuclei of the progenitor cells; C: Quantification of the number of positively stained cells for survivin demonstrated significantly reduced survivin expression in the gastric mucosa of aging patients (70 years of age or older) vs young patients as reflected by significantly fewer positively stained cells. Reproduced with permission from Tarnawski *et al.*^[1].

plain the increased susceptibility of aging human gastric mucosa to injury as a consequence of the mechanisms leading to the imbalance between pro-apoptotic PTEN and anti-apoptotic survivin, which were established in our studies utilizing a rat model^[1].

A subsequent study by another group^[3] fully confirmed our findings including the basal atrophy of gastric glands in gastric mucosa of aging rats and the increased expression of *egr-1*, PTEN, and caspase-9 and also showed reduced mRNAs for CGRP and neuronal NOS.

Table 1 Structural, functional and biochemical abnormalities of aging gastric mucosa

Partial atrophy of gastric glands in the basal one-third of the mucosa and their replacement with connective tissue
Degenerative changes in parietal and chief cells and accumulation of disorganized collagen fibrils in connective tissue immediately adjacent to capillary blood vessels. The latter most likely interferes with transport of oxygen and nutrients
Reduced sensory innervation and abolished hyperemic response to mild and moderate irritants
Reduced bicarbonate and prostaglandin generation and secretion
Reduced (by 60%) mucosal blood flow and profound hypoxia of epithelial cells
Increased expression of <i>egr-1</i> and its transcriptional activity-most likely responsible for activation of the <i>PTEN</i> gene
Increased expression of <i>PTEN</i> mRNA and protein (pro-apoptosis protein) and reduced expression of survivin (anti-apoptosis protein); this imbalance results in increased apoptosis
Increased apoptosis
Other abnormalities: reduced telomerase activity, cellular senescence, increased lipid peroxidation, impaired hypoxia sensor in endothelial (and epithelial?) cells, increased reactive oxygen species, downregulated or mutated Klotho protein in some submucosal neural elements, and dysregulated mitochondrial-nuclear communication
Decreased importin- α expression in endothelial cells of gastric mucosa leading to reduced activation and expression of vascular endothelial growth factor, which is a pro-angiogenic factor and protects gastric endothelial cells

All the above changes underlie increased susceptibility of aging gastric mucosa to injury by a variety of factors including aspirin and other non-steroidal anti-inflammatory drugs, ethanol, ischemia/reperfusion and others, and these changes most likely impair injury healing. PTEN: Phosphatase and tensin homologue deleted on chromosome ten.

It is important to point out that the use of rats for the studies on aging has several advantages^[1]. Fisher F-344 rats obtained from the National Institute on Aging (NIA, United States) have a very similar, almost identical, genetic background; and, we can analyze the entire mucosa and gastric wall in a standardized fashion, which is impossible in human endoscopic biopsy specimens^[1]. Importantly, we can ensure the absence of *H. pylori* and viral infections because the F-344 strain is tested for these and the animals sent to investigators are *H. pylori* and viral free. Moreover, absence of damaging environmental factors, such as smoking or drugs (*e.g.*, NSAIDs) in rats eliminates possible confounding influences that are difficult, if not impossible, to completely control for with human biopsy specimens^[1]. Therefore, the rat model utilized in our studies can differentiate aging-related mucosal changes from mucosal changes resulting from various environmental factors^[1].

Investigating available mucosal protective agents we found that hydrotalcite (Al and Mg containing antacid) exerted a protective action on aging gastric mucosa in rats, similar to that afforded by ZnSO_4 ^[1], and significantly reduced injury of aging gastric mucosa induced by ethanol and NSAIDs by preserving endothelial cells of mucosal blood vessels and epithelial progenitor cells^[46]. Since hydrotalcite is clinically available in some countries (*e.g.*, China and most European countries), this finding may have an important clinical application.

As described above, there is increased apoptosis and increased executioner caspase activity in gastric mucosa of aging rats^[1,3]. A recent study demonstrated that melatonin given for 3 wk significantly reduced caspase-3 levels in gastric mucosa of aging rats^[47]. In another study, cell proliferation, telomerase activity and lipid peroxidation were examined in gastric mucosa of young and aging rats^[48]. Telomerase activity was significantly reduced in aging *vs* young rats while lipid peroxidation was increased. Treatment with melatonin for 3 wk significantly increased telomerase activity and reduced lipid peroxidation in gas-

tric mucosa of aging rats^[48]. The authors concluded that melatonin may reverse changes in aging gastric mucosa by inhibiting the replicative cellular senescence through both a stimulatory effect on telomerase activity and a suppressive effect on lipid peroxidation^[48].

Significance and clinical implications of increased susceptibility of aging gastric mucosa to injury

In many countries (*e.g.*, China, Japan, Western Europe and United States) the population is aging. For example, in the United States it is estimated that approximates 16% of the population will be ≥ 65 years of age by 2020^[49]. This aging population is increasingly using aspirin for cardiovascular and cerebrovascular events and/or prophylaxis; and, is using aspirin and other NSAIDs (the most widely used drugs worldwide) for arthritis and musculoskeletal ailments^[29,50]. Clinical studies demonstrated that patients' age is a significant predictor of gastric injury and its complications^[26,29]. In a long-term prospective study of 34701 arthritic patients, Laine *et al*^[26] examined the risk factors for NSAIDs-associated upper gastrointestinal events. They found that an age of ≥ 65 years was a significant predictor of NSAIDs-induced risk of bleeding, perforation, obstruction or ulcer and their complications. They concluded that age ≥ 65 years, prior upper GI clinical events and low dose aspirin are main risk factors for these complications^[26].

Another recent study^[29] listed age > 70 years among the major factors associated with increased risk of upper GI complications in patients on a low dose aspirin prophylaxis/treatment. Since, aspirin and NSAIDs are the most widely used drugs worldwide and cause a higher rate of gastric complications in elderly patients, the issue of aging gastropathy has important clinical implications. Moreover, since gastric mucosal defense is impaired in aging, it is likely that injury caused by other noxious factors such as ethanol, bile reflux, chemotherapeutic agents, *etc.* is also increased in aging individuals. Importantly increased injury susceptibility of aging gastric mucosa can

be potentially reduced or reversed pharmacologically *e.g.*, by using prostaglandin analogs (misoprostol), Al-Mg containing antacids (hydrotalcite), and/or GI sparing novel NSAIDs^[51], melatonin and others.

***H. pylori* and gastric mucosal defense in relation to aging**

There is relatively little information pertaining to this topic and the available information is mainly related to peptic ulcer disease, GI bleeding, *H. pylori* and NSAIDs in relation to aging. The interactions and a molecular crosstalk between *H. pylori* and human gastric mucosa (without focus on aging) were recently reviewed by Ricci *et al.*^[52]. In general, the prevalence of *H. pylori* infection increases with age and is present in 40%-60% of asymptomatic elderly individuals and in more than 70% of elderly patients with gastroduodenal diseases^[53-55]. Since gastric mucosal defense in aging individuals is impaired, not surprisingly peptic ulcer disease in elderly patients is an increasingly frequent occurrence^[53-55].

The outcome of infection and its pathological consequences depend on the type of *H. pylori* (*e.g.*, Cag A+), duration of infection, changes in the gastric mucosa (*e.g.*, superficial gastritis, atrophic gastritis, metaplasia, dysplasia), gastric acid secretory status, and many other variables. *H. pylori* infection may temporarily activate cyclooxygenase 2 and, consequently, the generation of protective prostaglandins, which in experimental conditions may reduce acute mucosal damage by ethanol or acid^[56]. However, most studies indicate that *H. pylori* infection (especially Cag A+) has a negative effect on gastric mucosal defense by reducing surface hydrophobicity, impairing mucin production rate, impeding the tightening of tight junctions between the surface epithelial cells in response to acid, disrupting the gastric mucosal “barrier” and inducing loss of survivin and a decrease in gastric cell viability^[57-62].

A recent study^[63] evaluated gastric mucosal “barrier” defects using confocal laser endomicroscopy and TEM in *H. pylori* (-) *vs H. pylori* (+) patients. In gastric mucosa (outside intestinal metaplasia) the paracellular permeability was significantly (18-fold) increased in *H. pylori* (+) patients *vs H. pylori* (-) patients^[63]. After eradication of *H. pylori* the paracellular “barrier” dysfunction significantly improved indicating a causal relationship between *H. pylori* infection and gastric mucosal “barrier” dysfunction^[63]. In intestinal metaplasia areas of the gastric mucosa, mucosal permeability was increased in both *H. pylori* (+) and *H. pylori* (-) patients^[63]. In elderly patients using NSAIDs *H. pylori* infection is associated with an increased ulcer incidence^[53-55,64-67] and *H. pylori* eradication reduces peptic ulcer incidence in NSAIDs users, especially those new to NSAIDs and within the Asian population^[65]. The same paradigm applies to low dose aspirin users^[66,67]. Therefore from the practical point of view *H. pylori* eradication should be recommended for elderly patients before starting chronic NSAIDs therapy and especially before instituting low dose aspirin therapy in *H. pylori* (+) patients

with preserved gastric acid secretion^[66,67].

While the precise effects of *H. pylori* infection on mucosal defense in aging gastric mucosa has not been examined, based on the existing experimental and clinical studies indicating that *H. pylori* impairs gastric mucosal defense, one can speculate that *H. pylori* infection will further decrease mucosal defense in aging individuals. The correlation between *H. pylori* infection and mucosal defense in aging stomach deserves in our opinion a separate editorial article.

Molecular abnormalities in aging-future directions

Previous and more recent studies identified hypoxia, increased reactive oxygen species, and abnormal expression of various factors such as PTEN, survivin, caspases 3 and 9, in aging gastric mucosa. However, the key switch that triggers these changes will need to be elucidated through future studies. It is conceivable, and perhaps very likely, that some of the impairments resulting from aging pertain not only to the gastric mucosa but also other tissues; and, that some key targets and mediators may be similar, *e.g.*, in endothelial cells from various tissues. While some cellular and molecular targets and mechanisms operating in aging tissues have been identified, *e.g.*, increased reactive oxygen species, mitochondrial dysfunction, reduced sir-tuin 1, impaired nuclear-mitochondrial communication^[68], deficiency of the anti-aging transmembrane protein, Klotho, dysfunction of the hypoxia sensor (HIF-1 α)^[42,69] and impairment of the metabolic sensor (AMPK)^[42,69], the fundamental master switch triggering these events still remains elusive and requires further research.

CONCLUSION

Aging gastric mucosa-“aging gastropathy” has impaired mucosal defense and increased susceptibility to injury by aspirin, NSAIDs, ethanol and other injurious factors. In the last decade research uncovered novel mechanisms underlying impairment of mucosal defense in aging gastric mucosa including partial atrophy of gastric glands, reduced gastric mucosal blood flow with resulting profound hypoxia, increased expression of *egr-1* and PTEN, reduced expression of survivin and significantly increased apoptosis due to increased expression of activated caspase-3 and caspase-9. Other abnormalities identified in aging gastric mucosa include reduced expression of growth factors (*e.g.*, VEGF), impaired hypoxia sensor, decreased telomerase activity, cellular senescence, and increased lipid peroxidation. These findings provide a better understanding of aging gastropathy, which because of an increasing aging population and increased use of aspirin and other NSAIDs (most widely used drugs) worldwide has major clinical implications and impact. While some cellular and molecular targets and mechanisms operating in aging tissues have been identified, the fundamental master switch triggering these events still remains elusive and requires further research.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Characteristics of gastric cancer in Asia

Rubayat Rahman, Akwi W Asombang, Jamal A Ibdah

Rubayat Rahman, Akwi W Asombang, Jamal A Ibdah, Division of Gastroenterology and Hepatology, University of Missouri School of Medicine, One Hospital Drive, Columbia, MO 65212, United States

Author contributions: Rahman R wrote the manuscript; Asombang AW was involved in reviewing the literature and manuscript; Ibdah JA provided overall intellectual input into the design and edited the manuscript.

Correspondence to: Jamal A Ibdah, MD, PhD, Professor, Director, Division of Gastroenterology and Hepatology, University of Missouri School of Medicine, One Hospital Drive, CE 405, Columbia, MO 65212, United States. ibdahj@health.missouri.edu

Telephone: +1-573-8827349 Fax: +1-573-8844595

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and linkage to *H. pylori* in Asia.

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Key words: Gastric cancer; Asia; Epidemiology; Gastric cancer incidence; Gastric cancer mortality

Core tip: Gastric cancer (GC) is the third most common cancer and the second most common cause of cancer death in Asia. Highest incidence rates are observed in Eastern Asia and varies in other parts of Asia. Mortality rates are slowly declining but remain a significant public health problem. The seroprevalence of *Helicobacter pylori* is very closely related to the incidence of GC in Asia. In contrast to Western world, management of GC is focused on prevention and early detection in Eastern Asia.

Abstract

Gastric cancer (GC) is the fourth most common cancer in the world with more than 70% of cases occur in the developing world. More than 50% of cases occur in Eastern Asia. GC is the second leading cause of cancer death in both sexes worldwide. In Asia, GC is the third most common cancer after breast and lung and is the second most common cause of cancer death after lung cancer. Although the incidence and mortality rates are slowly declining in many countries of Asia, GC still remains a significant public health problem. The incidence and mortality varies according to the geographic area in Asia. These variations are closely related to the prevalence of GC risk factors; especially *Helicobacter pylori* (*H. pylori*) and its molecular virulent characteristics. The gradual and consistent improvements in socioeconomic conditions in Asia have lowered the *H. pylori* seroprevalence rates leading to a reduction in the GC incidence. However, GC remains a significant public health and an economic burden in Asia. There has been no recent systemic review of GC incidence, mortality, and *H. pylori* molecular epidemiology in Asia. The aim of this report is to review the GC incidence, mortality,

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INTRODUCTION

Gastric cancer (GC) is a heterogeneous, multi factorial disease. It endangers human physical and psychosocial wellbeing, causing a significant public health and economic burden both in the developed and developing countries^[1,2]. According to International Agency for Research on Cancer, the global and regional burden of GC is enormous^[3]. The incidence and mortality vary widely according to geographic areas, socio-cultural and economic entities. GLOBOCAN 2008 data revealed that about one million new cases of GC were estimated to have diagnosed in 2008, making it the fourth most common malignancy in the world, after lung, breast, and

Table 1 Gastric cancer incidence, mortality and prevalence in the world as reported in GLOBOCAN 2008^[3]

	Total incidence (in 1000s)	Incidence rate per 100000	Total mortality (in 1000s)	Mortality rate per 100000	5-yr prevalence (in 1000s)	Prevalence
World	988	14.0	737	10.3	1598	5.5%
Asia	727	18.5	530	13.4	1216	10.4%
Europe	145	10.3	116	7.9	201	2.4%
Northern America	24	4.2	12	2.1	37	0.8%
Africa	22	4.0	21	3.8	30	2.1%

Table 2 Male gastric cancer incidence, mortality and prevalence in the world as reported in GLOBOCAN 2008^[3]

	Total incidence (in 1000s)	Incidence rate per 100000	Total mortality (in 1000s)	Mortality rate per 100000	5-yr prevalence (in 1000s)	Prevalence
World	640	19.7	463	14.2	1050	7.8%
Asia	484	25.9	342	18.3	819	15.7%
Europe	86	14.5	68	11.3	121	2.9%
Northern America	15	5.8	7	2.8	23	1.0%
Africa	12	4.7	11	4.5	16	3.0%

Table 3 Female gastric cancer incidence, mortality and prevalence in the world as reported in GLOBOCAN 2008^[3]

	Total incidence (in 1000s)	Incidence rate per 100000	Total mortality (in 1000s)	Mortality rate per 100000	5-yr prevalence (in 1000s)	Prevalence
World	348	9.1	273	6.9	548	3.6%
Asia	243	11.7	188	8.9	396	6.2%
Europe	59	7.0	47	5.3	79	1.9%
Northern America	9	2.8	5	1.5	13	0.6%
Africa	10	3.3	9	3.2	13	1.5%

colorectal cancers. More than 70% of GC cases occur in developing countries with half the world's total cases occurring in Eastern Asia^[1,2]. Overall, GC incidence and mortality have fallen dramatically over the past 70 years. Despite its recent decline, GC is the second leading cause of cancer death in both sexes worldwide. The highest mortality rates occur in Eastern Asia while the lowest occur in Northern America. In Asia, GC is the third most common cancer after breast and lung. But is the second most cause of cancer death in Asia after lung cancer^[2,3]. Although the incidence and mortality rates of GC are slowly declining in Asia, it still remains a significant health problem. Asia is the world's largest and most populous continent. With approximately 4.3 billion people, it accounts for 60% of the world's current human population. Asia's growth rate is very high for the modern era and has quadrupled during the last 100 years. It is estimated that Asia's growth rate will remain high^[3,4]. There has been no recent systemic review of GC incidence, mortality and *Helicobacter pylori* (*H. pylori*) molecular epidemiology in Asia.

CURRENT EPIDEMIOLOGY OF GC

More than 727000 cases of GC were diagnosed in Asia in

2008, accounting for 11.9% of all the cancers diagnosed. It is the third most common cancer in Asia after breast and lung cancer^[3]. Table 1 shows that the age-standardized rate (ASR) of incidence is highest in Asia (18.5%) compared to other continents. The ASR of mortality is also highest (13.4%) and it is second only to lung cancer (19.15) in Asia. It is the third most prevalent cancer in Asia after breast and colorectal cancers^[3]. The incidence and mortality rates of GC are also the highest in both males and females in Asia compared to other continents (Tables 2 and 3)^[3,5].

The incidence and mortality rates are also higher in Eastern Asia compared to other regions in Asia. This region includes China, Japan and South Korea, the three countries with the highest GC incidence and mortality rates, while the lowest rates occur in South-Central Asia^[1,3,5,6]. The age adjusted GC incidence, mortality, and prevalence rates in the various regions in Asia are summarized in Table 4. In all the four parts of Asia, the rates are lower in females than males as shown in Tables 5 and 6^[1-5].

CURRENT INCIDENCE TREND OF GC IN ASIA

More than half of the world's population live in Asia^[2]

Table 4 Gastric cancer incidence, mortality and prevalence in Asia as reported in GLOBOCAN 2008^[3]

	Total incidence (in 1000s)	Incidence rate per 100000	Total mortality (in 1000s)	Mortality rate per 100000	5-yr prevalence (in 1000s)	Prevalence
Eastern Asia	601	30.2	418	20.3	1071	15.2%
South-Eastern Asia	43	8.6	35	7.1	56	3.9%
South-Central Asia	68	4.8	63	6.5	68	2.6%
Western Asia	14	9.4	13	8.3	19	4.2%

Table 5 Male gastric cancer incidence, mortality and prevalence in Asia as reported in GLOBOCAN 2008^[3]

	Total incidence (in 1000s)	Incidence rate per 100000	Total mortality (in 1000s)	Mortality rate per 100000	5-yr prevalence (in 1000s)	Prevalence
Eastern Asia	408	42.4	274	28.1	732	21.0%
South-Eastern Asia	24	10.9	20	8.9	32	6.2%
South-Central Asia	42	6.7	39	6.3	42	4.3%
Western Asia	9	12.6	8	11.2	12	5.8%

Table 6 Female gastric cancer incidence, mortality and prevalence in Asia as reported in GLOBOCAN 2008^[3]

	Total incidence (in 1000s)	Incidence rate per 100000	Total mortality (in 1000s)	Mortality rate per 100000	5-yr prevalence (in 1000s)	Prevalence
Eastern Asia	193	18.3	144	13.0	338	9.5%
South-Eastern Asia	18	6.7	15	5.6	23	2.6%
South-Central Asia	26	3.9	23	3.6	26	1.6%
Western Asia	5	6.7	4	5.9	7	3.0%

China, Japan and South Korea have reported the highest GC incidence rate both in males and females in the world. More than half of the total cases of GC are diagnosed in East Asia each year^[1,2,5,6]. Overall, the incidence rate trend of GC in Asia is declining in the last two decades. Although the incidence rate of GC remains somewhat unchanged in some countries of Asia, the overall incidence rate of GC in East Asia is declining^[7]. In China, the incidence rate of GC in males declined from 41.9 per 100000 in 2000 to 37.1 per 100000 in 2005^[8,9]. While from 2000 to 2005, GC incidence rate decreased from 19.5 to 17.4 per 100000, respectively in females^[1,8,9]. In Japan, the incidence rate of GC declined from about 80 to 60 per 100000 from 1980 to 2000. In 2008, the incidence rate of GC in Japan was 31.1 per 100000 both in males and females^[5,10,11]. In South Korea, the incidence rate of GC also declined to 65.6 per 100000 in males and 25.8 per 100000 in females^[2,6,12]. Many countries of South-East Asia (Singapore, Thailand, and Malaysia) have also observed a slow decline of GC incidence rate over the last few decades^[2,3].

The overall incidence rate of GC in South-Central Asia is low in comparison to the other parts of Asia. The incidence of GC in India (3.8 per 100000) is overall less than the worldwide incidence. The age-adjusted rate of GC among urban registries in India is 3.0-13.2 per 100000^[2,3]. Overall, the incidence rate of GC in India is decreasing. However, this declining trend has not been seen in certain parts of India. Among the major population-based cancer registries in India, only Mumbai

and Chennai have reported a decline in incidence. The incidence rate of GC in other parts of South-Central Asia like Pakistan, Bangladesh and Sri Lanka is also decreasing slowly^[2-4,13,14].

Western Asia is the land of multiple ethnic groups, principally from three main backgrounds: Semitic (Arabs and Jews), Indo-European (Persians and Kurdish) and Turkic (Turkish and Turkmens). Its geographic location, which has been under continuous influences from Asia, Europe and Africa, has variable incidence rates of GC. The GC rate differs in this region from very high in Iran (26.1 per 100000) to low in Israel (12.5 per 100000)^[3,15,16]. GC occurs nearly 7 times more frequently in Iran than in Iraq^[17]. In Jordan, the overall incidence rate is 4.8 per 100000 (males 5.6 and females 4.1)^[3,15,16]. The incidence rate of GC and its trend remains stable or improving slowly in most of the countries in Western Asia^[3,15-17].

GC MORTALITY IN ASIA

The overall GC mortality rate and its variations according to geographic areas closely follow the distribution of the GC incidence rate in the world as well as in Asia. In Asia, the mortality rate is higher in males than females^[3]. Similar to the incidence rate, GC mortality rate is the highest in Eastern Asia (Table 4)^[3]. Mortality rates also vary in different countries in Asia (Table 7). China has the highest mortality rate from GC (30.1 per 100000) followed by Japan (20.5 per 100000) and South Korea (13.8 per 100000). The mortality rate is moderate to low in South-

Table 7 Incidence, mortality and prevalence of gastric cancer in selected countries in Asia as reported in GLOBOCAN 2008^[3]

Country	Incidence rate per 100000	Mortality rate per 100000	Prevalence
China	29.9	22.3	5.6%
Japan	31.1	13.5	18.1%
South Korea	12.1	9.8	7.0%
Indonesia	9.4	8.8	4.8%
Malaysia	8.4	7.4	3.3%
Thailand	3.5	2.5	1.5%
India	3.8	3.6	1.5%
Bangladesh	5.2	5.0	2.8%
Pakistan	6.3	5.9	3.0%
Iran	15.6	14.1	8.8%
Iraq	3.6	3.5	1.9%
Jordan	4.8	4.5	2.2%
Saudi Arabia	3.3	3.0	2.0%
Israel	8.6	4.7	2.4%

Eastern Asia (Malaysia 8.5 per 100000), while relatively low in South-Central Asia (India 4.6 and Bangladesh 5.7 per 100000). The mortality rates vary in Western Asia (Iran 19.9, Israel 6.7, Jordan 5.2 and Iraq 3.7 per 100000)^[1,3,5,6,8,15].

CURRENT MORTALITY TREND OF GC IN ASIA

In the new millennium, there have been some distinct and progressive changes in the pattern of gastrointestinal cancer - especially in GC in Asia. Despite all the recent changes in screening, diagnosis, treatment and surveillance, GC still remains the second most common cause of cancer mortality in Asia^[3]. The mortality rate of GC remains statistically unchanged in some countries of Asia, however, a significant decline in the mortality rate of GC in Eastern Asia led to an overall decline in the mortality rate in Asia^[7]. In China, the highest mortality rate was observed in rural areas, especially in Gansu, Henan, Hebei, Shanxi and Shaanxi Provinces in the middle-western part of China^[8,9]. Although there was a slight increase from the 1970s to early 1990s, a significant decline in GC mortality was noticed in almost the entire population during the last decade in China. Between 2000 and 2005, Mortality from GC declined in males, while the number slightly increased in females, despite the significant declining trend in mortality rates among all age groups in China. In 2008, the mortality rates of GC in China were 30.1 in males and 14.6 in females per 100000^[1,7,8].

There has been a significant change in the mortality trend of GC in Japan. This country has had a significant decline in the mortality rate of GC from 1980 to 2010. In 2008, the overall mortality rate was 14.7 per 100000 (20.5 and 12.6 per 100000 in males and females, respectively)^[2,3,11]. Similar decline was also observed in South Korea^[2,3,12]. The mortality rate also declined in most of the countries in Eastern and South-Eastern Asia^[2,3,7,18].

There was a decline in the mortality rates of GC in

urban areas of India (overall 3.6, male 4.6, and female 2.7 per 100000 in 2008). But in many rural areas, the mortality rate still remains high and unchanged. The mortality rates in other countries in South-Central Asia remain low and unchanged. Iran has the highest mortality rate of GC in South-Central and Western Asia. The overall mortality rate is 14.1 per 100000 (male 19.9 and female 8.2) and is slowly declining. The mortality rate remains low and improving slowly in Jordan (overall 4.5, male 5.2 and female 3.8 per 100000) and Israel (overall 4.7, male 6.7 and female 3.0 per 100000)^[3,13-15].

EPIDEMIOLOGY OF *HELICOBACTER PYLORI* INFECTION IN ASIA

It is well postulated that the seroprevalence of *H. pylori* is very closely related to the incidence of GC. There is a difference in the seroprevalence of *H. pylori* infection between countries and within specific regions and communities of individual countries, not only in Asia but also in other countries of the world. In tandem with the socioeconomic development in many countries, a temporal decrease in the seroprevalence rate has been reported^[2].

The seroprevalence rates of *H. pylori* infection in under-developed or developing countries are higher than in developed countries. The highest seroprevalence rate is reported in Bangladesh (92%) followed by Kuwait (84%) and India (79%)^[2,19,20]. On the other hand, the seroprevalence rates in developed countries are reported to be lower. Among East Asian countries, the overall seroprevalence rate is 58.07% in China, 39.3% in Japan, 59.6% in South Korea and 54.5% in Taiwan^[21-24]. Among South-east Asian countries, the reported seroprevalence rate was 35.9% in Malaysia, 31% in Singapore, 75% in Vietnam and 57% in Thailand^[2,7,18,25-27]. The seroprevalence rates are also high in many countries of the South-Central and Western Asia - 78% in Jordan, 77% in Iran, 78% in Iraq, 75% in Saudi Arabia and 72% in Israel^[15,28]. In addition, a temporal effect was also evident with the younger population having low prevalence rates similar to developed Western countries^[2].

A temporal effect in *H. pylori* seroprevalence rate has also been noted in Asia^[29,30]. In a study from Guangzhou province in China, it was found that the overall *H. pylori* seroprevalence rate had decreased from 62.5% in 1993 to 47% in 2003. Among children aged 1-5 years, the seroprevalence rate was 19.4% and this rose to 63.2% among subjects aged 40-50 years. In Japan, the overall seroprevalence rate was 72.7% in 1974, decreased to 54.6% in 1984 and was 39.3% in 1994. In South Korea, the seroprevalence rate decreased from 66.9% in 1998 to 59.6% in 2005^[31]. In addition to a temporal decline in the overall seroprevalence rate over time, the younger population generally has a lower seroprevalence rate. Current evidence suggests that most *H. pylori* infection is acquired in childhood. The data from Asia also indicates that the rate of *H. pylori* infection has been decreasing over the last 40-50 years, with an overall decline in *H. py-*

lori seroprevalence, similar to that of Western developed countries^[2,32-34]. Evidence related to the survival of *H. pylori* outside the gastric niche is limited. Principal route of transmission yet to be confirmed. There is strong evidence that indicates the prevalence of *H. pylori* infection has a strong correlation with access to clean water, suggesting a transmission route to the host^[35,36].

The Indian enigma is a subset of the Asian enigma, which refers to the observations that there are regions where *H. pylori* infection is high yet the GC incidence is relatively low. The term was coined based on the epidemiological observation. The regions where these observations are made are India, Bangladesh, Pakistan and Thailand^[2,4,5]. It is to be acknowledged that there are still gaps in our appreciation of the process of gastric carcinogenesis. There is also a lack of data documenting the precise gastric histology in these populations with low GC but high *H. pylori* seroprevalence rates^[37,38].

MOLECULAR EPIDEMIOLOGY OF *H. PYLORI* IN ASIA

Different *H. pylori* strains transpire across diverse geographic regions, and the differences in these strains have been correlated with the variation in GC epidemiology. Six main geographic strains were identified, the hpEastAsia is the strain from East Asian countries^[39]. It has been perceived that populations with high GC rates correspond almost exactly to populations with the hpEastAsia strain. In South Asian countries where *H. pylori* seroprevalence rates are high but GC prevalence rates are low, the strains were predominantly hpAsia^[29,39-41].

In the milieu of GC carcinogenesis, bacterial virulence factors that have been implicated include the cytotoxin-associated gene A antigen (CagA), vacuolating cytotoxin (VacA), and outer membrane proteins (OMP)^[41]. Huang *et al.*^[42] performed a meta-analysis of 16 studies with 2284 cases and 2770 controls to scrutinize the relationship between CagA seropositivity and the risk of GC and indicated that infection with cagA-positive strains of *H. pylori* amplified the risk for GC over the risk associated with *H. pylori* infection alone. CagA protein has been classified into Western and Eastern types. The East Asian strain has been documented to be more virulent than the Western CagA with respect to clinical outcomes^[42,43]. Azuma *et al.*^[44] validated that in the grades of inflammation and mucosal atrophy were significantly higher in patients infected with Eastern CagA-positive strains than in those infected with Western CagA-positive strains. Satomi *et al.*^[45] revealed that in Okinawa, Japan, where both Western and East Asian CagA were present, the prevalence of East Asian CagA-positive strains was significantly higher in patients with GC (84.6%) than in patients with duodenal ulcer (27.3%). There are increased frequencies of GC in individuals with type O blood and in secretors [expressing Le (b) antigen], but other studies have not found any relationship between blood groups and this infection^[46,47]. The prevalence of *H. pylori* infec-

tion in intestinal-type GC is higher than in the diffuse type and in the control group. An association was found between *H. pylori* infection and GC located distally (antrum/pylorus)^[48].

MANAGEMENT AND OUTCOME OF GC IN ASIA

GC management has been principally focused on the management of advanced GC in western populations, where the risk of GC tends to fall into the low-risk category. But this statement does not hold true for the highest-risk continent - Asia, where resources have focused on preventive strategies as well as management of early stage GC^[49]. It is widely accepted that GC, like many other malignancies, progresses through a cancer cascade^[50]. However, why certain individuals and families have a greater propensity to move along the cascade towards GC, is most certainly a multi-factorial process, and arises from complex and multifaceted interactions between host factors, *H. pylori* and environmental factors. Diets high in salt and nitrates carry the highest risks, with salt in particular demonstrating an ability to augment the effects of carcinogens. Fresh fruits and vegetables are associated with a reduced risk of GC. But fortification of the diet with ascorbic acid or use of multivitamins does not appear to confer the same protection. Studies focusing on sustained measurable alterations in diet sufficient to affect GC incidence and prevalence are underway^[51-53].

However, *H. pylori* infection has proven to be an interesting target and multiple studies have indicated that *H. pylori* infection is a necessary, but not a sufficient causal factor in the development of GC^[51]. Unfortunately, four randomized placebo-controlled trials evaluating the impact of *H. pylori* screening and eradication on GC prevalence did not show a significant reduction in GC development; however, there was a non-significant trend towards risk reduction for GC with *H. pylori* eradication. The strategy of population screening and treatment of *H. pylori* infection appears to be the strategy of choice in high GC risk populations in Eastern Asia^[54-57].

China, Japan and South Korea are the in champions in the management of early GC, and this has primarily been driven by need. Given that these countries have some of the highest GC, screening is done through barium meal (Japan), gastroscopy (Japan and South Korea) and serum pepsinogen/gastroscopy (China)^[55-57]. Studies from the eastern Asia examining the techniques of endoscopic mucosal resection and endoscopic submucosal dissection for early stage GC have proven that with improving technical expertise and careful surveillance, the outcome is excellent with high 5-year survival^[58]. However, the detection of early GC is difficult and only systematic population screening has been shown to increase early detection and confer a survival advantage^[59,60]. Health economics modeling indicates that population endoscopic screening for early GC is cost effective in moderate- to high-risk populations and may not be economically prudent for other

part of Asia^[59,61].

In the management and outcome of advanced GC, the majority of the studies have been carried out in the Western world, as most of the cases of GC are diagnosed at advanced stages^[62]. The backbone of the management of advanced GC is chemotherapy, which is comparable between Western world and Asia^[63]. The S1-cisplatin doublet remains the standard chemotherapy regimen for advanced GC in Japan, replacing 5FU-cisplatin. S1-cisplatin is also available in South Korea, Singapore, Taiwan, Philippines and China for this indication, but is not approved in North America^[64,65]. Fluoro-pyrimidines show less toxicity in Asian populations, possibly secondary to polymorphisms in genes encoding drug-metabolizing enzymes, translating into more options for advanced GC treatments in Asian populations. Better survival differences after gastrectomy for GC favoring Asians patients may be explained by different disease patterns, the related need for fewer extensive procedures, and fewer patient risk factors in Eastern Asia. In other parts of Asia, the outcomes after surgery are variable^[9,58,66]. The overall 1 and 5-year survivals are comparable between Asia, Europe, and North America according to gender and stage. there are slight variations of outcomes among different regions of Asia, depending on socioeconomic condition and access to medical care^[9,65,67].

CONCLUSION

GC is exerting a significant health and economic burden in many countries of Asia^[3]. The gradual and consistent improvement in socioeconomic condition in Asia has brought about an overall decrease in *H. pylori* seroprevalence rates and thus improvement of GC incidence^[2,7]. Nonetheless, differences in still exist between developed and less developed countries of Asia^[7,68]. There is now better understanding of the process of gastric carcinogenesis, and the role of bacterial virulence factors interrelating with host immune factors. In order to address the high clinical burden of GC, a recent Asia-Pacific Gastric Cancer Consensus meeting has strongly recommended a strategy for *H. pylori* screening and eradication in high-risk populations to reduce GC incidence. On the other hand, there have been continuous efforts to improve the screening, early diagnosis, surgical and medical management and surveillance of GC^[7].

Similar to the Western world, the incidence and prevalence of *H. pylori* infection and GC in Asia has decreased over the past few decades. Multiple factors have played interactive roles in this regard. With a better understanding of the molecular epidemiology of *H. pylori* infection, GC carcinogenesis and overall improved management of GC, the clinical outcome is slowly improving^[69,70]. However, more elaborative and circumferential steps have to be taken for primary, secondary and tertiary prevention of GC in Asia which in turn will eventually decrease the global burden of GC.

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Gastric cancer research in Mexico: A public health priority

Clara Luz Sampieri, Mauricio Mora

Clara Luz Sampieri, Instituto de Salud Pública, Universidad Veracruzana, Av. Luis Castelazo Ayala, Col. Industrial Ánimas, 91190 Xalapa, Veracruz, México

Mauricio Mora, Facultad de Medicina, Universidad Veracruzana. Médicos y Odontólogos, Col. Virginia Cordero, 91120 Xalapa, Veracruz, México

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Correspondence to: Dr. Clara Luz Sampieri, Instituto de Salud Pública, Universidad Veracruzana. Av. Luis Castelazo Ayala, Col. Industrial Ánimas, 91190 Xalapa, Veracruz, México. csampieri@uv.mx

Telephone: +52-228-8418900 Fax: +52-228-8418935

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with gastric cancer and the development of the disease (16/48); relationship between the Epstein-Barr virus and pathologies associated with gastric cancer and the development of the disease (3/48); molecular markers for the development of diseases associated with gastric cancer and gastric cancer (15/48). Mexico requires a program for the prevention and control of gastric cancer based on national health indicators. This should be produced by a multidisciplinary committee of experts who can propose actions that are relevant in the current national context. The few studies of gastric cancer conducted on the Mexican population in national institutes highlight the poor connection that currently exists between the scientific community and the health sector in terms of resolving this health issue. Public policies for health research should support projects with findings that can be translated into benefits for the population. This review serves to identify national research groups studying gastric cancer in the Mexican population.

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Abstract

This study aimed review studies conducted on Mexican patients diagnosed with gastric cancer and/or diseases associated with its development, in which at least one Mexican institute has participated, and to assess their contributions to the primary and secondary prevention of this disease. A search of the Medline database was conducted using the following keywords: gastric/stomach cancer, Mexico. Studies of the Mexican population were selected in which at least one Mexican Institute had participated and where the findings could support public policy proposals directed towards the primary or secondary prevention of gastric cancer. Of the 148 studies found in the Medline database, 100 were discarded and 48 were reviewed. According to the analysis presented, these studies were classified as: epidemiology of gastric cancer (5/48); risk factors and protectors relating to gastric cancer (9/48); relationship between *Helicobacter pylori* and pathologies associated

Key words: Gastric cancer; Mexico; Research; Prevention; Public health

Core tip: The few studies of gastric cancer in the Mexican population included in this review highlight the poor connection between the scientific community and the health sector in terms of resolving this health issue. Public policies for health research should support projects for the creation of gastric cancer research networks that include experts from different disciplines. These networks could generate, among other products, an official Mexican standard (Norma Oficial Mexicana) for gastric cancer as well as strategies for its prevention, control and treatment.

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INTRODUCTION

Mexico: A country of inequality

Mexico, according to the most recent data from the World Bank, had a gross domestic product of 1178 billion United States dollars in 2012; in 2011, 6.2% of its spending was invested in health, while 5.3% was spent on education in 2010 and 0.4% was allocated to science and technology in 2009^[1]. It is considered a country of medium-high income, with a life expectancy at birth of 77 years^[1]. In 2012, the population of Mexico was 120.8 million and it was estimated that 83% of the rural population had access to a water supply^[1]. At the beginning of the 1970s, the number of births per year per 1000 individuals was greater than 7, but in 2009 it was 2.4^[2]. In 2010, 6.5 million people were reported as vulnerable in terms of income and 46% of the population was considered to be in a situation of multidimensional poverty^[1].

Cancer in Mexico

Cancer has been one of the most important diseases in Mexico since the end of the twentieth century, representing a public health problem, not only in terms of its grave clinical manifestations and high mortality, 75.4 per 100000 habitants in 2011^[2], but also in the variety of individual and environmental risk factors with which it is associated^[3]. In 2011, in populations of age 20 and over, the malignant tumors that caused the highest number of deaths in women were: breast (13.8%); cervical-uterine (10.4%); stomach (7.0%); bronchopulmonary (6.4%); liver-intrahepatic bile duct (5.5%) and colon (4.3%), while in men these were: prostate (16.9%); bronchopulmonary (12.8%); stomach (8.6%); liver-intrahepatic bile duct (5.3%) and colon (5.3%)^[2].

Despite the high mortality of cancer in Mexico, few studies have provided indicators, such as magnitude, transcendence and vulnerability, of utility to the planning of this public health problem. Of the studies that do exist, the foremost are: (1) Tovar-Guzmán *et al*^[4] (1999), who report that during the period 1980 to 1995, the crude mortality rate of prostate cancer increased from 3.16 to 6.75 cases per 100000 men of age 40 years and over. The age-adjusted rate for the same period was 2.71 to 7.01 cases per 100000 men of age 40 years and over. The standardized mortality ratio (SMR) for the different states of Mexico showed a loose relationship among different regions, with high SMR values in the states of Baja California Sur at 183.28 (95%CI: 158.36-208.18), Jalisco at 161.81 (95%CI: 156.18-167.44) and Aguascalientes at 152.21 (95%CI: 136.115-168.27), while the lower SMR values corresponded to Quintana Roo at 47.87 (95%CI: 35.86-59.98), Guerrero at 57.69 (95%CI: 52.89-62.49) and Estado de Mexico at 59.91 (95%CI: 57.46-62.36)^[4]; (2) Tovar-Guzmán *et al*^[5] (2001), who report a general

increase in the rate of gastric cancer mortality during the years 1980 to 1997, from 4.43 cases per 100000 habitants (95%CI: 4.27-4.59) in 1980, to 6.46 (95%CI: 6.28-6.64) cases in 1997^[5]. Interestingly, these authors found a differential trend in mortality per gender, which probably reflected the regional socioeconomic conditions of the country^[5]. Male:female ratio was 1.2:1.0. The SMR per state showed that the states with the highest rates were Yucatan at 149.96 (95%CI: 142.64-157.29), Sonora at 144.67 (95%CI: 138.55-150.80), Zacatecas at 135.95 (95%CI: 128.79-143.10) and Michoacan at 135.57 (95%CI: 131.03-139.71), while the states with the lowest SMR values were Quintana Roo at 56.02 (95%CI: 47.95-64.09); Estado de Mexico at 57.57 (95%CI: 56.05-59.10) and Guerrero at 73.64 (95%CI: 70.00-77.28). For females, the highest index of potential years of life lost (IPYLL) was found in Chiapas at 192.52 (95%CI: 189.3-195.7), Oaxaca at 155.48 (95%CI: 152.8-158.2) and Yucatan at 130.01 (95%CI: 126.6-133.4), while the lowest IPYLL was found in the states of Durango at 64.06 (95%CI: 61.6-66.5), Sinaloa at 69.11 (95%CI: 67.1-71.1) and Nuevo Leon at 71.00 (95%CI: 69.3-72.6)^[5]. For males, the highest IPYLL was in Chiapas at 169.51 (95%CI: 166.8-172.2), Sonora at 159.02 (95%CI: 156.1-162.0) and Chihuahua at 125.74 (95%CI: 123.4-128.1), while the lowest IPYLL were in the states of Quintana Roo at 73.19 (95%CI: 68.7-71.7), Estado de Mexico at 77.05 (95%CI: 76.2-77.9) and Guerrero at 82.48 (95%CI: 80.6-84.4)^[5]; and (3) Tovar-Guzmán *et al*^[6] (2008), who state that over the period 1980 to 2004 cervical-uterine cancer had a crude mortality rate of 20.2 in 1980, 24.2 in 1989 and 14.4 in 2004 per 10000 women of age 25 years and over. The age-adjusted mortality rate was 12.8 in 1980, 15.6 in 1988 and 8.8 in 2004 per 100000 women of age 25 years and over. The highest SMR values were found in the states of Colima at 164.6 (95%CI: 153.3-175.8), Nayarit at 151.2 (95%CI: 143.4-159.0) and Yucatan at 150.6 (95%CI: 144.7-156.5), while the lowest values were detected in Estado de Mexico at 59.8 (95%CI: 58.6-61.0), Distrito Federal at 68.3 (95%CI: 66.9-69.7) and Nuevo Leon at 71.9 (95%CI: 69.2-74.6)^[6]. The IPYLL due to cervical-uterine cancer during this period ranged from 168.8 (95%CI: 156.0-181.5) in Colima, 154.4 (95%CI: 146.9-161.9) in Tabasco and 149.9 (95%CI: 141.3-158.4) in Nayarit, to 61.6 (95%CI: 60.2-63.0) in Distrito Federal, 64.9 (95%CI: 63.5-66.3) in Estado de Mexico and 68.4 (95%CI: 65.5-66.3) in Nuevo Leon^[6].

Gastric cancer in Mexico

In Mexico, despite the fact that gastric cancer represents the third highest cause of death by cancer in people of age 20 years or more^[2], and is a disease that is subject to epidemiological surveillance^[7], no specific program exists for its prevention, nor is there an official Mexican standard (Norma Oficial Mexicana) for its prevention, detection, treatment and control. An official clinical practice guide was only published in 2009 for the diagnosis and treatment of gastric adenocarcinoma in adult patients^[8].

It is important to highlight that, in terms of biologi-

cal behavior and epidemiology, gastric cancers constitute a highly heterogeneous group of tumors, a fact that is likely to cause difficulty for the prediction of patient outcome using classifications. Perhaps the best-known classification for gastric cancer is the system of Lauren, which distinguishes two groups of tumors: intestinal and diffuse. The intestinal type typically presents cohesive neoplastic cells that form gland-like tubular structures, and a defined pattern of histological changes in healthy gastric mucosa^[9]. In the diffuse type, there is no neoplastic cell cohesion, so cells infiltrate and thicken the stomach wall without the formation of a discrete mass^[9]. The intestinal type is normally diagnosed in older people and its development depends on environmental factors^[9]. In contrast, the diffuse type usually occurs in young people, and is associated with individual factors^[9]. No specific histological type of gastric cancer predominates in Mexico, according to the Lauren classification^[10], and gastric cancer exhibits different behavior in patients under 30 years of age. Nevertheless, delays in diagnosis and behavior of the tumor are the most important factors in prognosis^[11].

Gastric cancer is one of the main causes of hospital morbidity in Mexican males; the highest rate is found in the population of 75-79 years of age (47 per 100000 males in this age group), followed by the population of 65 to 74 years of age (38 per 100000)^[2]. The most recent data, produced by the now defunct Histopathological Register of Malignant Neoplasms (RHNM, by its Spanish acronym), reported that gastric cancer constituted 3% of cancer cases diagnosed in Mexico during the year 2000, with three cases recorded per 100000 inhabitants^[12].

The high mortality^[5,13], low survival and the considerable deterioration in life quality of the people suffering this disease, mean that gastric cancer represents a public health problem in Mexico that requires research aimed at proposing health interventions. In theory, prevention strategies could be effective, given the following factors: (1) prolonged latency period during which intervention should be possible^[14]; (2) infection with *Helicobacter pylori* (*H. pylori*), which commonly begins in infancy or early childhood and persists as a chronic gastritis, is a principal cause of gastric cancer^[14]; while chronic infection with *H. pylori* is a major force behind the precancerous process, *H. pylori* eradication only produces a modest retardation of the precancerous process^[14]; and (3) antioxidant micronutrients may play an etiological role^[14]. While there have been no studies on the incidence of environmental and inherited gene defects in gastric cancer in Mexico, it is clear that potentially modifiable factors associated with the development of the disease can play an important role in its prevention. According to Anand *et al.*^[3], only 5%-10% of all cancers are caused by an inherited gene defect; although all cancers are a result of multiple mutations, these mutations are the result of interaction with the environment. In terms of population attributable risk in gastric cancer, a study conducted in Italy indicated that approximately 8% of stomach cancers could be related to this familial component^[15]. Most cancers are not heredi-

tary in origin and potentially modifiable factors, such the consumption of alcohol and tobacco, diet and infections, can have an important effect on their development^[3].

Given the relevance of gastric cancer to public health in Mexico, this study aimed to review those studies that have been conducted on Mexican patients with a diagnosis of gastric cancer, and/or associated diseases, in which at least one Mexican institute has participated, and that have generated knowledge of utility to the primary and secondary prevention of the disease. In this context, it is important to highlight that the scientific, technological and commercial sectors in Mexico have been tasked with researching "Malignant neoplasms in Children and Adults" with the support of the Sectoral Fund for Research in Health and Social Security (Fondo Sectorial de Investigación en Salud y Seguridad Social, SSA/IMSS/ISSSTE). This was done with the aim of reducing the morbidity, mortality and most prevalent complications among the susceptible population, as well as to improve the life quality of cancer patients and reduce the costs of their care^[16]. The products expected from this priority line of research include effective strategies of prevention, procedures for early diagnosis, new schemes of treatment; strategies to reduce complications and mortality or improve life quality and proposals for molecular markers^[16]. These research funds are administered by the National Council of Science and Technology (Consejo Nacional de Ciencia y Tecnología, CONACyT)^[17]. The National Health Institutes, a group of twelve institutions belonging to the Ministry of Health (Secretaría de Salud) that conduct scientific research in the field of health, and specifically the National Cancerology Institute (Instituto Nacional de Cancerología, INCan), has the task of developing excellent medical care, teaching and oncological research in Mexico^[18].

RESEARCH METHODS

Search strategy

The Medline database was searched on the 21st of August 2013, using the following combinations of key words: (1) gastric cancer, Mexico; and (2) stomach cancer, Mexico. English and Spanish language was selected as a limit. A total of 148 articles were obtained: 111 in English and 37 in Spanish.

Inclusion and exclusion criteria

The abstract of each article was carefully revised to verify the following criteria: (1) inclusion criteria: the study must have involved at least 10 Mexican gastric cancer patients, with associated pathologies or precursor lesions of gastric cancer, that were in Mexico at the time of the study; at least one of the authors of the study had to be ascribed to a Mexican institute; the studies had to have findings that could support the proposal of public policy directed towards the primary or secondary prevention of gastric cancer. The objectives of prevention were considered as follows: primary, avoiding the occurrence

Table 1 Epidemiological studies of gastric cancer in Mexico

Ref.	Year	Institute of adscription of corresponding author-city	Period of study	Main finding	Source
[23]	2013	IMSS Mexico City	NA	There is no association between altitude and the incidence and mortality of gastric cancer	Epidemiological observations
[20]	2012	UV Veracruz, Veracruz	2005-2009	From a total of 1803 cases of digestive tract cancers, gastric cancer was the second most common, with 302 cases (16.76%)	Hospital registries from 5 institutions of Veracruz state
[22]	2012	INCan Mexico City	1993-2002	From a total of 767464 cases of digestive system cancers, gastric cancer was the sixth most common with 27659 cases (4%); the third most common in males and seventh in females	Data-base of the histopathological register of malignant neoplasms in Mexico (RHNM)
[21]	2003	INCMNSZ Mexico City	1978-2001	A total of 90% of the cases were diagnosed in people of age 41 years and more From a total of 11276 cases of digestive system cancers, 3830 (34%) were of gastric cancer	Hospital registries from 6 institutions of Mexico City
[5]	2001	INSP Cuernavaca, Morelos	1980-1997	Increase in adjusted mortality rate Gender-based differential trend in the magnitude and prematurity of mortality	INEGI

H. pylori: *Helicobacter pylori*; NA: Non applicable; IMSS: Mexican Institute of Social Security/Instituto Mexicano del Seguro Social; UV: University of Veracruz/Universidad Veracruzana; INCan: National Institute of Cancerology/Instituto Nacional de Cancerología; RHNM: Histopathological Register of Malignant Neoplasms/Registro Histopatológico de Neoplasias Malignas; INCMNSZ: National Institute of Medical Science and Nutrition Salvador Zubiran/Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán; INSP: National Institute of Public Health/Instituto Nacional de Salud Pública; INEGI: National Institute of Statistics and Geography/Instituto Nacional de Estadística y Geografía.

of the disease and secondary, detection of the disease at early stages, before presentation of symptoms; and (2) exclusion criteria: literature reviews; case studies; studies of tertiary prevention (considered as the reduction of incapacitation and restoration of patient functionality) of gastric cancer, diagnostic tests, treatment or detection in the environment of *H. pylori*; basic science *in vitro* or *in vivo* models in which the authors omitted recommendations for the primary or secondary prevention of gastric cancer; studies that did not specify the number of gastric cancer cases or patients with preneoplastic lesions; and studies of epidemiological description in a medical unit.

Applying these criteria, 100 studies were discarded and 48 were reviewed. A further 35 studies were incorporated into the introduction and conclusions sections of this review.

GASTRIC CANCER RESEARCH IN MEXICO

While support exists in Mexico for research into strategies of prevention, diagnosis and control of cancer^[16], and gastric cancer is a public health problem because of its high mortality and high percentage of late-stage detection^[2,5,13,19], there are few studies that provide results to support the development of public policies directed to the prevention and control of this disease. The paucity of research into gastric cancer of any form is reflected in the fact that in the official clinical practice guide for diagnosis and treatment of gastric adenocarcinoma in adult patients^[8], produced only five years ago by the Ministry of Health, only two of the 33 references correspond to studies conducted on Mexican patients and in Mexican institutes.

The trend of mortality in cancer, including gastric cancer, has remained relatively stable in Mexico for at least 40 years^[2,5,13]. In this context, it is notable that of the total number of publications found in the Medline database with the key words stomach/gastric cancer and Mexico, 73% (108/148) were published after the year 2000. Publications from the 1970s and 1980s are practically non-existent.

Nonetheless, data generated by the National Institute of Statistics and Geography (Instituto Nacional de Estadística y Geografía, INEGI) has made a considerable contribution to understanding trends in gastric cancer mortality in Mexico. Epidemiological studies of gastric cancer are scarce in Mexico; to the best of our knowledge, only one study has presented indicators with which to prioritize public health problems since 2001^[5]. Other studies analyzed hospital registers^[20,21], a now defunct official database^[22] or investigated the possible relationship between altitude and the risk of development of the disease^[23], given that altitude has been reported as a factor associated with gastric cancer in other Latin American countries (Table 1).

The natural history of gastric cancer includes a long period of latency^[14]: the latency period for alcohol consumption for the development of gastric cancer has been estimated theoretically at 20 years^[24], during which intervention is possible. Research into strategies for screening subjects that are or have been exposed to risk factors that increase the probability of developing gastric cancer is therefore of great importance. Among these risk factors, the potentially modifiable factors related to lifestyle (dietary habits, smoking, and alcohol consumption) present a great window of opportunity in terms of primary prevention of the disease.

The studies found in the Medline database relating to

Table 2 Studies of risk and protection factors in gastric cancer in the Mexican population

Ref.	Year	Institute of adscription of corresponding author-city	Main finding	Quantity and type of groups studied
[25]	2012	UV Xalapa, Veracruz	Protective effect against gastric cancer: use of mouthwash, refrigeration of food and regular consumption of fruit and vegetables	49 gastric cancer 162 controls
[27]	2012	INSP Cuernavaca, Morelos	Risk of gastric cancer: omission of breakfast and failure to refrigerate food Risk of gastric cancer: moderate to high capsaicin consumption synergistically in genetically susceptible individuals (IL-1B-31C allele carriers) infected with more virulent <i>H. pylori</i> (CagA positive) strains	158 gastric cancer 317 controls
[28]	2009	INSP Cuernavaca, Morelos	Protective effect against gastric cancer: higher intake of cinnamic acids, secoisolariciresinol and coumestrol. Main sources of these molecules: pears, mangos, beans, carrots, squash and legumes	257 gastric cancer 478 controls
[29]	2003	INSP Cuernavaca, Morelos	Risk of gastric cancer: high consumption of capsaicin (90-250 mg of capsaicin per day, 9-25 jalapeno peppers per day), compared to low-level consumption (0-29.9 mg of capsaicin per day, 0-3 jalapeno peppers per day); this effect is independent of <i>H. pylori</i> status	234 gastric cancer 468 controls
[30]	1999	INSP Cuernavaca, Morelos	Protective effect against gastric cancer: intake of polyunsaturated fat, fiber and vitamin E, independent of the histological type of the tumor (intestinal or diffuse) Risk of gastric cancer: consumption of saturated fat and cholesterol	220 gastric cancer 752 controls
[26]	1999	NCI ¹ Bethesda, MD, United States	Protective effect against gastric cancer: intake of yellow and orange vegetables. Risk of gastric cancer: consumption of fresh and processed meat, dairy products, fish and salty snacks	220 gastric cancer 752 controls
[31]	1998	INSP Cuernavaca, Morelos	No association with risk of gastric cancer: consumption of foods prepared with corn, wheat or rice	220 gastric cancer 752 controls
[32]	1998	INSP Cuernavaca, Morelos	Risk of gastric cancer: wine consumption at least 10 glasses per month. No association with risk for gastric cancer: consumption of beer and distilled alcoholic beverages including brandy, rum and tequila	220 gastric cancer 752 controls
[33]	1994	INSP Cuernavaca, Morelos	Potential risk of gastric cancer: chili pepper consumption	220 gastric cancer 752 controls

¹In collaboration with the INSP. *H. pylori*: *Helicobacter pylori*; UV: University of Veracruz/Universidad Veracruzana; INSP: National Institute of Public Health/Instituto Nacional de Salud Pública; NCI: National Cancer Institute.

factors associated with the development of gastric cancer in the Mexican population have practically all been conducted in just two national institutions: the University of Veracruz (Universidad Veracruzana, UV)^[25] and the National Institute of Public Health (Instituto Nacional de Salud Pública, INSP). This latter institute has, on certain occasions, benefited from international collaboration^[26-33]. Among the factors associated with the development of gastric cancer in Mexico, those which stand out as presenting the highest risk are omission of breakfast^[25] and the high consumption of capsaicin^[27], saturated fat^[30], cholesterol^[30], and fresh^[26] and processed meat^[25,26]. Factors that protect against the development of the disease are: high consumption of fruit^[25,28] and vegetables^[28] (Table 2).

In 1984, Marshall and Warren discovered the etiological role of *H. pylori* in gastritis and peptic ulcers, for which they received the Nobel Prize in 2005^[34]. Infection by *H. pylori* is mainly acquired in infancy via fecal-oral and oral-oral pathways, and it has been estimated that 50% of the world's population could be infected with this bacterium, increasing to 80% of the population within some developing countries^[34].

H. pylori produces gastritis in almost all infected individuals, with a minority progressing towards chronic atrophic gastritis^[35]. Gastritis is an inflammation of the gastric mucosa, which does not imply serious complications^[34]; in contrast, chronic atrophic gastritis is characterized by a loss of the parietal and principal cells that drives a reduction in the secretion of peptic acid and increases the risk of developing gastric cancer^[34].

The progression, severity and consequences of infection by *H. pylori* depend on an interaction of multiple factors. Those relating to the host include genetic background or physiological and immunological state. Factors relating to the bacteria include bacterial genomic plasticity, capacity for adaptation to the individual conditions of the host, modulation of the reaction to the host immune system response and production of various virulence factors, such as vacuolating cytotoxin and the cytotoxin-associated antigen A (CagA)^[36].

The study of the relationship between *H. pylori* and the development of gastric cancer is without doubt a complex process in Mexico, for many reasons: (1) the diversity of reported strains^[37,38]; (2) association with modifiable factors^[39]; (3) host effect in progression of the

Table 3 Studies of *Helicobacter pylori* in pathologies associated with the development of gastric cancer and gastric cancer in a Mexican population

Ref.	Year	Institute of adscription of corresponding author-city	Main finding	Quantity and type of groups studied
[39]	2013	ISSSTE Culiacan, Sinaloa	Association between alcohol consumption and <i>H. pylori</i> infection. No relationship between <i>H. pylori</i> and smoking and coffee consumption	269 <i>H. pylori</i> positive 269 <i>H. pylori</i> negative
[46]	2013	IMSS Mexico City	Association between <i>H. pylori</i> and p53 expression and between p53 and intestinal metaplasia	104 patients with no evidence of acute or clinically significant gastric pathology
[41]	2013	INSP Cuernavaca, Morelos	IgG2 response to CagA could be used as a novel serological marker to identify patients with <i>H. pylori</i> -associated gastric cancer	46 intestinal metaplasia 41 gastric cancer 50 controls
[47]	2013	INSP Cuernavaca, Morelos	No association between CagA and gastric cancer	67 gastric cancer 368 non atrophic gastritis 124 preneoplastic lesion
[48]	2012	UNAM Mexico City	Correlation of antibody subclass titers with Th1/Th2 markers may aid pathology characterization and diagnosis	14 gastric cancer 5 peptic ulcer 13 bleeding peptic ulcer 12 dyspepsia
[49]	2012	IMSS Mexico City	Failure to express cag19 and cag24 <i>in vivo</i> in precancerous lesions might serve as a biomarker of the risk of development of gastric cancer	11 gastric cancer 10 non atrophic gastritis 10 duodenal ulcer
[40]	2011	INSP Cuernavaca, Morelos	Vac-A neutralizing antibodies might serve as a biomarker of the risk of development of gastric cancer and duodenal ulcer	90 intestinal metaplasia 60 gastric cancer 52 duodenal ulcer 145 non atrophic gastritis
[43]	2009	UNAM Tlalnepantla, Estado de Mexico	Patients with chronic gastritis had a high incidence of infection by <i>H. pylori</i> ; 44% of the <i>H. pylori</i> strains may be considered as highly virulent since they possessed two or three of the virulence markers analyzed: vacA s1 cagA babA2	238 chronic gastritis
[50]	2009	IMSS Mexico City	30 genes are significantly associated with non-atrophic gastritis, duodenal ulcer, or gastric cancer and may serve as risk biomarkers	10 non atrophic gastritis 10 duodenal ulcer 9 gastric cancer
[51]	2008	UNAM Mexico City	<i>H. pylori</i> is uniformly distributed across the stomach in dyspepsia and has preference for fundus and corpus in gastric cancer. <i>H. pylori</i> genotype diversity across the systematic whole-organ and tumor is remarkable. There is insufficient evidence to support the association of one isolate with a specific disease, due to the multistrain nature of <i>H. pylori</i>	16 gastric cancer 14 dyspepsia
[38]	2008	INSP Cuernavaca, Morelos	<i>H. pylori</i> infection and CagA are risk markers for intestinal metaplasia. In gastric cancer, prevalence of these risk markers decreases, probably reflecting the fact that infection reduces when advanced atrophy and metaplasia develops	368 non atrophic gastritis 126 precancerous lesions 65 gastric cancer 59 duodenal ulcer
[52]	2004	UANL Nuevo Leon, Nuevo Leon	Absence of the HLA-DQA1*0503 allele could be a host risk factor for the development of gastric cancer. Infection with <i>H. pylori</i> CagA ⁺ , VacA ⁺ strains represents a significant risk in terms of the development of gastric cancer	22 gastric cancer <i>H. pylori</i> positive 8 high grade dysplasia <i>H. pylori</i> positive 77 matched controls <i>H. pylori</i> positive
[37]	2004	INSP Cuernavaca, Morelos	There is no association between nitrite and ascorbic consumption or interactions of these nutrients with seropositivity to <i>H. pylori</i> CagA ⁺ . Seropositivity to <i>H. pylori</i> CagA ⁺ strains may be an independent factor in diffuse gastric cancer	211 gastric cancer 454 controls
[53]	2001	SU ¹ California, United States	In regions with a high prevalence of chronic atrophic gastritis, serological screening with CagA alone is an effective test for identifying eligible subjects	178 <i>H. pylori</i> positive 155 <i>H. pylori</i> CagA ⁺
[45]	1997	INSP Cuernavaca, Morelos	<i>H. pylori</i> infection present in 87.2% of cases and 82.5% of controls	109 gastric cancer 177 controls
[44]	1993	INCan Mexico City	In a high-risk population, precursor lesions for adenocarcinoma are universally associated with <i>H. pylori</i> infection	245 symptomatic patients

¹In collaboration with INCan, UNAM Mexico City and the Colegio de la Frontera Sur, San Cristobal de las Casas, Chiapas. *H. pylori*: *Helicobacter pylori*; ISSSTE: Institute of Social Security and Services of State Employees/Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado; IMSS: Mexican Institute of Social Security/Instituto Mexicano del Seguro Social; INSP: National Institute of Public Health/Instituto Nacional de Salud Pública; EBV: Epstein-Barr virus; UNAM: National Autonomous University of Mexico/Universidad Nacional Autónoma de México; UANL: Autonomous University of Nuevo Leon/Universidad Autónoma de Nuevo León; SU: Stanford University; INCan: National Institute of Cancerology/Instituto Nacional de Cancerología.

Table 4 Studies of the Epstein-Barr virus in pathologies associated with the development of gastric cancer and gastric cancer in a Mexican population

Ref.	Year	Institute of adscription of corresponding author-city	Main finding	Quantity and type of groups studied
[54]	2013	IMSS Mexico City	Co-infection with EBV and <i>H. pylori</i> in pediatric patients is associated with severe gastritis	333 pediatric patients with chronic abdominal pain
[56]	2005	INCan Mexico City	EBV was detected in 7.3% of cases, all pertaining to patients > 50 years of age. Among Latin-American countries, Mexico has the lowest frequency of EBV associated gastric carcinoma	330 gastric cancer
[55]	1999	INCan Mexico City	EBV is detected in 8.15% cases, six occur in males and five in females	135 gastric cancer

H. pylori: *Helicobacter pylori*; IMSS: Mexican Institute of Social Security/Instituto Mexicano del Seguro Social; INCan: National Institute of Cancerology/Instituto Nacional de Cancerología; EBV: Epstein-Barr virus.

infection^[40,41]; (4) contrasting socioeconomic, sanitary and climatological conditions of the country, which could affect the presence of the bacterium in the environment^[42]; and (5) the differential occurrence of bacterial strains in diseases associated with the development of gastric cancer^[43], precancerous lesions^[38,44] and gastric cancer^[38]. Of the studies selected according to the criteria used in this review, 16/48 (33%) focused on the relationship between *H. pylori* and the development of gastric cancer, and have made a considerable contribution towards the understanding of this complex phenomenon (Table 3).

Contributions made by studies conducted in Mexico that could support the design of strategies for the prevention and control of gastric cancer (Table 3) include the knowledge that, in regions with a high prevalence of chronic atrophic gastritis, serological screening with CagA is an effective test for identifying eligible subjects^[45] and, in high-risk populations, precursor lesions for gastric cancer are universally associated with *H. pylori* infection^[44].

The role of the Epstein-Barr virus (EBV) in relation to the development of gastric cancer in Mexico has been little studied; however, pediatric patients co-infected with EBV and *H. pylori* produce more severe clinical charts^[54] and its incidence in gastric cancer is low^[55,56] (Table 4).

International studies have suggested certain molecules as markers in gastric cancer: some of these findings have been confirmed in Mexico, for example, adhesion molecules, such as E-cadherin^[57]; tumor suppressor genes, for example p53^[58]; extracellular matrix remodeling genes; matrix metalloproteinases (MMPs), such as MMP-9^[59]; inflammatory molecules, TNF^[60] and IL-8^[61]; cell growth factors and their receptors, such as human epidermal growth factor receptor 2^[62]; and enzymes that participate in the metabolism of the methyl groups, such as methylenetetrahydrofolate reductase^[63] (Table 5). However, most of these molecules have failed to become popular as prognostic tools in gastric cancer, probably because of limitations in their reliability, sensitivity and specificity. However, these are problems that could be solved by adopting methods to optimize reproducibility: avoiding sampling variability, increasing the sample size of tumors,

extending the number of genes analyzed and creating partnership platforms to study multicenter trials^[64], as well as following international recommendations in relation to the design and execution of studies^[65,66].

DISCUSSION

As part of the Mexican Ministry of Health, the National Council for the Prevention and Treatment of Cancer in Infancy and Adolescence (Consejo Nacional para la Prevención y el Tratamiento del Cáncer en la Infancia y la Adolescencia) directs actions for the prevention of cancer in people below 18 years of age^[75]. However, a specific program for gastric cancer is required, based on national health indicators and featuring a consensus for the timely detection of the disease. Experience in countries with a high incidence of gastric cancer, such as China and Japan, has shown that mass screening of the asymptomatic population using endoscopy and actions of vigilance in higher risk subjects have been cost-effective strategies: they have been able to detect between 50% and 80% of cases in the early stages^[76]. Thus, individuals identified as being at highest risk can be monitored endoscopically to detect dysplasia and early cancer^[14]. In countries where the incidence of gastric cancer is not so high, for example the United States and Canada, mass screening with endoscopy is not recommended: analysis of cost-effectiveness shows no justification for the application these programs^[76].

In Mexico, no studies have been conducted on the prevalence of gastric cancer in each stage of the disease. One retrospective cohort study conducted in the INCan in Mexico City in 2001 reported that, in a set of 834 patients with gastric cancer, only 21 (2.5%) were diagnosed in the early stages of the disease^[77]. It is important to clarify that these data relating to the incidence of early stage gastric cancer came from a reference hospital, for which reason they should not be taken to reflect the national trend. To elucidate trends in gastric cancer per stage in Mexico, implementation of a system of epidemiological vigilance is necessary at each different level of care. Data

Table 5 Studies of molecular markers for the development of gastric cancer and gastric cancer in a Mexican population

Ref.	Year	Institute of adscription of corresponding author-city	Main finding	Quantity and type of groups studied
[67]	2013	UG Guadalajara, Jalisco	EGFR-R521K and ERBB2-1655V polymorphisms are not suitable as markers for identifying individuals at risk of developing gastric cancer	155 gastric cancer 121 controls 103 general population
[62]	2013	INCMSZ Mexico City	HER2 amplification is restricted to intestinal gastric cancer.	269 gastric cancer
[59]	2010	UV Xalapa, Veracruz	HER2 amplification is suitable as a marker for screening gastric cancer histotype MMP9 expression is enhanced in gastric cancer compared to normal mucosa, and has potential as a molecular marker	6 gastric cancer 11 superficial gastritis
[68]	2010	UNAM Mexico City	Claudin 6, 7, and 9 expression is related to gastric carcinogenesis, and detection of these is a useful prognostic marker in intestinal and diffuse gastric cancer	70 gastric cancer
[60]	2010	IMSS Mexico City	Polymorphisms in TNF and HSP70 have a severity dose-response as risk markers from preneoplastic lesions to gastric cancer, probably because of their association with an intense and sustained inflammatory response	228 non atrophic gastritis 98 intestinal metaplasia 63 gastric cancer 58 duodenal ulcer 132 controls
[63]	2009	INSP Cuernavaca, Morelos	In subjects with high consumption of folate, choline and vitamin B6, and 5,10-methyle netetrahydrofolate reductase (MTHFR) 677 TT genotype, there is a reduction in diffuse gastric risk compared to MTHFR 677 CC + CT carriers. In subjects with low consumption of methionine and MTHFR 677 TT genotype, there is a reduced risk of diffuse gastric cancer compared to MTHFR 677 CC + CT carriers. Carriers of the MTHFR 677 TT genotype with a low consumption of folate have a significantly increased risk of development of intestinal gastric cancer	248 gastric cancer 478 controls
[69]	2007	UANL Monterrey, Nuevo Leon	There is no association between the MTHFR C677T polymorphism and development of gastric cancer	51 gastric cancer 83 controls
[57]	2007	INCMSZ Mexico City	The -160 C/A polymorphism of E-cadherin has a direct effect on the risk of diffuse gastric cancer at a young age	39 gastric cancer younger than 45 years of age 78 controls
[61]	2007	UANL Monterrey, Nuevo Leon	The IL-8-251*A allele could be related to the development of gastric cancer	78 gastric cancer 259 controls
[70]	2006	INSP Cuernavaca, Morelos	High prevalence of MTHFR 677T allele may be a contributor to the high rate of morbidity and mortality in gastric cancer	201 gastric cancer 427 controls
[71]	2006	LSU ¹ New Orleans, United States	Identification of the IL-1B-31 promoter polymorphism is a useful marker for the risk of intestinal type gastric cancer in subjects with CagA ⁺ <i>H. pylori</i> infection	183 gastric cancer 377 controls
[58]	2005	NYU2 New York, United States	Carrying the Arg/Arg genotype in the codon 72 exon 4 of p53 is associated with risk of development of gastric cancer	65 gastric cancer 182 controls
[72]	2005	UANL Monterrey, Nuevo Leon	Carrying the proinflammatory IL-1B-31*C allele is associated with increased risk of gastric cancer	63 gastric cancer 215 controls
[73]	2004	INCan Mexico City	There is an association of major histocompatibility complex HLA-DQA1*0601 and HLA-DQB1*0501 alleles in gastric cancer compared to chronic gastritis and the healthy condition. These HLA-DQ alleles may be conferring susceptibility for the development of gastric cancer	20 gastric cancer 40 <i>H. pylori</i> -associated chronic gastritis 90 controls
[74]	2003	UANL Monterrey, Nuevo Leon	Carrying the pro-inflammatory IL-1B-31*C allele is associated with an increased risk of gastric cancer and high-grade dysplasia	33 gastric cancer 8 high-grade dysplasia 25 controls

¹In collaboration with INSP; ²In collaboration with UANL. *H. pylori*: *Helicobacter pylori*; UG: University of Guadalajara/Universidad de Guadalajara; INCMSZ: National Institute of Medical Science and Nutrition Salvador Zubiran/Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán; UV: University of Veracruz/Universidad Veracruzana; UNAM: National Autonomous University of Mexico/Universidad Nacional Autónoma de México; IMSS: Mexican Institute of Social Security/Instituto Mexicano del Seguro Social; INSP: National Institute of Public Health/Instituto Nacional de Salud Pública; UANL: Autonomous University of Nuevo Leon/Universidad Autónoma de Nuevo León; LSU: Louisiana State University; NYU: New York University; INCan: National Institute of Cancerology/Instituto Nacional de Cancerología.

from such a system would generate indicators permitting the design of programs of prevention and control. In terms of gastric cancer prevention in Mexico, it should be considered that, in regions of high-prevalence of chronic atrophic gastritis, serological screening with

CagA is an effective test for identifying eligible subjects^[45] and that, in high-risk populations, precursor lesions for gastric cancer are universally associated with *H. pylori* infection^[44]. Moreover, the scientific evidence provided by randomized trials in China^[78] and Mexico^[79] shows that,

while curing *H. pylori* infection produces a modest deceleration of the precancerous process, it does not prove that eradication of *H. pylori* decreases cancer risk^[14]. Understanding the modifiable factors associated with gastric cancer in the local population is also of great importance in terms of prevention of the disease (Table 2).

One window of opportunity in Mexico could be conducting studies in which questionnaires are used to identify risk profiles in specific groups of the population. This could be done with the aim of monitoring more closely those people that have an elevated risk of developing gastric cancer. In this context, the Gail model for breast cancer in the United States^[80] and the model of oral cancer risk factors in rural Sri Lanka^[81] indicate the utility of this type of strategy, because it allows the relatively simple and cost-effective identification of people with a high risk of developing cancer, who can then be subjected to special control^[80,81]. In China, good results have been obtained from the combined application of a questionnaire regarding risk factors for colorectal cancer and an immunochemical fecal occult blood test to identify subjects at risk of suffering cancer^[82]. In Mexico, a risk model for gastric cancer would be difficult to establish because of the wide variety of factors associated with its development and to the broad diversity of socio-cultural, climatological and dietary conditions that exist in the country. Another challenge would be the validation of such a model, because it implies a prolonged period of monitoring of a large cohort of subjects, who would have to submit to invasive study by endoscopy. The creation of research networks is necessary within Mexico, which should include the health sector and the academic community, to approach this health problem with a multidisciplinary focus and propose actions for its prevention and control within a national context.

The few studies of gastric cancer in the Mexican population included in this review reveal little or no linkage between the scientific community and the health sector to resolve this health problem. Public policies in health research should direct initiatives for the formation of research networks that include experts from different disciplines. Such networks could generate, among other academic products, an official Mexican standard (*Norma oficial Mexicana*) for the prevention, detection, treatment and control of gastric cancer. This review should serve as a guide to identify the national research groups interested in the study of gastric cancer in the Mexican population.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Role of gene polymorphisms in gastric cancer and its precursor lesions: Current knowledge and perspectives in Latin American countries

Miguel Angel Chiurillo

Miguel Angel Chiurillo, Laboratorio de Genética Molecular “Dr. Jorge Yunis-Turbay”, Decanato de Ciencias de la Salud, Universidad Centroccidental Lisandro Alvarado, Barquisimeto 3001, Venezuela

Author contributions: Chiurillo MA designed and wrote the review.

Correspondence to: Miguel Angel Chiurillo, MD, PhD, Laboratorio de Genética Molecular “Dr. Jorge Yunis-Turbay”, Decanato de Ciencias de la Salud, Universidad Centroccidental Lisandro Alvarado, Barquisimeto 3001, Estado Lara, Venezuela. mchiurillo@ucla.edu.ve

Telephone: +58-251-2591985 Fax: +58-251-2591804

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Abstract

Latin America shows one of the highest incidence rates of gastric cancer in the world, with variations in mortality rates among nations or even within countries belonging to this region. Gastric cancer is the result of a multifactorial complex process, for which a multistep model of carcinogenesis is currently accepted. Additionally to the infection with *Helicobacter pylori*, that plays a major role, environmental factors as well as genetic susceptibility factors are significant players at different stages in the gastric cancer process. The differences in population origin, demographic structure, socio-economic development, and the impact of globalization lifestyles experienced in Latin America in the last decades, all together offer opportunities for studying in this context the influence of genetic polymorphisms in the susceptibility to gastric cancer. The aim of this article is to discuss current trends on gastric cancer in Latin American countries and to review the available published information about studies of association of gene polymorphisms involved in gastric cancer susceptibility from this region of

the world. A total of 40 genes or genomic regions and 69 genetic variants, 58% representing markers involved in inflammatory response, have been used in a number of studies in which predominates a low number of individuals (cases and controls) included. Polymorphisms of *IL-1B* (-511 C/T, 14 studies; -31 T/C, 10 studies) and *IL-1RN* (variable number of tandem repeats, 17 studies) are the most represented ones in the reviewed studies. Other genetic variants recently evaluated in large meta-analyses and associated with gastric cancer risk were also analyzed in a few studies [*e.g.*, prostate stem cell antigen (*PSCA*), *CDH1*, *Survivin*]. Further and better analysis centered in gene polymorphisms linked to other covariates, epidemiological studies and the information provided by meta-analyses and genome-wide association studies should help to improve our understanding of gastric cancer etiology in order to develop appropriate health programs in Latin America.

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Key words: Latin America; Gastric cancer; Precancerous lesions; Gene polymorphisms; Single nucleotide polymorphisms

Core tip: This article is a review about the current state of art of studies carried out in Latin America using gene polymorphisms to assess gastric cancer susceptibility. Latin America shows one of the highest incidence rates of gastric cancer in the world, with variations in mortality rates among nations or even within countries belonging to this region. Moreover, Latin America is a region with a particular genetic background, high rates of *Helicobacter pylori* infection and lifestyles condition. This review also gives special emphasis on the importance of the studies conducted in gastric precancerous diseases.

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EPIDEMIOLOGY OF GASTRIC CANCER IN LATIN AMERICA

Gastric cancer is one of the most lethal types of cancer, accounting in 2008 for about 800000 deaths, but its incidence varies substantially worldwide^[1]. There were approximately 870000 noncardia gastric cancer cases and 74.7% of them have been attributed to *Helicobacter pylori* (*H. pylori*) infection^[1]. Although the rates of gastric cancer have been declining over the past 50 years in most Western countries, gastric cancer is still the fourth most common malignancy and the second leading cause of death due to cancer worldwide. The highest incidence (more than two-thirds) of gastric cancer is observed in East Asia, Eastern Europe, and the Andean region of South America, while North America, Northern Europe and North and East Africa show the lowest recorded rates^[1].

Latin American countries display some of the highest mortality rates worldwide. For males the estimates age-standardized mortality rates (ASMR) are led by Honduras (25.9%), Ecuador (24.1%), Costa Rica (23.6%), Chile (23.1%) and Guatemala (22.3%), while for women the highest rates are found in Guatemala (22.0%), Honduras (19.0%), Ecuador (17.5%), Peru (17.1%) and Costa Rica (10.6%). In contrast, lower ASMR are observed for both sexes in Puerto Rico, Cuba, Dominican Republic, Mexico and Argentina^[1,2].

Significant variations in the incidence of gastric cancer have been observed between different ethnic groups living in the same region; for example, African-Americans, Hispanics and Native Americans are affected more than Caucasians in the United States^[3]. Moreover, in a comparison between Japanese migrants to the United States and Brazil, Japanese migrants to the United States show a significantly lower incidence rate than Japanese living in Japan, while Japanese migrants to Brazil show a similar rate to the latter group, suggesting that the geographical distribution of gastric cancer may not be solely attributable to ethnic differences^[4].

Recently, Torres *et al*^[5], with the apparent association between altitude and the incidence of gastric cancer in the countries of Western Latin America along the Pacific Rim, proposed that the altitude may be a surrogate for the clustering of host, bacterial, dietary, and environmental factors related to gastric cancer risk. The relation appears to be strongest in the mountainous regions of Central America and Andean South America, but it is absent in Chile, where risk is more strongly associated with the age of *H. pylori* acquisition and socio-economic

determinants. Among possible explanations for this association could be that the host genetic background, as well as *H. pylori* genotypes, may cluster more readily in certain isolated mountainous communities^[5].

CARCINOGENESIS: MULTISTEP MODEL

It has been proposed a multistep cascade model for the development of intestinal-type gastric adenocarcinoma, which consists of a progression from chronic superficial (non atrophic) gastritis, to chronic atrophic gastritis to intestinal metaplasia, and finally, gastric adenocarcinoma^[6]. This model hypothesizes the sequence of precancerous lesions as a dynamic process from an initial superficial inflammation caused by *H. pylori* infection to a fully malignant neoplasm of the stomach. Thus, the chronic infection of the gastric mucosa by the *H. pylori* is a major attributable risk factor of gastric cancer^[7].

More than 50% of the population worldwide is infected with *H. pylori* with a higher prevalence in developing countries and in groups with poor socio-economic conditions. The improvements in living conditions in developed countries has determined a declining in the prevalence of infection, while remaining high, about 80%, in the developing world. In Latin American countries it has been reported a prevalence of *H. pylori* infection ranging from 70% to 90%^[8]. However, less than 2% of *H. pylori* carriers develop gastric cancer^[9]. Moreover, the incidence of gastric cancer in areas of Africa and South Asia with high prevalence of *H. pylori* infections is much lower than in other countries^[10].

Consistent with the multifactorial pathogenesis, the observed differences in the clinical outcomes and gastric cancer prevalence worldwide may be due to environmental factors (mainly diet, smoking and alcohol use) often playing a dominant role. Moreover, the influence of host factors, especially those governing the severity of the immune response, is also relevant.

GENE POLYMORPHISMS, GASTRIC CANCER AND ETHNICITY

The identification and discrimination of host genetic variants influencing susceptibility in populations with high incidence of gastric cancer has been a major challenge. These genetic variants may modulate the effects of exposure to environmental factors by regulating multiple biological pathways during gastric carcinogenesis.

Common susceptibility genetic variants have been identified as significantly associated with gastric cancer risk by candidate-gene studies, such as inflammatory [interleukin (IL)-1 β , IL-8 and tumor necrosis factor- α (TNF- α)] and anti-inflammatory cytokines (IL-10), DNA repair genes and metabolic enzymes (such as the glutathione S-transferase family, cytochrome P450 superfamily, and metabolism of folate and arachidonic acid)^[11-20].

Moreover, recent genome-wide and large scale gene association studies have focused on analyzing regions of

the genome in which have been detected candidate genes involved in cell proliferation, differentiation, and survival, such as *MUC1*^[21], *PLCE1*^[22], *PTGER4*, *PRKAA1*, *ZBTB20*^[23,24], prostate stem cell antigen (*PSCA*)^[25,26], genes participating in EGFR and FAS-mediated signaling pathway^[27,28] and DNA repair pathways^[29]. Chromosome 9p21.3 and 10q23 regions have been identified as genetic susceptibility loci for multiple disease phenotypes including gastric cancer^[22,30].

The current understanding of host genetic polymorphisms and gastric cancer susceptibility is based largely on studies in Asians and Caucasians (from Europe and North America) populations. Moreover, ethnicity has been proposed as a factor modifying the risk of cancer^[2].

The present-day population of Latin American countries is historically and anthropologically admixed, as the result of a mixing process between Native Americans, Europeans (mostly Spaniards, Portuguese and Italians) and Sub-Saharan Africans (mainly from Western Africa), whom came into contact for five centuries^[31]. The populations of Latin America experienced different admixture processes with varying degrees of ancestral population proportions that came in different migration waves^[32,33]. Therefore, in studies of genetic association to diseases, the addition of a population structure estimate could be very effective to identify and correct possible effects of the population substructure.

Genetic admixture studies have recently helped to identify variants associated with prostate and oral cancer in African-American and Hispanics populations, respectively^[34,35]. A similar approach was applied recently by Pereira *et al*^[36] using a panel of 103 ancestry informative markers (AIM) to test if individual Native American, European and African ancestries are risk factors for gastric cancer in an urban admixed sample in Peru. This work determined that Native American individual ancestry is associated with gastric cancer, but this was explained by the association of socioeconomic variables with both gastric cancer and Native American ancestry. Therefore, indicating that the high incidence of gastric cancer in the Peruvian population, with a very high Native American ancestry, does not seem to rely on a genetic basis. More recently, a study carried out in the Northern region of Brazil examined the effect of population substructure, by the analysis of a panel with 48 AIMs, on the association between five single nucleotide polymorphisms (SNP) of N-acetyltransferase 2 (*NAT2*) gene and the susceptibility to breast and gastric cancer^[37]. They detected a higher African contribution in the study group with cancer, and a significant association of *NAT2* 282*T allele carriers with gastric cancer.

GASTRIC CANCER AND GENETIC VARIANTS IN LATIN AMERICAN COUNTRIES

The present overview included studies carried out on

humans that were found in the databases of PubMed/MEDLINE, LILACS and SciELO, published up to 25 October 2013 and with no restriction regarding language. This review includes the analysis of 61 articles reporting studies of association between genetic variants and the risk of gastric cancer and/or known precancerous lesions. All studies correspond to case-control comparisons, including healthy, non-cancer (counting precancerous lesions), asymptomatic and population-based controls^[12,37-96].

All reviewed studies were conducted with Latin American populations: Brazil (22), Chile (1), Colombia (6), Costa Rica (7), Honduras (1), Mexico (16), Peru (1) and Venezuela (7). Two-thirds of the articles considered the detection of *H. pylori* infection by urease test, culture, histology, serology or polymerase chain reaction (PCR). Some studies also included the typing of genetics variants of bacterial virulence factors by molecular methods. Regarding host genetic variants, studies evaluated 69 polymorphisms from 40 genes or genomic regions, including 2 microsatellites or variable number of tandem repeat (VNTR), 2 gene deletions, 3 insertion/deletions and 62 nucleotide substitutions (21 of them in protein coding regions, resulting in 3 synonymous and 18 nonsynonymous substitutions). Figure 1 shows a summary of gene polymorphisms included in the Latin American studies classified by functional categories: inflammatory response, mucosal protection, metabolic enzymes and transporters, oxidative damage, cellular adhesion, DNA repair, oncogene/tumor suppressor/stability genes, apoptosis.

Techniques used for detecting gene polymorphisms were dot blot hybridization, sequencing, conventional polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism, PCR-single-strand conformation polymorphism, real-time PCR with fluorescent probes, PCR-sequence specific oligonucleotide probe, PCR-Sequence-Specific Primer, Amplification-refractory mutation system-PCR, and KASParTM SNP genotyping system.

The largest number of studies investigating the association between gene polymorphisms and gastric cancer (and premalignant lesions) risk in different countries of the region includes the evaluation of interleukin-1 family variants: *IL-1B* (*IL-1B*-511 C/T, 14 studies -31 T/C, 10 studies, +3954, 8 studies) and *IL-1RN* (VNTR, 17 studies). Table 1 shows the main characteristics of these studies.

Genetic variants in inflammation-related genes, especially cytokines and their receptors are thought to influence the first stage of the precancerous cascade and are related to a more intense inflammatory response after gastritis associated to *H. pylori* infection^[7]. The inflammatory-related genes that have been most frequently studied in relation to gastric cancer, sometimes with conflicting results, are the interleukin genes *IL-1B*, *IL-1RN*, *IL-8* and *IL-10*. SNPs within these and other functional cytokine regions that markedly influence expression and secretion profiles may modify the intensity of the inflammatory re-

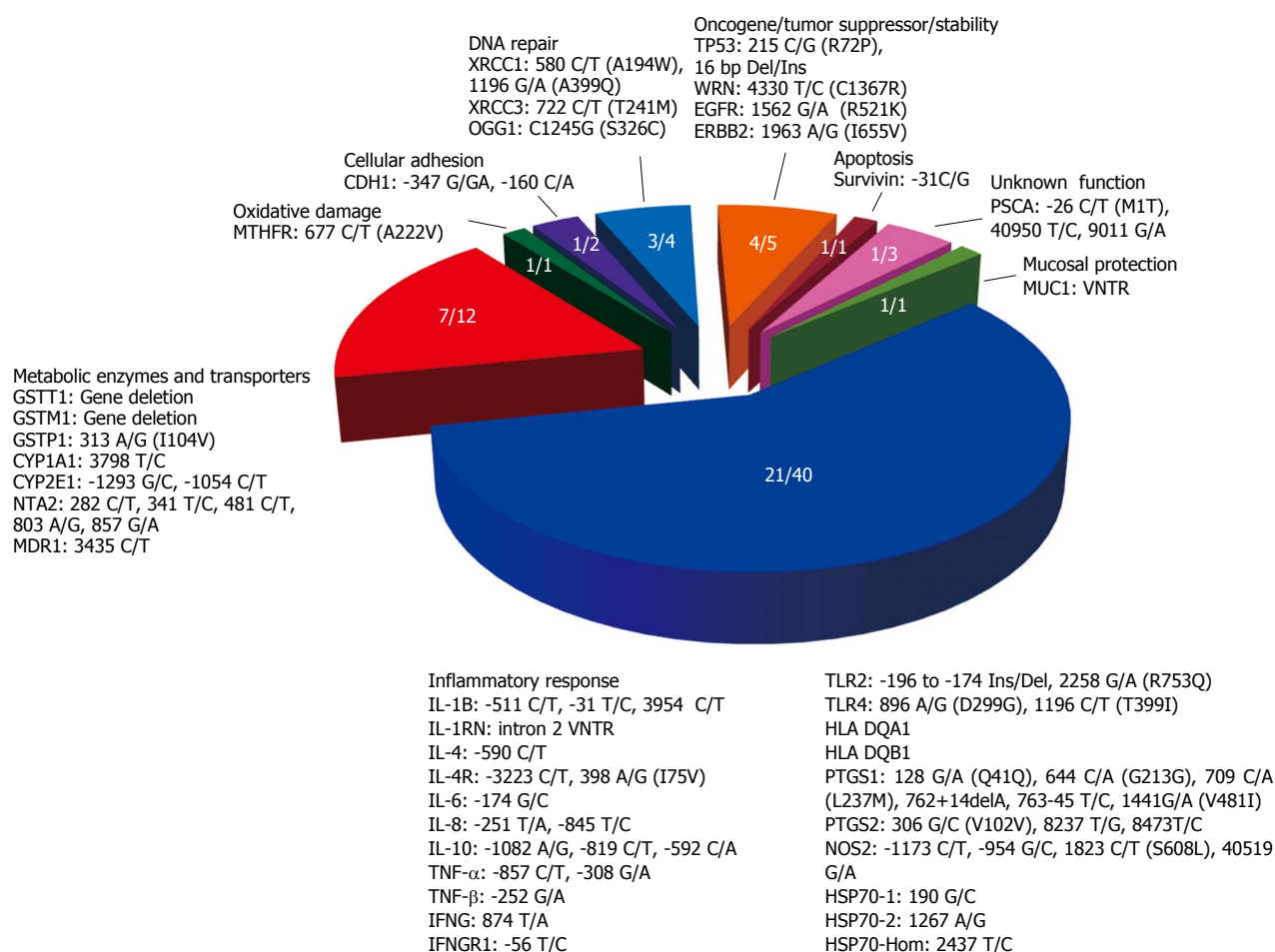


Figure 1 Graphic summary of gene polymorphisms included in the Latin American studies classified by functional categories. The fractions in each section of the cake indicated total number of genes/number of polymorphisms examined in each group^[12,37-96].

sponse to infectious agents, thereby contributing to variations in gastric cancer risk^[97].

Since the first study of El-Omar *et al*^[98] in 2000, a significant number of studies have evaluated the association between genetic variations in the *IL-1* gene cluster (*IL-1B*-511 C/T, *IL-1B*-31 T/C, *IL-1B*+3954 C/T and *IL-1RN* intron 2 VNTR) and gastric cancer. This association (significantly with noncardia or with intestinal type of gastric cancer) has a fundamental principle: alleles *IL-1B*-31*C, -511*T, and *IL-1RN**2, lead to high-level expression of IL-1 β , reduction of acid output, corpus-predominant colonization by *H. pylori*, pangastritis and atrophic gastritis, which are considered precursors as well as risk factors for gastric cancer^[98]. Furthermore, *H. pylori* infection induces IL-1 β production, and the consequent hypochlorhydria favors further colonization by pH-sensitive *H. pylori*^[99]. In addition, global meta-analyses have suggested race-specific associations of some cytokine variants in Caucasian and Asian populations^[97,100-104].

A recent meta-analysis, showed that the profile of *IL-1B* risk alleles in Latin Americans mirrors that found in Asian populations with low or no associations with gastric cancer^[105]. For example, in a high-incidence region of gastric cancer in Honduras, a sample of healthy (population-based) controls of Hispanic mestizo origin, had the

IL-1B-511*T⁺ and *IL-10*-1082*A⁺ genotypes prevalence among the highest reported^[45].

The meta-analysis of Xue *et al*^[103] showed that *IL-1B*-511*T and *IL-1RN**2 alleles were significantly associated with an increased risk of developing gastric carcinoma among Caucasians, but not in Asians or Hispanics. On the other hand, in the case-controls comparisons carried out in 2013 by Bonequi *et al*^[105], in which were analyzed studies from Brazil, Colombia, Costa Rica, Honduras, Mexico, Peru and Venezuela, it was identified the *IL-1RN**2 allele associated with a moderate increased risk for gastric cancer (overall OR = 1.51, 95%CI: 1.15-1.99), supporting its involvement in gastric carcinogenesis as has been previously reported in non-Asian populations^[104].

Among pro-inflammatory cytokines, IL-8 acts as a potent chemoattractant and activator of neutrophils that may play a role in gastric cancer pathogenesis^[14,106]. IL-8 exhibits several functional polymorphisms, among them the *IL-8*-251 A/T SNP in the promoter region is associated with an increase in synthesis of that interleukin by gastric epithelial cells^[14,106].

A study conducted in Mexico showed that the *IL-8*-251*A allele is a risk factor for the development of non-cardia gastric cancer^[60]. Similarly, Vinagre *et al*^[95] observed

Table 1 Association studies of *interleukin -1* gene cluster and gastric cancer/precancerous lesions

Year	Study population	Number of populations	Target genes and variants	Main findings associated with increased susceptibility
2004	Brazil Gatti <i>et al</i> ^[70]	56 GC; 56 ChrG	<i>IL-1B</i> -511C/T; -31T/C <i>IL-1RN</i> intron 2 VNTR	There was no association
2005	Costa Rica Alpizar-Alpizar <i>et al</i> ^[40]	58 GC; 41 nonneoplastic lesions; 58 cancer free patients; 41 healthy controls	<i>IL-1B</i> -511C/T; -31T/C; +3954C/T <i>IL-10</i> -1082G/A; -819C/T; -592C/A <i>IL-1RN</i> intron 2 VNTR	Carriers of the <i>IL-1B</i> +3954*T allele had an increased risk for developing GC (OR = 3.7 ² , 95%CI: 1.34-10.2). <i>IL-1RN</i> heterozygote genotype (*2/*L) was associated with GC (OR = 2.94 ² , 95%CI: 1.09-7.93).
2005	Mexico Garza-González <i>et al</i> ^[12]	63 distal GC; 215 non-cancer lesions	<i>IL-1B</i> -31T/C <i>IL-1RN</i> intron 2 VNTR <i>TNF-α</i> -308G/A	Presence of <i>IL-1B</i> -31*C allele was associated with increased risk of distal GC (OR = 7.63 ¹ , 95%CI: 1.73-46.94)
2005	Brazil Rocha <i>et al</i> ^[73]	168 GC <i>H. pylori</i> +; 541 asymptomatic controls	<i>IL-1B</i> -511C/T; -31T/C <i>IL-1RN</i> intron 2 VNTR <i>TNF-α</i> -308G/A	<i>IL-1RN</i> *2 was associated with noncardia GC (OR = 1.93, 95%CI: 1.06-3.49)
2006	Mexico Sicinschi <i>et al</i> ^[58]	183 GC; 377 controls	<i>IL-1B</i> -31T/C; +3954 C/T <i>IL-10</i> -592C/A <i>IL-1RN</i> intron 2 VNTR	<i>IL-10</i> -592*C allele carrier was associated with intestinal-type of GC (OR = 2.08 ¹ , 95%CI: 1.07-4.05). Subjects with <i>IL-1B</i> -31 CC genotype and <i>H. pylori</i> CagA positive serology had an increased risk of intestinal-type GC (OR = 3.19 ² , 95%CI: 1.05-9.68)
2006	Honduras Morgan <i>et al</i> ^[45]	170 GC; 162 healthy controls	<i>IL-1B</i> -511C/T <i>IL-10</i> -1082G/A <i>IL-1RN</i> intron 2 VNTR <i>TNF-α</i> -308 G/A	<i>IL-1B</i> -511 TT + <i>IL-10</i> -1082 AA combination increased risk of GC (OR = 2.6, 95%CI: 1.0-6.8)
2007	Costa Rica Con <i>et al</i> ^[43]	58 AG; 31 corpus AG; 23 IM	<i>IL-1B</i> -511C/T; +3954C/T <i>IL-10</i> -1082G/A; -592C/A <i>IL-1RN</i> intron 2 VNTR	<i>IL-1B</i> +3954*T carrier and <i>IL-1RN</i> homozygous *2 allele were associated with IM (OR = 3.4 ¹ , 95%CI: 1.2-10.00 and OR = 3.1 ² , 95%CI: 1.1-9.00, respectively)
2008	Costa Rica Sierra <i>et al</i> ^[42]	25 ABG; 76 AAG; 253 NAG; 21 Normal mucosa; 21 healthy controls	<i>IL-1B</i> +3954C/T <i>IL-1RN</i> intron 2 VNTR	No association was found
2009	Peru Gehmert <i>et al</i> ^[46]	133 GC vs 133 NAG 86 NAG vs 43 ChrAG	<i>IL-1B</i> -511C/T <i>IL-1RN</i> intron 2 VNTR	<i>IL-1B</i> -511*C allele carrier and CT and CC genotypes were associated with AG (OR = 5.6 ¹ , 95%CI: 2.02-15.51; OR = 4.8 ² , 95%CI: 1.65-13.83; OR = 11.2 ² , 95%CI: 2.27-55.37, respectively) and GC (OR = 2.36 ¹ , 95%CI: 1.34-4.11; OR = 2.17 ² , 95%CI: 1.23-3.84; OR = 4.15 ² , 95%CI: 1.33-12.93, respectively)
2009	Brazil Melo Barbosa <i>et al</i> ^[24]	177 gastric benign pathologies; 100 asymptomatic controls	<i>IL-1B</i> -511C/T; -31T/C <i>IL-1RN</i> intron 2 VNTR <i>TNF-α</i> -308 G/A	Carriers of <i>IL-1RN</i> *2 allele with <i>H. pylori</i> CagA-positive serology had a greater risk of developing GU (OR = 8.82, 95%CI: 1.762-44.181) and GC (OR = 16.76, 95%CI: 1.99-140.71)
2009	Costa Rica Con <i>et al</i> ^[44]	52 GC; 191 non-cancer <i>H. pylori</i> positive patients	<i>IL-1B</i> -511C/T; +3954C/T <i>IL-10</i> -1082G/A; -592C/A <i>IL-1RN</i> intron 2 VNTR	<i>IL-1B</i> +3954 TC (OR = 2.1 ² , 95%CI: 1.0-4.3), <i>IL-1RN</i> *2/*L (OR = 3.5 ² , 95%CI: 1.7-7.3), <i>IL-10</i> -592 AA (OR = 3.1 ² , 95%CI: 1.2-8.2) and <i>IL-10</i> -592 CA (OR = 3.2 ² , 95%CI: 1.5-6.8) genotypes, as well the <i>IL-1B</i> +3954 TC, <i>IL-1RN</i> *2/*L, <i>IL-10</i> -592 CA (OR = 4.7, 95%CI: 1.7-13.0) combination were associated with GC
2009	Venezuela Cañas <i>et al</i> ^[84]	84 GC; 84 ChrG	<i>IL-1B</i> -511T/C; +3954C/T <i>IL-10</i> -592C/A <i>IL-1RN</i> intron 2 VNTR	<i>IL-1B</i> +3954*C carrier and <i>IL-1RN</i> *2/*2 genotype were associated with GC (OR = 6.2 ¹ , 95%CI: 1.3-28.8 and OR = 7.0 ² , 95%CI: 2.3-21.5, respectively). The <i>IL-1RN</i> *2/*2 genotype was also associated with a well/moderately-differentiated adenocarcinoma (OR = 8.1 ² , 95%CI: 2.5-26.8)
2010	Mexico Martínez-Carrillo <i>et al</i> ^[56]	100 ChrG; 28 GU 102 healthy controls	<i>IL-1B</i> -511C/T; -31T/C	The <i>IL-1B</i> -511 TC genotype and the -511*C allele were associated with ChrG (OR = 3.1 ² , 95%CI: 1.4-6.8 and OR = 3.0 ¹ , 95%CI: 1.4-6.3, respectively). The subjects carrying -31*T were found to be at a higher risk of having ChrG (OR = 2.8 ¹ , 95%CI: 1.3-5.8). The <i>IL-1B</i> -511*C/-31*T haplotype was associated with ChrG (OR = 2.1, 95%CI: 1.2-3.8).
2010	Venezuela Chiurillo <i>et al</i> ^[85]	109 ChrG	<i>IL-1B</i> -511C/T; -31T/C; +3954C/T <i>IL-1RN</i> intron 2 VNTR	Carriage of <i>IL-1B</i> -511*T (OR = 5.4, 95%CI: 1.9-15.8) and -31*C (OR = 5.1, 95%CI: 1.8-14.7) alleles combined with iceA2+ <i>H. pylori</i> genotype increased the risk of ChrAG with severe histopathological changes.
2011	Colombia Martínez <i>et al</i> ^[88]	46 GC; 99 NAG	<i>IL-1B</i> -511C/T <i>IL-1RN</i> intron 2 VNTR	<i>IL-1B</i> -511 TT carriers had increased risk of GC (OR = 11.31 ² , 95%CI: 1.20-106.54)
2011	Colombia Martínez <i>et al</i> ^[91]	58 GC; 89 DU (54 with precancerous lesions); 194 ChrG and normals	<i>IL-1B</i> -511C/T <i>IL-1RN</i> intron 2 VNTR <i>IL-10</i> -1082G/A; -819C/T; <i>TNF-α</i> -308G/A	Genotype <i>IL-1B</i> -511 TT was associated with GC (OR = 4.69 ² , 95%CI: 1.22-18.09)

2011	Venezuela Chiurillo <i>et al</i> ^[86]	121 ChrG	<i>IL-1B</i> -511C/T; -31T/C; +3954C/T	There was association with severe histological changes only considering <i>H. pylori</i> genotypes
2012	Mexico López-Carrillo <i>et al</i> ^[57]	158 GC; 317 clinical controls	<i>IL-1B</i> -31T/C	<i>IL-1B</i> -31*C allele carriers who were both <i>H. pylori</i> CagA positive and with moderate to high Capsaicin consumption had increased risk of GC (OR = 3.41 ¹ , 95%CI: 1.12-10.43)
2013	Brazil Mattar <i>et al</i> ^[94]	19 GC; 71 clinical controls; 196 inflammation of the upper gastrointestinal tract; 28 GU; 76 DU	<i>IL-1RN</i> intron 2 VNTR	The carriage of <i>IL-1RN</i> *2/*2 was an independent risk factor for GC (OR = 5.81, 95%CI: 1.06-31.98). The carriage of allele *2 had an independent protective effect on DU (OR = 0.45, 95%CI: 0.22-0.91)
2013	Brazil de Oliveira <i>et al</i> ^[79]	200 GC; 229 ChrG; 240 healthy individuals	<i>IL-1RN</i> intron 2 VNTR <i>TNF-α</i> -857C/T <i>TNF-α</i> -308G/A <i>TNF-β</i> -252G/A <i>IL-8</i> -251T/A <i>IL-8</i> -845T/C <i>IL-10</i> -592C/A TLR2-196 to -174 Ins/Del TLR4+896A/G (D299G); +1196C/T (T399I)	Association with GC was observed for <i>IL-1RN</i> *2 (OR = 2.60 ¹ , 95%CI: 1.65-4.10), <i>TNF-α</i> -857*T (OR = 1.70 ¹ , 95%CI: 1.08-2.67), <i>IL-8</i> -845*C (OR = 3.46 ¹ , 95%CI: 1.69-7.07), <i>IL-10</i> -592*A (OR = 2.34 ¹ , 95%CI: 1.47-3.70), TLR2 -196 to -174 *Del (OR = 2.20 ¹ , 95%CI: 1.28-3.78) and TLR4+896*G (OR = 2.09 ¹ , 95%CI: 1.08-4.02) alleles. Association with ChrG was observed with <i>IL-1RN</i> *2 (OR = 1.88 ¹ , 95%CI: 1.25-2.83) and <i>IL-10</i> -592*A (OR = 3.00 ¹ , 95%CI: 1.99-4.50) alleles

¹ Dominant; ² Co-dominant. *H. pylori*: *Helicobacter pylori*; GC: Gastric cancer; ChrG: Chronic gastritis; ChrAG: Chronic atrophic gastritis; DU: Duodenal ulcer; NAG: Non-atrophic gastritis; GU: Gastric ulcer; AAG: Atrophic antral gastritis; ABG: Atrophic body gastritis; IM: Intestinal metaplasia.

that the AA ($P = 0.026$) and AT ($P = 0.005$) genotypes were most frequent in the group of patients with gastric adenocarcinoma from the state of Pará, Brazil. Furthermore, they also found the *IL-8* -251*A allele associated with the risk for developing gastric cancer. On the contrary, also in Brazil but in the state of São Paulo, Felipe *et al*^[96] found the *IL-8* -251 AT genotype and *T carriers associated with an increased risk of gastric cancer. These authors also observed that individuals with AA genotype may have protective effect for gastric cancer, while patients harboring the TT genotype presented a lower median survival time. Whereas Garcia de Oliveira *et al*^[79], in another region of the state of São Paulo, found only the *IL-8* -845 T/C SNP ($P < 0.001$) associated with risk for gastric cancer.

These results could suggest that the association between *IL-8* -251 A/T polymorphism and gastric cancer is likely influenced by environmental factors, and even ethnicity, considering that the geographical conditions and the proportion of the genetic ancestral contributions differ between the northern and southeast regions of Brazil^[107,108]. Moreover, a recent meta-analysis suggest the potential influence of ethnicity in the association of *IL-8* -251 A/T polymorphism with gastric cancer, since it is generally stronger in Asian than in Caucasian population^[109].

There are three functional promoter SNPs in the *IL-10* locus: -1082 A/G, -819 C/T and -592 C/A. In this locus only the -592 C/A SNP was found associated with gastric cancer in Latin American studies. Con *et al*^[44] in Costa Rica and Garcia de Oliveira *et al* in Brazil^[79] found that *IL-10*-592 AA and CA genotypes were individually associated with gastric cancer. Contrary, Sicinschi *et al*^[58] in Mexico identified the *IL-10*-592 CC genotype associated with more than double of the risk of the intestinal-type gastric cancer. A recent meta-analysis based on 12

previous studies concluded that the *IL-10*-592 C/A polymorphism was not a risk factor for gastric cancer. However, when stratifying the data by race, the *IL-10*-592 AA genotype was found to be a protective factor against the development of this neoplasm in Asians but not among Caucasians and Latinos^[110].

Toll-like receptors (TLR2 and TLR4), involved in *H. pylori* recognition in gastric mucosa, also have polymorphic variants that modulate their functional pattern^[111]. Hence, some reports have studied SNPs in *TLRs* that are associated with impaired immune response and induction of a potent inflammatory response in the gastric mucosa, being then associated with susceptibility of gastric diseases. In Mexico two studies evaluated the association of *TLR4* +896A/G and +1196C/T SNPs with gastric cancer and precancerous diseases^[60,93]. Although no association with gastric cancer was found in these studies, Trejo-de la *et al*^[93], including also analysis of *TLR2* +2258 G/A SNP, showed that patients with *TLR4* polymorphisms expressed significantly lower levels of *IL-1β*, *IL-6*, *IL-8* and *GRO-α*; and higher levels of *TNF-α*, *IL-10*, *MCP-1* and *MIP-1α*. Moreover, Silva's research group in two recent reports investigated *TLR2* -196 to -174 del, *TLR4* +896A/G and *TLR4* +1196C/T polymorphisms at risk of chronic gastritis and gastric cancer in a Brazilian population in the state of São Paulo^[78,79]. In both studies *TLR2* -196 to -174*del and *TLR4* +896*G alleles showed an association with increased risk for gastric cancer.

The study of Garcia de Oliveira *et al*^[79] mentioned above evaluated ten inflammatory-related gene polymorphisms in 669 samples (200 of gastric cancer, 229 of chronic gastritis, and 240 of healthy individuals), of which *IL-1RN* L/2 ($P < 0.001$), *TNF-α*-857 C/T ($P = 0.022$), *IL-8*-845 T/C ($P < 0.001$), *IL-10*-592 C/A ($P < 0.001$), *TLR2* ins/del ($P < 0.001$), and *TLR4*+896 A/G ($P = 0.033$) polymorphisms were observed associated with

the risk of gastric cancer using a dominant model. In addition, a combined analysis of these six polymorphisms revealed a profile with two to four combined genotypes, which confers a higher risk of gastric carcinogenesis.

Regarding polymorphisms in inflammation-related genes, three genes encoding heat shock proteins (HSP) were also evaluated in two studies. Partida-Rodríguez *et al*^[61] studied *HSP70-1*+190 G/C, *HSP70-2*+1267 A/G and *HSP70-Hom*+2437 T/C SNPs in 447 Mexican patients, including 228 with non-atrophic gastritis, 98 with intestinal metaplasia, 63 with gastric cancer, 58 with duodenal ulcer, and 132 asymptomatic individuals. They also evaluated in this analysis the *TNF-α*-308 G/A and *TNF-β*-252 G/A polymorphisms. Compared with the asymptomatic group, they found significant association of *TNF-β*-252*A and *HSP70-1**C alleles with gastric cancer. More recently, Ferrer-Ferrer *et al*^[41] in 2013 addressed the possible association between *HSP70-2*+1267 A/G and *HSP70-Hom*+2437 T/C polymorphisms and the risk of developing gastric cancer in a high-risk population in Costa Rica. These authors found that the GA genotype of *HSP70-2*+1267 was associated with increased risk of gastric cancer as compared to the GG genotype.

With regard to tumor-suppressor genes, seven studies conducted the analysis of the *TP53* Arg72Pro polymorphism related to the risk of gastric cancer. In Mexican patients, Pérez-Pérez *et al*^[55] identified association of the Arg/Arg genotype with the increased risk of distal gastric cancer. Similarly, in Venezuela, individuals carrying the Arg allele had an elevated risk of developing gastric cancer, while the Arg/Arg genotype was associated with poorly-differentiated gastric cancer^[83]. However, the association of gastric cancer with *TP53* Arg72Pro polymorphism in Latin American countries was not consistent in the meta-analysis of Bonequi *et al*^[105]. Differences in distribution of *TP53* Arg72Pro genotypes could be associated with the location, stage, and histological differentiation of gastric cancer. Moreover, a meta-analysis suggests that the *TP53* codon 72 polymorphism (Pro allele) may be associated with gastric cancer, particularly among Asians^[112].

A Brazilian case-control study evaluated the effect of a functional SNP (-31C/G) of *Survivin*, which is involved in the regulation of apoptosis and cell cycle control^[75]. Although this study included a small sample size, results suggest that the presence of the *C allele of *Survivin* gene promoter -31 C/G polymorphism in combination with D17S250 microsatellite instability (a marker of *TP53* gene) may be used as risk factor for gastric cancer in this population. Involvement in gastric carcinogenesis of *Survivin* can be taken from the observation that overexpression of this protein in gastric cells reduces cell death after infection with *H. pylori*^[113].

The *CDH1* gene, encoding E-cadherin protein, is now established as a tumor suppressor in gastric cancer^[114]. Polymorphisms at positions -347 G/GA and -160 C/A reduce the transcriptional activity of *CDH1*, although their association with susceptibility to gastric cancer is controversial^[115,116]. Medina-Franco *et al*^[62] ana-

lyzed a sample of 39 Mexican patients younger than 45 years old with diagnosis of diffuse gastric cancer and observed association with -160 CA and AA genotypes. Moreover, Borges *et al*^[69] observed in Brazilian patients carrying *CDH1* -160*A and -347*GA alleles an increased probability of developing gastric cancer, especially of the diffuse-type.

SNPs in the *PSCA* gene was found associated with gastric cancer risk in a Genome-wide association study (GWAS), and subsequently validated in other Asian and Caucasian populations^[26,117,118]. Although its function remains unknown, the expression of *PSCA* has been observed downregulated in the gastric tissue with intestinal metaplasia^[119]. Rizatto *et al*^[82] analyzed 3 SNPs in the *PSCA* gene (rs2294008 C/T, rs9297976 T/C and rs12155758 G/A) in gastric biopsies of 2045 subjects with gastric precancerous lesions and 180 cases of gastric cancer from a high-risk region of Western Venezuela. In this study the *T and *A alleles of rs2294008 and rs12155758, respectively, were found to be associated with gastric cancer.

GENE POLYMORPHISMS AND GASTRIC PRECANCEROUS LESIONS

The role of gene polymorphisms in precancerous lesions remains poorly understood, even for those that have been identified as associated with increased risk of gastric cancer. Identification of biomarkers of the precancerous process is needed for development of screening programs to prevent gastric cancer, as this may contribute to the understanding of gastric carcinogenesis.

Association between cytokine gene polymorphisms and gastric precancerous lesions were identified in a work carried out in Costa Rica by Con *et al*^[43], in which the *IL-1B*+3954*T and *IL-1RN* *2/*2 genotypes were associated with intestinal metaplasia. Whereas in Peruvians, Gehmert *et al*^[46] revealed an increased risk of atrophic gastritis associated with *IL-1B*-511*C allele. A Brazilian study in the state of São Paulo demonstrated the existence of an association of the anti-inflammatory cytokine variant alleles *IL-1RN**2 and *IL-10*-592*A with a higher risk of developing gastric cancer and chronic gastritis^[79]. In a recent meta-analysis Peleteiro *et al*^[120] showed an association of the *IL-1RN* *2/*2 genotype with the increased risk of gastric precancerous lesions, supporting a role for this polymorphism in the early stages of gastric carcinogenesis.

In the context of *H. pylori* infection, two studies of our group in Venezuela showed an association of chronic atrophic gastritis and severe histopathological changes with *IL-1B*-511*T, -31*C, +3954*C and *IL-1RN**2 polymorphisms only in presence of specific bacterial virulence genotypes^[85,86]. Similarly, Melo-Barbosa *et al*^[74] in Brazil, found that carriers of *IL-1RN**2 allele with *H. pylori* CagA-positive serology had a higher risk of developing gastric ulcer.

A research group have evaluated the prevalence of

gastric precancerous lesions in a large number of Venezuelans in relation with several genetic polymorphisms, most of them mediators of inflammation, and their interactions with other environmental factors. The first of them, by Kato *et al*^[81], studied *IL-10*, *IL-4* and *IL-4R* SNPs in 2033 patients. Authors identified the *IL-10*-1082*A low activity allele associated with intestinal metaplasia and dysplasia, while homozygous of the low activity allele (GG) of the 398 A/G polymorphism in the *IL-4R* gene had a modest increase in the risk of atrophic gastritis.

This group of researchers also evaluated genetic polymorphisms in other mediators of inflammation: *IFNG*, *IFNGR1*, *NOS2A*, *PTGS1*, *PTGS2*^[87]. A nonsynonymous substitution Ser608Leu of *NOS2A* gene (*A carriers) and the -56 C/T SNP located in the promoter of *IFNGR1* (CC genotype) were associated with higher risk of atrophic gastritis. Additionally, two SNPs of *PTGS2* were associated with risk of dysplasia (306 G/C -Val102Val- and 8473 T/C). More recently, in a further study of this group, the *T allele of the functional SNP rs2294008 in the *PSCA* gene was associated with atrophic gastritis and intestinal metaplasia^[82].

SNPs of *HSP70-1* (+190*C allele) and *HSP70-2* (+1267 GA genotype) were associated with an increased risk of duodenal ulcer in patients of Mexico and Costa Rica, respectively^[41,61]. Moreover, examination of *TLR4* +896A/G SNP in a Southeastern Brazilian population showed that the heterozygous AG genotype and allele *G frequencies were significantly higher in chronic gastritis and gastric cancer groups than in controls^[78].

CONCLUSION

Latin America is a territorial and cultural entity with a particular genetic complexity, but also characterized by wide socio-economic divergences and rapid changes in life styles throughout the continent with a strong trend towards urbanization of its population. Therefore, the study of the etiology of multifactorial diseases, such as gastric cancer, in this region appears to be a major challenge, but also an opportunity.

Given in Latin America the common scenario of a population with high rates of infection with *H. pylori*, persistent poverty, particular dietary habits, coupled with secular trends in environmental exposures and lifestyle, genetic can offer a useful tool to compare populations and assess gene-environment interactions that underline gastric cancer development.

Most studies here analyzed were conducted with samples from populations with high prevalence of *H. pylori* infection. Therefore, it is not surprising that most research in this region of the world have been carried out with gene variants involved in inducing a more intense inflammatory response after gastritis associated to *H. pylori* infection. Moreover, as has been raised in Asians, due to the distribution of *IL-1B* high-risk alleles in some Latin American populations shows an elevated prevalence,

could be suggested that they do not influence gastric cancer susceptibility in these populations, or in any case, its effect cannot be demonstrated.

Some research groups have been investigating the genetic contribution to gastric cancer in subjects of different ethnic backgrounds (mainly in Asians and Caucasians from United States and Europe), using previous GWASs information or conducting parallel GWASs with a large number of genes and new candidate loci for gastric cancer, as well as employing innovative techniques for genotyping and statistical analysis^[22,27-30]. Therefore, to the analysis of human genetic risk factors in our populations, it would be appropriate to exploit and replicate GWASs findings, since a simple extrapolation of results from these studies to the use of biomarkers in Latin American populations is not completely adequate.

Genetic studies in admixed populations are particularly susceptible to confusion due to population stratification resulting from the difference in ancestry between cases and controls. However, such confounding can be handled by estimating individuals' genetic ancestry using AIMs and then adjusting the analysis for individual ancestry. If in this region the human genetic background influences the high incidence of gastric cancer then can be expected that genetic variants harbored in admixed population account for this high incidence. Therefore, it would be possible to apply the genome-wide strategy of admixture mapping to detect these variants.

Finally, it is necessary to advocate for multicenter studies involving several Latin American research groups and large number of samples for the analysis of genetic polymorphisms in relation to precancerous lesions and environmental variables (lifestyle, dietary habits, *H. pylori* infection), in order to contribute to the understanding of gastric carcinogenesis and for the development of screening programs to prevent gastric cancer.

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Adjuvant chemotherapy for gastric cancer: Current evidence and future challenges

Rosalba Miceli, Gianluca Tomasello, Giacomo Bregni, Maria Di Bartolomeo, Filippo Pietrantonio

Rosalba Miceli, Unit of Medical Statistics, Biometry and Bioinformatics, Fondazione IRCCS Istituto Nazionale Tumori, 20133 Milan, Italy

Gianluca Tomasello, Medical Oncology Division, Azienda Istituti Ospitalieri di Cremona, 26100 Cremona, Italy

Giacomo Bregni, Maria Di Bartolomeo, Filippo Pietrantonio, Medical Oncology Department, Fondazione IRCCS Istituto Nazionale Tumori, 20133 Milan, Italy

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Correspondence to: Rosalba Miceli, PhD, Unit of Medical Statistics, Biometry and Bioinformatics, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian 1, 20133 Milan, Italy. rosalba.miceli@istitutotumori.mi.it

Telephone: +39-22-3903198 Fax: +39-22-3902095

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Abstract

Gastric cancer still represents one of the major causes of cancer mortality worldwide. Patients survival is mainly related to stage, with a high proportion of patients with metastatic disease at presentation. Thus, the cure rate largely depend upon surgical resection. Despite the additional, albeit small, benefit of adjuvant chemotherapy has been clearly demonstrated, no general consensus has been reached on the best treatment option. Moreover, the narrow therapeutic index of adjuvant chemotherapy (*i.e.*, limited survival benefit with considerable toxicity) requires a careful assessment of expected risks and benefits for individual patients. Treatment choices vary widely based on the different geographic areas, with chemotherapy alone more often preferred in Europe or Asia and chemoradiotherapy in the United States. In the present review we discuss

the current evidence and future challenges regarding adjuvant chemotherapy in curatively resected gastric cancer with particular emphasis on the recently completed landmark studies and meta-analyses. The most recent patient-level meta-analysis demonstrated the benefit of adjuvant chemotherapy over curative surgery; the same Authors also showed that disease-free survival may be used as a surrogate end-point for overall survival. We finally discuss future research issues such as the need of economic evaluations, development of prognostic or predictive biomarkers, and the unmet clinical need of trials comparing perioperative chemotherapy with adjuvant treatment.

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Key words: Gastric cancer; Adjuvant chemotherapy; Radiotherapy; Randomized trial

Core tip: Despite the benefit of adjuvant therapy has been clearly demonstrated, no general consensus has been still reached on the best treatment option. The narrow therapeutic index of adjuvant chemotherapy requires a careful assessment of expected risks and benefits for individual patients. Many issues, such as the role of postoperative radiotherapy and the best chemotherapy regimen, are still under investigation. Moreover, no prognostic or predictive factors beyond pathological stage have been prospectively validated. Despite researchers' efforts, this issue still represent an unmet medical need. In this review we describe the recently completed landmark studies and meta analyses, and we discuss the future challenges in this research field.

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INTRODUCTION

Gastric cancer (GC) is a major public health problem, because of its high incidence, morbidity and mortality rate. Despite a steady incidence decline over the last decades, GC still represents one of the major causes of cancer mortality worldwide^[1]. This is due to the high proportion of patients with metastatic disease at presentation or during the clinical course. Indeed, less than 5% of patients with advanced GC survive up to five years and the role of surgery as mainstay treatment is limited to approximately a quarter of all patients^[2].

Overall survival (OS) of patients who undergo surgery progressively diminishes as stage increases, ranging from 75% for stage I to 35% or less for stage II and beyond^[3]. Recurrences tend to occur at distant sites, suggesting the presence of micrometastatic disease at the time of surgery. Therefore, these observations led to the hypothesis that adjuvant chemotherapy should improve outcomes in curatively resected stage II-III GC.

Despite the benefit of adjuvant therapy has been clearly demonstrated, no general consensus has been still reached on the best treatment option. The narrow therapeutic index of adjuvant chemotherapy (*i.e.*, limited survival benefit with considerable toxicity) requires a careful assessment of expected risks and benefits for individual patients. Generally, surgery followed by chemoradiotherapy is the standard protocol in the United States, whereas perioperative or postoperative chemotherapy are recommended in the Europe and Asia. The difference of this approaches is mainly due to the fact that less than D2 lymph nodal dissection is routinely used in the United States, whereas D2 surgery is the standard treatment in Europe. Thus, optimal local control may be obtained by adding radiotherapy to D0-D1 surgery. Many issues, such as the role of postoperative radiotherapy and the best chemotherapy regimen, are still under investigation. Moreover, no prognostic or predictive factors beyond pathological stage have been prospectively validated. Despite researchers' efforts, this issue still represent an unmet medical need.

In this review we describe early randomized clinical trials (RCTs) of adjuvant chemotherapy for resected GC, with particular emphasis on the recently completed landmark studies and meta-analyses, and we discuss the future challenges in this research field.

CURRENT EVIDENCE

Role of D2 surgery

The extension of surgical dissection is an open issue in the treatment of potentially curable GC. Asian and Western surgeons have followed different paths in the last de-

cades in their approach to GC. D2 gastrectomy has been a standard of care in Eastern countries since the 1960s^[4]. In Europe this procedure became widely used after the publication of 15-year results of the Dutch D1D2 trial, showing better locoregional control and lower GC related deaths in the D2 arm^[5]. In 2008 Sasako *et al*^[6] published the results of a Japanese RCT comparing D2 lymphadenectomy alone *vs* D2 lymphadenectomy plus para-aortic nodal dissection, a procedure performed in Japan since the 1980s. However, patients undergoing wider, D3, dissection did not benefit in terms of disease-free survival (DFS) and OS and experienced more surgical complications. Nowadays, D2 resection is the recommended surgical approach for patients with resectable GC and it is the major determinant of patients' prognosis.

Adjuvant chemotherapy: An "historical overview"

The debate on surgical dissection is obviously directly linked to the use of adjuvant therapy.

Over the last few decades numerous RCTs have been conducted to evaluate the benefit of post-operative chemotherapy as compared to surgery alone^[7-10]. Most of them failed to demonstrate a statistically significant survival advantage for different reasons, including the lack of adequate statistical power to detect a survival difference, the use of obsolete surgical techniques or "suboptimal" chemotherapy regimens, and the delay in starting treatment after gastrectomy.

Among those RCTs demonstrating a benefit, most were performed in Asia and few in Western countries. For instance, a Spanish RCT evaluated the efficacy of the combination of mitomycin plus tegafur *vs* observation in patients with resected stage III GC^[11]. After a median follow-up of 37 mo, both OS and DFS were significantly better in the chemotherapy group. Five-year OS and DFS were 56% and 51% in the treatment group *vs* 36% and 31% in the control group.

Taking into consideration all the RCTs testing anthracycline-containing polychemotherapy regimens, disappointing results were reported. The only positive trial so far was a multi-institutional study conducted in Italy in the 90's which randomly assigned node-positive GC patients to receive epidoxorubicin, leucovorin and 5-fluorouracil for 7 mo or no treatment^[12]. After a median follow-up of 5 years, the median OS was 18 mo for untreated patients *vs* 31 mo for treated ones.

This positive experience opened the way to subsequent trials testing more intensive chemotherapy regimens in order to further improve clinical outcomes. The Italian cooperative research groups played a fundamental role in this scenario. In fact, three large RCTs were completed in the attempt of evaluating new polichemotherapy strategies for high-risk resected GC patients. The Italian Trials in Medical Oncology group conducted a RCT comparing D2 surgery alone *vs* D2 surgery followed by 2 cycles of etoposide, adriamycin, cisplatin and 2 cycles of Machover regimens. The results showed that at 5 years the sequential regimen led to a 7% re-

Table 1 Overall survival results of study based meta-analyses comparing post-operative chemotherapy *vs* surgery alone

Ref.	Studies analysed (n)	Pooled HR (95%CI)
Earle <i>et al</i> ^[16]	13	0.80 (0.66-0.97)
Mari <i>et al</i> ^[17]	20	0.82 (0.75-0.89)
Janunger <i>et al</i> ^[19]	21	0.84 (0.74-0.96)
Oba <i>et al</i> ^[20]	4	0.73 (0.60-0.89)
Liu <i>et al</i> ^[21]	19	0.85 (0.80-0.90)
Zhao <i>et al</i> ^[22]	15	0.90 (0.84-0.96)
Sun <i>et al</i> ^[23]	12	0.78 (0.71-0.85)

duction in mortality and 17% reduction in the disease relapse rate, neither of which were statistically significant. In fact the trial was designed to detect a 15% of difference in 5-year survival between the two arms. We want to emphasize in addition that the results obtained with an adequate surgical treatment were better than expected^[13].

The second trial was conducted by the Italian Oncology Group for Clinical Research and published in 2008^[14]. Patients with stage IB-IV, completely resected GC were randomized to receive chemotherapy with 4 cycles of cisplatin, epirubicin, and 5-FU/LV (PELF regimen) or follow-up alone. Ultimately, chemotherapy did not lead to a significant increase in either DFS (HR, in PELF arm *vs* follow-up arm = 0.92; 95%CI: 0.66-1.27) or OS (HR = 0.90; 95%CI: 0.64-1.26). In fact, 5-year OS was almost identical in chemotherapy and follow-up arms (47.6% *vs* 48.7%). Statistical concerns were raised for this trial, since it was underpowered to detect very modest differences in OS between the two arms. Higher than expected survival rates were registered in both groups. Similar results was obtained by a third study conducted by the Gruppo Oncologico dell'Italia Meridionale^[15].

The fourth study compared two different treatment arms: PELFW regimen, consisting of eight weekly administrations of cisplatin, leucovorin, epidoxorubicin, 5-fluorouracil, and glutathione with the support of filgrastim, and a regimen consisting of six monthly administrations of 5-fluorouracil and leucovorin (5-FU/LV)^[9]. Unfortunately, this study did not find any difference in mortality or relapse between treatment groups, failing to show any benefit from dose-dense or intensified strategies. Again, 5-year OS was unexpectedly high in both arms - approximately 50% - probably reflecting the high quality of resection procedures. Thus, an optimal surgery may have reduced the impact of chemotherapy on outcomes, as well as the critical planned difference in OS rates may have been inappropriate (expected 5-year survival of only 20% for the control arm).

Due to the large discordance in outcomes in published RCTs, subsequent study-based meta-analyses have been performed to evaluate to role of adjuvant chemotherapy and finally a survival benefit, albeit small, was demonstrated^[16-23]. Table 1 shows summary OS results of the meta-analyses in terms of pooled HRs comparing adjuvant chemotherapy *vs* surgery alone. All the studies

coherently showed a significant OS benefit for adjuvant chemotherapy; however, when analysing only the Western RCTs, the Janunger *et al*^[19] estimated a non-significant HR = 0.96 (95%CI: 0.83-1.12). On the opposite side, the Oba *et al*^[20] including only Japanese studies estimated a HR as low as 0.73.

Milestone meta-analysis

In 2010 the GASTRIC Group published a patient-level meta-analysis to quantify the potential benefit of adjuvant chemotherapy over curative surgery in terms of both OS and DFS^[10]. The results obtained using individual patient data are potentially more reliable than those carried out on aggregate data. Table 2 shows the summary results in terms of pooled HRs; the overall estimates were practically overlapping for the two end-points and demonstrated reduced risks in the chemotherapy group. The HRs were translated in a small absolute benefit: for OS, 5.8% at 5 years and 7.4% at 10 year, whereas for DFS the Authors could estimate only a 5.3% absolute benefit at 5 years. Sub-group analyses by type of regimen showed that the greatest benefit was associated with monotherapy; however, such estimates were based only on two RCTs, one of which was Japanese. Even if no significant heterogeneity was detected across Europe, Asia, and United States, as we have already pointed out, the HRs are usually lower in Asian RCTs as compared to Western ones.

In 2013 the Cochrane Collaboration published a further study-level meta-analysis reviewing RCTs of post-surgical chemotherapy *vs* surgery alone^[24]. A significant improvement of OS (HR = 0.85; 95%CI: 0.80-0.90; 34 studies) and DFS (HR = 0.79; 95%CI: 0.72-0.87; 15 studies) was confirmed for adjuvant chemotherapy. Based on these results, the Authors recommended to offer adjuvant chemotherapy as a routine option - when-ever possible - following GC curative resection.

If considering OS results, the HRs obtained in the three study-level meta-analyses with the highest number of RCTs^[17,19,24]; were consistent with those obtained in the individual-level meta-analysis^[10].

From the literature to the bedside: new landmark studies

New insights confirming the effectiveness of fluoropyrimidine-based adjuvant chemotherapy were made available by two landmark Asian RCTs.

The ACTS-GC study was aimed at confirming the effectiveness on OS of 1-year adjuvant chemotherapy with the oral fluoropyrimidine S-1 following D2 gastrectomy^[25]. After a median follow-up of 3 years, 3-year OS was 80.1% in the S-1 group and 70.1% in the surgery alone group. S-1 reduced the risk of death by 32% (HR = 0.68; 95%CI: 0.52-0.87, *P* = 0.003). In the 5-year follow-up update, OS was 71.7% in the S-1 arm and 61.1% in the surgery-alone arm, therefore S-1 reduced the risk of death by 33% (HR = 0.67; 95%CI: 0.54-0.83). The 5-year relapse-free survival (RFS) was 65.4% in the S-1 arm and 53.1% in the surgery-only arm^[26]. The

Table 2 GASTRIC group meta-analysis^[10]

Studies (<i>n</i>)		Comparison	Pooled HR (95%CI)	
For OS analysis	For DFS analysis ¹		OS	DFS
17	14	Overall	0.82 (0.76-0.90)	0.82 (0.75-0.90)
2	1	Mono Chemotherapy <i>vs</i> surgery	0.60 (0.40-0.84)	0.49 (0.29-0.84)
3	2	Fluorouracil + mitomycin C + other without anthracyclines <i>vs</i> surgery	0.74 (0.58-0.95)	0.69 (0.48-0.98)
3	2	Fluorouracil + mitomycin C + anthracyclines <i>vs</i> surgery	0.82 (0.71-0.95)	0.80 (0.69-0.94)

¹Analyses were performed on randomized clinical trials with available disease-free survival (DFS) data. OS: Overall survival.

Authors raised some doubts about the possibility of translating the advantages of such treatment to Western population because of different pharmacodynamics and surgery practices. However, following the footsteps of the ACTS-GC trial, assessing the efficacy of combining S-1 with other potentially active drugs such as platinum-derivatives or taxanes could be an interesting perspective.

Similarly, the CLASSIC RCT was designed to compare the efficacy of adjuvant capecitabine plus oxaliplatin (XELOX regimen) with D2 surgery alone in stage II or III GC patients^[27,28]. Three-year DFS was 74% in the chemotherapy group and 59% in the surgery only group (HR = 0.56; 95%CI: 0.44-0.72, $P < 0.0001$); the 5-year analysis confirmed such results: DFS was 68% *vs* 53% (HR = 0.58; 95%CI: 0.47-0.72, $P < 0.0001$). As regards OS, the 5-year rates were 78% in the XELOX group and 69% in the surgery alone group (HR = 0.66; 95%CI: 0.51-0.85, $P = 0.002$). However, the greater limitation of this study was that the beneficial effect deriving from the addition of oxaliplatin to fluoropyrimidine should be assessed by a specific RCT. In fact, a control arm constituted by surgery alone is not appropriate for future trials since the benefits of adjuvant chemotherapy were clearly demonstrated^[10]. Indeed, the ongoing POTENT study is moving along this line^[29]. This is a RCT that started enrolling in early 2013 and it is randomizing patients to receive oxaliplatin and S-1 for six cycles or S-1 for 1 year after surgery. The primary end point is OS, while secondary end points are DFS and safety.

A further research topic in the adjuvant setting is the possibility to improve outcome through a sequential, non cross-resistant polychemotherapy. This strategy may allow to sequentially administer several active agents in order to exploit different mechanisms of drug activity in the context of a relatively chemoresistant disease. In such a perspective, ITACA-S was a multicentre, Italian RCT aimed at comparing two different regimens in GC patients eligible for adjuvant chemotherapy^[30]. Patients in arm A received a polychemotherapy with 4 cycles of irinotecan plus 5-FU/LV (FOLFIRI regimen) followed by cisplatin and docetaxel for 3 cycles, while patients in arm B received monotherapy with 5-FU/LV alone (De Gramont regimen) for 9 cycles. After a median follow up of 49 mo, no significant difference was observed between the two arms in terms of DFS (HR = 0.98; 95%CI: 0.83-1.16, $P = 0.830$) and OS (HR = 1.00; 95%CI: 0.83-1.20, $P = 0.980$). Toxicity was consistent with literature, as previously reported^[31], and significantly

higher in the polychemotherapy arm.

Similarly, the Japanese SAMIT RCT compared 4 different adjuvant regimens: in arm A patients received UFT alone, in arm B received S-1 alone, while arm C and arm D patients received sequential therapy with paclitaxel followed by either UFT or S-1, respectively^[32]. The trial aimed at comparing UFT with S-1, and both single agents with a sequential, taxane-based regimen. After a median follow-up of 1875 d and 728 events, the results failed to show a statistically significant difference of DFS in the sequential arms as compared to single agent fluoropyrimidine arms (HR = 0.92; 95%CI: 0.80-1.07, $P = 0.273$). Comparing the data in arms A + C *vs* B + D, UFT-based chemotherapy was clearly less effective than S-1-based one in the study population.

As a matter of fact, sequential polychemotherapy does not seem to be the best strategy to improve GC patients' outcome in the adjuvant setting and, since fluoropyrimidine and platinum salts have synergistic activity, their upfront combination may hopefully be more effective than a single agent regimen.

Role of adjuvant chemoradiotherapy

Due to the high risk of local recurrence, different studies have been evaluating the potential benefit of radiotherapy alone or combined to chemotherapy as adjuvant treatments for GC^[33,34].

Early studies of adjuvant radiotherapy demonstrated reductions of local failure rate despite of lack of OS benefit^[35].

Much more impact on modern management of GC had the large US Intergroup INT0116 study^[36]. This trial randomly assigned stage IB-IV GC patients to surgery plus postoperative chemoradiotherapy or surgery alone. Chemotherapy with bolus 5-FU/LV was intermingled by a "sandwich" chemoradiation phase in which 5-FU/LV was given on the first four and the last three days of radiotherapy. With a median follow-up of 5 years, median overall survival was 27 mo for surgery alone and 36 mo for adjuvant chemoradiation. Three-year OS was 41% for the surgery-alone group and 50% for surgery followed by chemoradiation group. Local failures were reduced from 29% to 19% with the addition of adjuvant chemoradiation. After more than 10 years of follow-up a persistent benefit was demonstrated for the experimental strategy in terms of both OS (HR = 1.32; $P = 0.004$) and RFS (HR = 1.51; $P < 0.001$)^[37].

This hallmark trial was largely criticized due to the

fact that only 10% of patients had a D2 dissection and more than half of patients did not even have clearance/examination of the D1 (perigastric) nodes. Furthermore, most of the patients on this study had T3/T4 disease, and 85% had nodal metastases. This resulted in a lack of accurate tumor staging and consequently in a non proper arm-allocation at randomization - likely contributing to inferior survival and a 64 percent relapse rate in the surgery alone arm. Finally, approximately one third of patients in the chemoradiation group had to stop treatment prematurely because of toxicity.

Despite all these issues, the adjuvant strategy as proposed in this trial became very popular in North America and still represents a gold standard treatment in this setting. Moreover, a meta-analysis including RCTs which compared postoperative chemoradiotherapy *vs* postoperative chemotherapy^[38] concluded that postoperative chemoradiotherapy improved local relapse-free survival (HR = 0.53; 95%CI: 0.32-0.87) and DFS (HR = 0.72; 95%CI: 0.59-0.89) but not OS (HR = 0.79; 95%CI: 0.61-1.03). However, the study was based only on three Asian RCTs and the results may be not extendable to Western patients.

Following the promising results of the INT00116 trial, the CALGB 80101 aimed at assessing whether replacing 5-FU/LV with Epirubicin, Cisplatin and 5-FU (ECF regimen) in the adjuvant chemoradiotherapy setting would improve OS^[39]. However, there was no significant benefit from adding this polychemotherapy regimen to standard 5-FU/LV chemoradiation in terms of OS ($P = 0.800$). Similarly, the ARTIST trial was designed to compare postoperative treatment after D2 dissection with capecitabine plus cisplatin (XP) *vs* XP plus capecitabine-based chemoradiation. There was no significant difference in DFS between the two arms, although chemoradiation arm was associated with significantly prolonged DFS in the retrospectively identified, lymph node-positive subgroup. Estimated 3 year-DFS rate was 78.2% in the experimental arm *vs* 74.2% in the control arm ($P = 0.086$), while estimates were 77.5% *vs* 72.3% ($P = 0.037$). An ongoing phase III trial (ARTIST-II) was designed to compare chemotherapy alone *vs* chemoradiation in lymph node-positive, resected GC, aiming at prospectively confirm the ARTIST trial hypothesis-generating data^[40].

In conclusion, adjuvant chemoradiation may be offered to patients to reduce the risk of locoregional failure in patients with node positive disease or suboptimal surgery.

FUTURE CHALLENGES

Economic analyses

Usually, few RCTs perform concurrent economic analyses; recently, recommendations regarding such an issue included guidelines for data collection of costs, efficacy and proper sample size^[41]. However, prospectively collected information on economic costs require ensuring

proper information extraction from source documents, leading to difficulties in conducting trials aimed at investigating both treatment efficacy and related costs. Besides, in the planning phase another challenge is represented by the sample size, considering that the statistical power adequate to test the main study end-point may be not sufficient to address also economic questions. Moving beyond RCTs, it is even more difficult to gather sufficient information on treatment direct and indirect costs^[42].

There are several kinds of economical evaluations for comparative evaluation of treatments. The two most used in clinical settings are the cost-effectiveness analysis (CEA) and the cost-utility analysis (CUA), both used when the interventions being assessed are not of equal effectiveness. CEA and CUA are aimed at comparing the effectiveness and costs of two (or more) interventions and usually the comparison measure is expressed in terms of ratio (Incremental Cost Effectiveness or Cost-Utility Ratio, generically referred to as ICER), where the denominator is the gain in effectiveness of an intervention *vs* its comparator and the numerator is the differential cost. Since health is a function of both length and quality of life, in CUA the outcome measure captures both survival and health-related quality of life. The latter is measured by means of the quality adjusted life year (QALY). QALYs are calculated by multiplying survival time by an utility weight to adjust for the health-related quality of life experienced during that survival time.

Formal economic evaluations of adjuvant therapy for GC are very few. Earle *et al*^[43] performed a systematic review of CUA applications in oncology; from 1975 and 1997 they found 40 CUAs pertaining to cancer and none to GC. *Health Technology Assessment (HTA)* has published a number of reviews on economic analyses of adjuvant therapy, mainly in terms of costs-effectiveness evaluations. The majority of the studies were related to breast cancer, colorectal cancer, and lung cancer^[44-46], but none of them has evaluated GC adjuvant treatments.

In the study by Wang *et al*^[47] a cost-effectiveness analysis by Hisashige *et al*^[48] of adjuvant chemoradiotherapy for resected GC was performed based on the favourable results of the Intergroup 0116 trial^[36]. The costs of adjuvant therapy accounted for included those for radiotherapy, chemotherapy and toxicity management. Carrying out the analyses out from a payer's perspective (3% discount rate, lifetime time horizon), it was estimated an ICER of \$38400/QALY, *i.e.* one would expect to gain one more year of life lived in perfect health (QALY) for each additional \$38400 spent when treated with chemoradiotherapy.

Recently, the results of a cost-effectiveness analyses by Hisashige *et al*^[48] evaluating S-1 adjuvant chemotherapy were published, using as evidence of effectiveness the results of the ACTS-GC trial^[25]. They included the costs incurred for resources used during the trial and subsequent follow-up, including costs of adverse events and recurrences, being the latter the major component

in each of the two groups. The analyses were carried out from a payer's perspective with a 3% discount rate. Over a lifetime time horizon, the mean QALYs per patient were greater in the S-1 arm than in the surgery arm (8.65 *vs* 7.41). On the other hand, the S-1 arm incurred greater costs than the surgery arm (mean costs per patient: \$13057 *vs* \$9346). The ICER was \$3016 per QALY gained. Braithwaite *et al*^[49] noticed that such ICER estimate was far below the Japan threshold of willingness to pay for additional QALY (from \$53000 to \$56000), very far from the threshold of \$109000/QALY suggested by a recent review, and could be ranked to the top of the league table of cost-utility in oncology^[43]. In the latter table, the Hisashige *et al*^[48] ICER estimate ranked immediately before a study of second line treatment with docetaxel *vs* paclitaxel for patients with metastatic breast cancer (ICER = \$4100/QALY)^[50], and also before a study of adjuvant chemotherapy *vs* surgery alone in Duke's B or C colorectal cancer patients (ICER = \$8100/QALY)^[51].

The issue of between study variability of ICER estimates is a current problem, especially because the choice of a threshold value for considering a treatment as cost-effective is depending on such variability. Hisashige *et al*^[48] estimated ICER was about 8% the value reported in the Wang *et al*^[47]. However, the two studies differentiate in many aspects; for instance, methodology, treatments administered and, besides, they have been performed in different locations, *i.e.*, United States and Japan, respectively. Location is one of most significant factors related to the ICER variability. The review by Bell *et al*^[52] examined cost-utility studies published between 1976 and 2001, 15% of which concerned neoplastic diseases. Most analyses reported favourable ICERs, which were statistically associated with location of the study (Europe, United States, Other), methodological quality (low, medium, high), and sponsorship (non-industry, industry, not specified). In particular, the likelihood to report ICERs below \$20000/QALY was two times more in studies industry sponsored than non-industry sponsored. Moreover, the studies conducted in Europe and the US rather than elsewhere were less likely to find ICERs below \$20000/QALY.

As noticed by Cleemput *et al*^[53], it is difficult to define a single ICER threshold value to be used as a policy-making tool, because it depends on many elements: who is making the decision, what the purpose of the analysis is, what the available resources are, thus different countries or studies reach disparate conclusions^[54]. Ternouth *et al*^[55] studied the trends in accepted ICER thresholds by disease type considering all published HTA appraisals from 2005 to 2010. Findings from Great Britain revealed that most accepted treatments have an ICER of about \$49000, but accepted ICERs for malignant disease cluster at a higher level, up to about \$102000. Data from Australian websites highlighted that for malignant disease the threshold tended to double.

Based on the above findings, the Wang *et al*^[47] ICER

of \$38400/QALY appears well in line with the Western studies and it is well below the thresholds accepted for malignant disease.

Prognostic and predictive factors

Prognostic factor are clinical or biologic characteristic measured at diagnosis proved to be associated with patients' prognosis (*i.e.*, recurrence rate, death rate, or other clinical outcomes) independently of treatment; they may be utilized for stratifying patients according to their risk with the aim of selectively administer adjuvant systemic treatments. Predictive factor are able to predict the likely benefit from treatment, either in terms of tumor shrinkage or survival, and can be utilized for identifying subpopulations of patients who are most likely to benefit from treatment. In summary, prognostic factors define the effects of patient or tumor characteristics on patient's outcome, whereas predictive factors define the effect of treatment on tumor^[56].

The prognostic stratification may be more effective when more factors are combined in a unique prognostic index. In two previous works of ours^[57,58] we have modified an existing index designed for prognostic classification of GC patients undergoing curative resection^[59]. Based on patient's age, tumor site, extent of wall invasion and nodal status, the original index classified patients in three prognostic categories: group I (5-year OS > 70%), group II (OS 30%-69%) and group III (OS < 30%). In the modified index we introduced the 1997 American Joint Commission on Cancer 4-level classification of nodal stage^[60]. The modified index was also internally and externally validated.

More advanced and complex tools are nowadays implemented for estimating patients' outcome, such as nomograms. One of the nomogram advantages is that it is possible to derive a "point" prediction of patient prognosis and, also, that there is no need to categorize continuous variables, such as patient's age or tumor size. One example in GC is the nomogram developed by Kattan *et al*^[61] which allows predicting the survival probability of GC patients up to nine years after R0 resection; the predictions were based on the following prognostic factors: patient's age and gender, tumor size, tumor primary location, tumor histology, depth of tumor invasion, percentage of positive nodes, percentage of negative nodes. Both the prognostic index^[57-59] and the nomogram^[61] were based on established clinical prognostic factors. However, such tools can potentially be improved by including powerful prognostic/predictive biomarkers.

Biomarker have great potential for use in clinical oncology; they can be different types of molecular entities (such as DNA, RNA or proteins), detected in different tissues or body fluids and associated with a disease process.

Many biomarkers are being evaluated in order to establish prognostic or predictive factors in GC and several have been identified for their potential key role, but their clinical use remains controversial^[62,63]. Indeed, both in

the setting of a single biomarker and of a multimarker predictive signature summarized by a categorical measure, the development and validation studies must be carefully designed. For prognostic biomarkers, provisional supportive data is possible through small retrospective studies, but it is difficult to achieve robust multi-site validation. For instance, Warneke *et al*^[64] investigated several biomarkers in a retrospective series of about 500 patients and some (*KRAS* mutation, persistent *H. pylori* infection, Mucin 2 and PIK3CA) were found to be associated with patient survival. Bria *et al*^[65] proposed a risk classification system comprising adenomatous polyposis coli gene, Fhit and HER2, together with 5 clinicopathological parameters. An external validation is warranted before applying the model in a clinical setting.

Our research group is conducting an ancillary study of the ITACA-S trial^[50], aiming at identifying the prognostic role of prospectively determined biomarkers on primary GC tissue. Among several candidates, our preliminary data showed that osteopontin (OPN) immunohistochemical expression is significantly associated with RFS and OS. Six-year RFS was 49.7%, in OPN negative group; 34.0% in OPN positive-focal and 22.9% in OPN positive-extended ($P < 0.001$). The corresponding figures for OS were 53.0%, 43.2% and 34.2% ($P = 0.002$). OPN was confirmed as significant prognostic factor also at multivariable analysis ($P = 0.001$ for RFS and 0.014 for OS), independently of treatment^[66].

Predictive biomarkers validation must be prospective in nature and requires more extensive data; the obvious strategy would be to conduct a properly designed RCT to test a biomarker by treatment interaction^[67]. In recent years, many molecular target agents have been investigated; however, at the moment no molecular biomarkers other than Human epidermal growth factor receptor type-2 (HER-2) for trastuzumab-based treatment^[68] have been validated.

Surrogate endpoints

In some situations, the end point of interest is expected to occur far into the future, making RCTs using such an end point infeasible. A surrogate end point is a substitute for the main clinical end point and potentially enable a more rapid assessment of intervention effectiveness, and, at times, with greater reliability and accuracy than classic end points such as survival. A surrogate end-point may be a different clinical end-point but also biomarkers may be employed as surrogate nonclinical end points in proof-of-concept studies. Surrogate end points are challenging to validate, and require data demonstrating both that the surrogate is prognostic for the true end point independently of treatment and that the effect of treatment on the surrogate reliably predicts its effect on the true end point^[67]. The statistical validation of biomarkers surrogacy presents major problems than validation of clinical surrogate end-points. Indeed, the supportive data for prognostic biomarkers is possible even through small retrospective studies, but it is more

difficult to demonstrate that the effect of treatment on the surrogate correlates with that of the true end point.

As regards GC, no biomarkers have been demonstrated as good surrogate end-point for OS. A meta-analysis by Oba *et al*^[69] examined the use of a clinical end point, *i.e.*, DFS as a surrogate end point for OS in adjuvant trials of GC. The Authors used the data achieved in a previous patient-level meta-analysis of theirs^[10] using the 14 RCTs in which DFS information could be retrieved, and demonstrated that DFS is an appropriate surrogate for OS in studies of GC in the adjuvant setting. The study also estimated the “surrogate threshold effect” (STE), defined as the minimum treatment effect on DFS necessary to predict a nonzero effect on OS, equal to 0.92; a future trial would require the HR CI upper limit (UL) for DFS to fall below 0.92 STE to predict a nonzero effect on OS. The association between 5-year OS and 4 or 5-year DFS was good; however, at 2 and 3 years, the number of DFS events did not allow obtaining precise estimates of STE. However, considering for instance the CLASSIC trial^[27,28], in the 5-year analysis UL was $0.72 < 0.92$; moreover, the 3-year DFS and 5-year OS estimates were super impossible, both in terms of rates and HRs, thus giving support to the establishment that XELOX effect on 3-year DFS reliably predicts that on 5-year OS.

CONCLUSION

The role of adjuvant chemotherapy is now clearly established in patients with resected GC. Future studies are needed to clarify the roles of various chemotherapy combinations and the ideal dosing schedule and to determine which subgroups of patients obtain a significant treatment benefit. Despite significant advances in treatment, mortality from GC remains high, and preventing this disease through global public screening programs is of paramount importance. Medical oncologists should keep an open mind, and individual treatment decisions should be reached after an assessment of patient suitability for adjuvant chemotherapy and after a full discussion of the risk-benefit profile. In fact, the appropriate selection of patients for adjuvant therapy depends largely on performance status and accompanying co-morbid conditions. Treatment of the elderly patient with GC a frequently debated topic. Most recent opinions suggest that physiologic (not chronologic) age should dictate which patients are most appropriate for therapy. Whether this may extend to the adjuvant setting would require prospectively designed RCTs. Molecular biomarkers could better identify which patients should be treated with, or spared by, chemotherapy and which drugs should be better used (assuming a differential sensitivity to a particular cytotoxic agent or regimen). This could help clinicians to increase the therapeutic index of adjuvant treatment and avoid potentially harmful treatment to patients who are not likely to gain a significant benefit. However, most available studies were limited by the small sample size and retrospective nature, with consequent methodologi-

cal limitations, and difficult in distinguishing the predictive or prognostic nature of analyzed factors. Finally, in view of the evidence of benefit from trastuzumab-based chemotherapy in patients with metastatic, HER-2 positive GC^[68], the addition of molecularly targeted agents to chemotherapy seems to be a logical next step to improve outcomes in the adjuvant setting.

Neoadjuvant chemotherapy has recently received increasing attention in an attempt to increase the rate of complete tumor resection, to combat systemic metastases, and to prolong survival in patients with GC. The available data indicate that neoadjuvant chemotherapy is feasible, does not increase post-operative morbidity and mortality, and it is able to increase the rate of R0 resection. This finding appears to translate into a survival benefit for those patients who respond to chemotherapy and have subsequent complete tumour resection. Randomized, controlled, prospective trials are therefore clearly warranted in order to compare neoadjuvant or perioperative chemotherapy with adjuvant treatment.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Role of human epidermal growth factor receptor 2 in gastric cancer: Biological and pharmacological aspects

Jan Trøst Jørgensen

Jan Trøst Jørgensen, Dx-Rx Institute, 3480 Fredensborg, Denmark

Author contributions: Jørgensen JT contributed solely this paper. Correspondence to: Jan Trøst Jørgensen, MSc Pharm, PhD, Dx-Rx Institute, Baunevaenget 76, 3480 Fredensborg, Denmark. jan.trost@dx-rx.dk

Telephone: +45-40-747846 Fax: +45-48-480528

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Abstract

Amplification of the human epidermal growth factor receptor 2 (*HER2*) gene and overexpression of the HER2 protein is found in 15%-20% of patients with gastric and gastroesophageal junction cancer. The degree of HER2 overexpression and amplification varies with the location of the carcinoma, with higher expression in the gastroesophageal and proximal parts compared to the distal parts of the stomach. Further, HER2 overexpression and amplification also seems to be related to the Lauren histological classification, with higher levels found in the intestinal phenotype compared to the diffuse and mixed types. The prognostic properties of HER2 overexpression and amplification are still under debate, but a large number of studies seem to indicate that HER2 is a negative prognostic factor. The usefulness of HER2 targeted therapy in gastric cancer was demonstrated in the ToGA trial, where HER2-positive patients with advanced gastric and gastroesophageal junction adenocarcinoma were randomized to receive 5-FU/capecitabine and cisplatin, either alone or in combination with trastuzumab. A statically significant gain in overall survival was seen in patients who received the combined treatment of trastuzumab and chemotherapy. Patients with a strong overexpression of the HER2 protein (IHC3+) specifically benefited from the treatment, with a median overall survival of 17.9 mo. As a consequence of the positive results of the ToGA trial, patients

with advanced gastric or gastroesophageal junction adenocarcinoma are now routinely tested for HER2. The ToGA trial must be characterized as a landmark in the treatment of gastric cancer and it has paved the way for a number of new HER2 targeted compounds such as pertuzumab, ado-trastuzumab emtansine, lapatinib, afatinib, and dacomitinib, which are currently undergoing phase II and III clinical testing. Overall, this review will discuss the current status of HER2 in gastric and gastroesophageal junction cancer and the future direction in relation to HER2 target therapy.

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Key words: Human epidermal growth factor receptor 2; Gastric cancer; Prognostic; Companion diagnostics; Trastuzumab; Pertuzumab; Ado-trastuzumab emtansine; Lapatinib

Core tip: Amplification of the human epidermal growth factor receptor 2 (*HER2*) gene and overexpression of the HER2 protein can be detected in 15%-20% of patients with gastric and gastroesophageal junction (GEJ) cancer. Recently, HER2 has proven to be an important target for treatment with trastuzumab in these patients, and a positive HER2 status seems to possess both prognostic and predictive properties. A number of new compounds directed towards HER2 and other members of the HER family is currently under development. This review will discuss the current status of HER2 in gastric and GEJ cancer and the future direction in relation to HER2 target therapy.

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INTRODUCTION

In gastric and gastroesophageal junction (GEJ) cancer, human epidermal growth factor receptor 2 (HER2) overexpression has become an important selective biomarker for treatment with trastuzumab (Herceptin®, Roche/Genentech)^[1]. The gene for the HER2 protein (also known as ErbB-2, c-erbB2, or Her2/neu) is a proto-oncogene located on the chromosome 17q. This gene encodes a 185-kDa transmembrane tyrosine kinase receptor protein, which is a member of the HER family that consists of HER1 (EGFR), HER2, HER3, and HER4. HER2 forms both homo- and heterodimers and serves as a critical dimerization partner for other members of the HER family, and leads to activation of downstream signaling pathways associated with cell proliferation, differentiation, survival, and angiogenesis^[2]. Amplification of the *HER2* gene and overexpression of HER2 in gastric cancer was first described in 1986^[3-5], and since then a large number of studies has confirmed these findings^[6].

Gastric cancer is the fourth most commonly diagnosed cancer and the second most common cause of cancer-related death worldwide^[7]. Despite some advances in the prevention and treatment of the disease, the 5-year survival still remains around 20%-25% in most parts of the world. Although the incidence of gastric cancer is declining, the prognosis for the disease remains poor. The poor survival rate is mainly explained by the advanced stage of the disease at the time of diagnosis. If screening for gastric cancer was performed, as in Japan, the tumors could be detected at an earlier stage and thus surgical resection performed, which has shown to increase the 5-year survival significantly^[8]. When the disease becomes metastatic the treatment is largely palliative, and different combinations of chemotherapy have resulted in a median overall survival of 8-10 mo^[9]. Based on data from the ToGA trial, trastuzumab, in combination with chemotherapy, was approved in 2010 for treatment of patients with HER2 overexpressing metastatic gastric or GEJ cancer, and thus became the first targeted anti-cancer drug for treatment of this serious disease^[10,11]. This short review will discuss HER2 status as a prognostic and selective biomarker in gastric and GEJ cancer, as well as current and future HER2 directed therapies.

HER2 AND GASTRIC CANCER

Different slide-based assays are available for the detection of overexpression of the HER2 protein, which is measured by immunohistochemistry (IHC), or amplification of the *HER2* gene, which is measured by fluorescence *in situ* hybridization (FISH) or other ISH methods. Examples of a positive HER2 status by IHC and FISH are shown in Figure 1. Due to differences in tumor biology, HER2 testing in gastric cancer differs from breast cancer. The gastric cancer tissue more frequently shows HER2 heterogeneity and incomplete membrane staining, and as a consequence of this a specific gastric cancer testing protocol has been developed^[12,13]. Based on the results

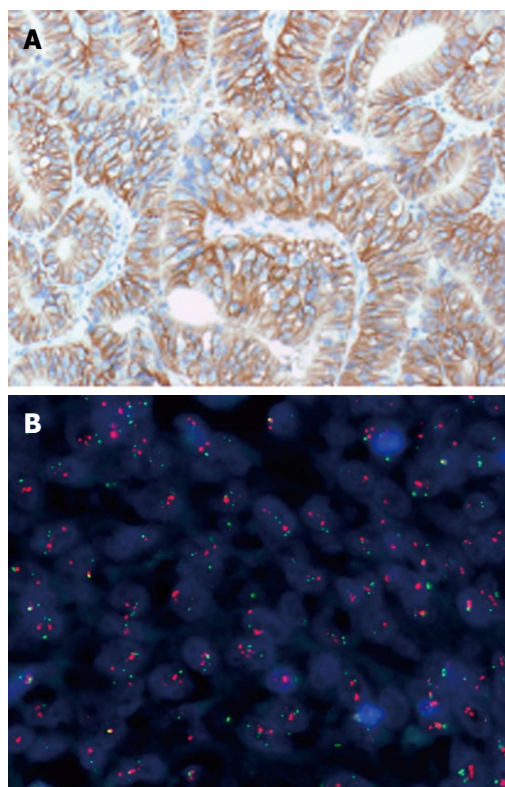


Figure 1 Human epidermal growth factor receptor 2 positive gastric adenocarcinoma. A: Immunohistochemistry (HercepTest™, Dako); B: Fluorescence *in situ* hybridization (FISH) (Human epidermal growth factor receptor 2 FISH pharmDx™ Kit, Dako).

from the ToGA trial, which will be discussed later, IHC is the primary test in gastric and GEJ cancer, with FISH being used as a reflex test in cases of an equivocal IHC2+ result. In Table 1 are shown the interpretation and scoring guideline for the HercepTest™ (Dako), which, together with the *HER2* FISH pharmDx™ Kit (Dako), are the only companion diagnostic assays that are currently approved by the United States FDA in relation to testing of gastric and GEJ cancer patients for whom treatment with trastuzumab is under consideration. The reason for this is that these two assays were those used to select the patients enrolled in the ToGA trial^[10]. As HER2 positivity in the ToGA trial was defined as being either IHC3+ or FISH+ and both tests were performed on almost all patients, the United States FDA requires that both assays are used in order to determine the HER2 status^[14].

The prevalence of HER2 overexpression in gastric cancer varies a lot from study to study. In a larger literature survey based on 11860 patients from 38 individually published studies, the calculated weighted mean was 17.9% (95%CI: 14.8-20.9). The corresponding range for these studies was from 4.4% to 53.4%. This survey also looked at *HER2* amplification; however, here the number of patients was somewhat lower. The prevalence estimate was based on 1597 patients from 8 different published studies and the calculated weighted mean was 12.2% (95%CI: 9.5-14.8). The corresponding range for these studies was from 8.7% to 18.1%^[6]. The explanation

Table 1 Interpretation and scoring of human epidermal growth factor receptor 2 immunohistochemistry for gastric cancer, as approved by the United States Food and Drug Administration in relation to the HercepTest (Dako)

Score	Surgical specimen staining pattern	Biopsy specimen staining pattern	HER2 overexpression assessment
0	No reactivity or membranous reactivity in < 10% of tumor cells	No reactivity or no membranous reactivity in any (or < 5 clustered) tumor cells	Negative
1+	Faint/barely perceptible membranous reactivity in ≥ 10% of tumor cells; cells are reactive only in part of their membrane	Tumor cell cluster (≥ 5 cells) with a faint/barely perceptible membranous reactivity irrespective of percentage of tumor cells stained	Negative
2+	Weak to moderate complete, basolateral or lateral membranous reactivity in ≥ 10% of tumor cells	Tumor cell cluster (≥ 5 cells) with a weak to moderate complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained	Equivocal
3+	Strong complete, basolateral or lateral membranous reactivity in ≥ 10% of tumor cells	Tumor cell cluster (≥ 5 cells) with a strong complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained	Positive

HER2: Human epidermal growth factor receptor 2.

for the large variation found in the HER2 positivity rate for the IHC studies is likely to be multifactorial, and here the difference in the populations studied may play a role. However, the most important factor is probably the use of non-standardized assays using different antibodies and the application of different scoring and interpretation criteria for the stained slides^[6].

In the screening program related to the ToGA trial, 3807 patients were screened, which makes it the largest single study conducted on the prevalence of HER2 positivity in gastric and GEJ cancer. This program showed an overall HER2 positivity rate of 22.1%, although with a large variation from country to country. The highest prevalence rate (33.2%) was found in Australia and the lowest (5.9%) in Taiwan^[11].

A number of studies have shown that HER2 overexpression and amplification are related to the Lauren histological classification, with higher levels found in the intestinal phenotype compared to the diffuse and mixed types^[6,11,15-21]. This was also confirmed in the ToGA screening program, where the HER2 positivity rate was found to be statistically significantly ($P < 0.001$) higher in the intestinal phenotype (32.2%) compared to the diffuse (6.1%) and mixed (20.4%) types^[11]. Furthermore, the degree of HER2 overexpression seems to vary with the location of the carcinoma, with higher expression in the proximal part and the GEJ compared to distal parts of the stomach^[21,22]. Again, the ToGA screening program confirmed this observation with a HER2 positivity rate of 33.2% when the cancer is located in the GEJ, compared to 20.9% when located in the stomach. Again, this difference in HER2 positivity related to tumor site was statistically significant ($P < 0.001$)^[11]. A few studies have also shown that the expression of HER2 increases with disease progression^[23-26].

TOGA TRIAL

The ToGA trial must be characterized as a landmark in the treatment of gastric cancer. Following the successful completion of the study, trastuzumab, in combination

with chemotherapy, became the first targeted drug to be approved for this indication. The study was designed as an open labeled, randomized multicenter phase III study in HER2-positive patients with histologically confirmed inoperable locally advanced, recurrent, or metastatic adenocarcinoma of the stomach or GEJ. HER2 positivity was defined as being either IHC positive (3+) or positive by *HER2* FISH (*HER2*/CEN17 ratio ≥ 2). However, both an IHC and FISH test were performed on almost all patients. After inclusion in the study, patients were randomized to receive chemotherapy (5-FU or capecitabine and cisplatin) or chemotherapy plus trastuzumab. More than 3800 patients were screened for the study and 584 of these were randomized. The primary endpoint in the study was overall survival (OS), with secondary endpoints including overall response rate (ORR) and progression free survival (PFS)^[10].

For the primary endpoint, the combination of chemotherapy plus trastuzumab was shown to be statistically superior to chemotherapy alone. The median OS increased from 11.1 to 13.8 mo ($P = 0.0046$), with a hazard ratio (HR) of 0.74 (95%CI: 0.60-0.91). The secondary endpoints of ORR and PFS showed superiority in favor of the combined treatment with chemotherapy and trastuzumab. The overall tumor response rate was 47% in combined treatment with chemotherapy and trastuzumab, compared to 35% in the group with chemotherapy alone. A pre-planned exploratory analysis looking at the effect in the different HER2 IHC categories (0, 1+, 2+, 3+) showed that the survival benefit provided by trastuzumab seemed to be dependent on the level of HER2 protein overexpression. The single subgroup of patients with the greatest survival benefit was the one with a HER2 test result of IHC3+. Here, the median OS increased to 17.9 mo for the group treated with the combination of trastuzumab and chemotherapy compared to chemotherapy alone, which achieved an OS of 12.3 mo. Overall, the survival gain for the group of patients with IHC3+ expressing tumors was nearly 6 mo. The HR for this group of patients was 0.58 (95%CI: 0.41-0.81)^[10]. The results of the subgroup analysis for the different IHC scores are

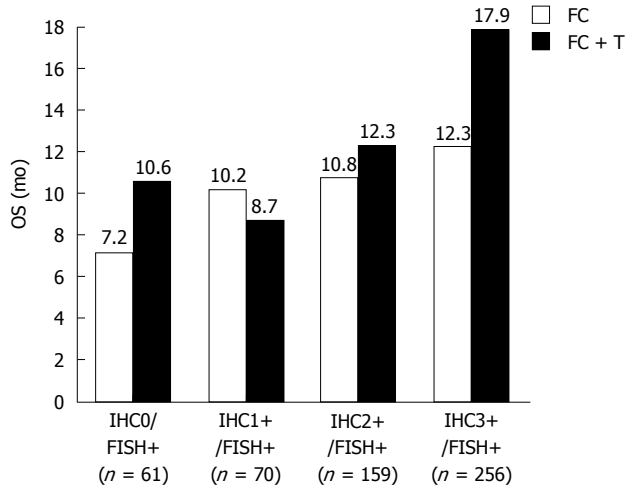


Figure 2 Median overall survival in months for the four individual human epidermal growth factor receptor 2 immunohistochemistry scores for the two treatment groups. OS: Overall survival; FC: Fluorouracil/capecitabine plus cisplatin; FC + T: Fluorouracil/capecitabine plus cisplatin plus trastuzumab^[10]; FISH: Fluorescence *in situ* hybridization; IHC: Immunohistochemistry.

Table 2 Positive human epidermal growth factor receptor 2 status by immunohistochemistry and/or fluorescence *in situ* hybridization for the patients enrolled in the ToGA trial^[10,11,27]

HER2 status	n
IHC0/FISH+	61
IHC1+/FISH+	70
IHC2+ FISH+	159
IHC3+/FISH+	256
IHC3+/FISH-	15
IHC3+/FISH no results	16
IHC no results/FISH+	7
Total	584

N: Number of patients. HER2: Human epidermal growth factor receptor 2; IHC: Immunohistochemistry; FISH: Fluorescence *in situ* hybridization.

shown in Figure 2.

Based on the information from the pre-planned exploratory analysis, a post-hoc explorative analysis was performed on a subpopulation of the originally included patients. This population comprised the patients who were IHC3+ positive or IHC2+ positive, and those who were FISH positive. A total of 446 patients fulfilled these criteria, and the median OS for the group of patients who had received chemotherapy plus trastuzumab increased to 16.0 mo compared to 11.8 mo for the patients on chemotherapy alone. The HR for this analysis was 0.65 (95%CI: 0.51-0.83). The median follow-up for all the patients in the ToGA trial was reported to be 17.1 mo^[10].

Concerning the selective properties of the two HER2 companion diagnostic assays, explorative analysis showed that the IHC test should be used as the primary test for selection of patients for treatment with trastuzumab. As shown in Figure 2, the effect of trastuzumab seems to be dependent on the degree of HER2 protein overexpression, with the best median OS in the group of

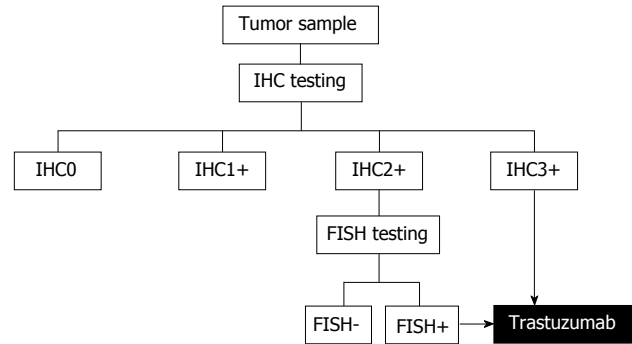


Figure 3 Human epidermal growth factor receptor 2 testing algorithm developed based on the results of the ToGA trial. Immunohistochemistry (IHC) is the primary test with reflex testing with Fluorescence *in situ* hybridization (FISH) in case of an equivocal IHC result (IHC2+)^[27].

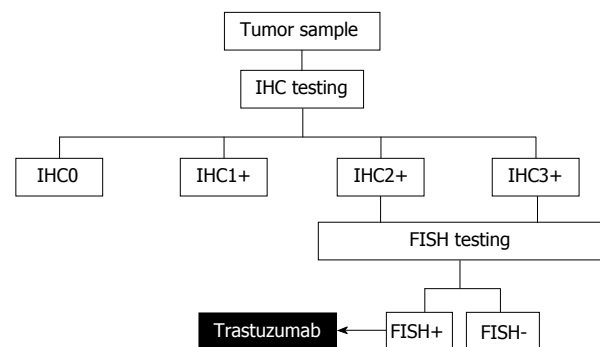


Figure 4 Human epidermal growth factor receptor 2 testing algorithm with fluorescence *in situ* hybridization reflex testing for both immunohistochemistry 2+ and immunohistochemistry 3+. This testing algorithm was recommended by the United States Food and Drug Administration in relation to approval of trastuzumab for advanced gastric cancer. FISH: Fluorescence *in situ* hybridization; IHC: Immunohistochemistry.

patients with IHC3+. Furthermore, when looking at the HER2 test results for the patients enrolled in the ToGA trial, the agreement between overexpression of the HER2 protein and amplification of the gene is found to be somewhat lower in gastric cancer than that normally observed in breast cancer. A relatively high number of HER2 FISH positive cases were found among the IHC0 and IHC1+ tumors as shown in Table 2^[10,11,27]. Based on the subgroup analyses in the ToGA trial, a specific HER2 testing algorithm was developed as shown in Figure 3^[27]. However, since nearly 95% (533/584) of the patients enrolled in the ToGA trial had tumors that were HER2 amplified, it has been argued that the criteria for treatment with trastuzumab should be both gene amplification and protein overexpression. In relation to the approval of trastuzumab for treatment of advanced gastric cancer this was, in fact, the position taken by the United States FDA, who recommended that reflex testing with FISH should be considered for both IHC2+ and IHC3+^[14]. An algorithm taking this into consideration is shown in Figure 4. A recent survey made in the United States also showed that FISH reflex testing was performed for both IHC0 and IHC1+ at some cancer centers^[28]. So, despite recommendations from both sci-

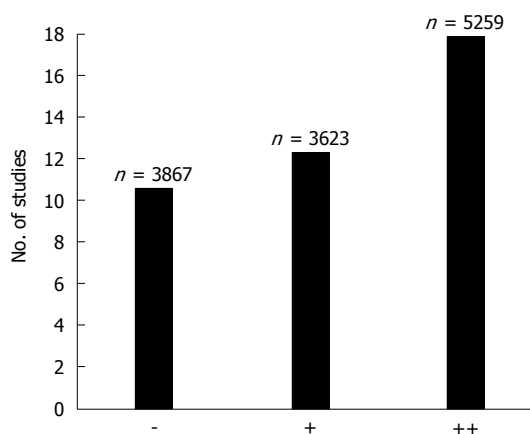


Figure 5 The number of studies and patients (*n*) in each of the three scoring categories. Symbols: Two pluses (++) indicate the strongest association with the Human epidermal growth factor receptor 2 (HER2)+ status, one plus (+) indicates a somewhat weaker association with the HER2+ status, and minus (-) indicates that no associations with the HER2+ was found^[6].

entific and regulatory sides there still seems to be no real consensus with respect to which testing algorithm to use.

HER2 AS A PROGNOSTIC MARKER

As described above, the primary analysis of the ToGA trial showed a median OS of 13.8 mo for the group of patients that received the combined treatment of chemotherapy and trastuzumab compared to 11.1 mo for the group that was assigned to chemotherapy alone. In the discussion section of the paper published in the *Lancet* in 2010, it was mentioned that the OS of 11.1 mo in the group of patients receiving chemotherapy alone was longer than expected. As a possible explanation for this, it was stated that HER2 overexpression might already be conferring a better prognosis across the groups of patients studied. However, it was also mentioned that HER2 overexpression leading to a better prognosis, is in contrast to recent studies that have showed an association between HER2-positive tumors, poor outcome, and aggressive disease. The authors further concluded that more studies were needed to address the issue of whether HER2 has an effect on prognosis in gastric cancer, and whether it confers a good or poor prognosis^[6,10].

In breast cancer, HER2 was found to be a negative prognostic factor very early on, and a number of studies have subsequently confirmed this^[2,29]. However, when it comes to gastric cancer there still seems to be no definite conclusion, despite the fact that the first studies demonstrating an association between a positive HER2 status and poor prognosis appeared more than 20 years ago^[26,30]. In order to address this issue, a systematic analysis of data from the literature was undertaken where a large number of studies on HER2 and gastric cancer were reviewed^[6]. The studies included in this analysis should fulfill the following two criteria: 1) the number of patients in each study should be ≥ 100 and the HER2 status should have been determined either by IHC or ISH, and

2) the selected articles should include an analysis of the association between the HER2 status and survival or relevant clinicopathological characteristics. Forty-two publications with a total of 12749 patients fulfilled the two criteria and were reviewed in detail. The studies described in these 42 articles were scored according to the strength of the association between the positive HER2 status and the prognostic information reported, using a three point categorical scale.

In 17 of these 42 studies (approximately 40%) an association between positive HER2 status and poor survival (++) was found, and an additional 13 studies (approximately 31%) similarly displayed a relationship with clinicopathological characteristics (+) such as serosal invasion, lymph node metastases, disease stage, or distant metastases. In the last 12 studies (approximately 29%), no association between positive HER2 status and poor survival or clinicopathological characteristics could be detected (-). Overall, 30 (71%) out of 42 studies showed an association between a positive HER2 status and poor survival and/or relevant clinicopathological characteristics. Figure 5 illustrates the number of studies and patients in each of the three scoring categories^[6].

Based on this analysis of the literature data, it was concluded that a clear trend towards a potential role for HER2 as a negative prognostic factor in gastric cancer was shown^[6], which is also in line with another recently published systematic review based on literature data^[31]. Furthermore, with reference to the publication of the ToGA trial in the *Lancet*, the data could not support the hypothesis that positive HER2 overexpression could act as a positive prognostic factor in gastric cancer^[10]. None of the articles that fulfilled the necessary criteria in the analysis supported this hypothesis. Possible confounding factors, such as a wider use of second line treatments and a possible better prognosis related to the intestinal phenotype, should be taken into consideration when interpreting the data from the ToGA trial^[32]. With regards to the latter, it is worth mentioning that approximately 75% of the patients included in the ToGA trial had tumors of the intestinal type^[10], which seems to be high compared to most of the studies reported in the analysis of data from the literature. This characteristic might have influenced the OS seen in the group of patients that received chemotherapy alone. In support of the hypothesis put forward in the *Lancet* article, one study was identified which showed that overexpression of HER2 resulted in a better prognosis compared to those who did not overexpress the protein. However, this study was not included in the analysis due to the number of patients being < 100 (thus failing one of the criteria)^[6]. Here, samples from 93 patients with advanced gastric carcinoma were investigated using IHC. Overexpression of HER2 was found in 10 patients (11%), and a multifactorial analysis showed a significantly better prognosis for those patients in relation to survival^[33]. However, after the finalization of the above described analysis of data from the literature, a relatively large study has recently been published. This study comprised 381 patients, with 78 (20%) of these being found

Phase I	Phase II	Phase III
ASLAN001		
HN781-36B		
MGAH22		
MM-111		
	Dacomitinib	
	Afatinib	
		Lapatinib
		Pertuzumab
		Ado-trastuzumab emtansine

Figure 6 Drugs targeting human epidermal growth factor receptor 2 in clinical development for treatment of gastric, esophageal, or gastroesophageal junction cancer. The individual compounds are listed according to the stage of development^[35].

Table 3 Overview of the phase II and III compounds and their targets

Compounds	Type of compound	Target(s)	Clinical phase
Dacomitinib (PF-00299804, Pfizer)	Irreversible pan-HER TKI	HER1 (EGFR) and HER2	II
Afatinib (Gilotrif, Boehringer Ingelheim)	Irreversible pan-HER TKI	HER1 (EGFR), HER2, and HER4	II
Pertuzumab (Perjeta, Roche/Genentech)	mAb	HER2 (subdomain II), HER2 hetero dimerization	II / III
Ado-trastuzumab emtansine (Kadcyla, Roche/Genentech)	ADC	HER2 (subdomain IV)	II / III
Lapatinib (Tyverb/Tykerb, GSK)	Reversible pan-HER TKI	HER1 (EGFR) and HER2	III

TKI: Tyrosine kinase inhibitors; mAb: Monoclonal antibody; ADC: Antibody-drug conjugate.

to be HER2 positive by IHC or ISH. When the HER2 status was correlated with survival data, patients with HER2 positive tumors had longer OS compared to the HER2 negative patients, however, the prognostic value disappeared in the multivariate analysis^[34].

Despite the data in gastric cancer not being as consistent as shown in breast cancer, the majority of studies seem to point towards HER2 overexpression and/or amplification as being an indicator of poor prognosis^[6]. In line with this conclusion, it has also been suggested recently that HER2 overexpression and/or amplification is a molecular abnormality that is linked to the development of gastric cancer^[32].

POTENTIAL HER2 TARGETED DRUGS IN GASTRIC CANCER

Following the initiation and success of the ToGA trial, a number of other HER2 targeted compounds have gone into clinical development for treatment of gastric, esophageal, or gastroesophageal junction cancer. These compounds represent small molecule tyrosine kinase inhibitors (TKI) and antibodies, as well as antibody-drug conjugates (ADC). Most of these compounds, together with the stage of development, are listed in Figure 6. However, in this review, emphasis will be placed on the drugs that are further advanced in clinical development (phase II and III). An overview of these compounds and their targets are given in Table 3. Phase I compounds will only be described briefly.

Phase I compounds

As is the case for the other phases, the compounds in

phase I clinical development can be divided into small molecule inhibitors and antibodies. According to ClinicalTrials.gov there are two small molecule pan HER inhibitors, ASLAN001 (Aslan Pharmaceuticals) and HM781-36B (Hanmi Pharmaceuticals), in phase I. When it comes to the antibodies, two compounds are in phase I development, the HER2 monoclonal antibody MGAH22 (MacroGenics) and the bi-specific antibody MM-111 (Merrimack Pharmaceuticals) directed towards HER2 and HER3^[35].

Dacomitinib

Dacomitinib (PF-00299804, Pfizer) is an oral pan-HER TKI. The compound irreversibly inhibits HER1 (EGFR) and HER2 tyrosine kinase, as well as blocking HER1/HER2, HER2/HER3, and HER3/HER4 heterodimerization^[36,37]. In different preclinical models, dacomitinib has shown significant growth-inhibitory effects in HER2-amplified gastric cancer cells, such as SNU-216 and NCI-N87. Furthermore, the combination of dacomitinib with chemotherapeutic agents (such as 5-FU and cisplatin) or targeted agents (such as trastuzumab) showed a synergistic effect^[36]. However, a clinical phase II study in HER2 positive (IHC3+ or FISH+) patients with advanced gastric cancer, where dacomitinib was given as monotherapy, showed a response rate of only 7.4% and an OS of 7.1 mo. The relatively modest clinical effect may be explained by the advanced stage of the disease and that the patients had been heavily pretreated^[38].

Afatinib

Afatinib (Gilotrif, Boehringer Ingelheim) is another oral irreversible pan-HER TKI that targets HER1 (EGFR),

HER2, and HER4^[39]. The compound has recently obtained FDA approval for first-line treatment of patients with metastatic non-small cell lung cancer whose tumors have tested positive for *EGFR* mutations. The FDA has also approved the Therascreen *EGFR* RGQ Kit (Qiagen), a companion diagnostics assay, for use in the detection of *EGFR* exon 19 deletions or exon 21 substitution mutations^[40]. In relation to gastric cancer, afatinib has demonstrated antitumor activity in a HER2 positive xenograft mouse model^[41]. Additionally, results from a small clinical phase II study in HER2 positive patients with esophago-gastric (EG) cancer has recently been presented. Based on data from this study, the investigators concluded that single agent afatinib showed clinical efficacy in patients with trastuzumab refractory EG cancer. However, this conclusion must be regarded as preliminary, as it was only based on data from 7 patients and more patients are expected to be enrolled in the study^[42].

Lapatinib

Lapatinib (Tyverb/Tykerb, GSK) is an oral TKI, but, in contrast to both dacomitinib and afatinib, its inhibitory effect on HER1 (EGFR) and HER2 is reversible. Lapatinib is currently approved for treatment of HER2 positive metastatic breast cancer in combination with capecitabine (Xeloda, Roche) or for HER2 positive postmenopausal women with hormone receptor positive metastatic breast cancer in combination with letrozole (Femara, Novartis)^[43]. The antitumor effect of lapatinib has been investigated in different gastric cancer cell lines, and it was shown to induce a selective and potent growth inhibition in the two HER2-amplified gastric cancer cell lines SNU-216 and NCI-N87. Furthermore, in the same model lapatinib combined with 5-fluorouracil, cisplatin, oxaliplatin, or paclitaxel showed an additive or synergistic effect^[44]. These results provide the rationale for the clinical development of lapatinib for the treatment of HER2-positive gastric cancer. A phase II clinical trial was performed in patients with unresectable gastric adenocarcinoma, although in this protocol HER2 positivity was not an inclusion criterion. A total of 47 patients were enrolled in the study and 44 received lapatinib as monotherapy until disease progression or unacceptable toxicity. The response rate was relatively modest with 5 patients (11%) having a confirmed or unconfirmed partial response. The median OS was 4.8 mo (95%CI: 3.2-7.4)^[45]. Data from the LoGIC phase III trial where lapatinib plus chemotherapy (capecitabine and oxaliplatin) was compared to chemotherapy alone in patients with HER2 positive advanced gastric, esophageal, or gastroesophageal junction adenocarcinoma has recently been presented. The median OS for the lapatinib plus chemotherapy group was 12.2 mo compared 10.5 mo for the group that received chemotherapy alone. The primary endpoint for the study with regards to HR for OS was not reached (HR: 0.91, 95%CI: 0.73-1.12, $P = 0.35$). The response rate was 53% for the combined group receiving lapatinib and chemotherapy, compared to 40% for the group receiving

chemotherapy alone. However, a pre-specified subgroup analysis in Asian patients and patients < 60 years showed a significant improvement with a HR of 0.68 and 0.69, respectively. A total of 545 HER2 positive patients were randomized in the LoGIC study^[46]. Another phase III clinical trial, TYTAN, in Asian patients with advanced gastric cancer is still ongoing. In said study, patients with HER2 amplified tumors are randomized to lapatinib plus paclitaxel or paclitaxel alone^[47]. Based on the clinical data presented so far, the future role of lapatinib in gastric cancer must be regarded as unclear.

Pertuzumab

Pertuzumab (Perjeta, Roche/Genentech) is a humanized monoclonal antibody that binds to sub-domain II of the extracellular part of the HER2 protein, thereby blocking its ability to form heterodimers with other members of the HER family, including HER1 (EGFR), HER3, and HER4. Trastuzumab also binds to the extracellular part of the HER2 protein, albeit to a different sub-domain (IV), and it does not possess an inhibitory effect in relation to dimerization of HER2 with the other HER receptors^[48,49]. Pertuzumab has a mechanism of action that is complementary to that of trastuzumab, and the combination of these two monoclonal antibodies has been demonstrated to be effective as a first-line treatment in metastatic breast cancer^[50]. This has recently led to regulatory approval of the compound for treatment of HER2 positive metastatic breast cancer in combination with trastuzumab and docetaxel. In gastric cancer, a tumor mouse xenograft model using the HER2 positive NCI-N87 cells has been used to demonstrate the preclinical antitumor activity of pertuzumab. Based on this model, a significantly enhanced antitumor efficacy of pertuzumab in combination with trastuzumab was shown compared to monotherapy with each of the two compounds. Similar antitumor efficacy was shown using another HER2 positive cell line (4-1ST), thus paving the way for the clinical development of pertuzumab in gastric cancer^[51]. Both clinical phase II and III trials in HER2 positive metastatic gastric or gastroesophageal junction adenocarcinoma have been initiated, which include the large international JACOB study. In this study, pertuzumab plus trastuzumab and chemotherapy (cisplatin, 5-FU/capecitabine) are compared to placebo plus trastuzumab and chemotherapy. It is planned that the JACOB study should enroll 780 patients at approximately 200 sites in 35 countries worldwide^[52-54].

Ado-trastuzumab emtansine

Ado-trastuzumab emtansine (Kadcyla, Roche/Genentech) is a novel ADC specifically designed for the treatment of HER2-positive cancer. It is composed of the potent cytotoxic agent DM1 (a thiol-containing maytansinoid anti-microtubule agent) conjugated to trastuzumab via a specific linker molecule. Ado-trastuzumab emtansine binds to the HER2 protein (sub-domain IV) with an affinity similar to that of trastuzumab. It is hypothesized

that after binding to the receptor protein, ado-trastuzumab emtansine undergoes receptor-mediated internalization, followed by intracellular release of DM1, which then exerts its cytotoxicity in the tumor cell^[55]. Ado-trastuzumab emtansine has been compared with lapatinib plus capecitabine in a phase III trial in HER2-positive breast cancer patients with metastatic disease. Although these patients had previously been treated with a taxane and trastuzumab, the study showed that ado-trastuzumab emtansine significantly improved PFS and OS compared to the combination of lapatinib plus capecitabine^[56]. Following the successful completion of phase III, ado-trastuzumab emtansine has recently been approved for treatment of patients with HER2-positive metastatic breast cancer who have previously received trastuzumab and/or a taxane. In gastric cancer ado-trastuzumab emtansine has been tested in a number of different preclinical *in vitro* and *in vivo* HER2 positive cell models. Using the NCI-N87 and OE-19 cells lines *in vitro*, ado-trastuzumab emtansine was found to be more effective than trastuzumab. In a mouse xenograft model using the same cell lines, a similar positive anti-tumor effect was found *in vivo*^[57]. In another preclinical study, ado-trastuzumab emtansine showed pronounced antitumor activity *in vivo* in two other HER2 expressing cell lines (SCH and 4-1ST). Furthermore, the effect of combining ado-trastuzumab emtansine with pertuzumab has also been investigated using NCI-N87 xenografted cells, and here the combination showed a significant antitumor effect, whereas the use of the individual compounds as monotherapy did not^[58]. Additionally, the positive preclinical findings led to the initiation of a clinical development program, and currently a phase II/III trial has been initiated in order to evaluate efficacy and safety of ado-trastuzumab emtansine compared to taxane treatment in patients with HER2-positive advanced gastric cancer^[59].

CONCLUSION

The ToGA trial must be regarded as a landmark, not only did the study show that trastuzumab is effective in treating HER2 overexpressing gastric cancer, but it also gave us important information on the pathophysiological characteristics of the disease. As a consequence, HER2 testing of patients with advanced gastric cancer and treatment with trastuzumab has now become standard in most countries. The ToGA trial also demonstrated that overexpression of HER2 in gastric cancer possesses selective properties in relation to treatment with trastuzumab in a similar manner to what is known from breast cancer. When it comes to prognostics properties there still seems to be some discussion about the value. However, a recent large systematic analysis of data from the literature showed a clear trend towards a potential role of HER2 as a negative prognostic marker in gastric cancer. Hopefully, future additional data will clarify this issue. Despite the controversies around the prognostic properties of HER2, there seems to be much more consensus

regarding the importance of the receptor as a therapeutic target in gastric cancer. A number of new compounds targeting HER2 and other members of the HER family are under development, and several of these have already reached phase III clinical studies. Pertuzumab and ado-trastuzumab emtansine, as well as some of the small molecule pan HER inhibitors, might be potentially useful for HER2 positive gastric cancer patients that have developed resistance to trastuzumab.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Targeting receptor tyrosine kinases in gastric cancer

Asahiro Morishita, Jian Gong, Tsutomu Masaki

Asahiro Morishita, Jian Gong, Tsutomu Masaki, Department of Gastroenterology and Neurology, Kagawa University School of Medicine, Kagawa 761-0793, Japan

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Correspondence to: Asahiro Morishita, MD, PhD, Department of Gastroenterology and Neurology, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki-cho, Kida-gun, Kagawa 761-0793, Japan. asahiro@med.kagawa-u.ac.jp

Telephone: +81-87-8912156 Fax: +81-87-8912158

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Abstract

Molecularly targeted therapeutic agents are constantly being developed and have been shown to be effective in various clinical trials. One group of representative targeted oncogenic kinases, the receptor tyrosine kinases (RTKs), has been associated with gastric cancer development. Trastuzumab, an inhibitor of ERBB2, has been approved for the treatment of gastric cancer, although other receptor tyrosine kinases, such as epidermal growth factor receptor, vascular endothelial growth factor, platelet-derived growth factor receptor, c-Met, IGF-1R and fibroblast growth factor receptor 2, are also activated in gastric cancer. The promising results of the trastuzumab clinical trial for gastric cancer resulted in the approval of trastuzumab-based therapy as a first-line treatment for human epidermal growth factor receptor 2-positive patients. On the other hand, the trial examining bevacizumab in combination with conventional chemotherapy did not meet its primary goal of increasing the overall survival time of gastric cancer patients; however, a significantly higher response rate and a longer progression-free survival were observed

in the bevacizumab arm of the trial. Other clinical trials, especially phase III trials that have tested drugs targeting RTKs, such as cetuximab, panitumumab, gefitinib, erlotinib, figitumumab, sorafenib, sunitinib and lapatinib, have shown that these drugs have modest effects against gastric cancer. This review summarizes the recent results from the clinical trials of molecularly targeted drugs and suggests that further improvements in the treatment of advanced gastric cancer can be achieved through the combination of conventional drugs with the new molecularly targeted therapies.

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Key words: Receptor tyrosine kinases; Gastric cancer; Epidermal growth factor receptor; Trastuzumab; Cetuximab; Lapatinib; Panitumumab; Erlotinib; Bevacizumab

Core tip: Since the finding of receptor tyrosine kinases (RTKs) about thirty years ago, its functions have been examined over the years as key regulators of proliferation, differentiation, and metastasis. Several RTKs are activated in advanced gastric cancer (AGC) and various RTK inhibitors have been developed as tailored therapy. The results of recent clinical trials evaluate the effectiveness of targeting RTKs. Unfortunately, recent progress in the development of RTK-targeted therapy for AGC patients has been modest. To provide maximal therapeutic benefits, well-designed clinical trials and combinations with appropriate drugs are required. In addition, new predictive biomarkers are immediately obliged to guide the selection of a drug-sensitive patients' population.

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INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death worldwide^[1,2]. The high mortality rate is due to the lack of effective therapy for advanced stages of the disease. Conventional therapy options for gastric cancer include surgery, chemotherapy, radiation therapy and combination treatments. In the early stages, the disease can often be cured through complete surgical removal of the tumor^[3]. However, because gastric cancer results in few symptoms during the early stages, most patients are usually diagnosed after the cancer has progressed to an advanced stage. Moreover, even after surgical resection, tumors will recur in many patients, resulting in short survival times. The 5-year survival rate of gastric cancer has remained at 20%-25% in the western world^[4]. Therefore, the high mortality rate underscores the need for effective medical treatments for patients with advanced stages of gastric cancer^[3].

Receptor tyrosine kinases (RTKs) consist of ligand-binding extracellular domains which identify the subfamilies of RTKs, a transmembrane domain and a tyrosine kinase motif, and the activation of these kinases has been shown to play an important role in the control of many fundamental process, such as growth, differentiation, adhesion, migration and apoptosis^[5-8]. The activation and overexpression of RTKs were initially reported in various cancers^[9,10]. Currently, RTK inhibitors have been validated through clinical trials, and some agents have received regulatory approval, such as trastuzumab for the treatment of advanced breast cancer^[10], gefitinib for non-small cell lung carcinoma^[11] and cetuximab for metastatic colon cancer^[12]. Unlike other solid tumors, which are predominantly associated with specific signaling pathways, such as the HER-2 pathway in breast cancer, the genetic and molecular pathogenesis of gastric cancer may be more complex^[13,14]. In gastric cancer, although the amplification of RTKs, such as ErbB2, c-Met and fibroblast growth factor receptor (FGFR) 2, is associated with cancer progression, the only approved inhibitor is trastuzumab, an ErbB2-targeting antibody^[15]. Trastuzumab came into use after the release of promising efficacy results from the Trastuzumab for Gastric Cancer (ToGA) trial^[16]. Additionally, other RTKs have also emerged as potential targets for the future treatment of gastric cancer.

In this review, we delineate the underlying molecular basis of the RTK pathways and summarize the current results of the clinical phase III trials (Table 1) and ongoing clinical trials that are targeting RTKs in patients with gastric cancer. Additionally, we also present future possibilities for the improvement of RTK inhibitor efficacy and for the identification of new strategic targets for gastric cancer treatment.

RTKS IN CELLULAR SIGNALING

RTKs are transmembrane glycoproteins that are activated by binding to their cognate ligands, resulting in the

Table 1 Phase III trials of targeted Receptor tyrosine kinases in advanced gastric cancer

Clinical trial	Line of treatment	RTK inhibitor	Chemotherapy	Status
ToGA	First	Trastuzumab	FP or XP	Completed
AVAGAST	First	Bevacizumab	XP	Completed
EXPAND	First	Cetuximab	XP	Completed
REAL-3	First	Panitumumab	EOX	Completed
LoGIG	First	Lapatinib	OX	Ongoing
TYTAN	Second	Lapatinib	T	Ongoing

ToGA: Trastuzumab for gastric cancer; AVAGAST: Avastin in gastric cancer; EXPAND: Erbitux in combination with xeloda and cisplatin in advanced esophago-gastric cancer; REAL-3: Randomized ECF for advanced and locally advanced esophagogastric cancer 3; LoGIG: Lapatinib optimization study in ErbB2 (HER-2) positive gastric cancer; TYTAN: Lapatinib (Tykerb) with paclitaxel (Taxol) in Asian ErbB2+ (HER+) gastric cancer study; F: 5-fluorouracil; P: Cisplatin; X: Capecitabine; E: Epirubicin; T: Paclitaxel.

phosphorylation of tyrosine residues on the receptor and downstream signaling proteins. Fifty-eight of the 90 known protein tyrosine kinases are also receptors^[5]. Various RTKs have been normally associated with intracellular signal transduction including growth, differentiation, adhesion, migration, and apoptosis (Hubbard and Till). In various types of cancer, many signaling pathways including cell proliferation, differentiation, and metabolism pathways, are activated by RTK dimerization^[6,17]. In general, RTK activation occurs through ligand-induced dimerization, in which a bivalent ligand and two receptor molecules form a dimeric complex^[18]. Two main processes are required for RTK activation: the enhancement of the intrinsic catalytic activity and the creation of binding sites to recruit downstream signaling proteins. Importantly, tyrosine autophosphorylation is critical for both of these processes. Autophosphorylation of tyrosine residues located in the activation loop of the kinase domain stimulates kinase activity, whereas autophosphorylation in the juxtamembrane, kinase insert and carboxy-terminal regions generates docking sites for modular domains that recognize the phosphotyrosine residues in specific sequences^[19].

RTKS IN GASTRIC CANCER

The RTK family consists of 58 kinases, and each is characterized by ligand-binding extracellular domains which identify the subfamilies of RTKs, a transmembrane domain and a tyrosine kinase motif^[5]. Of those kinases, the known RTKs are separated into 21 families, such as the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) and FGFR families, which are characterized by similar structures and the potential of dimerization in gastric cancer^[20]. Additionally, it has been reported that the expression of platelet-derived growth factor (PDGF) and platelet-derived growth factor receptor (PDGFR) are involved in gastric cancer growth^[21]. Each RTK inhibitor is dia-

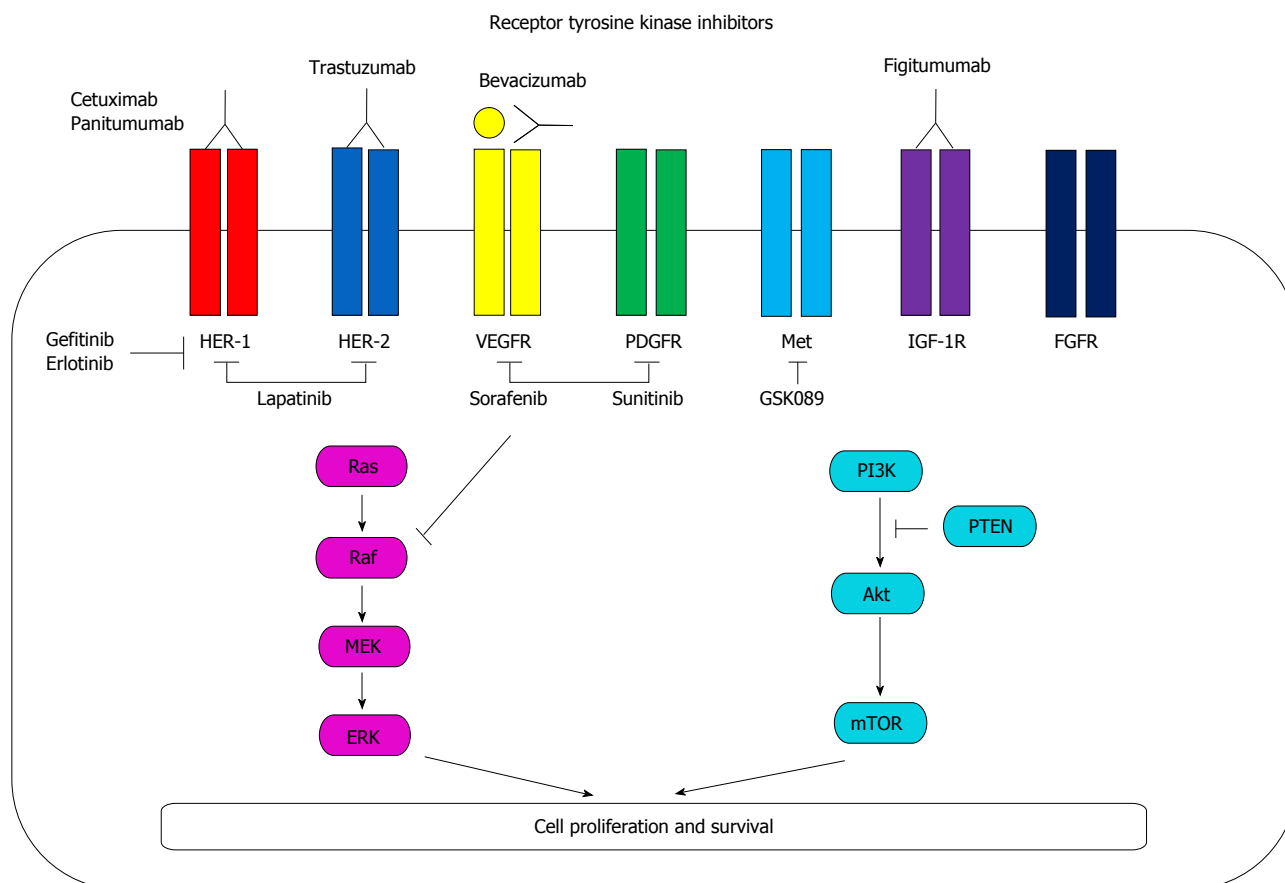


Figure 1 Receptor tyrosine kinase inhibitors. VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; IGF-1R: Insulin-like growth factor 1 receptor; FGFR: Fibroblast growth factor receptor; HER: Human epidermal growth factor receptor; PTEN: Phosphatase and tensin homolog.

grated in Figure 1 and inactivates various RTKs. Among these RTK inhibitors, monoclonal antibodies, such as cetuximab and panitumumab, trastuzumab and figitumumab directly bind to each RTK and inhibit its signaling. Bevacizumab also binds vascular endothelial growth factor (VEGF) and inhibit VEGFR signaling. Gefitinib and erlotinib competes with the binding of ATP to the tyrosine kinase domain of EGFR and lapatinib blocks phosphorylation of HER-1 and HER-2. Sorafenib inhibits the enzyme RAF kinase and VEGFR-2/PDGFR-beta signaling cascade. Sunitinib also blocks VEGFR-2/PDGFR-beta and c-kit. In addition, GSK089 inhibits c-Met and blocks its signaling (Figure 1).

Common alterations and mutations of RTKs have been identified in gastric cancer. Interestingly, Deng *et al.*^[22] showed that druggable alterations in RTKs occurred in 37% of gastric cancer patients; the most frequently amplified RTK was FGFR2 (9.3%), followed by KRAS (8.8%), EGFR (7.7%) and ERBB2 (7.2%)^[22]. Furthermore, the RTK amplification status was shown to be an independent marker of poor prognosis in gastric cancer patients according to Cox multivariate analysis, and this result was independent of chromosomal instability.

Recently, together with Tyro3, Axl and Mer receptor tyrosine kinases are aberrantly expressed in numerous human cancers. It has been reported that Axl and Mer inhibition constitutes a novel therapeutic strategy that may

enhance the efficacy of standard chemotherapy in glioblastoma multiforme^[23], non-small cell lung cancer^[24,25] and breast cancer^[26]. In gastric cancer, combination of Axl and Mer expressions correlated inversely with patient prognosis^[27]. Inhibition of these RTKs may provide potential targets for AGC.

In our recent study, the levels of EGFR, ErbB2, FGFR1, FGFR2, insulin R and EphA4 were increased in human gastric cancer tissues compared with normal mucosa according to a protein array^[28]. Additionally, ErbB2 was most activated in the human gastric cancer cell lines MKN45, MKN74, MKN1 and MKN7^[28]. Therefore, these findings suggest that these molecules may be potential targets for selective therapy in gastric cancer. We will summarize the clinical trials for the newly established modalities related to RTK expression in gastric cancer.

RTK TARGETED THERAPY

The insufficient effect of chemotherapy on advanced gastric cancer has resulted in the development of new biological therapies that modulate various targets of signal transduction pathways that are overexpressed in gastric cancer. A large number of molecularly targeted drugs have been clinically developed to inhibit angiogenesis as well as to specifically inhibit the human epidermal growth factor receptor, platelet-derived growth factor receptor

and c-MET receptor.

TARGETING HER-2

HER-2 (ErbB2) is a member of the ErbB2/HER family, which is comprised of four receptors including HER-1 (EGFR), HER-2, HER-3 and HER-4. Of these receptors, the targeting of HER-2 has been the most successful for the treatment of advanced gastric cancer. HER-2 overexpression is observed in 10%-38% of gastric cancer patients^[29-31]; however, the effect of HER-2 expression on the prognosis of AGC remains controversial^[32-35]. Recently, Bang *et al.*^[16] reported that HER-2-positive patients using Immunohistochemistry (IHC) scoring system had a superior outcome when treated with conventional chemotherapy with trastuzumab, which selectively binds to HER-2 and inhibits its downstream signaling pathway, in the ToGA trial. Although this result suggests HER-2 is not a negative prognostic factor, it might be confounded by various factors, such as the second line therapy or intestinal subtype. Additionally, IHC scoring system in gastric cancer is different from that of breast cancer^[36].

First-line trastuzumab-based trials in AGC patients were reported in 2006^[37]. Prior to the ToGA trial^[16], three phase II trials evaluating the effectiveness of trastuzumab in AGC patients were presented. The results from the first phase II trial of trastuzumab combined with cisplatin and docetaxel showed that a radiological response was observed in 4/5 HER-2-positive patients (defined as IHC3+ or FISH+) with metastatic gastric cancer or gastroesophageal junction carcinoma patients^[37]. In the second phase II trial, HER-2-positive (defined as IHC2+ and FISH+ or IHC3+) AGC or gastroesophageal junction adenocarcinoma patients were treated with 75 mg/m² cisplatin and trastuzumab (8 mg/kg loading dose followed by 6 mg/kg for future cycles) every 21 d until disease regression, and there was a 35% response rate in the 17 evaluable patients who received a median of two cycles of treatment^[38]. Taken together, the overall response rate (ORR) was 35%-44% in the trial arm consisting of trastuzumab combined with conventional chemotherapy.

The ToGA trial was an open-label, international, phase III, randomized controlled trial that was undertaken in 24 countries^[16]. In total, 594 patients with gastric or gastroesophageal junction cancer that overexpressed HER-2 protein (as determined by immunohistochemistry or gene amplification by fluorescence *in situ* hybridization) were randomly assigned to the study treatments (trastuzumab plus chemotherapy, *n* = 298; chemotherapy alone, *n* = 296); of these patients, 584 were included in the primary analysis (*n* = 294 and *n* = 290, respectively). The median overall survival in the trastuzumab plus chemotherapy arm was 13.8 mo (95%CI: 12-16) compared with 11.1 mo (95%CI: 10-13) in the chemotherapy alone arm (HR = 0.74, 95%CI: 0.60-0.91, *P* = 0.0046). The study met not only the primary endpoint of improved overall survival but also the secondary endpoint of improved response rates and progression-free survival. Ad-

ditionally, a 2.7 mo gain in median survival was observed in the intent-to-treat population. In the ToGA study, no significant overlapping toxicity was evaluated, except for cardiac dysfunction^[16]. Trastuzumab is correlated with an increased risk of cardiotoxicity^[39], similar to anthracyclines, which are frequently used in the treatment of breast and gastric cancers. Trastuzumab-related cardiac dysfunction is largely reversible by removal of the antibody^[40] and has been classified as type II chemotherapy-related cardiac dysfunction^[41]. In the ToGA study, the left ventricular ejection fraction was monitored every 12 wk during treatment. The regimen was well tolerated, and the hematological toxicity for the chemotherapy doublet was within the expected levels. Interestingly, no additional toxicity was observed, except for an asymptomatic reduction in the left ventricular ejection fraction to below the normal range, which was reported in 5.9% of the patients. Notably, although this patient group has a relatively short life expectancy, the addition of trastuzumab did not compromise the patients' quality of life^[42].

TARGETING EGFR

The EGFR is intrinsically expressed in various organs, including the skin, gut and renal tissues. EGFR overexpression is observed in 27%-64% of gastric cancers, especially in the more proximal tumors^[43,44], and is correlated with older age, more aggressive histology and higher disease stage; additionally, EGFR expression is a poor prognostic factor^[43].

Cetuximab (Erbix, Imclone Systems) is a recombinant humanized murine monoclonal antibody against EGFR and is the most investigated anti-EGFR therapy in gastric cancer. In the first line phase II trials, six non-randomized trials investigated the addition of cetuximab to doublet chemotherapy^[45-49]. The response rate of the above studies ranged from 41% to 63%, and the median overall survival ranged from 9 to 16.6 mo. A randomized phase II study comparing the addition of cetuximab to three discrete chemotherapies was reported at ASCO 2010. None of the treatment arms that included cetuximab exhibited a better survival outcome compared with the conventional control arms. In 2011, preliminary data from another phase II study demonstrated that there was no clinically significant benefit associated with the addition of cetuximab to docetaxel and oxaliplatin^[50]. Additionally, the results of the large, randomized, phase III EXPAND study (NCT00678535), which investigated the addition of cetuximab to cisplatin and capecitabine chemotherapy, were presented in 2013^[51]. The median progression-free survival (PFS) for the 455 patients administered the capecitabine-cisplatin plus cetuximab treatment was 4.4 mo (95%CI: 4.2-5.5) compared to 5.6 mo (95%CI: 5.1-5.7) for the 449 patients treated with capecitabine-cisplatin alone (HR = 1.09, 95%CI: 0.92-1.29; *P* = 0.32). Additionally, 83% of the patients in the chemotherapy plus cetuximab group and 77% of the patients in the chemotherapy group experienced grade 3-4

diarrhea, hypokalemia, hypomagnesemia, rash and hand-foot syndrome.

Panitumumab is a humanized monoclonal antibody that targets EGFR. Van Cutsem *et al.*^[52] reported a phase III trial of panitumumab plus best supportive care compared to best supportive care alone in patients with advanced colorectal cancer that failed to respond to 5-FU, irinotecan and oxaliplatin. However, there are very few reports of this agent being used to treat AGC patients. Recently, the results of a randomized, open-label, phase III trial for patients with previously untreated advanced esophagogastric cancer (REAL3) were revealed; this study examined two groups of esophagogastric cancer patients treated with epirubicin, oxaliplatin and capecitabine with or without panitumumab^[53]. The median overall survival of the 275 patients with advanced esophagogastric adenocarcinoma in the epirubicin, oxaliplatin and capecitabine (EOC) treatment group was 11.3 mo (95%CI: 9.6-13.0) compared to 8.8 mo (95%CI: 7.7-9.8) in the 278 patients treated with modified-dose EOC plus panitumumab (mEOC+P) (HR = 1.37, 95%CI: 1.07-1.76; *P* = 0.013). The main adverse events that were observed during this trial were grade 3-4 diarrhea (48/276 mEOC+P patients 17% *vs* 29/266 EOC patients 11%), rash (29/276 mEOC+P patients 11% *vs* 2/226 EOC patients 1%), mucositis (14/276 mEOC+P patients 5% *vs* 0/226 EOC patients) and neutropenia (35/276 mEOC+P patients 13% *vs* 74/226 EOC patients 28%)^[53]. On the other hand, other EGFR monoclonal antibodies, such as matuzumab and nimotuzumab, resulted in even shorter PFS times when combined with chemotherapy (compared with chemotherapy alone) in randomized phase II trials^[54,55].

Gefitinib and erlotinib, which are EGFR tyrosine kinase inhibitors (TKIs), were assessed in phase II trials; however, these drugs produced unsatisfactory results when used as monotherapies for gastric cancer patients. These inhibitors were effective first-line treatments against gastroesophageal cancer (GEJ), but were not effective for gastric cancer patients, when examined in a phase II study^[56]. On the other hand, combination therapy with 5-FU, oxaliplatin and erlotinib demonstrated that the ORR was greater than 50% in patients with esophageal or GEJ cancer^[57].

COMBINED TARGETING OF HER-2 AND EGFR

Lapatinib is a receptor tyrosine kinase inhibitor that inhibits both HER-2 and EGFR. A phase II trial demonstrated that lapatinib could achieve an ORR of 7% and a 20% rate of disease stabilization^[58]. Regarding adverse events, one patient each experienced grade 4 cardiac toxicity and vomiting in 47 patients with metastatic gastric cancer. Additionally, two patients experienced grade 4 fatigue. In another study, lapatinib had restricted single-agent activity. Only two of 21 previously treated patients had durable, stable disease^[59]. Remarkably, these disappointing results were expected, as lapatinib was adminis-

tered to both HER-2+ and HER-2- patients.

Two phase III trials are ongoing to determine the utility of lapatinib as a first- and second-line treatment for AGC patients. The first trial, the Lapatinib Optimization Study in ErbB2 (HER-2)-Positive Gastric Cancer (LoGIC) trial, is investigating lapatinib as a first-line treatment in combination with capecitabine and oxaliplatin^[60]. The second trial, the Lapatinib (Tykerb) with paclitaxel (taxol) in Asian ErbB2+ (HER-2+) Gastric Cancer (TYTAN) trial, is investigating second-line paclitaxel treatment with or without lapatinib in Asian patients^[61]. Importantly, HER-2-patients were excluded from the target AGC patients in these trials. The results of these interesting trials will determine whether lapatinib will be used to treat patients with AGC.

TARGETING VEGFR

Bevacizumab is a monoclonal antibody that inhibits vascular endothelial growth factor-A (VEGF-A), and the broad clinical activity of bevacizumab in antiangiogenic therapies has been reported^[62-66]. Various phase II trials of bevacizumab plus chemotherapy have been reported for AGC patients^[67-70]. An ORR of 42%-67% was achieved, and the median TTP was 6.6-12 mo, whereas the OS time was 8.9-16.2 mo. Grade 3-4 thromboembolic diseases were reported in approximately 25% of the patients, and gastric perforation was observed in up to 8% of the patients in the phase II trials.

The phase III Avastin in Gastric Cancer (AVAGAST) trial was designed to evaluate the efficacy of adding bevacizumab to first-line capecitabine-cisplatin treatment for advanced gastric cancer. In total, 774 patients were randomly separated and administered capecitabine and cisplatin with or without bevacizumab^[71]. In this trial, cisplatin was administered for the six cycles; capecitabine and bevacizumab were administered until the disease progressed or unacceptable toxicity developed. The primary end point was overall survival (OS). The ORR significantly improved with the addition of bevacizumab (46% *vs* 37%; *P* = 0.0315), and the median PFS was also significantly longer (6.7 *vs* 5.3 mo; HR = 0.80; 95%CI: 0.68-0.93; *P* = 0.0037)^[71]. Additionally, the clinical outcomes were different depending on the geographical region. Survival was extended in Pan-American patients who were treated with bevacizumab; however, this was not the case for Asians or Europeans, despite the better prognosis of the latter. Differences in population genetics, patient selection and second-line chemotherapy may explain these results. Furthermore, biomarker studies will elucidate the reasons underlying the differences in efficacy. Interestingly, Ohtsu *et al.* reported that angiogenic markers, such as plasma VEGF-A and tumor neuropilin-1, can have predictive value for the clinical outcomes of patients with gastric cancer treated with bevacizumab in the AVAGAST randomized phase III trials^[72]. These results may lead to a better understanding of the study outcome. The most common grade 3-5 adverse events in both arms of

the trial were neutropenia, anemia and appetite loss, and these events occurred at similar rates with or without bevacizumab^[71].

COMBINED TARGETING OF VEGFR AND PDGFR

Sorafenib and sunitinib are multitargeted TKIs that inhibit angiogenesis by targeting VEGFR, PDGFR and other signaling pathways. Sorafenib is a multitarget inhibitor of BRAF, VEGF, PDGFR and the Ras/Raf/MERK/ERK pathway. A phase II study was performed to assess the combination of oxaliplatin and sorafenib as a second-line therapy for AGC after treatment with cisplatin and fluoropyrimidine first-line therapy. Among 40 AGC patients, the CR was 2.5%, and the SD was 47.2%. The median PFS was 3 mo (95%CI: 2.3-4.1), and the median OS was 6.5 mo (95%CI: 5.2-9.6). The median OS was 9.7 mo when the time-to-progression during the first-line chemotherapy was > 6 mo and was decreased to 5.6 mo when the time-to-progression was < 6 mo ($P = 0.04$)^[73]. Grade 3-4 neutropenia (9.8%), thrombocytopenia (7.3%) and neurotoxicity (4.9%) were reported. The combination of oxaliplatin and sorafenib in AGC patients previously treated with cisplatin and fluoropyrimidine appeared safe; however, these results did not support the implementation of a phase III trial^[73].

Sunitinib suppresses PDGFR, Kit, rearranged during transfection (RET), Flt-3 and VEGFR. A phase II study of single-agent sunitinib as a second-line treatment for AGC patients treated with one prior chemotherapy regimen was performed, and 2.6% of the enrolled patients had a partial response, whereas 25 patients (32.1%) had stable disease. The median PFS was 2.3 mo, and the median OS was 6.8 mo. Grade 3-4 thrombocytopenia and neutropenia were reported in 34.6% and 29.4% of the patients, respectively^[74]. In another phase II trial, disease stabilization was reported in five out of 14 patients^[75]. These results suggested that single-agent sunitinib did not have sufficient clinical value as a second-line treatment for AGC. Sunitinib is unlikely to be further developed into a first-line treatment or to be used in combination with chemotherapy due to the complete failure of sunitinib to change the survival outcome of other solid tumors when combined with chemotherapy^[76].

TARGETING OTHER RTKS AND NEW COMBINED TARGETING OF HER2 AND VEGF

The overexpression and activation of c-Met, an RTK for hepatocyte growth factor, induces proliferation and anti-apoptotic signals^[77]. c-Met was found to be overexpressed in human gastric cancer cells both *in vitro*^[78] and *in vivo*^[79]. Amplification of the *MET* gene can be used to determine the response to Met inhibition *in vitro*^[55]. A phase

II study of GSK1363089 (GSK089, formerly XL880), a c-Met TKI, demonstrated that this compound had minimal activity in metastatic gastric cancer patients, and liver dysfunction, fatigue and venous thromboembolism were reported as adverse events^[80].

Insulin-like growth factor 1 receptor (IGF-1R) expression is correlated with poor outcome in AGC patients^[81], and treatment with the IGF-1R antibody figitumumab in conjunction with docetaxel was well tolerated and in a phase I trial of advanced solid tumor patients^[82]. FGFR may be a targetable RTK, as the secretion of the FGF family by fibroblasts stimulates the proliferation of scirrhous gastric cancer cells^[83]. Additionally, mutations in FGFR are associated with the development of gastric cancer^[84], and selective inhibitors of FGFR may be used in clinical trials for AGC patients^[85].

In addition, AXL receptor tyrosine-kinase family, such as axl/ufo and nyk/mer protein kinases, co-operatively correlates with the cancer progression, metastasis and patients' prognosis in gastric cancer^[27]. The specific ligand growth arrest-specific gene 6 (Gas6) binds to Axl and Gas6-Axl signaling pathway enhanced cellular survival and invasion and suppressed apoptosis via Akt family during gastric carcinogenesis^[86]. Gas6-Axl signaling could be a potential therapeutic target in gastric cancer.

Recently, Singh *et al.*^[87] reported combined blockade of HER-2 and VEGF brings about greater growth inhibition in HER-2 overexpressing gastric cancer xenografts. This result suggests that new combination therapy using inhibitors of HER-2 and VEGF may represent a new approach for the treatment of HER-2 positive AGC patients.

CONCLUSION

Effective RTK inhibitors have been developed over the years, and their potential usefulness will increase further as preclinical data using gastric cancer models continue to demonstrate their effectiveness. Several RTKs are activated in advanced gastric cancer; therefore, targeting these RTKs may lead to tailored therapy. Unfortunately, recent progress in the development of RTK-targeted therapy for AGC patients has been modest. Well-designed clinical trials and combinations with appropriate drugs are required to provide maximal therapeutic benefits. Furthermore, new predictive biomarkers are immediately needed to guide the selection of a potentially drug-sensitive cohort of patients.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

State-of-the-art preoperative staging of gastric cancer by MDCT and magnetic resonance imaging

Joon-Il Choi, Ijin Joo, Jeong Min Lee

Joon-Il Choi, Department of Radiology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea

Ijin Joo, Jeong Min Lee, Department of Radiology and Institute of Radiation Medicine, Clinical Research Institute, Seoul National University Hospital, Seoul National University College of Medicine, Seoul 110-744, South Korea

Author contributions: Choi JI and Lee JM designed and performed the research; Choi JI, Lee JM and Joo I wrote the manuscript.

Correspondence to: Jeong Min Lee, MD, PhD, Department of Radiology and Institute of Radiation Medicine, Clinical Research Institute, Seoul National University Hospital, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea. jmsh@snu.ac.kr

Telephone: +82-2-20723107 Fax: +82-2-7436385

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Abstract

Gastric cancer is one of the most common and fatal cancers. The importance of accurate staging for gastric cancer has become more critical due to the recent introduction of less invasive treatment options, such as endoscopic mucosal resection or laparoscopic surgery. The tumor-node-metastasis staging system is the generally accepted staging system for predicting the prognosis of patients with gastric cancer. Multidetector row computed tomography (MDCT) is a widely accepted imaging modality for the preoperative staging of gastric cancer that can simultaneously assess locoregional staging, including the gastric mass, regional lymph nodes, and distant metastasis. The diagnostic performance of MDCT for T- and N-staging has been improved by the technical development of isotropic imaging and 3D reformation. Although magnetic resonance imaging (MRI) was not previously used to evaluate gastric cancer due to the modality's limitations, the

development of high-speed sequences has made MRI a feasible tool for the staging of gastric cancer.

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Key words: Gastric cancer; Multidetector row computed tomography; Magnetic resonance imaging; Preoperative staging; The tumor-node-metastasis staging

Core tip: With the technical development of multiplanar imaging and 3D reformation, the diagnostic performance of Multidetector row computed tomography for T-staging has improved. N-staging of advanced gastric cancer has also improved, but the diagnostic effectiveness of N-staging is limited for patients with early gastric cancer. The limitations of magnetic resonance imaging (MRI) once prevented its use in evaluating gastric cancer; however, the development of high-speed sequences has made MRI a feasible tool. The intrinsic strength of MRI is the ability to produce contrast in soft tissue, and the use of tissue-specific contrast agents may aid in gastric cancer staging.

Choi JI, Joo I, Lee JM. State-of-the-art preoperative staging of gastric cancer by MDCT and magnetic resonance imaging. *World J Gastroenterol* 2014; 20(16): 4546-4557 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4546.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4546>

INTRODUCTION

Although its prevalence is decreasing in Western countries, gastric cancer remains the second most common cause of cancer-related death. Gastric cancer is more common in Asian countries, particularly China, Japan, and South Korea^[1-3]. Complete surgical resection was

Table 1 T-staging of gastric cancer, American Joint Committee on Cancer 7th manual

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma <i>in situ</i> : intraepithelial tumor without invasion of the lamina propria
T1	Tumor invades the lamina propria, muscularis mucosae, or submucosa
T1a	Tumor invades the lamina propria or muscularis mucosae
T1b	Tumor invades the submucosa
T2	Tumor invades the muscularis propria
T3	Tumor penetrates the subserosal connective tissue without invasion of the visceral peritoneum or adjacent structures. T3 tumors also include those extending into the gastrocolic or gastrohepatic ligaments or into the greater or lesser omentum, without perforation of the visceral peritoneum covering these structures
T4	Tumor invades the serosa (visceral peritoneum) or adjacent structures
T4a	Tumor invades the serosa (visceral peritoneum)
T4b	Tumor invades adjacent structures, such as the spleen, transverse colon, liver, diaphragm, pancreas, abdominal wall, adrenal gland, kidney, small intestine, and retroperitoneum

once thought to be the only successful option for curing gastric cancer^[4]. However, the development of endoscopic procedures that can be used to treat early gastric cancer, such as endoscopic mucosal resection (EMR) and endoscopic submucosal dissection, has reduced the morbidity and mortality rates with minimally invasive curative therapy^[5]. Furthermore, the recent development of chemotherapeutic agents can prolong the survival of patients with advanced disease. Multiple treatment options make the choice of the appropriate treatment for each patient more important, and the accurate staging of a patient's disease can have a major role in determining the final clinical outcome. The tumor-node-metastasis (TNM) staging system, which is a generally accepted staging system in clinical practice, has been shown to accurately predict patient prognosis^[6]. Traditionally, deep tumor infiltration into an adjacent structure (T4) and the presence of multiple, metastatic lymph nodes (N3 or N4) or distant metastases limited the resectability of gastric cancer, and the major function of preoperative staging was to detect these conditions. However, due to the widespread use of EMR for treating early gastric cancer, more precise and accurate staging is required, and differentiating between T1 and T2 (or even between T1a and T1b) is currently necessary for endoscopists to determine the appropriate prognosis^[5,7]. Additionally, because the presence of nodal metastases is a contraindication for EMR, the accuracy of N-staging (N0 *vs* N1) now receives more attention.

The standard imaging modalities used for the preoperative staging of gastric cancer include computed tomography (CT) and endoscopic ultrasonography (US). Endoscopic US is regarded as the most accurate imaging tool for evaluating tumor depth, and CT is the principal imaging modality used for staging because of its ability to detect distant metastases. Magnetic resonance imaging (MRI) and diagnostic laparoscopy are other imaging tools that can be successfully used to stage gastric cancer.

The currently used multidetector row computed tomography (MDCT) with 16 or more channels and thin collimation can provide 1-mm-thick, high-resolution imaging, and the effect of motion is very limited due to the high image acquisition speed of MDCT. Isotropic imaging can be used to obtain multiplanar reformation

(MPR) images at any angle, and virtual gastroscopy or CT gastrography with 3D reformation is also available. Virtual gastroscopy provides 3D-reconstructed, endoluminal images, such as those used in CT colonography. These benefits have improved the diagnostic performance of MDCT when detecting and staging gastric cancer in daily, clinical practice. Technical developments improving MRI, such as parallel imaging and fast sequences, have increased its use in abdominal imaging. Although the reported results of MRI for abdominal imaging have not been completely successful, the intrinsic strength of MRI as a contrast imaging method may allow for T-staging accuracy that is comparable to that of MDCT. Additionally, new contrast agents can also enhance MRI performance in N- and M-staging of gastric cancer.

In this manuscript, we provide the review of the recently revised TNM staging system for gastric cancer and then discuss the performance of MDCT and MRI in the preoperative staging of gastric cancer. We will also discuss some of the state-of-the-art techniques that can be used to assess gastric cancer, with special emphasis on preoperative staging.

SEVENTH EDITION OF THE AMERICAN JOINT COMMITTEE ON CANCER STAGING SYSTEM FOR GASTRIC CANCER

The latest version of TNM staging by the Union for International Cancer Control and the American Joint Committee on Cancer (AJCC) is the seventh edition, released in 2009, and is summarized in Tables 1, 2 and 3^[8]. This staging system has been periodically revised based on newly collected scientific data. In the latest revision, tumors arising at the esophagogastric (EG) junction and those arising in the stomach within 5 cm of the EG junction and involving the EG junction are considered esophageal cancer. The T-staging system has also been modified in a manner similar to other bowel tumors (*i.e.*, esophagus and small and large bowel); T2 is now defined as a tumor invading the muscularis propria and T3 as a tumor invading the subserosal connective tissue. Stage T2b as defined in the 6th edition was restaged as T3 in

Table 2 N-staging of gastric cancer, American Joint Committee on Cancer 7th manual

NX	Regional lymph node(s) cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 2 regional lymph nodes
N2	Metastasis in 3 to 6 regional lymph nodes
N3	Metastasis in 7 or more regional lymph nodes

the 7th edition, and T3 as defined in the 6th edition was restaged as T4a. As early gastric cancer (EGC) is traditionally defined as a tumor confined to the mucosa and submucosa, cancers staged T1a and T1b are considered EGC by definition. The previously adopted Japanese classification for N-staging, with designations based on the anatomical location of the involved regional lymph nodes, is not used now^[9,10]. Currently, the number of cancer-involved lymph nodes is the only factor determining the N-stage. The advantages of this method are its relative ease and independent reporting by pathologists and the strong correlation between the number of affected lymph nodes and patient survival rates. However, the current method for N-staging can be biased by the number of collected lymph nodes; several authors reported that the total number of collected lymph nodes can influence a patient's prognosis^[11,12]. Regardless, this opinion is not yet reflected in the AJCC 7th edition, a version in which the number of lymph nodes for each N-staging was decreased (Table 2). Retropancreatic, para-aortic, hepato-duodenal, retroperitoneal, and mesenteric lymph nodes are considered distant metastases (M1) in gastric cancer.

MDCT

MDCT protocols

A fasting time of at least six hours is required for complete gastric emptying. Patients usually receive 10-20 mg of butylscopolamine bromide intramuscularly or intravenously 10-15 min before CT scanning to minimize peristaltic movement^[13,14]. Any history of glaucoma, urinary outflow obstruction, or cardiac arrhythmia is checked to avoid the use of butylscopolamine in patients with contraindications. To obtain optimal distention of the stomach, several types of oral contrast agents are used. Among them, positive contrast agents, such as diluted barium or water-soluble iodine, mask the enhancement of the gastric mucosa and inhibit 3D reformation, both of which are critical for cancer staging^[15,16]. Therefore, negative (air) or neutral (water or methyl cellulose) contrast agents are generally used. Negative and neutral agents help to depict the detailed enhancement pattern of gastric wall layers^[17,18]. In 3D reformation and virtual gastroscopy, air is the preferred oral contrast agent. A recent study reported that MDCT using gas distention and 3D CT gastrography provided T-staging of preoperative gastric cancer comparable to hydro-CT (*i.e.*, using water as the oral contrast agent) but that gas distention was more effective for lesion detection. We therefore recom-

Table 3 Stage and prognostic group of gastric cancer, American Joint Committee on Cancer 7th manual

Stage X	TX	NX	MX
Stage I A	T1	N0	M0
Stage I B	T2	N0	M0
	T1	N1	M0
Stage II A	T3	N0	M0
	T2	N1	M0
	T1	N2	M0
Stage II B	T4a	N0	M0
	T3	N1	M0
	T2	N2	M0
	T1	N4	M0
Stage III A	T4a	N1	M0
	T3	N2	M0
	T2	N3	M0
Stage III B	T4b	N0 or N1	M0
	T4a	N2	M0
	T3	N3	M0
Stage III C	T4b	N2 or N3	M0
	T4a	N3	M0
Stage IV	Any T	Any N	M1

mend air as the preferred oral contrast agent^[15]. In our medical institution, effervescent granules with a minimal amount of water are orally administered immediately prior to CT scanning to obtain optimal gastric distention using air^[13]. Each patient receives intravenous contrast agent at a rate of 3-4 mL/s using a power injector, and CT images are obtained approximately 70 s following the injection, which is the optimal time for evaluating the enhancement of gastric tumor and hepatic metastases. In some medical institutions, early arterial phase imaging can be added to assess possible vascular anomalies in the vessels supplying the stomach^[19,20]. The left posterior oblique and right decubitus positions are used for evaluating the entire distended stomach. In the left posterior oblique position, the lower part of the stomach, including the antrum, is distended, and residual fluid collects in the fundus of the stomach. In the right decubitus position, the upper part of the stomach, including the fundus, is fully extended^[21]. When using water as the oral contrast agent, the supine and prone positions are recommended. An MDCT unit with 16 or more channels is recommended to acquire isotropic imaging with less than 1.25-mm collimation. Two-dimensional axial, coronal, and sagittal images provide a quick view of the stomach and tumor and allow for instant evaluation of the tumor location and depth. Three-dimensional rendering, such as virtual gastroscopy or CT gastrography, can provide additional information on depth perception, fold change, and superficial lesions^[22,23]. In our medical institution, axial CT images are reconstructed using a 3-mm section thickness and a reconstruction interval of 2-3 mm; an additional image set using a 1-mm section thickness and reconstruction interval is reconstructed for 3D rendering. Coronal and sagittal MPR images are also reconstructed with a 3-mm section thickness and interval. Virtual gastroscopy^[24] and barium-study-looking 3D CT gastrography using a surface-shaded, volume-rendering technique^[13] are

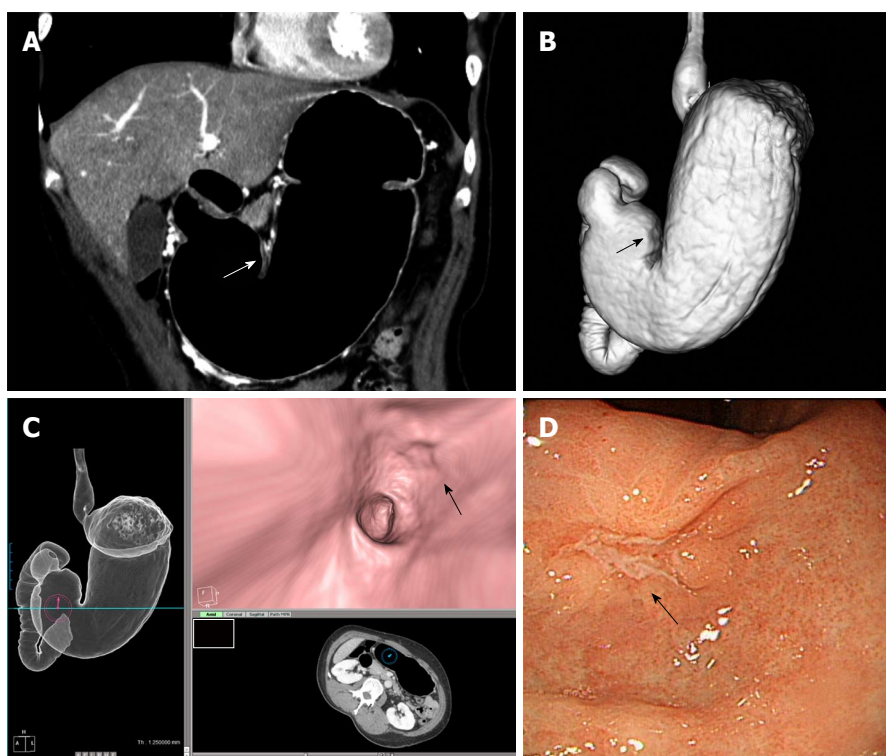


Figure 1 T1a gastric cancer in a 53-year-old female patient. A: Coronal 2D image shows small mucosal enhancement (arrow) in the lesser curvature side of the antrum; B: Computed tomography gastrography shows a small mucosal irregularity (arrow) in the same area; C: Virtual gastroscopy delineates a shallow, depressed lesion (arrow); D: Endoscopy reveals a small mucosal irregularity confined to the mucosa (arrow). Endoscopic submucosal dissection was performed and pathological examination revealed pT1a early gastric cancer.

reconstructed with thin-section, isotropic data (Figure 1).

Lesion detectability and T-staging

Pathologically, the gastric wall consists of the following five layers: mucosa, submucosa, muscularis propria, subserosa, and serosa. However, on CT images, the gastric wall is observed as three layers: well-enhancing mucosa, submucosa as a low attenuated stripe, and musculoserosal layers of slightly elevated attenuation^[25]. Gastric cancers manifest as having enhancing wall thickening on CT, and the destruction of normal gastric wall structures can suggest the possible depth of invasion. Therefore, the extent of gastric wall thickening and the degree of enhancement can influence the detection rate and T-staging accuracy. T1 tumors are sub-staged as T1a and T1b: a stage T1a tumor is confined to the mucosa, and T1b tumors have invaded the submucosa. Because the incidence of lymph node involvement is much higher in T1b tumors than in T1a tumors (17.9% *vs* 2.2%) and endoscopic procedures for a tumor invading the submucosa are challenging, tumor sub-staging has a substantial clinical impact^[14,26-28]. EUS is known to be effective for differentiating between T1a and T1b tumors^[29]. On MDCT images, T1a tumors are usually not visible, and T1b tumors more frequently show mucosal thickening and enhancement. To differentiate between T1b and T2, T1b tumors show a low-attenuated stripe at the base of the tumor, which suggests a submucosa layer, while T2 tumors show loss of a low-attenuated stripe, which indicates involvement of the entire

submucosal layer^[14] (Figure 2). T3 tumors have subserosal invasion, and discrimination between a gastric mass and the outer layer is visibly impossible, and smooth outer margin of the outer layer or a few small linear strandings in the perigastric fat plane can be noted^[30]. Stage T4a tumors also demonstrate serosal involvement, which makes differentiating between T3 and T4a using MDCT very difficult (the gastric serosa is not delineated on CT images). In addition, the amount of adipose tissue in the subserosal area varies from person to person^[16] (Figures 3 and 4). In our experience, T4a tumors frequently show micronodules or dense, band-like stranding and can be found in the perigastric area. Stage T4b tumors show direct extension into an adjacent organ or structure and show obliteration of the fat plane between the gastric mass and adjacent organs.

Two meta-analyses of preoperative gastric cancer staging have been reported^[31,32]. However, these two studies are collections of data from the 1990s to 2006 or 2009 and have not been adapted to the updated AJCC staging system. Additionally, most of the data used in the two meta-analyses were obtained using MDCT with fewer than 4 channels. Kwee *et al.*^[31] reported that the diagnostic accuracy of MDCT for overall T-staging varied between 77.1% and 88.9%. Sensitivities and specificities for serosal invasion (T4a or T4b in the AJCC 7th edition) have been reported to be between 82.8% and 100% and between 80% and 96.8%, respectively^[13,17,33,34]. The authors also reported that the overall T-staging and identification

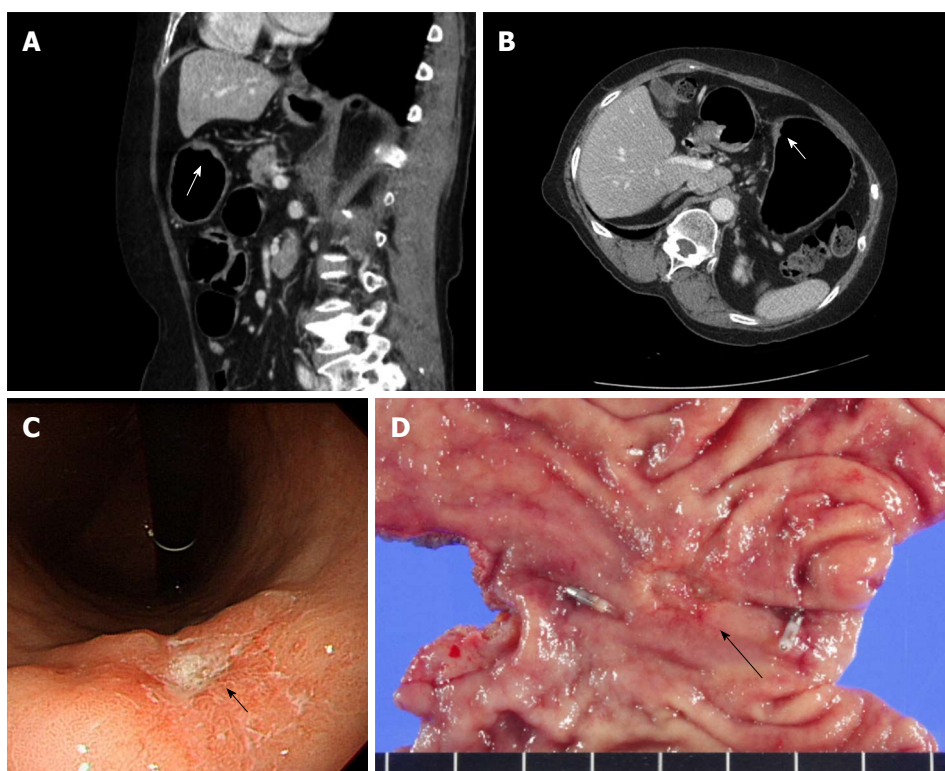


Figure 2 T2 gastric cancer in a 66-year-old female patient. A: Sagittal 2D image shows enhancing wall thickening with ulceration (arrow) in the lesser curvature side of the low body of the stomach; B: Left posterior oblique axial 2D image also delineates enhancing wall thickening (arrow) in the lesser curvature side of the low body of the stomach. The enhancing lesion involves the entire gastric wall layer, and no low attenuated stripe is visible at the base of the tumor; C: Endoscopy reveals a protruding mass with ulceration (arrow). The impression of the endoscopist was early gastric cancer; D: Subtotal gastrectomy was performed and pathological examination revealed pT2 gastric cancer (arrow).

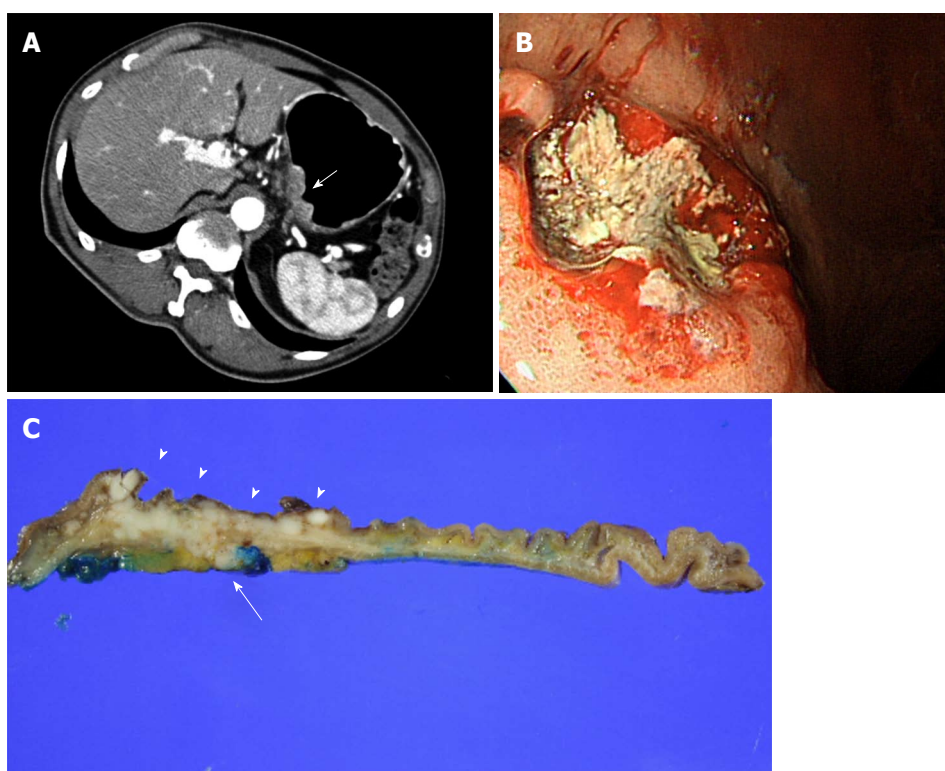


Figure 3 T3 gastric cancer in a 63-year-old male patient. A: Right decubitus 2D axial image shows thickening of the gastric wall (arrow) involving the entire layer. Perigastric infiltrations are noted outside of the tumor; B: Endoscopy reveals a large ulcerative tumor; C: Surgical specimen showing the tumor (arrowheads) and tumor extension to perigastric fat (arrow).

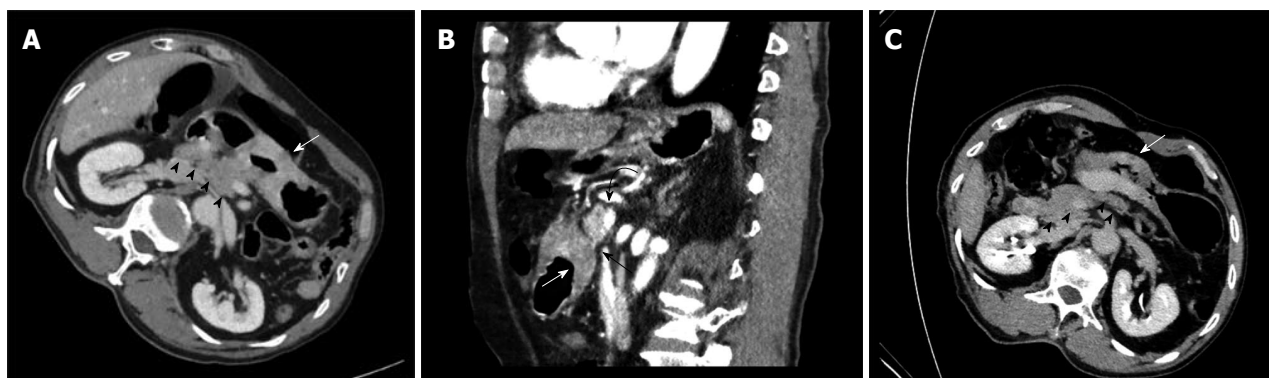


Figure 4 T4a gastric cancer in a 72-year-old male patient. A: Left posterior oblique axial 2D image shows prominent wall thickening of the gastric body (arrow) abutting the pancreas (arrowheads); B: Sagittal 2D image delineates reserved fat plane (black arrow) between the gastric mass (white arrow) and pancreas (curved arrow); C: Right decubitus 2D image delineates a change in positions between the mass (arrow) and pancreas (arrowheads). This finding is called a "sliding sign" and is considered evidence of non-invasion of an adjacent organ on imaging.

of serosal invasion were comparable using EUS and MDCT^[31]. In their meta-analysis, Seevaratnam *et al.*^[32] only reported the accuracy of overall TNM staging, indicating that T-staging was more accurate in ≥ 4 channel MDCT with MPR images than in scanners with < 4 channels.

The detection rate of early gastric cancer (T1) has recently been studied by several researchers using advanced technology. Yu *et al.*^[35] reported that 98% of the gastric cancers not visualized on optimally performed 2D CT imaging were EGC without LN involvement. Another study of early gastric cancer evaluated hydro-stomach CT (water as the oral contrast agent) without 3D reformation using blinded reviews and unblinded reviews in which the reviewer had knowledge of the tumor location, and no significant difference in EGC detection was found between blinded and unblinded reviews. The sensitivities and specificities for the blinded and unblinded reviews were 19%-27% and 98%-100%, respectively^[36]. The results of these studies are disappointing, though a recent study using 64-channel MDCT did report a 90%-94.7% detection rate for EGC^[30,37]. Other studies have also discussed the additional value of virtual gastroscopy for detecting EGC, reporting a sensitivity of 78.7%-84.0%^[13,38]. Although virtual gastroscopy cannot delineate the color change of mucosa and image interpretation is time consuming for radiologists, adding virtual gastroscopy information to 2D images can enhance the diagnostic performance of MDCT for the detection of EGC. Coronal and sagittal MPR images without virtual gastroscopy or CT gastrography were not helpful for improving the EGC detection rate or accuracy of T-staging^[39]; however, 3D reformation images do improve EGC detection. Using 3D reformation of MDCT images can help clinicians make correct treatment decisions. Shallow tumors are good candidates for less invasive procedures, such as EMR or laparoscopic surgery.

Kim *et al.*^[30] reported on the diagnostic performance of 64-channel MDCT using 2D MPR images and virtual gastroscopy for T-staging according to the AJCC 7th edition guidelines. In that study, the sensitivities for correct T-staging were 62.5%-93.0%, and the specificities

were 90.5%-97.9%; the overall T-staging accuracy was 77.2%^[30]. Using the 6th edition AJCC staging guidelines, Chen *et al.*^[40] reported an improved T-staging accuracy of 89% when using 2D and MPR images compared to 73% accuracy when using only 2D images. Kim *et al.*^[39] also reported improved T-staging in advanced gastric cancer (AGC) patients when MPR images were added. These results suggest that the use of both MPR images and axial images improves the T-staging accuracy, especially in AGC.

N-staging

To determine the optimal treatment method for each patient, accurate N-staging is as important as T-staging. N-staging provides critical information that is needed to appropriately predict a patient's prognosis. For EMR or endoscopic submucosal dissection, N0 should be confirmed using EUS or MDCT. Additionally, as extensive lymphadenopathy detected surgically is known to be associated with higher morbidity and mortality, aggressive surgical procedures should be avoided for the patients with extensive lymphadenopathy. Indeed, proper evaluation of the lymph node status could be very helpful for determining the optimal treatment options and for planning the extent of lymphadenectomy. Lymph node status is an important prognostic factor for predicting the overall survival rate of patients with gastric cancer^[28,41]. The reported five-year survival rates based on the 6th AJCC system for N0, N1, N2, and N3 are 86.1%, 58.1%, 23.3%, and 5.9%, respectively^[28]. As enlarged lymph nodes are frequently the only measurable lesions in patients with gastric cancer, evaluation of a patient's treatment response to chemotherapy might depend on the observable lymph node status.

However, the results of studies evaluating the accuracy of MDCT N-staging are somewhat disappointing. According to the meta-analysis by Kwee *et al.*^[42], the sensitivity and specificity of MDCT N-staging varied between 62.5% and 91.9% and 50.0% and 87.9%, respectively^[13,37,39,40]. These poor and variable results may be due to the lack of standard CT criteria for diagnos-

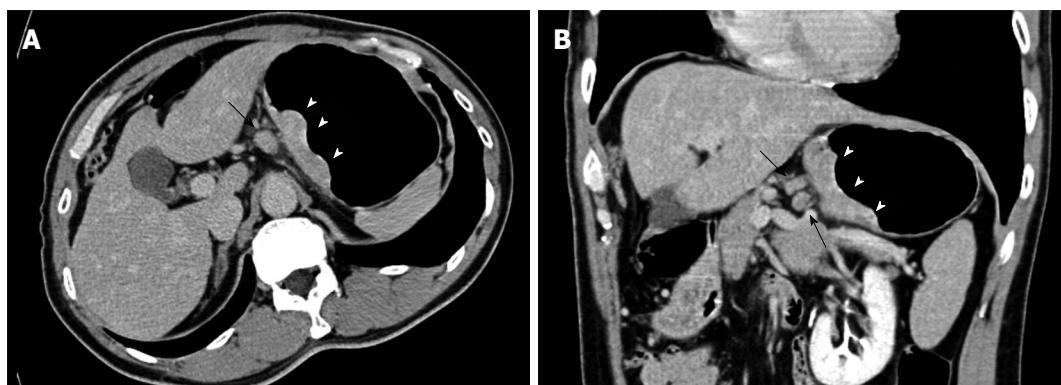


Figure 5 A prominent lymph node in a 57-year-old male patient with advanced gastric cancer. A: Left posterior oblique axial 2D image shows a large gastric mass (arrowheads) in the posterior wall of the gastric body and an enlarged lymph node (arrow). The LN diameter was 12 mm; B: Coronal 2D image shows a gastric mass (arrowheads) and two enlarged lymph nodes (arrows).

ing metastatic lymph nodes. Although many radiologists classify malignant lymph nodes as those with short axis diameters of 6–8 mm for perigastric lymph nodes^[43], other criteria are frequently used, including roundness and central necrosis, heterogeneous enhancement, more than 1 cm without fatty hilum, marked enhancement (over 80 or 100 HU), and clustering of more than three lymph nodes^[13,37,39,40] (Figure 5). To date, the accuracy of predicting lymph node metastasis has not been satisfactory using any criteria, and there is still no worldwide consensus for diagnosing metastatic lymph nodes using CT. N-staging of gastric cancer is one of the inherent limitations of CT. Although there is a clear correlation between the lymph node size and metastasis, microscopic nodal metastases in normal-size lymph nodes and lymph node enlargement resulting from reactive or inflammatory change are common in gastric cancer patients^[16,44,45]. Microscopic metastases can frequently be found in normal-sized lymph nodes of patients with EGC, which makes accurate N-staging more difficult in EGC cases than in patients with AGC^[14,44].

The use of MPR images and 3D reformation of isotropic MDCT data did not prevent inaccurate N-staging of gastric cancer. In recent studies, N-staging accuracy was not found to be improved by MPR images or virtual endoscopy^[13,40]. Another study reported improved N-staging performance when MDCT with MPR images was used in AGC cases, though there was no improvement in the EGC N-staging accuracy^[39]. Therefore, MPR images are expected to be helpful for the evaluation of the preoperative N-staging of AGC.

Some updated techniques for effective lymph node evaluation in gastric cancer have been reported. Kim *et al.*^[46] reported on the feasibility of mapping the sentinel node (the initial draining node from the tumor) using ethiodized oil in an animal and human study. This CT lymphography technique may help to minimize lymph node dissection in patients with EGC. Quantitative measurement of iodine concentrations using dual-energy spectral CT with monochromatic images has also been reported to improve the accuracy of N-staging^[47].

M-staging

The presence of distant gastric cancer metastases is a contraindication for surgical resection. Distant metastases can be classified into the following three groups: hematogenous metastases, lymphatic metastases, and peritoneal carcinomatosis. The liver is the most common location for hematogenous metastases in gastric cancer, and the lungs, bones, and adrenal glands are other organs affected by metastases. Tumor involvement in the lymphatic pathway also informs M-staging. Metastasis in distant lymph nodes, such as the retropancreatic, para-aortic, or retroperitoneal lymph nodes, may be classified as distant metastases (M1). Lymphatic metastases can also invade the liver or lungs. Compared with the limited field of view of EUS or MRI, MDCT is an ideal modality for evaluating distant metastases in patients with gastric cancer. A meta-analysis of data from 1994 to 2010 reported CT sensitivities and specificities for hepatic metastases and peritoneal carcinomatosis of 74% and 99% and 33% and 99%, respectively^[48]. However, most of the data used in this meta-analysis were obtained prior to 2005, and the performance of contemporary MDCT might be improved. Pan *et al.* reported more than 96.6% accuracy in M-staging 350 patients with gastric cancer using MDCT^[49]. As the meta-analysis results indicate, peritoneal carcinomatosis is one of the weak areas for accurate M-staging using CT. Preoperative detection of peritoneal carcinomatosis can prevent unnecessary laparotomy, and some surgeons prefer staging laparoscopy when peritoneal carcinomatosis is suspected^[50,51]. One study reported that the sensitivity and specificity for detecting peritoneal carcinomatosis were 50.9% and 96.2% with 16- and 64-channel MDCT, respectively^[51]. Known CT findings of peritoneal carcinomatosis include ascites, soft-tissue plaques or nodules on the peritoneal surface and bowel wall, prominent intra-abdominal fat stranding, and irregular peritoneal thickening with enhancement^[52] (Figure 6). In addition, a larger gastric tumor size (3–4 cm or more), T3 or T4 staging, and Borrmann type 3 or 4 suggest peritoneal carcinomatosis^[51–53]. The presence of greater than 50 mL of ascites is correlated with peritoneal carcinomatosis in 75%–100%

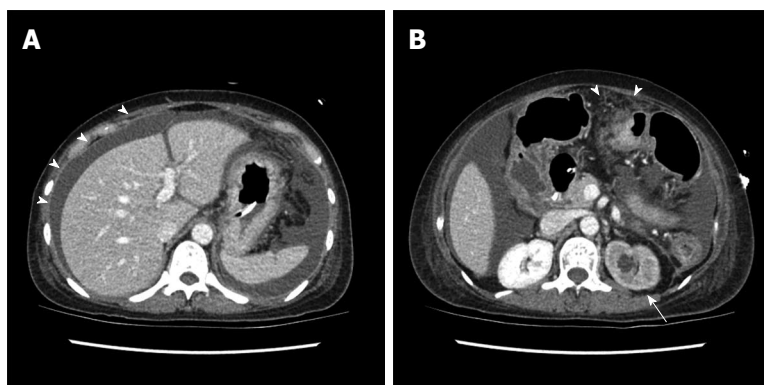


Figure 6 Peritoneal carcinomatosis in a 48-year-old female patient with advanced gastric cancer. A: Axial 2D CT image shows a large volume of ascites and peritoneal thickening (arrowheads); B: Axial 2D CT image at a lower level than (A) delineates ascites, omental infiltration and nodules (arrowheads), and hydronephrosis of the left kidney (arrow).

of gastric cancer patients^[52]. Yajima *et al*^[54] also reported that the presence of ascites on the CT scans of AGC patients predicts peritoneal carcinomatosis with 51% sensitivity and 97% specificity.

MRI

MRI protocols

With recent technological MRI developments, such as the 3.0 T field strength scanner, multichannel phase-arrayed coils, parallel imaging techniques, a more powerful gradient system, and new rapid three dimensional gradient echo techniques, higher quality MR images with reduced blurring and higher spatial resolution can be obtained within a single breath-hold^[55]. Given that MR can provide higher intrinsic soft tissue contrast than CT, there have been several studies demonstrating the value of MR in evaluating gastric cancer, especially in patients for whom contrast-enhanced CT is contraindicated due to renal dysfunction or hypersensitivity to iodinated contrast media^[56]. In addition, several studies have demonstrated that diffusion-weighted imaging may be helpful for staging malignant tumors of the intestines and for detecting lymph node metastases^[57]. However, although high-speed MR techniques can solve some of the disadvantages of MRI for detecting gastric cancer (such as blurring and lower spatial resolution), MRI is not yet widely accepted as a standard imaging modality for staging gastric cancer. Therefore, there is no generally accepted protocol for gastric MRI. However, in general, the use of butylscopolamine bromide to decrease bowel motion and the use of air or water as an oral contrast agent are the same in MRI and MDCT scanning. The supine or prone position is generally accepted as a method for distending the area of the stomach where a tumor may be located. The fat-suppressed, T1-weighted, gradient echo sequence, single-shot fast spin echo or turbo spin echo T2-weighted images, and true fast imaging with steady-state precession (True-FISP) are common sequences used for the detection of gastric cancer. Gadolinium-chelate contrast agents can also be injected for post-contrast imaging using 3D spoiled gradient echo sequences. Axial or coronal images can both be acquired, and protocols may vary in different medical institutions. These images are acquired using phased array coils to increase the signal-to-noise

ratio.

In our institution, the MRI protocol for gastric cancer includes the following sequences: half-Fourier acquisition single-shot turbo spine-echo (HASTE) T2-weighted imaging with and without fat saturation, true-FISP, T1-weighted 3D gradient-recalled-echo (GRE) in- and out-of-phase imaging, and T1-weighted fat-suppressed 3D GRE imaging. Diffusion-weighted images are obtained using multiple *b* values of 0, 100, 500 and 1000 mm²/s. Dynamic gadolinium contrast-enhanced imaging during the arterial, portal, hepatic venous, and equilibrium phases are obtained. The spectral selection attenuated inversion technique is used for fat suppression (Figure 7).

Staging

Compared to those using MDCT, there are only a small number of MR studies of gastric cancer patients, largely due to the intrinsic limitations of MR, such as the susceptibility to bulk motion (*e.g.*, respiration, pulsation, and peristalsis), high cost, and lower spatial resolution compared to MDCT or EUS. However, the excellent soft-tissue contrast of MRI might be helpful for accurate T-staging, and continuous technical improvements, such as parallel imaging, have made gastric MRI feasible and have resulted in the publication of several studies on this subject.

However, *in vitro* studies have reported conflicting results. Palmowski *et al*^[58] performed an *in vitro* study with resected specimens, and reported that 1.0 Tesla MRI with T1- or T2-weighted images could visualize three layers of the gastric wall (mucosa, sub-mucosa, and muscularis propria) and, in some cases, five gastric wall layers. Gastric cancer was localized in 96% of the study patients, though the accuracy of T-staging was only 50%, primarily due to the overstaging of T2 to T3. However, Sato *et al* reported 100% accuracy and clear visualization of all of gastric wall layers in an *in vitro* study using 1.5 Tesla MRI^[59]. Kim *et al*^[60] also reported the results of an *in vitro* study using 1.5 Tesla MR. In their study, T1-weighted images depicted three layers of the gastric wall, and the T-staging accuracy was 74%; they also reported 47% accuracy for gastric cancer N-staging. That study considered lymph nodes 8 mm or larger at the short diameter to be positive. An endoluminal MRI coil attached to endoscopy has also been developed as a T-staging method,

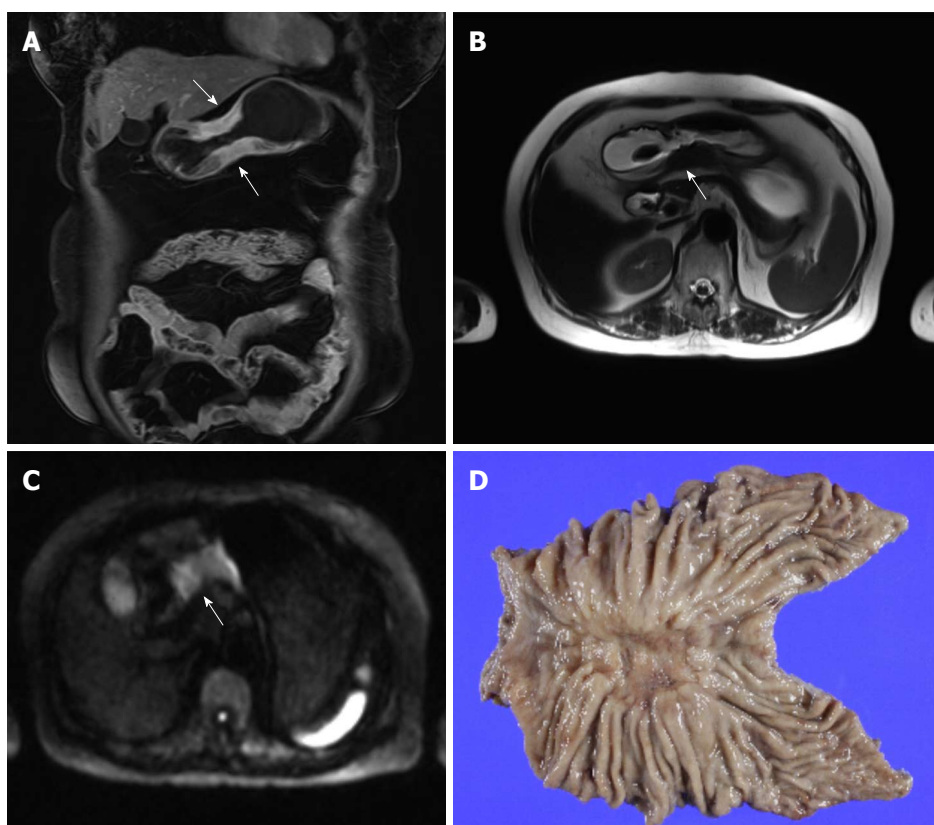


Figure 7 Magnetic resonance imaging of gastric cancer in a 71-year-old female patient. A: Coronal image of contrast enhanced magnetic resonance imaging (MRI) shows enhancing wall thickening of the gastric body (arrows); B: T2-weighted image delineates a low echoic mass (arrow) destroying layers of the gastric wall. The outer border of the mass is irregular; C: Diffusion-weighted image with a b-value of 1000 shows a high signal intensity mass (arrow) with diffusion restriction; D: Subtotal gastrectomy was performed, and pathological examination revealed pT3 cancer.

and an ex vivo study reported significantly more accurate T-staging than EUS^[61].

In early 2000, a few reports of in vivo studies were published. The diagnostic accuracy of MRI for T-staging varied between 71.4% and 82.6%, and the sensitivity and specificity for detecting serosal invasion varied between 89.5% and 93.1% and between 94.1% and 100%, respectively^[31,62,63]. Kang *et al*^[62] reported the rapid enhancement of gastric cancer compared to normal mucosa after the injection of gadolinium chelates and also reported 83% T-staging accuracy. Additionally, these authors reported a 52% detection rate of regional lymph node involvement. Kim *et al*^[64] compared spiral CT and 1.0 Tesla MRI and reported that MRI was slightly superior for T-staging. Sohn *et al*^[63] reported comparable results using spiral CT and 1.5 T MRI for both T- and N-staging.

Recently, 64-channel MDCT and 1.5 Tesla MRI with contemporary sequences were compared in two studies, with the T-staging accuracy being comparable for MDCT and MRI^[65,66]. However, one study reported that MRI was superior for detecting T1 tumors (50% *vs* 37.5% accuracy for MRI and MDCT, respectively)^[65]. The cumulative results of these studies indicate that MDCT and contemporary MRI show similar performance in T- and N-staging.

Tissue-specific contrast MR agents can be used for an accurate N-stage diagnosis. T1 lymphotropic contrast

agents, including gadofluorine M, or such T2* agents as ultra-small, superparamagnetic iron oxide, might be helpful for differentiating metastatic lymph nodes^[67,68].

Peritoneal carcinomatosis can be evaluated on delayed, post-contrast MRI images^[69]. Diffusion-weighted imaging is now widely accepted for abdominal MRI and adding diffusion-weighted imaging to delayed gadolinium-enhanced imaging can improve the detection rate of peritoneal carcinomatosis^[70].

CONCLUSION

Accurate preoperative staging of gastric cancer is important for treatment planning and prognosis prediction. Due to the development of less invasive treatment options, gastric cancer should be preoperatively staged with accuracy. The technical progress of MDCT and the continuous development of 3D imaging processes have improved MDCT performance in the preoperative staging of gastric cancer. The EGC detection rates can be improved through the use of virtual gastroscopy and CT gastrography. MPR images of MDCT can provide coronal or sagittal images and increase the accuracy of the tumor depth diagnosis. With the development of high-speed techniques, MRI evaluation of gastric cancer is now feasible, and some studies have reported results that are comparable to or better than MDCT. Gastric cancer

N-staging is an unsolved problem for both MDCT and MRI, and generalized standards for metastatic lymph nodes should be established. Additionally, tissue specific contrast agents will improve future N-staging. MDCT is the primary imaging modality used for the detection of distant gastric cancer metastasis, even though the technique has some limitations (such as peritoneal carcinoma detection). With continuing technical development, MDCT and MRI will play major roles in the future preoperative staging of gastric cancer.

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Ethnic differences in gastric cancer genetic susceptibility: Allele flips of interleukin gene

Juwon Kim, Yoonjung Kim, Kyung-A Lee

Juwon Kim, Department of Laboratory Medicine, Yonsei University Wonju College of Medicine, Gangwon-do 220-701, South Korea

Yoonjung Kim, Kyung-A Lee, Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul 135-720, South Korea

Author contributions: Kim J, Kim Y and Lee KA wrote and edited the manuscript.

Correspondence to: Kyung-A Lee, MD, PhD, Department of Laboratory Medicine, Yonsei University College of Medicine, 211 Eonju-ro, Gangnam-gu, Seoul 135-720, South Korea. kal1119@yuhs.ac

Telephone: +82-2-20193531 Fax: +82-2-20194822

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Abstract

Polymorphisms in promoter regions of inflammatory cytokines have been widely studied, and potentially functional polymorphisms have been discovered. Conflicting results from meta-analyses of interleukin (*IL*)-1 β and *IL*-10 polymorphisms show differences in gastric cancer susceptibilities between Caucasian and Asian populations. In particular, we note the suggestion of an allele flip in *IL*-1 β and *IL*-10 gene polymorphisms. In Asian populations, the *IL*-1 β -1464G/-511C/-31T haplotype indicates risk for gastric cancer, while the opposite haplotype, *IL*-1 β -1464C/-511T/-31C is the risk-related allele in Caucasians. Furthermore, while *IL*-10-1082G/-819C/-592C is associated with gastric cancer in Asians, *IL*-10-1082A/-819T/-592T is linked to gastric cancer risk in Caucasians. These seemingly contradictory results may be attributed to distinct carcinogenic mechanisms underlying the different gastric cancer subtypes. The allele flip observed in *IL*-10 and gastric cancer appears to reflect allelic heterogeneity, similar to that observed in *IL*-1 β . In this review, we focus on the allele flip phenomenon observed between different ethnic groups in

an effort to resolve certain controversial results from recent studies on interleukin polymorphism. In addition, we re-emphasize the importance of stratifying gastric cancer subtypes based on anatomical site and Lauren classification to prevent false associations arising through dilution of true ones.

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Key words: Allele flip; Gastric cancer; Interleukin gene

Core tip: In Asian populations, the highly expressed interleukin (IL)-1 β haplotype may increase risk for gastric cancer. Abundant IL-1 β expression determined by this haplotype may suppress gastric acid production in response to chronic *Helicobacter pylori* (*H. pylori*) infection, resulting in atrophic gastritis, the precursor of non-cardia gastric cancer. Conversely, the less expressive *IL*-1 β haplotype associates with gastric cardia cancer in Caucasians. Only low levels of IL-1 β are produced in response to *H. pylori* infection and gastric acid secretion is increased. Induction of gastroesophageal reflux disease may then promote cardia cancers.

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INTRODUCTION

Gastric cancer is the fourth most common cancer diagnosis in men worldwide, and the mortality rate is one of the highest among cancers. The incidence rate is especially high in East Asian countries including Japan, China and Korea^[1,2]. Of the various factors that contribute to

gastric cancer, including infectious, dietary, environmental and genetic factors, the chronic inflammatory state induced by *Helicobacter pylori* (*H. pylori*) infection is currently regarded as the most prevalent. Of note, however, only a small proportion of *H. pylori*-infected individuals develop gastric cancer, which implies that individual susceptibility, possibly genetic, is also involved^[1,3,4].

Associations of chronic inflammation with carcinogenic processes have prompted researchers to investigate the role that *H. pylori*-related inflammation may play in gastric cancer development. Thus, it was found that inflammatory cytokines, such as interleukin (IL)-1 β , also encoded by *IL-1B*, IL-1 receptor antagonist (*IL-1RA*), and tumor necrosis factor (TNF)- α are upregulated during *H. pylori* infection^[5-8]. Interest now converges on *IL-1B*, *IL-1RN*, *IL-8* and *IL-10*, encoding IL-1 β , IL-1RA, IL-8, and IL-10. The potent proinflammatory cytokine IL-1 β participates in a variety of cellular activities, including cell proliferation, differentiation and apoptosis^[9], in the amplification of immune response to infection, and as a potent inhibitor of gastric acid secretion^[10]. Studies on single nucleotide polymorphisms (SNPs) in the *IL-1B* promoter region reveal significant associations with gastric cancer that some meta-analyses support^[10,11]. Polymorphisms of *IL-10* and *IL-8* are also associated with gastric cancer risk^[10].

Other meta-analyses, however, present conflicting results with respect to *IL-1B* and *IL-10* polymorphisms and gastric cancer susceptibilities between Caucasian and Asian populations. On review of multiple meta-analyses, an “allele flip” between Asian and non-Asian groups is observed; most prominently in polymorphisms of *IL-1B* and *IL-10*. The allele flip refers to an inverse risk relationship of an allele in different groups or settings, for example, an allele found to be protective in one situation, but risk-related in another^[12]. A genuine allele flipping results from variations in allele frequencies and linkage disequilibrium (LD) that produce different patterns of risk association of a marker allele or haplotype across different ethnic groups^[13]. Alternatively, multiple loci may interact to create a disease phenotype^[14]. Finally, the phenomenon may be caused by allelic heterogeneity and locus heterogeneity, wherein different populations exhibit associations with alleles at different loci, through differences in genetic background or environment^[12]. Despite extensive review using meta-analysis, no clear explanation of allele flipping among these interleukin genes between different ethnic groups has emerged.

Here we concentrate on allele flips of *IL-1B* and *IL-10* polymorphisms in association with gastric cancer development in Asian and Caucasian groups. Of particular interest are the etiological significance of flipping in relation to genetic susceptibility and the incidence of gastric cancer at different anatomical sites.

GASTRIC CANCER EPIDEMIOLOGY AND CLASSIFICATION

Gastric cancer may be classified by histopathological

criteria as proposed by Lauren *et al.*^[15] into two principal types, intestinal and diffuse, which differ in histology, pathogenesis, epidemiology, genetic profile, and prognosis^[16]. Based on anatomical site, stomach cancer may be classified as cardia or non-cardia (fundus, antrum, pylorus lesser curvature, and greater curvature)^[17]. Non-cardia intestinal gastric cancer is strongly associated with chronic inflammation related to *H. pylori* infection^[3,4]. For tumors arising in the proximal region of the stomach, namely the gastric cardia/gastroesophageal junction, inflammation due to chronic gastric acid secretion may be the driving force in carcinogenesis^[18,19]. Gastric cardia cancers are usually of the intestinal type^[20]. As proposed by Hansen *et al.*^[21], the cardia cancers may comprise two distinct etiological subtypes, one non-cardia-like gastric cancer and the other resembling esophageal adenocarcinoma. The types of gastric cancer are summarized in Table 1. Diffuse-type gastric cancer is thought to arise through genetic changes in gastric cancer stem cells or epithelial precursor cells and usually lacks defined premalignant lesions^[22]. Recently, Shah *et al.*^[20] proposed a classification of gastric cancer based on clinical and epidemiological data into three principal types: proximal nondiffuse gastric cancer, diffuse gastric cancer, and distal nondiffuse gastric cancer. These distinctions are supported by gene expression analysis.

In the United States, rates of gastric cancer at all sites decreased from 1978 to 2005, while cardia cancer rates increased in the early years and plateaued^[17]. With respect to histological type, intestinal-type cancer decreased at all sites except at the gastric cardia^[17]. The declining prevalence of *H. pylori* infection has most likely contributed to downward trends in intestinal-type gastric cancer at non-cardia sites, particularly in Caucasians^[17]. Among Asians living in the *H. pylori* endemic region, there is ample evidence that non-cardia types outnumber cardia types, although the incidence of cardia-type gastric cancers has increased in recent years^[23].

IL-1 GENE POLYMORPHISM

Non-cardia, intestinal type gastric cancer

The IL-1 family includes the cytokines IL-1 α , IL-1 β and IL-1RA, encoded by three genes, *IL-1A*, *IL-1B* and *IL-1RN* on chromosome 2q14^[24,25]. Three polymorphisms in the promoter region of *IL-1B*, including *IL-1B*-1464 (G/C; rs1143623; previously known as -1476), *IL-1B*-511 (C/T; rs16944), and *IL-1B*-31 (T/C; rs1143627), are widely studied in association with gastric cancer risk. Studies of the *IL-1B* polymorphism have revealed an increased risk of gastric cancer with proinflammatory phenotype in Caucasian carriers of *IL-1B*-31C and *IL-1B*-511T^[11]. In meta-analyses, however, the *IL-1B* and *IL-1RN* polymorphisms imply different levels of risk for Asians and Caucasians. A landmark meta-analysis by Persson *et al.*^[26] revealed a consistent negative association of *IL-1B*-31C with gastric cancer in Asians. Other data show an even more significant increase in risk for non-cardia gastric cancer related to *IL-1B*-511T and *IL-1RN**2 alleles, but

Table 1 Differing risk associations for cytokine polymorphisms according to race and tumor type

Tumor type	Non-cardia or cardia with atrophy (non-cardia-like), intestinal		Cardia (esophageal), intestinal		Diffuse	
	Asian	Non-asian	Non-asian	Asian	Asian	Non-asian
Race						
Direction of association						
<i>H. pylori</i> infection	↑	↑	↓	↓	↑	↑
Gastric acid secretion	↓	↓	↑	↑	-	-
IL-1β production	↑	↑	↓	↓	-	-
IL-10 production	-	↓	-	↑	-	-
Allele flips of IL-1B and IL-10 (risk haplotype)						
IL-1B-1464/-511/-31	GCT	CTC	CTC	-	-	-
IL-10-1082/-819/-592	-	ATA	-	GCC	-	-
Genetic factors other than IL-1B and IL-10 ¹	IL-8, ZBTB20, PRKAA1	IL-1RN			MUC1, PSCA	IL-1RN, TNFA
Molecular classification by gene expression analysis ^[20]	Distal non-diffuse		Proximal non-diffuse		Diffuse	

¹Information based on meta-analyses^[10,26] and GWAS^[69-71,81]. ↑: Increase; ↓: Decrease. *H. pylori*: *Helicobacter pylori*; IL: Interleukin; PSCA: Prostate stem cell antigen.

only among Caucasians, while the *IL-1B*-511T allele may be protective against gastric cancer among Asians^[27,28]. In addition, complete LD between *IL-1B*-31 and -511 has been found^[11]. In East Asian populations, *IL-1B*-31TT homozygosity may be associated with increased risk for intestinal-type gastric cancer^[29].

Another polymorphism in the *IL-1B* promoter region at -1464 may be associated with gastric cancer, and -1464G is a putative risk allele in Asians^[30]. Moreover, -1464G is closely linked to *IL-1B*-511C/-31T alleles previously designated as risk alleles in Asians. In contrast, -1464C, in LD with *IL-1B*-511T/-31C and a risk allele among Caucasians, is associated with decreased risk of gastric cancer in the Chinese population^[31]. In atrophic gastritis, a precursor lesion in gastric cancer, -1464CC, may be associated with atrophic gastritis in the antrum among Caucasians^[32]. The haplotype associated with gastric cancer risk in Asians (*IL-1B*-1464G/-511C/-31T) may imply the opposite level of risk in Caucasians, among whom the *IL-1B*-1464C/-511T/-31C is the putative risk allele.

However, in a country such as China, comprising multiple ethnic groups with diverse geographical and historical roots, allelic heterogeneity with respect to gastric cancer prevalence and *H. pylori* infection status is apparent^[33]. Of note, -511TT is associated with an increased risk of gastric cancer in low-risk regions of China, an association that might be less obvious in high-risk regions^[33]. This is similar to the situation wherein -511TT is associated with increased gastric cancer risk in a Caucasian population with lower gastric cancer risk and *H. pylori* infection, whereas -511CC is the risk allele among Asian population, namely China, Korea and Japan, where gastric cancer risk is high. Therefore, it is plausible that *IL-1B*-1464G/-511C/-31T are the risk alleles for gastric cancer among Asians, while the exact opposite is true for Caucasians, indicating the existence of a genuine allele flip in the *IL-1B* gene polymorphisms with respect to gastric cancer risk.

To test the influence of haplotype on IL-1β expres-

sion, Chen *et al.*^[34] investigated the effect of SNPs in the *IL-1B* promoter region in terms of haplotype context. Of note, the SNPs at -1464, -511 and -31 in the promoter region expressed functional activities that were influenced by haplotype context^[34]. This observation was confirmed in a subsequent study *in vivo*, by the finding of a positive association between haplotype pairs containing *IL-1B*-1464G/-511C/-31T and levels of IL-1β expression in Caucasian subjects, despite previous understanding of *IL-1B*-511T/-31C as the proinflammatory allele^[35,36]. In an *in vitro* study, -31T expressed stronger promoter activity than -31C by virtue of retaining the TATA sequence, and showed greater binding affinity for transcription factors as well^[36]. The haplotype consisting of *IL-1B*-1464G/-511C/-31T shows a positive association with lung cancer risk and higher *IL-1B* gene expression in Caucasians^[37]. In evaluating transcriptional activities of individual SNPs, the -1464C SNP had higher transcriptional activity by itself^[38]. Placing the SNPs in haplotype context, however, the G allele at -1464 in the -1464G/-511C/-31T haplotype combination expressed higher transcriptional and translational activities, underscoring the influence of other SNPs in the genetic environment on individual SNPs^[38].

It is also possible that the allele flip seen in *IL-1B* polymorphisms results from allelic heterogeneity reflecting differences in clinical backgrounds between Asian and Caucasian populations, such as the prevalence of *H. pylori*-related premalignant gastric lesions and cancer arising at different anatomical sites. The presence of *H. pylori* infection is strongly associated with risk of non-cardia intestinal gastric cancer. The *IL-1B*-1464G/511C/-31T haplotype, with high mucosal IL-1β expression, is believed to be a proinflammatory allele that produces IL-1β in excess in response to *H. pylori* infection, and suppresses gastric acid secretion. Prolonged hypochlorhydria provides an environment favorable to *H. pylori* survival, leading to atrophic gastritis or intestinal metaplasia, and subsequently to non-cardia intestinal-type gastric cancer; the predominant subtype among East Asians. The direct

suppression of gastrin secretion by excess IL-1 β expression in association with the -31T allele may be directly observed^[39].

In non-cardia intestinal-type gastric cancer in Caucasians, *IL-1RN**2 may be an essential factor. IL-1RA, encoded by the *IL-1RN* gene, is a naturally occurring anti-inflammatory cytokine that competes with the binding of IL-1 to its receptor^[40]. The second intron of *IL-1RN* includes a penta-allelic 86-bp variable number tandem repeat producing two repeats (*IL-1RN**2) or three to six repeats (*IL-1RN**L)^[40]. As El-Omar *et al*^[11] have suggested, the presumably proinflammatory *IL-1RN**2 and *IL-1B*-31C haplotypes, presenting risk factors for *H. pylori*-related gastric cancer, may be in strong LD in Caucasian populations. Accordingly, low acid secretion shows a significant positive association with *IL-1RN**2, and homozygosity for this allele increases risk of hypochlorhydria. In an *in vitro* study, *IL-1RN**2 evidently increased production of IL-1B, regardless of the allele type of *IL-1B*, indicating that *IL-1RN**2 has a decisive role, not the IL-1B polymorphisms^[41]. In the Human Genome Epidemiology Network (HuGE) meta-analysis, the association of *IL-1RN**2 with gastric cancer detected appears to be confined to non-Asian populations, because overall frequency of the *IL-1RN**2 allele among Asians is low, if measurable^[26,32].

Cardia, intestinal-type gastric cancer

Studies of gene associations in cardia cancer are conducted mostly with Caucasian subjects because non-cardia, intestinal-type cancer predominates among Asians. In an unusual investigation, Kamangar *et al*^[42] first divided cancer into cardia and non-cardia gastric adenocarcinoma and then tested associations with *H. pylori*. *H. pylori* showed a strong positive risk association with non-cardia gastric cancer but an inverse association with cardia gastric cancer risk^[42]. Some studies have found that *H. pylori* infection is associated with decreased risk of adenocarcinoma arising near the esophagogastric junction^[42-47]. This may be explained by the tendency of *H. pylori* colonization to induce gastric atrophy, with reduced acid secretion, thereby reducing acid reflux into the esophagus, and reducing risk of esophageal or junction cancer^[42,48]. The cardia cancers were positively associated with gastroesophageal reflux disease (GERD)^[49]. The *IL-1B*-1464C/-511T/-31C allele among Caucasians is associated with low levels of IL-1 β expression in response to *H. pylori* infection or other inflammatory stimuli, and could not efficiently suppress gastric acid. Increased acid production following a subsequent inflammatory response would then produce GERD-like symptoms and promote cardia cancers.

IL-10 GENE POLYMORPHISM

IL-10, encoded by the *IL-10* gene at chromosome 1q31.1, is an anti-inflammatory cytokine. Three polymorphisms in the *IL-10* promoter, namely *IL-10*-1082

(G/A; rs1800896), -819 (C/T; rs1800871), and -592 (C/A; rs1800872), are shown to influence inflammation in response to infection at the transcriptional level^[50-52]. An allele flip in *IL-10* polymorphisms with respect to gastric cancer risk is also observed. In Caucasian populations, risk for non-cardia gastric cancers in association with the -1082AA genotype may be increased twofold, while the -1082G allele is the risk allele in cardia gastric cancer in studies of Asians, independent of *H. pylori* infection^[53-55]. In a meta-analysis, *IL-10*-1082G carriers showed a significant increase in risk of developing gastric cancer, especially for cardia-type gastric cancer among Asians^[56,57]. A recent meta-analysis by Yu *et al*^[58] showed a significantly negative association of *IL-10*-819TT with gastric cancer risk in Asians, in accordance with the previous finding that *IL-10*-819CC is a risk allele^[59]. Furthermore, identification of *IL-10*-592AA as a protective allele for total gastric cancer incidence in Asians supports *IL-10*-1082G/-819C/-592C as the risk haplotype^[60,61].

Evidence indicates that selection mechanisms operating on the *IL-10* region differ among ethnic groups. In Asian populations, with relatively high prevalence of chronic *H. pylori* infection, *IL-10*-1082A, is found more frequently than in Caucasian populations. Relatively low IL-10 expression by *IL-10*-1082A promotes elimination of *H. pylori* infection, and this may exert positive selective pressure on the haplotype. In Caucasian populations *H. pylori* infection is less prevalent, and greater IL-10 production would be advantageous in defense against infectious and inflammatory diseases^[55]. This may explain the relatively high frequency of the *IL-10*-1082G allele in Caucasian populations^[55]. Evidence for balancing selection within the IL-10 promoter region is consistently reported in studies of European populations^[62,63].

In Caucasian populations with low rates of *H. pylori* infection and premalignant lesions, the -1082A allele imposes risk for gastric cancer through low IL-10 production and consequent excess of proinflammatory cytokines. This promotes inflammation of the gastric mucosa, which may increase frequency of the mutation^[55,64]. These findings are consistent with observations of carcinogenesis in non-cardia cancer. In Asian populations, wherein *H. pylori* infection and premalignant lesions such as atrophic gastritis and intestinal metaplasia are more common, high-expression *IL-10*-1082G may suppress cytotoxic anti-tumor T-cell activity and thereby promote tumor progression^[55,65]. In high-risk populations, IL-10 may play an essential role in advanced stages of gastric cancer^[55]. In the Taiwanese population, *IL-10*-1082G may be linked to gastric cancer risk and advanced cancer, and cardia location of gastric cancer may be associated with a high-producing *IL-10* genotype^[66]. Carriers of the *IL-10*-1082G/-819C/-592C haplotype may be susceptible to virulent *H. pylori* strains with a high capability to colonize and adapt, and also to gastric cancer development^[67]. As compared to carriers of the *IL-10*-1082A/-819T/-592A haplotype, those with the *IL-10*-1082G/-819C/-592C carriers show higher mucosal levels of IL-10 mRNA,

which may result in a diminished proinflammatory response and capacity to control *H. pylori* infection^[67]. Actually, it appears that the *IL-10* genotype at -1082 is sufficient to establish a risk relationship with gastric cancer, because the *IL-10*-1082 genotype correlates well with mucosal *IL-10* mRNA levels, and *IL-10*-1082G fully represents the high-expression *IL-10*-1082G/-819C/-592C haplotype^[55,67]. Consequently, the allele flip of *IL-10* observed in gastric cancer represents allelic heterogeneity, similar to that observed in *IL-1B*.

GENETIC FACTORS OTHER THAN *IL-1B* AND *IL-10*

Diffuse-type gastric cancer

Intestinal-type gastric cancer follows a multistep progression that usually initiates in chronic gastritis, whereas diffuse-type gastric cancer lacks defined premalignant lesions; diffuse-type gastric cancer is therefore suspected to be more influenced by genetics factors^[22,68]. Genome-wide association studies (GWASs) reveal some additional gastric cancer susceptibility loci^[69]. Detected in a Japanese GWAS, an SNP (rs2976392) in the prostate stem cell antigen (*PSCA*) gene, which encodes a glycosylphosphatidylinositol-anchored cell surface antigen, shows a significant association with diffuse-type gastric cancer^[70]. Two SNPs (rs2070803 and rs4072037) in mucin 1 (*MUC1*) also show positive risk associations with diffuse-type gastric cancer in Asian populations^[69-71]. We found that normal T cell expressed and secreted (*RANTES*)-403A presents a significant increase in risk for diffuse-type gastric cancer in an Asian male population, when stratified by Lauren classification and sex^[72].

Non-cardia, intestinal-type gastric cancer

The chemokine *IL-8* participates in the initiation and amplification of acute inflammatory reactions as well as in the maintenance of chronic inflammatory processes^[73]. Evidence now links *IL-8* to tumorigenesis, angiogenesis and metastasis^[74-76]. A meta-analysis has shown an increased risk of *IL-8*-251A (T/A; rs4073) allele in several cancers, including gastric cancer, among Asians, but no such correlations among Europeans, suggesting racial differences in disease susceptibility with respect to *IL-8* polymorphisms^[77]. A case-control study has also shown no significant association between *IL-8*-251 polymorphism and increased risk of gastric cancer, whereas the association remains in Asians^[78]. In gastric cardia adenocarcinoma (non-cardia like), but not esophageal squamous cell carcinoma, the AGT/AGC haplotypes of *IL-8* polymorphisms showed a fourfold increase in relative risk in a high-risk Chinese population^[79]. Unfortunately, most association studies on *IL-8* polymorphisms have focused on a single polymorphism at -251, without considering its haplotype structure. The *IL-8*-251A allele resides on two different haplotypes, and only one of these is associated with disease^[80]. In other words, information regarding the *IL-8* haplotype structure is essential in determining the

true relationship between *IL-8* polymorphisms and gastric cancer development.

Finally, the rs13361707 SNP in the first intron of protein kinase, AMP-activated, $\alpha 1$ catalytic subunit (PPKAA1) and rs9841504 in the intron of zinc finger and BTB domain containing protein 20 (ZBTB20) emerge as susceptibility loci from GWAS analysis^[81].

CONCLUSION

Here, we aimed to summarize our rapidly evolving understanding of polymorphic structure in the interleukin promoter region and the involvement of *IL-1B* and *IL-10* polymorphisms in gastric cancer development. Analysis of these polymorphisms offers possible explanations for the allele flip observed in associations of *IL-1B* and *IL-10* with gastric cancer risk. The epidemiology of gastric cancer subtypes suggests a difference in genetic background between Asian and Caucasian groups. Among Asians, the *IL-1B*-1464G/-511C/-31T haplotype presents a risk allele for gastric cancer. This corresponds physiologically to increased *IL-1 β* expression in response to chronic *H. pylori* infection, which may inhibit gastric acid production and promote atrophic gastritis and non-cardia gastric cancer. Then, what about the gastric cardia cancer, which affects only a minority of Asians? In Asians, the highly expressive *IL-10* allele may serve to augment the inflammatory response to colonization by virulent strains of *H. pylori*, and, following malignant transformation high *IL-10* production, may tend to suppress anti-tumor cytotoxic T-cell response, thereby contributing to tumor progression. *IL-1 β* expression influences the initiation of cancer in response to *H. pylori* infection, whereas *IL-10* influences tumor progression after malignant transformation. Conversely, the less-expressive haplotype of *IL-1B* is associated with gastric cancer risk in Caucasians, specifically cancer of the gastric cardia. In this setting, low levels of *IL-1 β* produced in response to *H. pylori* infection may increase gastric acid secretion, promoting gastric cardia cancers through induction of GERD. Concerning non-cardia gastric cancers in Caucasians, the less expressive *IL-10* haplotype may promote metaplasia in the distal portion of the stomach by augmenting inflammatory response, while the *IL-1RN**2 polymorphism contributes by activating *IL-1 β* production. In conclusion, stratifying gastric cancer subtypes according to both anatomical site and the Lauren histological classification is essential in establishing genetic risk factors, because different subtypes follow different pathways of development and failure to consider this may produce false associations.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Endoscopic treatment for early gastric cancer

Yang Won Min, Byung-Hoon Min, Jun Haeng Lee, Jae J. Kim

Yang Won Min, Byung-Hoon Min, Jun Haeng Lee, Jae J. Kim, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea

Author contributions: Min YW drafted the manuscript; Min BH and Lee JH performed critical revision of the manuscript; Kim JJ designed the study and edited the manuscript.

Correspondence to: Jae J. Kim, MD, PhD, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea. jjkim@skku.edu

Telephone: +82-2-34103409 Fax: +82-2-34106983

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Endoscopic mucosal resection is an effective treatment modality with comparable results to surgery for selected cases of EGC. Endoscopic submucosal dissection (ESD) increases *en bloc* and complete resection rates and reduces the local recurrence rate. Recently, favorable outcomes of ESD have been reported in patients meeting expanded criteria of endoscopic treatment for EGC. This review will describe the techniques, indications and outcomes of endoscopic treatment for EGC.

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Abstract

Gastric cancer remains one of the most common causes of cancer death. However the proportion of early gastric cancer (EGC) at diagnosis is increasing. Endoscopic treatment for EGC is actively performed worldwide in cases meeting specific criteria. Endoscopic mucosal resection can treat EGC with comparable results to surgery for selected cases. Endoscopic submucosal dissection (ESD) increases the *en bloc* and complete resection rates and reduces the local recurrence rate. ESD has been performed with expanded indication and is expected to be more widely used in the treatment of EGC through the technological advances in the near future. This review will describe the techniques, indications and outcomes of endoscopic treatment for EGC.

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Key words: Early gastric cancer; Endoscopic mucosal resection; Endoscopic submucosal dissection

Core tip: Gastric cancer remains one of the most common causes of cancer death. However the proportion of early gastric cancer (EGC) at diagnosis is increasing.

INTRODUCTION

Gastric cancer remains one of the most common causes of cancer death worldwide, although its incidence and mortality rate are decreasing^[1,2]. Gastric cancer has become a relatively rare cancer in North America and in most Northern and Western Europe, but not in Eastern Europe, Russia and selected areas of Central and South America or East Asia^[2]. Given that the high incidence of gastric cancer, the National Cancer Screening Program recommends that men and women aged 40 years and over undergo upper endoscopy or upper gastrointestinal series every other year in Korea^[3] and similarly, gastric cancer screening has been conducted nationwide for all residents aged 40 years and over in Japan^[4]. As a result, the proportion of early gastric cancer (EGC) at diagnosis is increasing. The prognosis of EGC is excellent with a 5-year survival rate of over 90%^[5,6]. Furthermore, with the improved detection rate of EGC, the endoscopic treatment has become widespread due to advances in the instruments available and endoscopist's experience^[7-9]. This review will describe the techniques, indications and

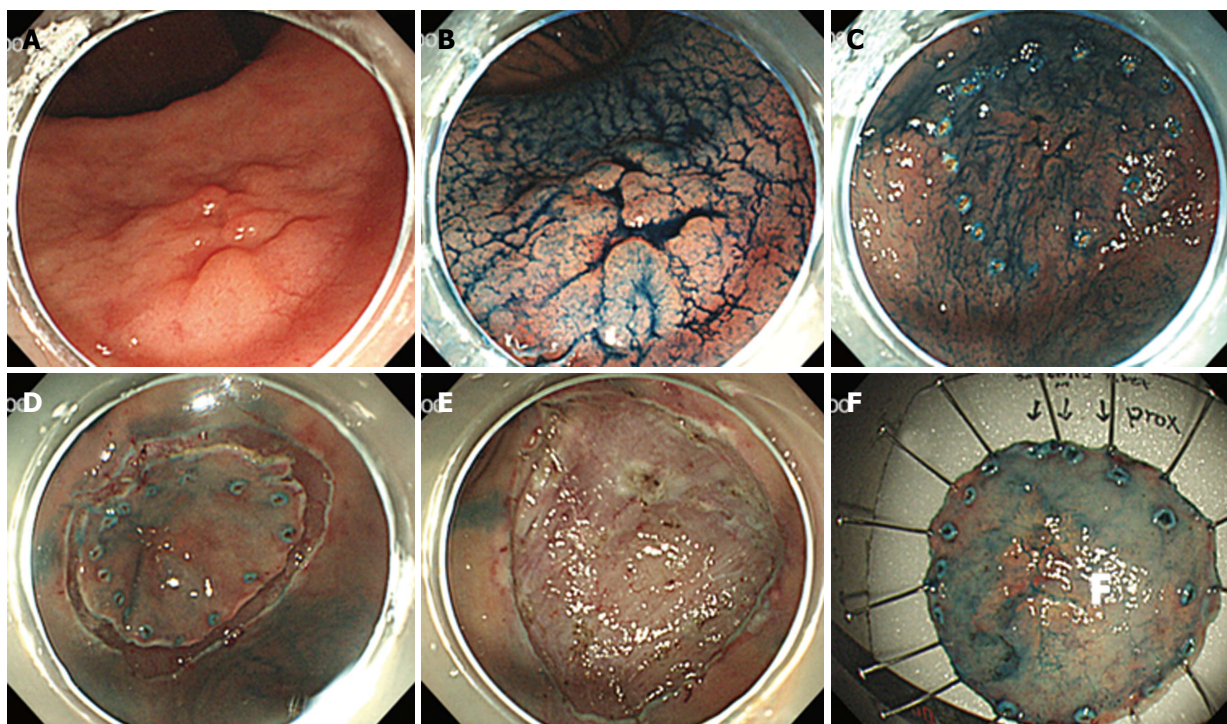


Figure 1 Endoscopic submucosal dissection procedure. A: On the lesser curvature side of the antrum, a 1.2-cm type IIa + IIc early gastric cancer is observed; B: Indigo-carmines is sprayed along the extent of tumor to aid visualization. C: Marking outside the lesion; D: After injection of saline mixed with diluted epinephrine (1:100000) and indigo-carmines into the submucosal layer, circumferential mucosal pre-cutting is performed using a knife; E: After dissection of the submucosal layer, an artificial ulcer is observed; F: Fixation of the tissue specimen.

outcomes of endoscopic treatment for EGC.

TECHNIQUES OF ENDOSCOPIC TREATMENT FOR EGC

Endoscopic mucosal resection

The technique and instruments of endoscopic polypectomy were developed in Japan^[10]. Since then, the strip biopsy was described as an extension of endoscopic snare polypectomy in 1984^[11]. This technique uses a double channel endoscope. After submucosal injection under the lesion, a snare is inserted through one channel and is used to remove the lesion, while a grasper, inserted through the other channel, is used to lift the lesion. In 1988, Endoscopic mucosal resection (EMR) after circumferential pre-cutting was described^[12]. In this technique, cutting around the lesion with a needle knife is done after submucosal injection using hypertonic saline mixed with diluted epinephrine, and then the lesion is removed by a snare. In 1992, EMR using transparent cap (EMR-C) was developed^[13]. This technique uses a transparent plastic cap that is connected to the tip of an endoscope. After submucosal injection, the lesion is sucked into the cap while a specialized crescent shaped snare, which is deployed at the tip of the cap, is closed. With this technique intramucosal cancers 2 cm or less in diameter can be safely removed^[14]. After that, EMR with ligation (EMR-L) was described^[15]. In this technique, a standard variceal ligation device is used to capture the lesion. After sucking

the lesion into the cap, lodged band is deployed underneath the lesion. The banded lesion is then resected by a snare. EMR-L is also safe and effective treatment modality for selected EGCs^[16]. As described above, EMR-C and EMR-L are relatively simple and effective treatment modality for EGC. However, it is apt to fragment tumors that are larger than 1.5-2.0 cm in diameter, which results in incomplete histological diagnosis and possibly in an increased risk of local recurrence^[17-19]. To overcome this drawback ESD, which is particularly effective for *en bloc* resection of tumors regardless of their size, was developed in late 1990s.

Endoscopic submucosal dissection

Endoscopic submucosal dissection (ESD) permits *en bloc* resection of larger lesions than that can be treated with EMR^[20-26]. This technique consists of several steps (Figure 1)^[27,28]. The marking around the lesion is done and circumferential mucosal pre-cutting is performed after submucosal injection. To distinguish clearly between the muscle layer and the submucosal layer and allow better hemostasis, normal saline mixed with diluted epinephrine and indigo-carmines is often used as submucosal injection solution. The injection is repeated a few times until the target mucosa is sufficiently raised. After lifting of the lesion, submucosal layer under the lesion is dissected with lateral movement using various knives. Several knives have been developed and used for ESD, which include needle knife, insulation-tipped diathermic knife (IT knife), hook knife, flex knife, triangle tip knife, flush

Table 1 Criteria for endoscopic treatment in patients with early gastric cancer

	Mucosal cancer				Submucosal cancer	
	No ulceration		Ulceration		SM1	SM2
Size (mm)	≤ 20	> 20	≤ 30	> 30	≤ 30	Any size
Histology						
Intestinal type	A	B	B	D	B	D
Diffuse type	C	D	D	D	D	D

SM1: Cancer invasion into the upper third of the submucosa; SM2: Cancer invasion into the middle third of the submucosa. A: Conventional indications; B: Expanded indications; C: Surgery, but need for further consideration; D: Surgery (gastrectomy + lymph node dissection); Data from Soetikno *et al*^[8].

knife, splash knife, IT-2 knife and dual knife^[6,28-30]. After resection of the lesion, visible vessels in the artificial ulcer is treated with hemostatic devices to prevent delayed bleeding.

INDICATIONS

Determining an indication for endoscopic treatment appears to be the most important step in managing patients with EGC. To select appropriate patients with EGC and to achieve a complete resection, the exact margin and depth of tumor could be determined through endoscopic evaluations. The horizontal extent of tumor can be determined with standard endoscopy and chromoendoscopy (CE). In some cases with unclear margins even with CE, magnifying endoscopy with narrow-band imaging could be useful to identify the precise margin^[31]. The depth of tumor invasion can also be assessed with standard endoscopy and CE. In addition, endoscopic ultrasonography could be used to further ascertain the depth. However, the accuracy of endoscopic ultrasonography in assessing the depth of invasion in EGC was reported to range from 71% to 78%^[32,33].

Conventional indication

The standard criteria^[8] for selection of patients with EGC who are appropriate for the endoscopic treatment are below: (1) well or moderately differentiated adenocarcinoma and/or papillary carcinoma; (2) confined to the mucosa; (3) smaller than 2 cm for superficially elevated type lesions; (4) smaller than 1 cm for the flat and depressed type lesions; (5) without ulcer or ulcer scar; and (6) without venous or lymphatic involvement^[34]. The rationale for this guideline is based on the knowledge that patients meeting the criteria are expected free from lymph node (LN) metastasis^[35].

Expanded indication

Expansion of the criteria for selection of patients with EGC who are appropriate for the endoscopic treatment has been proposed in Japan from clinical observations that the too strict absolute indication leads to unnecessary surgery^[36]. From the surgical data involving 5265

patients who underwent gastrectomy for EGC, Gotoda *et al*^[7] were able to further define the risk of LN metastasis in certain groups of patients with EGC and showed four groups with a low risk of LN metastasis: (1) differentiated intramucosal adenocarcinoma without lymphovascular invasion less than 3 cm in diameter, irrespective of ulcer findings; (2) differentiated intramucosal adenocarcinoma without lymphovascular invasion and ulcer findings, irrespective of tumor size; (3) undifferentiated intramucosal cancer without lymphovascular invasion and ulcer findings smaller than 2 cm in diameter; and (4) differentiated adenocarcinoma with minute submucosal penetration (SM1, cancer invasion into the upper third of the submucosa) but without lymphovascular invasion smaller than 3 cm in diameter. These results have allowed the development of expanded criteria for endoscopic treatment for EGC^[8] (Table 1). In the study by An *et al*^[37], predictive factors for LN metastasis in EGC with submucosal invasion were identified and possibility of EMR was addressed in highly selected submucosal cancers with no lymphatic involvement, SM1 invasion, and tumor size < 1 cm. In a recent study by Lee *et al*^[38] to compare the therapeutic outcomes of conventional and expanded indications of ESD for differentiated EGC, the conventional indication group and expanded indication group did not differ with regard to the rates of local recurrence (0.7% *vs* 0%), metachronous recurrence (3.6% *vs* 3.3%) or cumulative disease-free survival. Survival outcome was similar in the subgroups classified by tumor depth and size.

The risk of LN metastasis is known to increase in undifferentiated cancer due to lymphovascular invasion^[39]. In the analysis of 3843 patients who underwent gastrectomy with LN dissection for solitary undifferentiated EGC, none of the 310 intramucosal cancers 20 mm or less in size without lymphovascular invasion and ulcerative findings was associated with LN metastases^[40]. Recently, favorable outcomes of endoscopic resection have been reported in selected patients with undifferentiated mucosal cancer or minimal submucosal invasion cancer (SM1)^[41-43]. However, these are all single center retrospective studies. Therefore, large scale, prospective studies are warranted to confirm the feasibility of ESD for undifferentiated gastric cancer.

To expand the indication of endoscopic treatment to submucosal invasion (SM1) differentiated EGC, the histological heterogeneity of gastric cancer is the important issue to be addressed. Based on morphological features and histological background, gastric carcinoma is divided into differentiated and undifferentiated type or intestinal and diffuse type^[44,45]. Gastric cancer shows remarkable heterogeneity in histological pattern, cellular phenotype, and genotype^[46]. In a retrospective study to compare the clinicopathologic features of node-positive and node-negative differentiated submucosal invasion differentiated gastric cancers, histological heterogeneity was the independent risk factor for LN metastasis^[47]. Thus, it is recommended to apply the endoscopic treatment to the

differentiated EGC without histological heterogeneity when it is considered in submucosal invasion cancer.

COMPLICATIONS

Bleeding

Bleeding is the most common major complication of endoscopic treatment for EGC. Most bleeding occurs during the procedure or within 24 h^[48]. Bleeding is divided into immediate (intraoperative) bleeding during procedure and delayed bleeding after procedure. Significant immediate bleeding occurs more often in the upper and middle thirds of the stomach than in the lower third of the stomach because of the larger diameter of the submucosal arteries in the upper and middle thirds of the stomach^[49]. However, bleeding can be successfully treated in most cases through coagulation of the bleeding vessels, or placement of metallic clips for severe bleeding. In terms of delayed bleeding, the incidence rates were reported to range from 0%-15.6% in the recent review involving 28 studies with at least 300 ESD cases for EGC^[50]. Delayed bleeding is associated to tumor location, larger tumor, recurrent lesion, macroscopic type (flat or depressed), old age (≥ 80 years) and longer procedure time^[51-53]. At first, delayed bleeding was reported to occur more frequently after ESD for lesions in the lower and middle thirds of the stomach compared to the upper third of the stomach^[55]. However, the reason remains unclear. In the recent study involving 1000 cases of ESD for early gastric neoplasms, delayed bleeding occurs more often in upper portion of the stomach than in lower portion (28.6% *vs* 13.8%, $P = 0.003$)^[51]. In relation to antiplatelet drugs, there is a controversy in the risk of bleeding after ES. Two Korean retrospective studies have reported conflicting results on the risk of bleeding after ESD for gastric neoplasms^[56,57].

Perforation

Perforation is less common major complication of endoscopic resection for EGC than bleeding and has been reported to range from 1.2% to 5.2%^[50]. Perforation is diagnosed when mesenteric fat or intra-abdominal space is directly observed during the procedure (frank perforation) or free air is found on a plain chest X-ray after the procedure without a visible stomach wall defect during the procedure (micro-perforation). Immediately recognized small perforations can be successfully treated without surgery with a combination of endoscopic clipping and broad spectrum antibiotics^[58-60]. However, large perforations would require immediate surgery. In cases of micro-perforation, management is not well established. In a retrospective study by Jeong *et al*^[61], 13 cases (3.18%) of micro-perforation after EMR for gastric neoplasms were reported. Among them, 11 cases were successfully treated only with fasting, nasogastric tube drainage and broad spectrum antibiotics. In severe pneumoperitoneum, respiratory deterioration and/or shock could occur. Decompression of the pneumoperitoneum must be per-

formed with a puncture needle in such cases^[60]. Instead of air insufflations, CO₂ insufflation during procedure could minimize such pneumoperitoneum caused by a perforation^[62,63].

Other complications

Stenosis after gastric ESD has been reported to range from 0.7% to 1.9%^[50]. In a retrospective study, stenosis occurred with 17% of cardiac resections and 7% of pyloric resections^[64]. Circumferential extent of the mucosal defect of $> 3/4$ and longitudinal extent > 5 cm were related to stenosis with both cardiac and pyloric resections. However, all affected patients ($n = 15$) were successfully treated by endoscopic balloon dilation.

Aspiration pneumonia after gastric ESD has been reported to range from 0.8% to 1.6%^[50]. However, the risk of aspiration pneumonia appears to increase in sedation with continuous propofol infusion with intermittent or continuous administration of an opioid^[65,66]. In addition, longer procedure time (> 2 h), male gender and old age (> 75 years) are associated with occurrence of aspiration pneumonia after ESD.

Pain after endoscopic resection is usually mild and dull in nature and can be controlled by proton pump inhibitor and opioids^[36].

OUTCOMES

EMR is often the procedure of choice for patients who meet the standard criteria for endoscopic resection of EGC. Studies have shown high survival and cure rates in patients with EGC who undergo EMR. In the analysis of 308 EGCs resected endoscopically, 89% of type IIa lesions less than 2 cm were resected curatively, while only 50% of those larger than 2 cm were resected completely. In type IIc, 83% of lesions less than 1 cm and 57% of those greater than 1 cm were excised completely by endoscopic resection. In type IIc, curative endoscopic resection was possible in 85% of differentiated carcinomas and 43% of undifferentiated carcinomas^[67]. These successful outcomes have allowed EMR to become the standard treatment for EGC in Japan^[36]. In a Japanese report of 131 patients with differentiated mucosal EGC less than 2 cm (without ulcerative change) that had been completely removed by EMR, two patients (1.5%) died of gastric cancer during the mean observation period of 58 mo. The disease-specific 5- and 10-year survival rates were 99% and 99%^[68]. However, EMR is associated with risks of local recurrence, especially when resections are not performed *en bloc*, or when the resection margins are involved by tumor. The risk of local recurrence after EMR ranged from 2% to 35% in Japanese series^[69]. In a recent cross-sectional, retrospective cohort study, maximum diameters exceeding 2 cm was the independent risk factor for piecemeal EMR and no recurrence was observed in the *en bloc* group^[70]. In a Korean multicenter, retrospective study, complete resection rate after EMR was (77.6%) and local recurrence rate 6.0% with a median

interval between EMR and recurrence of 17.9 mo (range 3.5-51.7 mo). No deaths were related to recurrence of gastric cancer during the overall median follow-up period of 39 mo^[71]. ESD increases *en bloc* and complete resection rates and reduces the local recurrence rate. In a retrospective study, EMR and ESD were compared with each other^[20]. *En bloc* and histologically complete resection rates were higher with ESD than with EMR, regardless of tumor size. Local recurrences were treated by incomplete EMR (*en bloc*, 2.9%; piecemeal, 4.4%) but no patient experienced recurrence after ESD. The outcomes of ESD show 94.9%-97.7% *en bloc* rates and 83.1%-97.1% 5-year survival rates^[30,51,72-75]. In a retrospective study of EGC that fulfilled the expanded criteria, *en bloc* resection was achieved in 94.9% (559/589) and 550 of 581 lesions (94.7%) were deemed to have undergone curative resection^[72]. *En bloc* resection was the only significant contributor to curative ESD. Patients with non-curative resection developed local recurrence more frequently. The 5-year overall and disease-specific survival rates were 97.1% and 100%, respectively^[72]. In the long-term outcomes of ESD for EGC, *en bloc* resection rate was 97.7% for all lesions treated by ESD. The incidence of positive horizontal and vertical margins was 3.7% and 3.4%, respectively. There were no deaths related to ESD. Local recurrence was observed in five patients (1.1%), and metachronous recurrences in 7.8% of the patients. The post-treatment 5-year survival was 83.1%. There were no deaths as a result of gastric cancer associated with sites treated by ESD^[73]. In a Korean multicenter, retrospective study, the rates of *en bloc* resection, complete *en bloc* resection, vertical incomplete resection and piecemeal resection were 95.3%, 87.7%, 1.8% and 4.1%, respectively^[51]. The rates of delayed bleeding, significant bleeding, perforation and surgery related to complication were 15.6%, 0.6%, 1.2% and 0.2%, respectively. In other Korean single center, retrospective study, *en bloc* and curative resection rates were 96.7% and 88.3%, respectively^[74]. The curative resection rate was significantly lower in the expanded group than in the standard group (82.1% *vs* 91.5%, $P = 0.001$). During a median follow-up of 24 mo, the local tumor recurrence rate was also higher in the expanded group than in the standard group (7.0% *vs* 1.8%, $P = 0.025$). Local recurrence was more frequent in lesions with non-curative resection than in those with curative resection (20.0% *vs* 1.3%, $P < 0.001$). The 5-year overall and disease-specific survival rates were 88% and 100%, respectively; the difference between the standard and expanded groups was not significant ($P = 0.834$).

CONCLUSION

EMR is an effective treatment modality with comparable results to surgery for selected cases of EGC. However, there is a risk of piecemeal resection with EMR in cases of large EGC, which is associated with higher recurrence rates. ESD increases *en bloc* and complete resection rates and reduces the local recurrence rate. Recently, favorable

outcomes of ESD have been reported in patients meeting expanded criteria of endoscopic treatment for EGC. However, its technical difficulty requires a long learning period and technical invasiveness increases the risk of complications. Thus, further efforts are needed to make ESD easier and safer, which could be achieved through the technological advances. In addition, standardization of the pathologic diagnosis is necessary for the more reliable ESD. Finally, confirmation of more long-term outcomes under the expanded indication is warranted for establishing an appropriate indication of ESD for EGC.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Diagnosis and evaluation of gastric cancer by positron emission tomography

Chen-Xi Wu, Zhao-Hui Zhu

Chen-Xi Wu, Zhao-Hui Zhu, Department of Nuclear Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

Author contributions: Wu CX and Zhu ZH wrote and edited the manuscript.

Correspondence to: Zhao-Hui Zhu, MD, PhD, Department of Nuclear Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, No. 1 Shuaifuyuan, Wangfujing Street, Dongcheng District, Beijing 100730, China. zhuzhh@pumch.cn

Telephone: +86-10-69154196 Fax: +86-10-65124875

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Core tip: This systematic review summarizes and discusses various aspects regarding positron emission tomography (PET), and PET/computed tomography application in gastric cancer, including diagnosis and its influencing factors, therapy evaluation, recurrence detection, current limitations and improvement, and so on.

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Abstract

Gastric cancer is the second leading cause of cancer mortality worldwide. The diagnosis of gastric cancer has been significantly improved with the broad availability of gastrointestinal endoscopy. Effective technologies for accurate staging and quantitative evaluation are still in demand to merit reasonable treatment and better prognosis for the patients presented with advanced disease. Preoperative staging using conventional imaging tools, such as computed tomography (CT) and endoscopic ultrasonography, is inadequate. Positron emission tomography (PET), using ¹⁸F-fluorodeoxyglucose (FDG) as a tracer and integrating CT for anatomic localization, holds a promise to detect unsuspected metastasis and has been extensively used in a variety of malignancies. However, the value of FDG PET/CT in diagnosis and evaluation of gastric cancer is still controversial. This article reviews the current literature in diagnosis, staging, response evaluation, and relapse monitoring of gastric cancer, and discusses the current understanding, improvement, and future prospects in this area.

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INTRODUCTION

Gastric cancer, a common malignancy with a poor prognosis, is listed as the second leading cause of cancer mortality worldwide^[1]. To date, a curative therapy for gastric cancer mainly relies on the complete resection of the tumor; thus, early diagnosis and accurate evaluation are important for decision-making regarding treatment and for the prognosis. The broad availability of gastrointestinal endoscopy has significantly improved the diagnosis of gastric cancer. Effective methods for the accurate staging and quantitative evaluation of gastric cancer are in demand to develop a reasonable selection of treatments for most patients presented with advanced gastric cancer. Accurate staging of the disease, including the full disclosure of the local invasion extent, lymph node involvement, and distant metastasis, is important for patient management and surgical planning. Conventional imaging tools for preoperative staging, including computed tomography (CT) and endoscopic ultrasonography (EUS), have so far been found inadequate due to their technical limitations^[2].

In recent decades, positron emission tomography (PET) using ^{18}F -fluorodeoxyglucose (FDG) as a tracer has proven useful in the diagnosis and evaluation of a variety of malignancies by providing metabolic information about tumors. In particular, most PET scanners have now been integrated with CT into a single system, significantly increasing diagnostic accuracy by combining metabolic and anatomic images. FDG PET/CT has shown great advantages in staging, therapeutic evaluation, and recurrence surveillance in various malignancies^[3]. As for gastric cancer, several clinical guidelines, including those of the National Comprehensive Cancer Network (NCCN), indicate that PET/CT is recommended in patients if metastatic cancer is not evident and is useful in demonstrating occult metastatic disease^[4]. The European Society for Medical Oncology (ESMO) guidelines for gastric cancer also suggest that PET imaging may improve staging through an increased detection of the involved lymph nodes or metastatic disease^[5]. However, the overall sensitivities of PET and PET/CT in the detection of gastric cancer are relatively low compared to those in most other malignancies. Due to such reasons as the physical FDG uptake and involuntary movements of the gastric wall, some types of gastric malignancies will affect the detection ability of FDG PET for gastric cancer. Thus, the value of FDG PET/CT in the diagnosis and evaluation of gastric cancer is still controversial^[6,7].

This article broadly reviews the current literature on FDG PET and PET/CT for the diagnosis, staging, therapeutic evaluation, and relapse monitoring of gastric cancer. An up-to-date understanding, recent improvements, and future prospects in this area are also discussed.

DETECTION AND EVALUATION OF PRIMARY GASTRIC CANCER

The detectability and diagnostic accuracy of FDG PET or FDG PET/CT in gastric cancer may be influenced by many factors, such as the tumor size, histological type, and location as well as the physiological FDG uptake by the gastric wall.

Influence of tumor size

Tumor size is one of the major factors influencing the FDG PET detection of primary gastric cancer. For small lesions, PET detection is always a challenge due to its limited spatial resolution. A study showed that FDG PET had a sensitivity of 76.7% for the detection of gastric cancer > 30 mm but only 16.8% for those less than 30 mm^[8]. Recent studies have indicated that tumor size was a major factor influencing the standardized uptake value (SUV) of gastric cancer on FDG PET^[9,10]. In another study, tumor invasion depth was found to be an independent factor for the FDG uptake in gastric lesions^[11]. Because late-stage tumors are usually larger in size with deeper invasion, advanced gastric cancer (AGC), in general, tend to yield a higher sensitivity in FDG PET imaging than early stage gastric cancer (EGC). Dassen

and colleagues summarized the sensitivity of FDG PET as ranging from 26% to 63% for EGC and from 93% to 98% for AGC^[7].

Influence of histological type

FDG PET may also have different sensitivities for the detection of different types of gastric malignancies. The biological characteristics vary among different types of gastric malignancies, which may significantly influence the uptake of FDG. Most studies reported that FDG PET had significantly lower sensitivities in detecting diffuse type, mucinous adenocarcinoma (MAC) or signet-ring cell carcinoma (SRC) than the intestinal-type gastric adenocarcinoma or tubular adenocarcinoma (TAC)^[8,12-15], although some studies using different patient groups obtained different results^[9,10,16].

The potentially lower FDG uptake in diffuse type gastric adenocarcinoma or MAC/SRC may be influenced by several factors, including the low-density diffuse infiltration of adenocarcinoma cells, existence of extracellular or intracellular metabolically inert mucus content, and low expression level of glucose transporter 1 (GLUT-1)^[12,13,17]. These factors cause the low sensitivity of FDG PET in these types of gastric cancer.

Influence of tumor location

The stomach regions can be divided into the gastroesophageal junction (GEJ) and the upper (or proximal), middle, and lower (or distal) parts. FDG PET detection of GEJ tumors was reported to be more sensitive than that of gastric adenocarcinomas at other stomach parts, possibly due to the higher incidence of intestinal types within GEJ cancers^[18]. Although some researchers reported that FDG PET had a similar detectability for gastric cancers located at the upper, middle, and lower parts of the stomach^[8,19], some others argued that gastric cancers located at the upper or proximal part were more readily detected than those at the lower or distal part of the stomach^[13].

Influence of the physiological uptake of the gastric wall

The physiological uptake can also influence PET detection of gastric cancer. Physiological accumulation is a common issue for the detection of malignancy using FDG PET or FDG PET/CT imaging. Under empty-stomach states, approximately 38.0% and 59.5% of normal gastric walls show moderate and intensive FDG uptake^[20], and the specificity was reported to be as low as 50%^[21] using FDG PET for gastric cancer due to the high incidence of normal gastric wall uptakes. Previously, it was reported that there were significant differences in the physiological FDG uptake among the three parts of the gastric wall (upper > middle > lower); thus, this technique may be more confidently used to diagnose a gastric malignancy at the distal part of the stomach^[22]. Recently, many studies have focused on methods to increase the sensitivity and specificity of FDG PET or PET/CT by reducing the physiological uptake in the gastric wall

through methods such as gastric distention^[20,21,23-27] or through medicines to inhibit gastric movement^[28,29], as will be discussed in the following sections.

Influence of tumor biology

For gastric cancer, one of the most frequently studied genes associated with FDG uptake is GLUT-1. Several studies have reported that tumorous GLUT-1 expression was positively associated with the FDG SUV of gastric cancer^[12,13,17]. Most recently, the expression of some hypoxia-related genes, such as hypoxia-inducible factor 1 (HIF-1) in gastric cancer cells, was also found to contribute to FDG uptake in gastric tumors^[10]. Actually, *GLUT-1* and *HIF-1* gene transcriptions are interrelated with one another, and they both play key roles in tumor cell metabolic changes^[30].

EVALUATION OF LYMPH NODE INVOLVEMENT

For N staging in gastric malignancies, one meta-analysis reported that the sensitivity and specificity of FDG PET or PET/CT ranged between 33.3%-64.6% and 85.7%-97.0%, respectively, although there was no significant diagnostic difference compared to AUS, CT or magnetic resonance imaging (MRI)^[31]. Other individual studies reported that FDG PET or PET/CT was less sensitive but more specific compared to commonly used CT and MRI^[16,32,33]. There are many reasons for the low sensitivity of FDG PET in detecting lymph node metastases. The first is the histological type of the primary tumor. As summarized before, the diffuse type or MAC/SRC was usually less or non FDG-avid. Therefore, metastases of the same cell types in lymph nodes were less likely to be detectable by FDG PET^[16,33]. Additionally, many studies have found that the SUV_{max} of the primary tumor was associated with the SUV_{max} of the lymph nodes^[33,34]. In a report, 60%-70% of lymph node metastases were not detected in patients with non FDG-avid primary tumors^[35]. The second reason is the size of metastatic lymph nodes. Some metastatic lymph nodes in gastric cancer could be as small as 3 mm^[36], which is beyond the detectability of most PET scanners. The PET scanners have a spatial resolution of 4-6 mm. Some small lymph nodes are even more difficult to discriminate because of the radioactive volume effect generated by the nearby primary tumor. Even by PET/CT, many lymph node metastases remain ambiguous^[34]. Other factors, such as the high physiological uptake background from the normal gastric wall, would also compromise the sensitivity of PET for N staging.

In spite of the low sensitivity, FDG PET or PET/CT usually showed a higher specificity than most other imaging modalities, including CT and MRI, in the N staging of gastric cancer. Because FDG PET and FDG PET/CT diagnose lymph node metastasis using glucose metabolism rather than the size change, it is very useful to dis-

tinguish the enlarged lymph nodes due to inflammation from cancer cell metastasis. Additionally, the different criteria for lymph node enlargement in CT and MRI images can also decrease the specificity of these modalities in the N staging of gastric cancer.

EVALUATION OF DISTANT METASTASIS

In general, the conventional tools for detecting distant metastasis are CT and histological confirmation. Among the many metastatic sites for gastric cancer, peritoneal metastasis is considered an operative contraindication and represents the most difficult type for treatment^[37]. Compared to CT, FDG PET usually showed a lower sensitivity for the diagnosis of peritoneal seeding^[14,34,38,39]. Reasons explaining these results include the following: (1) the small and diffuse growing patterns of metastasis seeding and (2) the diffuse histological type of gastric cancer, which is more likely to spread into the peritoneal cavity^[40]. Therefore, many studies suggest high quality CT as the preferred modality of choice for the diagnosis of peritoneal metastasis^[38]. Although the sensitivity is lower, FDG PET or PET/CT could still be useful for detecting peritoneal metastasis, especially when the CT results are equivocal. FDG imaging of peritoneal metastasis may also help to avoid unnecessary laparotomy in a considerable portion of patients. Just as in the recently published work by Smyth *et al.*^[14], although FDG PET/CT does not add benefit to high-quality contrast CT for identifying gastric cancer peritoneal metastases, the use of FDG-PET/CT in addition to CT, EUS and laparoscopy can avoid futile gastrectomy in almost 10% of patients, saving more than \$10000 per patient. The authors recommend its use in staging all potentially operable gastric cancer patients.

The frequently targeted distant solid organs include the liver, lungs and bones. In a study reported by Chung *et al.*^[41], FDG PET/CT imaging was able to detect solid organ metastasis (lungs, liver, bone, or adrenal gland) with a sensitivity of 95.2% and a specificity of 100%. In another study, FDG PET detection of the liver, lung and bone metastases was found to be satisfactory and accurate^[42]. Specifically, a study reported that FDG PET was sensitive for the detection of liver metastasis from gastric cancer^[43], although a meta-analysis reviewing CT, US, EUS, and FDG PET, FDG PET showed only a moderate ability in this aspect^[38]. For bone metastasis, whole-body bone scanning is a frequently used modality to evaluate the status of bone metastasis. In a study, the authors compared the value of FDG PET and whole-body bone scintigraphy for the detection of bone metastasis in gastric cancer patients. They found that the two modalities had a similar sensitivity and accuracy for detecting bone metastasis in gastric cancer, but FDG PET was superior for detecting synchronous bone metastasis^[44], with a sensitivity of 93.5%. However, Yoshioka *et al.*^[42] reported that FDG PET did not seem to be useful for the detection of bone metastasis, with a sensitivity of only 30%.

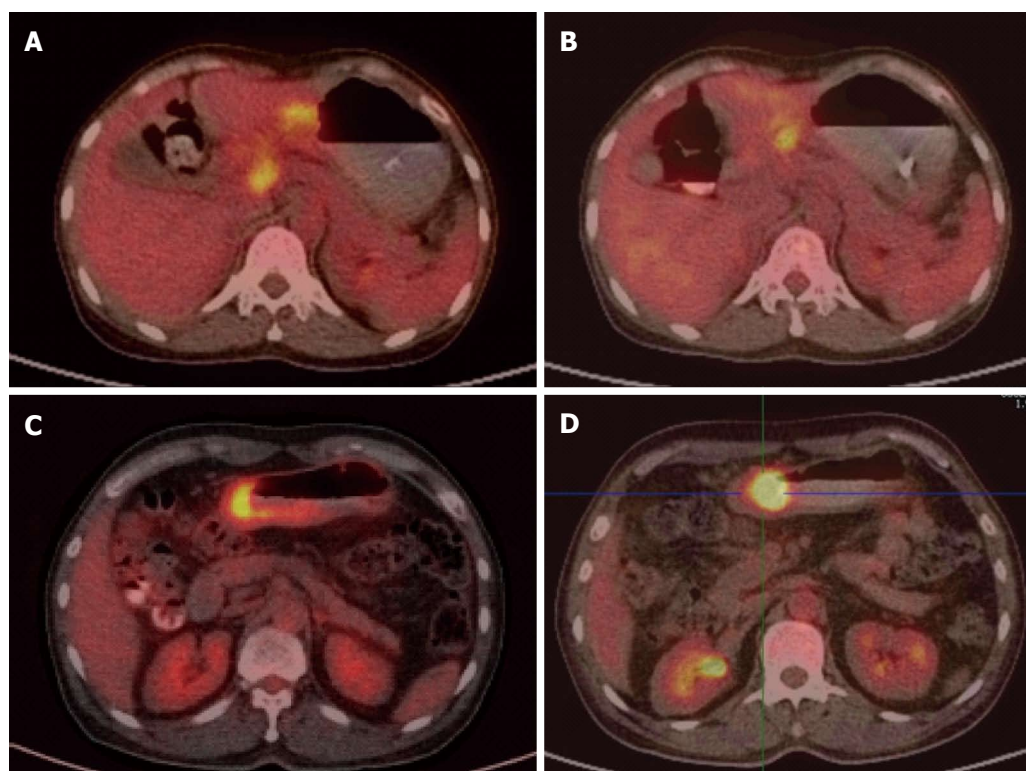


Figure 1 ^{18}F -fluorodeoxyglucose positron emission tomography/computed tomography for evaluation of response to neoadjuvant chemotherapy. ^{18}F -fluorodeoxyglucose (FDG) tomography/computed tomography (PET/CT) imaging of a responder patient before (A) and after three cycles of chemotherapy (B); After therapy, the patient showed significant tumor SUV reduction (A: $\text{SUV}_{\text{max}} = 4.1$, B: $\text{SUV}_{\text{max}} = 2.1$, $\% \Delta \text{SUV} = 37.3\%$), corresponding to his histological response (Grade 2b) according to the JRS GC (Japanese Research Society for Gastric Carcinoma) criteria; FDG PET/CT imaging of a non-responder patient before (C) and after three cycles of chemotherapy (D); The tumor SUV was the same after therapy (C: $\text{SUV}_{\text{max}} = 9.2$, B: $\text{SUV}_{\text{max}} = 9.7$, $\% \Delta \text{SUV} = -5.4\%$), corresponding to his histological response (Grade 0) according to the JRS GC criteria. SUV: Standardized uptake value.

RESPONSE EVALUATION AND RELAPSE MONITORING

Currently, the only curative treatment for gastric cancer is the surgical removal of gastric tumors with lymph node dissection. Recently, some treatment combinations, such as chemotherapy or radiotherapy, have been used in addition to surgical removal for gastric cancer patients. The evaluation of the therapy outcomes is, therefore, of great importance in managing patients, guiding future therapy improvements, and directing personalized treatments. Currently, FDG PET or PET/CT is emerging as an effective tool for therapeutic evaluation in many types of cancers, including gastric cancer. The following sections will discuss tumor response evaluation and tumor recurrence prediction using FDG PET or PET/CT in gastric cancer.

Tumor response evaluation or prediction

Although curative surgery remains the mainstay of gastric cancer treatment, the 5-year survival in these patients is only approximately 25%^[45]. To improve the relapse-free and overall survival in these patients, perioperative chemotherapy or radiochemotherapy has been gaining increasing interest in recent years for gastric cancer^[46-48]. For preoperative or so-called neoadjuvant chemotherapy, there has been accumulating evidence that it

might improve the survival in responding patients with locally advanced gastric cancer^[49,50]. However, for non-responders, a considerable number of complications following neoadjuvant chemotherapy and surgery as well as the minimal benefit from this additional therapy have to be considered^[51]. Depending on the different therapy regimes and evaluation methods, it has been reported that approximately 30%-60% of patients receiving preoperative chemotherapy were histological responders, including both total and partial responders^[46,52]. Therefore, it is important to distinguish those non-responding patients at an early phase of chemotherapies to prevent further ineffective and potentially harmful interventions.

In recent years, evidence has suggested that FDG PET or FDG PET/CT seems to be an effective noninvasive tool for response assessment in gastric cancer^[12,53-55]. Metabolic reduction early after the initial of neoadjuvant chemotherapy can be used to discriminate non-responders from responders for further therapeutic adjustments (Figure 1). FDG uptake changes in tumor sites seemed to be associated with subsequent histological tumor regression as well as with patient survival. In a phase II trial reported by Di Fabio *et al*^[53] using response evaluation criteria in solid tumors (RECIST) by CT as a standard response evaluation tool, they discovered that the sensitivity and specificity of FDG PET were satisfactory (83% and 75%, respectively) in evaluating gastric cancer

responses to neoadjuvant chemotherapy. In correlation with the prognosis, metabolic responders had a preferable prognosis compared to metabolic non-responders, and FDG PET evaluation was found to be even better than RECIST evaluation by CT in predicting the median time to disease progression (TTP) and overall survival. However, due to the low FDG uptake in some types of gastric cancer, it is sometimes still difficult or inaccurate to evaluate tumor responses based on SUV change in these cases. Therefore, in a retrospective study of Ott *et al.*^[55], the authors specifically described the FDG non-avid patients as a third metabolic group, aside from metabolic responders and non-responders. They suggested that the FDG non-avid group had a poor response rate and unfavorable prognosis similar to that of metabolic non-responders, indicating that neoadjuvant chemotherapy may not be useful in patients with low FDG uptakes at baseline PET imaging. In that study, they also found that FDG PET imaging analysis was in good accordance with the pathological analysis for tumor response and that metabolic responders (34.7%) also tended to have a more favorable prognosis compared to metabolic non-responders (65.3%) and FDG non-avid patients. In both of the studies described above, the PET evaluation of tumor response resulted in patient treatment strategy changes, during which non-responders either stopped previous chemotherapy plans and underwent earlier surgical removals or changed to other chemotherapy regimens.

Tumor recurrence prediction and surveillance

In many other types of malignancies, FDG PET/CT has been widely used for both preoperative prediction and post-surgery/treatment surveillance for tumor recurrence^[56-59]. For gastric cancer, the conventionally used recurrence prediction parameters include the stage of gastric cancer, depth of tumor invasion, and extent of lymph node metastasis^[60,61]. However, these factors are sometimes difficult to evaluate before surgery for gastric cancer; therefore, FDG PET/CT, as a noninvasive evaluation method, has been used to provide additional information to predict recurrence after an operation or treatment. Most studies found that FDG uptake in gastric cancer was an independent, significant prognostic factor for predicting cancer recurrence after curative surgical resection^[19,62,63]. In these studies, patients with lower uptakes of FDG in the gastric lesions before surgery had significantly lower incidences of tumor recurrence and better recurrence-free survival after the operation, especially those with intestinal type or TAC. In FDG non-avid diffuse type or MAC/SRC, a better prognostic tendency preferring lower FDG uptake was also discovered, but no exact conclusion was made^[62]. In addition, preoperative FDG PET/CT was reported as a predictor of the curability of gastric cancer. In a retrospective study by Hur *et al.*^[64], high FDG uptake in the primary tumor and positive FDG uptake in local lymph nodes at PET/CT were significantly associated with non-curative resection, suggesting that these patients should be subjected

to neoadjuvant chemotherapy or laparoscopic staging to avoid unnecessary laparotomy. However, a conflicting report suggested that the survival rate showed no significant difference between the patients with and without tumor FDG uptakes^[13], but this may be due to the effects of adjuvant chemotherapy before surgery, which was not performed in other studies.

For post-surgery surveillance, contrast-enhanced CT is the most commonly used imaging tool for gastric cancer, but it cannot always detect the presence and viability of tumor precisely, such as when differentiating recurrent tumors from post-surgical changes. With the increasing clinical use of PET/CT, some studies reported that FDG PET/CT was superior to contrast-enhanced CT in the detection of recurrent gastric cancer after initial surgery^[65], whereas others reported that these two imaging modalities shared a similar performance in the detection of gastric recurrence after surgery^[66]. Based on two recent meta-analyses, the sensitivity of FDG PET/CT in detecting gastric cancer recurrence after surgical removal was 78%-86%, whereas the specificity was 82%-88%^[67,68], and the results of PET imaging impacted patient management to different degrees, either by avoiding previously planned therapeutic procedures or by using previously unplanned treatment procedures^[65,69]. However, whether FDG PET/CT should be added in addition to CT examination for post-surgery gastric cancer recurrence surveillance is still debatable, as there was quite a large amount of evidence suggesting that the benefits from PET/CT imaging were not sufficient to outweigh its high cost compared to CT examination alone. In the study reported by Sim *et al.*^[66] the additional PET/CT on contrast CT did not increase diagnostic accuracy in the detection of recurrent gastric cancer in general, and contrast-enhanced CT was even more sensitive than PET/CT for detecting peritoneal seeding. An earlier study using FDG PET suggested that PET was not suited for the follow-up of gastric cancer after treatment^[70], but that might be due to the lower image quality at that time and the lack of image fusion, especially the anatomic localization by CT.

FDG PET IN OTHER TYPES OF GASTRIC NEOPLASMS

Gastric lymphoma

Primary gastric lymphoma (PGL) is the most frequent non-Hodgkin's lymphoma of extranodal origin, and it accounts for 3%-5% of all of the malignant tumors of the stomach^[71]. Histologically, PGL can be divided into diffuse large B-cell lymphoma (DLBCL) of the stomach and mucosa-associated lymphoid tissue (MALT) gastric lymphoma. The role of PET in PGL has been reported recently, and many studies have supported the usefulness of PET as a tool for response evaluation in PGL^[72-75]. One study used both CT and FDG PET/CT for the staging of patients with PGL and found that PET/CT correctly up-graded 22% and down-graded 14% of the patients, suggesting that PET/CT was more accurate

in staging PGL. In addition, the study found that FDG SUV_{max} was significantly associated with Lugano stage, indicating that PET imaging could reflect the aggressiveness of disease^[72], which was also supported by another study^[74]. In the study reported by Sharma *et al.*^[73], ¹⁸F-FDG PET/CT used in follow-ups seemed to be very accurate for the detection of relapse after treatment.

For the two major histological types of PGL, the FDG PET/CT detection rate was higher in the DLBCL subtype than in the MALT lymphoma, with sensitivities of 97%-100% and 39%-80%, respectively^[72,75,76]. Therefore, FDG PET or PET/CT has its limitations in detecting MALT lymphoma compared to other subtypes. Such limitations were reported by Yi *et al.*^[72] in the same study, showing that treatment-related ulcerative or mucosal lesions caused a high rate of false positive uptake, especially in patients with MALT lymphoma, indicating that PET/CT scans alone may not be enough to assess the response of PGL.

Gastrointestinal stromal tumors

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract, representing 1%-3% of gastrointestinal malignancies. Approximately 70% of GISTs occurs in the stomach, 20% in the small intestine, and less than 10% in the esophagus^[77]. GISTs derive from interstitial Cajal cells, and almost 100% of patients with GIST express c-kit receptor tyrosine kinase. Therefore, tyrosine kinase inhibitors, such as imatinib mesylate, now represent the standard treatment for patients with inoperable GISTs^[78]. The major application of FDG PET or PET/CT imaging to GIST is for therapeutic evaluation to provide early tumor response information, and a vast majority of studies have confirmed the value of FDG PET and PET/CT over morphological-based imaging modalities in this aspect^[79-85]. In some of these studies, the PET criteria correlate well with progression-free survival, while CT evaluation did not. Therefore, ¹⁸F-FDG PET has become the gold standard for the early assessment of tumor response to imatinib as well as other c-kit inhibitors in GISTs. In addition to the therapeutic evaluation, FDG PET and PET/CT have also been used to analyze the prognostic value of FDG SUV_{max} in GIST patients^[86,87], to differentiate GISTs from abdominal lymphoma by studying the metabolic heterogeneity differences^[88] and to study FDG kinetics and gene expression in GISTs^[89].

Gastric schwannomas

Schwannomas are tumors originating from nerves with a Schwann cell sheath. The stomach is the most common site of gastrointestinal schwannomas, accounting for 0.2% of all gastric neoplasms^[90]. There were several case reports of gastric schwannomas with FDG PET scans, and all of these cases showed high FDG uptakes, with SUV_{max} ranging from 5.8 to 7.1^[91-93]. Therefore, these studies indicated the necessity of differentiating between gastric schwannomas and GISTs, both of which will show up as

intensive FDG accumulations on PET images.

IMPROVEMENTS IN THE DIAGNOSIS OF GASTRIC CANCER USING PET OR PET/CT

As previously stated, the overall sensitivity and specificity of PET and PET/CT in the detection of gastric cancer are relatively lower than those in some other malignancies. The physiological uptake of FDG in the normal gastric wall and the existence of non FDG-avid histological types of gastric cancer may all contribute to this result. To improve the diagnosis and evaluation of gastric cancer using PET or PET/CT, several improvements have been applied in different aspects, either by decreasing the physiological uptake of the normal gastric wall, applying different time-point of imaging, or using more specific radio-tracers. In this section, therefore, we will mainly discuss improvements in the following three aspects.

Gastric distension

Because FDG is not a tumor-specific tracer, many benign lesions in the stomach, such as gastritis, leiomyoma, polyps, and even normal gastric walls, can have moderate to intense FDG uptakes. Therefore, when a positive uptake is observed in the stomach, the interpretation of the images should be carefully conducted, especially for post-treatment evaluation. To decrease the physiological uptake, gastric distension has been studied recently as a modified PET imaging protocol for patients with questionable stomach lesions that resulted in increased specificity and accuracy for the detection of gastric malignancies. Gastric distension can be achieved by the consumption of water, milk, food, or foaming agents before PET scanning^[20,21,23-27] (Table 1). After distension, the physiological uptake of the normal gastric wall was relatively decreased, thus increasing the tumor/background ratio, even for small size tumors (Figure 2). In addition, with water or milk as a negative contrast agent in the stomach, tumors could be more easily delineated. Some local lymph node metastases can also be detected with a lower gastric wall uptake background, improving the accuracy of staging^[23].

Dual-time point imaging

Another potential method for differentiating benign lesions in the stomach from malignancies is dual time-point PET scanning, which visualizes the trends of the FDG uptake changes. It is well recognized that for a malignant lesion, FDG uptake at late time-point (usually 2-3 h after FDG injection) PET scanning will be increased compared to the early time-point imaging result (45 min to 1 h after tracer injection). However, for physiological uptake or other non-malignant lesions, this value will most likely decrease or remain the same^[94]. This method has proven useful in the detection, staging and differentiation of various types of cancers, including breast cancer^[95],

Table 1 Gastric distention methods in ¹⁸F-fluorodeoxyglucose positron emission tomography and positron emission tomography/computed tomography imaging of gastric cancer

Ref.	Patients	n	Imaging modality	Distention methods	Sensitivity		Specificity	
					Before distention	After distention	Before distention	After distention
Tian <i>et al</i> ^[27]	With suspected gastric tumors	38	FDG PET	Oral intake of vesicant (2-3 g) with 40-60 mL water		83%		88%
Yun <i>et al</i> ^[26]	After gastrectomy for gastric cancer	30	FDG PET	Drinking at least 300 mL water	94%	88%	69%	92%
Zhu <i>et al</i> ^[25]	With proven primary gastric carcinomas	3	FDG PET	Intake of 100 g bread and 400 mL cow milk				
Zhu <i>et al</i> ^[20]	With proven gastric tumors	24	FDG PET	Intake of 300-400 mL cow milk		96%		
Kamimura <i>et al</i> ^[21]	With gastric carcinomas	16	FDG PET	Intake 400 mL water	100%	88%	50%	100%
Lee <i>et al</i> ^[23]	With proven gastric tumors	44	FDG PET/CT	Intake 500 mL water	50%	75%		
Ma <i>et al</i> ^[24]	With suspected gastric tumors	68	FDG PET/CT	Intake milk with diatrizoate meglumine	93%	91%	75%	92%

FDG: ¹⁸F-fluorodeoxyglucose; PET/CT: Positron emission tomography/computed tomography.

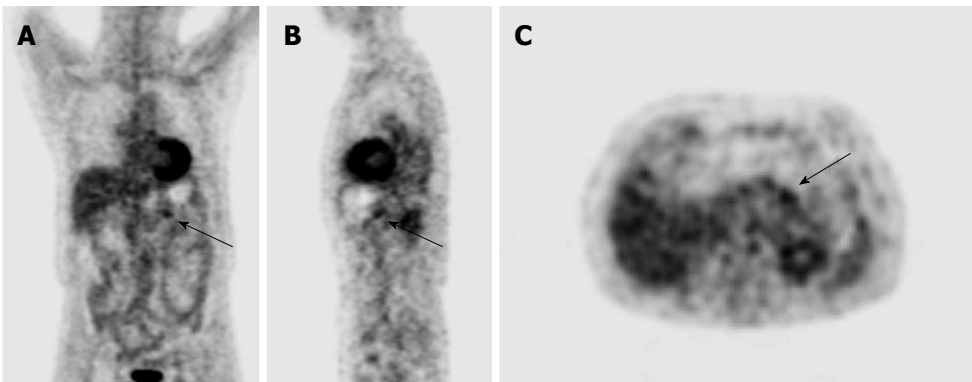


Figure 2 ¹⁸F-fluorodeoxyglucose positron emission tomography imaging of gastric cancer under gastric distention. A small size gastric tumor (arrow, 1.5 cm × 1.2 cm, highly differentiated gastric adenocarcinoma) was clearly observed with low background gastric wall uptake due to gastric distention (A-C).

lung cancer^[96], and colorectal cancer^[97]. For gastric cancer, limited studies have been reported. In the only report by Lan *et al*^[98] involving five gastric malignant tumors and three cases of gastritis, the SUV_{max} in the late time-point (2.5-3 h after FDG injection) increased by 4%-45% in all 5 malignant lesions, whereas two cases of gastritis had decreased uptakes, with the remaining one remaining at the same SUV level. The late time-point was especially useful when the early time-point SUV was equivocal. In the future, the exact value of dual time-point scanning for gastric cancer imaging awaits further proof.

Non-FDG tracers in the evaluation of gastric cancer

Targeting cell glucose metabolism using FDG is extensively used in PET oncologic imaging. However, due to the unsatisfactory imaging results of FDG PET or PET/CT in FDG non-avid gastric cancer, new PET imaging tracers are needed for the better detection of gastric cancer with higher sensitivity and specificity. Therefore, a new type of PET imaging tracer, ¹⁸F-FLT, has been devel-

oped and used to target cell proliferation in many *in vivo* imaging studies. The mechanism for the cell proliferating imaging using ¹⁸F-FLT proceeds in the following manner. After being taken up by the cell *via* both passive diffusion and facilitated transport by Na⁺-dependent carriers, ¹⁸F-FLT will be phosphorylated by thymidine kinase 1 (TK1) into ¹⁸F-FLT-monophosphate, which is trapped in the cell. However, because the enzymatic activity of TK1 is different in quiescent cells and proliferating cells, the accumulation of ¹⁸F-FLT-monophosphate will be higher in proliferating cells, such as malignant cancer cells, normal hepatocytes, and bone marrow cells^[99]. Recently, ¹⁸F-FLT PET and PET/CT imaging has been used in many types of cancers, such as colorectal cancer^[100], lung cancer^[101], brain tumors^[102] and gastrointestinal tumors^[103].

In gastric cancer, the use of ¹⁸F-FLT was reported to increase the detection rate, especially for FDG non-avid histological types. In a study reported by Herrmann and Herrmann *et al*^[104], ¹⁸F-FLT in the preoperative detection of gastric cancer had a sensitivity of 100%, while FDG

Table 2 Comparison of ^{18}F -FLT and ^{18}F -fluorodeoxyglucose positron emission tomography or positron emission tomography/computed tomography imaging for detection of gastric cancer

Ref.	Study purpose	Imaging modality	SUV		Sensitivity for detection of primary tumor		Sensitivity for detection of metastasis		Prognostic factor
			^{18}F -FLT	^{18}F -FDG	^{18}F -FLT	^{18}F -FDG	^{18}F -FLT	^{18}F -FDG	
Herrmann <i>et al</i> ^[104]	Preoperative evaluation	PET	Mean: 6.0	Mean: 8.4	100%	69%			
Kameyama <i>et al</i> ^[105]	Preoperative evaluation	PET	Mean: 7.0	9.4	95%	95%			
Kameyama <i>et al</i> ^[103]	Preoperative evaluation	PET	Mean: 2.1-8.0		90%				
Ott <i>et al</i> ^[106]	Neoadjuvant chemotherapy evaluation	PET	Before treatment: 6.1 After treatment: 5.3	Before treatment: 8.4 After treatment: 5.2					FLT uptake at 2-wk after treatment
Zhou <i>et al</i> ^[107]	Preoperative evaluation	PET/CT	Max: 5.5	Max: 8.4	92%	95%	Liver: 30% Bone: 20% Other organs: 90%-97%	Liver: 100% Bone: 100% Other organs: 91%-95%	

FDG: ^{18}F -fluorodeoxyglucose; PET/CT: Positron emission tomography/computed tomography; SUV: Standardized uptake value.

showed only a 69% sensitivity in the same population. In another study, ^{18}F -FLT showed a slight increase in the detection rate of primary gastric cancer, with a similar sensitivity to FDG (95.2% and 95.0%, respectively)^[105]. Importantly, in both studies, FLT was able to delineate gastric lesions that were negative in FDG images, most of which were non intestinal or diffuse types upon histology. Based on this advantage, Ott *et al*^[106] further investigated the value of ^{18}F -FLT PET imaging in predicting gastric cancer responses to neoadjuvant chemotherapy and patient prognosis. In that study, the SUV_{mean} of ^{18}F -FLT but not the FDG two weeks after chemotherapy was the only independent prognostic factor for gastric cancer patients. The unchanged high uptake of ^{18}F -FLT after treatment might indicate the failure of treatment because this suggested a constant proliferation at the tumor site. However, recently, another study came to the opposite conclusion, suggesting that ^{18}F -FLT PET had no added value in the preoperative staging of gastric cancer, especially for liver and bone metastasis, which had a much lower sensitivity than FDG PET^[107]. Indeed, the high physiological uptake of FLT in the liver and bone marrow can hamper the detection of some primary gastric tumors and bone metastasis sites, rendering FLT not suitable for M staging (Table 2). In the future, the exact value of ^{18}F -FLT in the diagnosis and evaluation of gastric cancer needs further investigation.

LIMITATIONS AND FUTURE PROSPECTS

In summary, the limitations of FDG PET and PET/CT in the diagnosis and evaluation of gastric cancer mainly come from three aspects: (1) the variety of histological differences in gastric cancer; (2) the physiological properties of the stomach; and (3) the spatial resolution of PET. Many FDG non-avid histological types greatly decrease the sensitivity of FDG PET and PET/CT in

gastric cancer detection, and new imaging tracers, including FLT, are currently under evaluation as alternatives. For the second limitation, gastric distention by different methods seems to be effective in decreasing background uptake. Furthermore, pharmaceutical interventions, including muscle relaxants and proton pump inhibitors, are also under further investigation for this purpose. As to the third limitation, currently the highest achievable spatial resolution of PET is 2.36 mm for clinical purposes and 0.83 mm for pre-clinical uses^[108]. The observation of early stage gastric cancer and metastatic lymph nodes similar to or below this range therefore remains difficult to achieve from PET images. In combination with CT, PET/CT appears to improve the accuracy of many diseases, including gastric cancer, but N staging in gastric cancer is still not satisfactory under current conditions. In the future, the spatial resolution of PET can be improved by optimizing the camera design within the physical fundamental limitations. In addition, the new generation of multimodality imaging equipment, such as PET/MR and PET/CT/MR, will hopefully provide complementary advantages in the diagnosis and evaluation of various diseases, including gastric cancer.

CONCLUSION

PET and PET/CT technology provides a useful tool for the diagnosis and evaluation of gastric cancer. These modalities can detect lymph node metastases and distant metastatic sites in other organs using one single image, can identify early tumor responses that may not be apparent using other modalities, and may have prognostic value that can change patient management. Although many problems remain, PET and PET/CT imaging remains promising, and with current and further improvements, PET and PET/CT imaging may make the diagnosis and evaluation of gastric cancer more standardized and accurate.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Inflammation-related factors predicting prognosis of gastric cancer

Wen-Jun Chang, Yan Du, Xin Zhao, Li-Ye Ma, Guang-Wen Cao

Wen-Jun Chang, Yan Du, Guang-Wen Cao, Department of Epidemiology, Second Military Medical University, Shanghai 200433, China

Xin Zhao, Li-Ye Ma, Department of General Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200438, China

Author contributions: Chang WJ, Du Y and Zhao X collected data, drafted the manuscript and contributed equally to this work; Ma LY critically read this paper; Cao GW supervised and revised the manuscript.

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Correspondence to: Guang-Wen Cao, MD, PhD, Professor of Medicine, Chairman, Department of Epidemiology, Second Military Medical University, 800 Xiangyin Rd., Shanghai 200433, China. gcgao@smmu.edu.cn

Telephone: +86-21-81871060 Fax: +86-21-81871060

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Abstract

Gastric cancer (GC), which is mainly induced by *Helicobacter pylori* (*H. pylori*) infection, is one of the leading causes of cancer-related death in the developing world. Active inflammation initiated by *H. pylori* infection and maintained by inherent immune disorders promotes carcinogenesis and postoperative recurrence. However, the presence with *H. pylori* in tumors has been linked to a better prognosis, possibly due to the induction of antitumor immunity. Tumor infiltrations of tumor-associated macrophages, myeloid-derived suppressor cells, neutrophils, Foxp3⁺ regulatory T cells are correlated with poor prognosis. Tumor infiltrating CD8⁺ cytotoxic T lymphocytes, dendritic cells, and CD45RO T cells are generally associated with good prognosis of GC, although some subsets of these immune cells have inverse prognosis prediction values. High ratios of Foxp3⁺/CD4⁺ and Foxp3⁺/CD8⁺ in tumors are as-

sociated with a poor prognosis; whereas high Th1/Th2 ratio in tumors predicts a good prognosis. High levels of interleukin (IL)-6, IL-10, IL-32, and chemokine C-C motif ligands (CCL)7 and CCL21 in circulation, high expression of CXC chemokine receptor 4, chemokine C-C motif receptor (CCR)3, CCR4, CCR5, CCR7, hypoxia-inducible factor-1 α , signal transducer activator of transcription-3, cyclooxygenase-2, and orphan nuclear receptor 4A2 in tumors are associated with an unfavorable prognosis. Increased serum levels of matrix metalloproteinases (MMP)-3, MMP-7, and MMP-11 and increased levels of MMP-9, MMP-12, and MMP-21 in tumors are consistently associated with poor survival of GC. Further emphasis should be put on the integration of these biomarkers and validation in large cohorts for personalized prediction of GC postoperative prognosis.

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Key words: Gastric cancer; Inflammation; Biomarker; Prognosis

Core tip: The prognosis of gastric cancer (GC) is not satisfactory, and is associated with *Helicobacter pylori* and/or Epstein-Barr virus infection, as well as host inflammation-related factors. In this article, we summarize the inflammation-related microbial and host factors that are reported to be associated with GC prognosis from different specimens and populations. So far, few simple panels have been clinically used for predicting GC prognosis. It is necessary to integrate different biomarkers with clinicopathological variables for personalized prediction of GC prognosis. The prognostic values of integrated predictors should be validated in large prospective cohorts before clinical application.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer in men and the fifth in women worldwide. Almost one million new cases are diagnosed annually. More than 70% of new cases and deaths occur in developing countries^[1]. To date, surgical resection remains the mainstay of curative treatment for GC. However, a subset of patients will develop local relapses and metachronous metastases after resection of the primary tumor. The overall 5-year survival rate of patients with GC in the United States is about 26%, while the rate improves to 63% if detected at an early stage. Similar low 5-year survival rates ($\leq 30\%$) are also seen in European countries. However, higher 5-year survival rates (up to 50%) are reported from East Asia such as Japan, mainly due to its early detection and treatment services^[2]. In addition, other measurable or unmeasurable factors including differences in proximal *versus* distal cancer incidences, environmental exposures, dominant pathological types, surgical factors, and neoadjuvant/adjuvant treatment protocols may also contribute to the differences in postoperative survival of GC patients. Because of the heterogeneity of GC prognosis, searching for more accurate predictors of GC prognosis has become a growing interest in GC research. Chronic infections of *Helicobacter pylori* (*H. pylori*) contribute to more than 75% of GC^[3], and about 10% of GC may be caused by Epstein-Barr virus (EBV) infection^[4]. Although a causal relationship of EBV infection with nasopharyngeal cancer has been identified, the association of EBV infection with GC has not been confirmed so far. Interestingly, *H. pylori* induce EBV reactivation in the gastric epithelium of GC patients latently infected with EBV^[5]. A population-based intervention trial has demonstrated that a selective cyclooxygenase-2 (COX-2) inhibitor, celecoxib, or *H. pylori* eradication alone had beneficial effects on the regression of advanced gastric lesions^[6]. Regular use of non-steroidal anti-inflammatory drugs in individuals with *H. pylori* infection can effectively reduce the risk of GC^[7], indicating chronic inflammation following *H. pylori* infection contributes to the onset of GC. Accumulating evidence indicates that inflammation-related factors also play an important role in recurrence and metastasis of some types of cancers including GC. Both systemic inflammatory responses, such as primary or modified Glasgow prognostic score^[8-11] and blood neutrophil-to-lymphocyte ratio^[11-15], and local inflammatory responses such as the infiltration of various immune cells and their subsets in tumors (*e.g.*, infiltrating S100A9⁺ inflammatory cells^[16]) are associated with the prognosis of GC. Here, we review and summarize the inflammation-related microbial pathogen (Table 1) and host (Table 2) factors that have been shown to be associated with GC prognosis.

MICROBIAL PATHOGEN FACTORS

H. pylori

Chronic infection with *H. pylori* is the major cause of GC. It is well established that *H. pylori* infection contributes greatly to the carcinogenesis of GC. However, the role of *H. pylori* infection in predicting the survival of GC patients is less well understood. Interestingly, a prospective study has demonstrated that GC patients with positive *H. pylori* infection frequently showed better relapse-free survival and better overall survival (OS) after curative resection^[17], which is contradictory to the notion that *H. pylori* acts as a risk factor of GC during the carcinogenesis process. Although this finding is in contrast to some studies^[18,19], other studies^[20,21] especially a recent meta-analysis containing 2454 cases^[22] have demonstrated that *H. pylori* infection is an independent protective factor for GC progression. This protective effect is also consistent among different ethnic groups, using various *H. pylori* evaluation methods and quality assessment measures^[22]. The suppressive effect of *H. pylori* on GC progression is possibly due to the induction of some antitumor immunity^[17]. CagA, CagE, VacA and protein modifications (*e.g.*, CagA phosphorylation) of *H. pylori* have been associated with gastric carcinogenesis^[23-26], but the association between these factors and GC prognosis is still unclear.

EBV

About 10% of GC cases are infected with EBV, while the prognostic value of EBV is poorly understood. Lymphoepithelioma-like carcinoma (LELC) is a special subtype of GC, and over 90% of LELC are EBV positive. LELC tends to have a lower frequency of lymph node metastasis and a better survival rate than other GC subtypes^[27,28]. A recent meta-analysis including 4599 GC patients from 13 studies has shown that EBV positivity in tumors by *in situ* hybridization is associated with lower mortality (HR = 0.72; 95%CI: 0.61-0.86) and might serve as a valuable prognostic factor^[29]. Furthermore, the protective effect is quite stable across patients or tumor types. However, these studies cannot clarify whether EBV infection itself or EBV-associated inflammatory responses and/or their interactions result in the protective effect. EBV-associated GC (EBV-GC) is a recently recognized entity defined by the presence of EBV in GC cells. After stratification of EBV-GC by host inflammatory response, it was found that EBV-GC patients with a Crohn's disease-like lymphocyte reaction had significantly longer OS and disease-free survival (DFS) than other EBV-GC patients, indicating that inflammation induced by EBV-GC could affect the prognosis of GC^[28]. Mechanisms of the heterogeneity of induced inflammatory responses by EBV-GC need to be explored further.

HOST INFLAMMATION-RELATED FACTORS

There is a renaissance of research into the connection

Table 1 Important pathogens associated with the prognosis of gastric cancer

Factors	Source	Sample	Sample size	Cut-off value or characterization	Time of measurement	Prognostic role
<i>H. pylori</i>	Germany	Sera	166	Positivity	Prior to gastrectomy	Increased OS and RFS ^[17]
<i>H. pylori</i>	China	Tumor tissue	162	Positivity	At the time of surgery	Decreased OS and RFS ^[18]
<i>H. pylori</i>	Brazil	Tumor tissue	68	Positivity	At the time of surgery	No difference ^[19]
<i>H. pylori</i>	South Korea	Sera	274	Negativity	At the time of surgery and adjuvant chemotherapy	Decreased OS ^[20]
<i>H. pylori</i>	Italy	Sera and tumor tissue	297	Negativity	At the time of surgery	Decreased OS ^[21]
<i>H. pylori</i>	Brazilian, Asian and Caucasian	Sera	2454	Positivity	At the time of surgery	Increased OS and DFS ^[22]
EBV	Taiwan	Sera	150	Positivity	Prior to gastrectomy	Increased OS ^[27]
EBV	Korea	Sera	123	Expression	At the time of surgery	Increased OS and DFS ^[28]
EBV	Asia, Europe and Latin America	Sera	4599	Positivity	At the time of surgery	Increased OS ^[29]

DFS: Disease-free survival; EBV: Epstein-Barr virus; *H. pylori*: *Helicobacter pylori*; OS: Overall survival; RFS: Relapse-free survival.**Table 2** Important inflammation-related host factors with prognostic values for gastric cancer

Factors	Source	Sample size	Cut-off value or characterization	Time of measurement	Prognostic role
In peripheral blood					
MIF	China	97	> 6600 pg/mL	Prior to gastrectomy	Decreased 5-year survival rate ^[45]
Th1/Th2 ratio	Japan	157	High	After curative gastrectomy	Increased DFS ^[61]
Th17	China	51	High levels	Prior to gastrectomy	Decreased OS ^[62]
Th22	China	51	High levels	Prior to gastrectomy	Decreased OS ^[62]
CD57 ⁺ T cells	Japan	48	≥ 18%	At the time of gastrectomy	Decreased OS ^[68]
NLR	South Korea	775	> 3.79	Prior to gastrectomy	Decreased 5-year survival rate ^[12]
NLR	China	46	> 2.5	Prior to gastrectomy	Decreased PFS and OS ^[13]
TLR9	China	314	TLR9-1486C	Prior to gastrectomy	Decreased OS ^[73]
IL-1B + IL-1RN	Italy	123	IL-1B-511C/T and IL-1B-31T/C + Wide-type IL-1RN	Prior to gastrectomy	Decreased PFS and OS ^[76]
IL-6	Poland	99	> 288.7 pg/mL	At the time of gastrectomy	Increased overall complications and infective complications ^[83]
IL-2R	Japan	96	High expression	Prior to gastrectomy	Decreased OS ^[88]
IL-32	Japan	182	Positive expression	At the time of gastrectomy	Decreased OS ^[89]
VAP-1	Japan	107	Low levels	Prior to gastrectomy	Decreased OS ^[91]
MDSCs	United Kingdom	25	Increasing percentage	Prior to gastrectomy	Increased the risk of death ^[50]
MMP-11	China	86	Low levels at the 75 th percentile in the total group	After chemotherapy	Decreased median survival time and 1-year survival rate ^[104]
MMP-12	China	165	Positive expression	Prior to chemotherapy	Decreased OS ^[105]
In tumor					
TAM	Japan, Germany, Ukraine	449	Positive expression	Prior to chemotherapy	Decreased OS ^[44]
CD68 ⁺ Mφ	Japan	111	High numbers	At the time of gastrectomy	Decreased OS ^[45]
Nitrotyrosine	China	66	Intermediate or high expressions	At the time of gastrectomy	Decreased 5-year survival rate ^[51]
CD33 ⁺ /p-STAT ⁺ cells	China	100	> 11 cells/HPF	After curative gastrectomy	Decreased 5-year survival rate ^[52]
DCs	Japan	174	High levels	At the time of gastrectomy	Increased 5-year survival rate ^[53]
DCs	Bulgaria	55	Low numbers	At the time of gastrectomy	Decreased 5-year survival rate ^[54]
CD208 ⁺	Japan	128	High expression levels	At the time of gastrectomy	Decreased postoperative outcome ^[55]
CD15 ⁺ TINs	Japan	115	< 21.60 cells/HPF	At the time of gastrectomy	Increased OS ^[56]
HIF-1α	Japan, China, South Korea, United Kingdom	1268	High expression	Prior to gastrectomy	Decreased OS ^[77]
HIF-1α	Japan, China, South Korea, United Kingdom	1555	High expression	Prior to gastrectomy	Decreased OS ^[78]
S100A9 protein	China	176	> 200 positive cells/HPF	At the time of gastrectomy	Increased OS ^[16]
Stroma FoxP3 ⁺ TILs	Germany	52	> 125.9/mm ²	At the time of gastrectomy	Increased NED-survival and OS ^[113]
Stroma CD68 ⁺ /Foxp3 ⁺	Germany	52	High cell ratios	At the time of gastrectomy	Increased median survivals ^[113]
Tc17	China	103	Percentage ≥ 2.75% or cell number ≥ 484.37 per million	At the time of gastrectomy	Decreased DFS and OS ^[60]
FOXP3 ⁺ Tregs	China	107	High numbers	At the time of gastrectomy	Increased OS ^[47]
CD45RO ⁺ T cells	Japan	101	High levels	At the time of gastrectomy	Increased OS and DFS ^[67]
Foxp3 ⁺ /CD8 ⁺ ratio	China	133	High	At the time of gastrectomy	Decreased OS ^[65]

Foxp3 ⁺ /CD4 ⁺ ratio	South Korea	180	High	At the time of gastrectomy	Loco-regional recurrence ^[66]
T-bet ⁺ TILs	China	152	High numbers	At the time of gastrectomy	Increased OS and DFS ^[69]
CD19 ⁺ cells	China	846	> 7.91% ± 2.98%	At the time of gastrectomy	Increased DFS ^[70]
CD20 ⁺ B cells	China	100	High density	Prior to gastrectomy	Increased OS and DFS ^[52]
Natural killer cells	Brazil	72	> 15 NK cells/10 HPF	At the time of gastrectomy	Increased OS and DFS ^[71]
COX-2	South Korea	457	Lack of expression	At the time of gastrectomy	Decreased OS and DFS ^[79]
STAT3	South Korea	100	> 10% stained cells	At the time of gastrectomy	Decreased OS and DFS ^[81]
NR4A2	China	245	Immunoreactive score ≥ 3	At the time of gastrectomy	Decreased OS and DFS ^[85]
IL-12	Japan	85	High density	At the time of gastrectomy	Increased OS and DFS ^[86]
IL-10	Poland	136	> 10 pg/mL	At the time of gastrectomy	Decreased OS and DFS ^[87]
Annexin A1	Taiwan	118	High expression	At the time of gastrectomy	Decreased OS ^[90]
CCL7 and CCL21	China	194	Higher expression	At the time of gastrectomy	Decreased OS ^[92]
CXCR4	China	97	Higher expression	At the time of gastrectomy	Decreased OS ^[94]
HighCXCR4/high SDF-1α	South Korea	221	Expression	At the time of gastrectomy	Decreased 5-year survival rate ^[95]
CCR3	Japan	48	Positive expression	At the time of gastrectomy	Decreased OS ^[96]
CCR5	Japan	60	Positive expression	At the time of gastrectomy	Decreased OS ^[96]
CCR4	South Korea	753	Positive expression	At the time of gastrectomy	Decreased 5-year survival rate ^[97]
CCR7	Japan	224	> 10% positivity	At the time of gastrectomy	Decreased OS ^[98]
CX3CL1	Japan	158	High expression	At the time of gastrectomy	Decreased DFS ^[100]
CCL18	China	59	High expression	At the time of gastrectomy	Increased OS and DFS ^[48]
MMP-9	China, Finland, The Netherlands, Poland, Spain	1700	High expression	At the time of gastrectomy	Decreased DFS ^[102]
MMP-21	China	296	High expression	At the time of gastrectomy	Decreased OS ^[106]
MMP 14	China	205	Positive expression	Prior to chemotherapy	Decreased OS ^[107]
MT1-MMP, CD11b ⁺ immunocytes and LNR	China	184	MT1-MMP positive, low CD11b ⁺ immunocytes and high LNR	At the time of gastrectomy	Increased OS ^[110]
Inflammation gene signature	Brazil	51	High expression pattern	At the time of gastrectomy	Decreased OS ^[112]

CCL: Chemokine (C-C motif) ligand; CCR: C-C chemokine receptor; CD: Cluster of differentiation; COX-2: Cyclooxygenase-2; CX3CL1: Chemokine (C-X3-C motif) ligand 1; CXCR4: C-X-C chemokine receptor 4; DFS: Disease-free survival; FDXP3: Forkhead box P3; HIF-1α: Hypoxia-inducible factors-1α; HPF: High power field; IL: Interleukin; MDSCs: Myeloid-derived suppressor cells; MIF: Migration inhibitory factor; MMP: Matrix metalloproteinase; NLR: Neutrophil lymphocyte ratio; NR4A2: Nuclear receptor subfamily 4, group A, member 2; PFS: Progression-free survival; SDF-1α: Stromal cell-derived factor-1α; STAT: Signal transducers and activators of transcription; TAM: Tumor associated macrophages; Th1: T helper cell type 1; Th2: T helper cell type 2; Th17: T help cell type 17; Th22: T help cell type 22; TIL: Tumor infiltrating lymphocyte; TIN: Tumor infiltrating neutrophils; TLR: Toll-like receptors; Treg: Regulatory T cells; VAP-1: Vascular adhesion protein-1.

between inflammation and cancer^[30-32]. Most current research support that acute inflammation triggered by tumor-infiltrating leukocytes does not exert normal immunoprotective mechanisms that lead to eradication of the evolving cancer (antitumor immunity). Excessively and chronically produced pro-inflammatory mediators may contribute to tumor promotion and progression^[31-34]. Inadequate pathogen eradication, prolonged inflammatory signaling, and defects in anti-inflammatory mechanisms can lead to chronic inflammation and benefit tumor development^[35]. In an inflammatory state, there is a high rate of cell turnover and the microenvironment is often highly oxidative and nitrosative, thus increasing the opportunities for DNA damage and somatic mutation. Chronic inflammation can promote an environment that is conducive to carcinogenesis, and it is involved in tumor initiation, promotion, and progression^[31,36-39]. The tumor microenvironment is created by the tumor and dominated by tumor-induced interactions^[40]. In the inflammatory microenvironment, there is a delicate balance between antitumor immunity and tumor-originated pro-inflammatory activity, which weakens antitumor immunity^[33,41]. The tumor not only manages to escape from the host immune system (tumor escape), but it effectively contrives to benefit from infiltrating cells by modifying

their functions to create the microenvironment favorable to tumor progression^[40]. The net outcome of a persistent inflammatory microenvironment is enhanced tumor promotion, accelerated tumor progression, invasion of the surrounding tissues, angiogenesis, and often metastasis^[31]. Cancer-associated inflammation is characterized by infiltration of immune cells including tumor infiltrating lymphocytes (TILs)^[42], expression of cytokines and chemokines, tissue remodeling, and angiogenesis. The diverse cells communicate with each other by means of direct contact or through cytokines and chemokines, therefore exerting their functions of tumor promotion or suppression. Cancer cells can also release chemokines and recruit immune cells to constitute the inflammatory microenvironment. The inflammation-related molecules such as nuclear factor-κB (NF-κB) and signal transducer activator of transcription-3 (STAT3), primary inflammatory cytokines, secondary inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs) form an inflammatory molecular network, playing an active role in maintaining tumor-promoting inflammation or antitumor immunity. Although tumor infiltrating immune cells and their interactions can reflect the host-tumor-pathogen immune response, immune cells and molecules in peripheral blood are also important for exploring the characteristics

of the complex tumor-related inflammation.

TIMs

TIMs are the major type of infiltrating inflammatory cells regulating antitumor immunity and are represented by mature cells such as macrophages, granulocytes, and dendritic cells (DCs), as well as by pathologically activated immature myeloid-derived suppressor cells (MDSCs)^[43]. Macrophages, one of the most important components of the inflammatory infiltration in tumors, include M1-like and M2-like subtypes. M1-like macrophages facilitate anti-tumor immunity, while M2-like macrophages promote tumor progression. M2-like macrophages are strongly affected by the tumor microenvironment, and are also termed tumor-associated macrophages (TAMs). A meta-analysis of 55 studies with 8692 patients has shown that higher TAM infiltration is associated with worse OS in several cancers, including GC (RR = 0.52; 95%CI: 0.35-0.77)^[44]. Thymidine phosphorylase (TP) expression is significantly correlated with the extent of infiltrating macrophages, and increased percentages of TP-positive macrophages and CD68⁺ macrophages in tumors also indicate poor outcomes in patients with GC^[45]. Macrophage migration inhibitory factor (MIF) can inactivate p53. Serum MIF positively correlates with MIF expression in GC, and increased serum MIF (> 6600 pg/mL) predicts a lower 5-year survival rate compared with those with lower serum MIF^[46]. However, GC patients with high intratumoral macrophages and regulatory T cells (Tregs) have better 5-survival rates than those with low intratumoral macrophages and Tregs^[47]. A high level of CCL18, mainly expressed in infiltrating macrophages that are preferentially located at the tumor invasion front, is also associated with favorable OS and DFS of GC patients^[48]. The possible explanation for this inconsistency could be the presence of heterogenic subpopulations of macrophages in the tumor microenvironment.

MDSCs are a heterogeneous population of cells characterized by their myeloid origin, immature state and the ability to suppress T cell responses. The MDSC population expands rapidly during inflammation and cancer, which is associated with advanced GC stage and reduced survival^[49,50]. Production of reactive oxygen species (ROS) and reactive nitrogen species is one of the major characteristics of all activated myeloid cells. Increased activity of free radical peroxynitrite is followed by ROS production, and peroxynitrite modification of chemokine (C-C motif) ligand 2 (CCL2) inhibits intratumoral migration of effector CD8⁺ T cells. Nitrosylation, a marker of peroxynitrite activity, has been reported to be associated with poor survival of GC patients^[51]. High CD33⁺/p-STAT⁺ cells representing a subset of MDSCs, are also associated with poor prognosis at stage IIIa GC^[52].

The major functions of DCs are to process and present antigens for the activation of T cells. Maintaining enough density of mature DCs in tumors prolongs the survival of patients with advanced GC^[53,54]. Contrary to the typical functions of DCs, intratumoral density of

CD208⁺ DCs has an inverse correlation with postoperative outcome in GC patients^[55]. Among immune cells, neutrophils have a protumorigenic role by promoting neoangiogenesis and reducing antitumor immune response. In GC patients, tumor infiltrating neutrophils with positive CD15 are independently associated with an unfavorable OS^[56]. S100A9, specifically expressed by inflammatory cells such as macrophages and neutrophils in early GC, is associated with a good prognosis^[16]. In addition, S100A9 secreted into gastric fluid also has a prognostic monitoring value for GC^[57].

TILs

TILs are another major component of infiltrating immune cells, and are represented by T cells, B cells, and natural killer (NK) cells. The subsets of T cells include CD8⁺ cytotoxic T cell (CTL), CD4⁺ T helper cell, CD45RO memory T cells, FOXP3⁺ Tregs, and nature killer T cells. CD8⁺ CTLs play an active role in directly killing tumor cells, indicating a favorable outcome^[58,59]. However, CD8⁺ T cells that produce interleukin (IL)-17 (Tc17 cells) promote the progression of inflammation and are possibly associated with poor prognosis^[60]. CD4⁺ lymphocytes include a group of heterogeneous T lymphocytes [e.g., T helper (Th)1, Th2, Th3, Th17, Treg, T follicular helper, and Th22] which can secrete diverse cytokines. Th1 cells (interferon γ -producing CD4⁺ T cells) can activate CTLs, and Th2 cells (IL4-producing CD4⁺ T cells) stimulate humeral immunity. Th1 activation is more effective than Th2 activation in inducing antitumor immunity. Consistently, high Th1/Th2 ratio in peripheral blood of GC significantly predicts a good postoperative prognosis^[61]. High circulating Th17 and Th22 cells are associated with tumor progression and poor survival in GC^[62]. CD4⁺ Tregs suppress effector T lymphocytes, which are characterized with positive Foxp3 expression. High Foxp3⁺ Tregs are correlated with GC progression and associated with a poor survival^[63-65]. The balances between Foxp3⁺ T cells and CD4⁺ T cells as well as Foxp3⁺ T cells and CD8⁺ T cells are important for the suppression of metastasis, and higher Foxp3⁺/CD4⁺ ratio^[66] and higher Foxp3⁺/CD8⁺ ratio^[65] in resected tumor specimens are associated with a poor prognosis. High CD45RO T cell infiltration is significantly related to postoperative prognosis in advanced GC but not in early GC^[67]. NK-like T cells comprising the subsets of CD56⁺ cells and CD57⁺ cells play an important role in modulating immune responses. In advanced GC, an increased proportion of CD57⁺ cells in the circulation indicates a poor prognosis^[68]. T-bet, a key master transcription factor for type 1 immune response, mainly expresses on CD4⁺, CD8⁺, and CD56⁺ TILs. High T-bet TILs in tumor are associated with a better DFS and OS of GC patients^[69]. The principal functions of B cells are to generate antibodies against antigens, but its functions related to tumor progression are less known. Recently, it has been reported that CD19⁺ and CD20⁺ B cells are associated with a favorable outcome in patients with GC^[52,70]. NK

cells directly clear tumor cells, representing an antitumor immunity. GC patients with high density of NK cells in the tumors exhibit a higher survival rate when compared to those patients with low density of NK cells, especially for those at advanced stages^[71].

TRANSCRIPTION FACTORS AND PRIMARY INFLAMMATORY CYTOKINES

In terms of cancer-related inflammation, a few molecules can serve as primary drivers (endogenous promoters), mainly including transcription factors such as NF- κ B and STAT3 and primary inflammatory cytokines such as IL-1, IL-6, and tumor-necrosis factor (TNF)- α . NF- κ B is a key orchestrator of innate inflammation and is aberrantly activated in many cancers. In GC, activated NF- κ B is frequently identified in early-stage tumors and usually predicts a favorable prognosis^[72]. The toll-like receptor (TLR)-MyD88 pathway and the primary inflammatory cytokines TNF- α and IL-1 α can activate NF- κ B. It has been reported that polymorphisms in NF- κ B pathway genes such as *TLR9*, *IL-1 β* , *IL-1Ra*, and *TNF- α* , are significantly associated with the prognosis of GC patients^[73-76]. NF- κ B can also be activated in response to hypoxia inducible factor (HIF)-1 α . Accumulating evidence indicates that the interactions and compensations between NF- κ B and HIF-1 α relate to immunity in the hypoxic condition. Two meta-analyses both reported that HIF-1 α expression was significantly correlated with poor prognosis of GC patients mainly from East Asian countries^[77,78]. NF- κ B induces the expression of inflammatory cytokines, adhesion molecules, and key enzymes in the prostaglandin synthase pathway such as COX-2. Immunohistochemical analysis has shown that COX-2 expression is an independent prognostic factor of DFS and OS of GC patients^[79]. Along with NF- κ B, STAT3 is a point of convergence for numerous oncogenic signaling pathways. In tumors, the maintenance of NF- κ B activation requires STAT3^[80]. STAT3 is constitutively activated both in cancer cells and immune cells, and higher STAT3 and STAT3 phosphorylation (Tyr705) in GCs indicate a poor prognosis^[81,82]. IL-6 is mainly produced by TIMs under the regulation of the NF- κ B signaling pathway. IL-6 is also linked with STAT3, and has multi-functions of growth-promoting and anti-apoptotic activities. Pre-operative high IL-6 levels have been proposed as a poor prognostic factor for recurrence and OS of GC patients^[83]. Nuclear receptor subfamily 4, group A, member 2 (NR4A2), a transcription factor belonging to the steroid orphan nuclear receptor superfamily, is also regulated by the NF- κ B signaling pathway and COX-2 derived prostaglandin E2^[84]. Expression of NR4A2 in GC cells confers chemoresistance of GC cell lines and predicts an unfavorable postoperative survival of GC patients, especially for those treated with postoperative chemotherapy^[85].

Cytokines, chemokines, and matrix metalloproteinases

Cytokines including IL-1, IL-6, and TNF- α are regula-

tors of host responses to infection and cancers, and play different roles in cancer-related inflammation network. Some cytokines facilitate the development of cancer-related inflammation, whereas others act as suppressors. T lymphocytes are a major source of cytokines. Cytokines produced by Th1 and Th2 are known as Th1-type cytokines (*e.g.*, TNF- α , IFN- γ , IL-12) and Th2-type cytokines (*e.g.*, IL-4, IL-5, IL-10, IL-13), and are characterized by pro-inflammatory and anti-inflammatory roles, respectively. High IL-12-positive cell density in surgical specimens may be a significant independent predictor of better prognosis of advanced GC patients^[86]. Conversely, an increased level of IL-10 is an independent unfavorable prognostic factor in patients with GC^[87]. The relative balance between Th1 and Th2 cytokines appears important in cancer-related inflammation. A high circulating soluble IL-2 receptor level is associated with worse prognosis of GC patients^[88]. IL-32 is a recently identified pro-inflammatory cytokine characterized by the induction of NF- κ B activation, and the expression of IL-32 is associated with more severe metastatic conditions in GC^[89]. Additionally, annexin A1 is a glucocorticoid-regulated anti-inflammatory protein. High tissue annexin A1 expression is an independent risk factor for poor OS in GC patients^[90]. Vascular adhesion protein-1 (VAP-1) regulates leukocyte tissue infiltration. Serum soluble VAP-1 is a candidate prognostic marker in GC, and low levels of serum VAP-1 are associated with poor prognosis in GC patients^[91].

Chemokines are 8-10 kDa secreted proteins with 20%-70% homology in structure, and share the common functional activity as being chemotactic for leukocytes. Over 40 chemokines have been identified so far. Although chemoattractants constitute a diverse array of molecules, they have to act together with a family of G protein-coupled receptors to communicate with leukocytes. Inflammatory chemokines are produced under pro-inflammatory stimuli (*e.g.*, IL-1, TNF- α , lipopolysaccharide, or pathogens) and determine the migration of inflammatory cells. CCL7 is a type of monocyte-specific chemokine, and CCL21 is a specific chemokine in DC cells and effector T cells. Over-expressed CCL7 and CCL21 in GCs are related to lymph node metastasis and poor prognosis^[92]. Stromal-derived-factor (SDF)-1 is strongly chemotactic for lymphocytes, and is found in GC metastasized to lymph nodes^[93]. CXCR4 is a receptor specific to SDF-1. Interestingly, upregulated intratumoral CXCR4 expression is associated with poor OS in patients with GC^[94], and high CXCR4/high SDF-1 α expression indicates the worst prognosis in GC patients^[95]. Chemokine (C-C motif) receptor 3 (CCR3), CCR4, CCR5, and CCR7 have been shown to have prognostic values for an unfavorable outcome in patients with GC^[96-98]. Intratumoral high CXCR4, CCL3, CCR4, CCR5, and CCR7 are associated with unfavorable prognosis. IL-8 is a chemokine produced by macrophages and other cell types. It induces chemotaxis in neutrophils to migrate toward the site of inflammation. Polymorphism of *IL-8* is associated with prognosis

in patients with GC, and the *IL-8* 251 A/A genotype may indicate a poor prognosis in GC patients^[99]. CX3CL1 is the only CX3C chemokine that can chemoattract NK cells, CD8⁺ T cells, monocytes, and dendritic cells, and is one of the independent prognostic factors of DFS in GC patients^[100].

An increased expression of the MMP family members is observed in almost every inflammation site. Studies in animal models have demonstrated that MMPs act broadly in the inflammation process, including regulation of inflammatory cytokine and chemokine activities, and generation of chemokine gradients. Pathogens such as *H. pylori* infection upregulate the expression of MMPs, which act on pro-inflammatory cytokines, chemokines and other proteins to regulate diverse aspects of inflammation. Elevated MMP-3 and MMP-7 in *H. pylori*-related GC can serve as biomarkers for a poor survival^[101]. *MMP-9* gene expression is a predictor of outcome in patients with metastatic GC^[102], which is further confirmed by a meta-analysis^[103]. Serum levels of MMP-11 in Chinese patients with advanced GC are not associated with the response to front-line chemotherapy, but could play an important role in predicting lymph node metastasis and prognosis^[104]. Increased MMP-12 and MMP-21 in tissues are associated with poor survival in patients with GC^[105,106]. MMP-14 is a negative prognostic marker for patients with GC^[107]. Although MMPs have been linked to GC prognosis, the precise mechanisms need to be clarified. It is possible that only some MMPs can truncate the inflammatory cytokines or chemokines and participate in the regulation of tumor-related inflammation.

CONCLUSION

The progression of GC after surgical resection is closely associated with microbial pathogens and host inflammatory factors. Positive *H. pylori* and/or positive EBV infection can serve as prognostic factors for a better survival of GC patients. Intratumoral TAMs, MDSCs, neutrophils, and Tregs are usually correlated with poor prognosis of GC. Tumor-infiltrating CD8⁺ CTLs, DCs, CD45RO T cells are generally correlated with better prognosis of GC, although some subsets of these cells have inverse prognostic prediction values. A high NF- κ B indicates a favorable prognosis, while high HIF-1 α , STAT3, NR4A2, and preoperative high IL-6 predict a poor prognosis. Polymorphisms of *TNF- α* , *IL-1 α* , and *TLR9*, which might affect the expression and/or function of these genes, are associated with the prognosis of GC patients. Increased IL-10, IL-32, CCL7, CCL21 and intratumoral high CXCR4, CCR3, CCR4, CCR5, and CCR7 are associated with unfavorable prognosis. Increased serum levels of MMP-3, MMP-7, MMP-11 and increased expression of MMP-9, MMP-12, and MMP-21 in tumors are consistently associated with poor GC survival.

In this article, we summarized the inflammatory factors associated with the prognosis of GC. As inflammation provides “fertile field” for the evolution of cancer-initiating cells, tumor growth-promoting molecules

predominantly expressed in cancer-initiating cells also represent a cluster of prognosis-predicting biomarkers and/or therapeutic targets^[108]. Since many studies are conducted in East Asian populations as summarized in Tables 1 and 2, the prognostic values of these molecules need to be tested in other populations. Furthermore, with the advancement of systems biology and vast amount of ‘omics’ data, it is of great importance to evaluate these data with clinical and pathological variables to more accurately predict cancer outcomes. Studies have already looked at combining gene expression data with clinicopathological data to better predict different types of cancer prognosis^[109-111]. However, only a few studies have been conducted in the field of GC research^[112,113]. Further emphases should be placed on the integration of diverse biomarkers and their validation in large cohorts for personalized prediction of GC postoperative prognosis.

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Invasive micropapillary carcinoma: A distinct type of adenocarcinomas in the gastrointestinal tract

Katarzyna Guzińska-Ustymowicz, Katarzyna Niewiarowska, Anna Pryczynicz

Katarzyna Guzińska-Ustymowicz, Katarzyna Niewiarowska, Anna Pryczynicz, Department of General Pathomorphology, Medical University of Białystok, 15-269 Białystok, Poland
Author contributions: Guzińska-Ustymowicz K and Niewiarowska K solely contributed to this paper; Pryczynicz A revised the paper; Guzińska-Ustymowicz K approved the final version to be published.

Correspondence to: Katarzyna Guzińska-Ustymowicz, MD, Department of General Pathomorphology, Medical University of Białystok, ul. Waszyngtona 13, 15-269 Białystok, Poland. kasia.guzinska@gmail.com
Telephone: +48-85-7485942 Fax: +48-85-7485996
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Abstract

Invasive micropapillary carcinoma (IMPC) is a rare histological type of tumor, first described in invasive ductal breast cancer, than in malignancies in other organs such as lungs, urinary bladder, ovaries or salivary glands. Recent literature data shows that this histological lesion has also been found in cancers of the gastrointestinal system. The micropapillary components are clusters of neoplastic cells that closely adhere to each other and are located in distinct empty spaces. Moreover, clusters of neoplastic cells do not have a fibrous-vascular core. The IMPC cells show reverse polarity resulting in typical "inside-out" structures that determines secretory properties, disturbs adhesion and conditions grade of malignancy in gastrointestinal (GI) tract. Invasive micropapillary carcinoma in this location is associated with metastases to local lymph nodes and lymphovascular invasion. IMPC can be a prognostic factor for patients with cancers of the stomach, pancreas and with colorectal cancer since it is related with disease-free and overall survival. The purpose of this review is to present the characterization of invasive micropapillary carcinoma in colon, rectum, stomach and others site of GI tract, and to determine the immunohistologi-

cal identification of IMPC in those localization.

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Key words: Invasive micropapillary carcinoma; MUC-1; Lymph node metastases

Core tip: We summarize the recent literature reports about individual cases and study groups of invasive micropapillary carcinoma. We postulated that invasive micropapillary carcinoma is still a great diagnostic challenge in pathomorphology and due to its high aggressiveness should be treated as a distinct histological subtype of carcinomas in gastrointestinal tract.

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INTRODUCTION

Invasive micropapillary carcinoma (IMPC) is a rare histological type of tumor, first described in invasive ductal breast cancer^[1]. Recent reports have confirmed that the micropapillary component can also occur in malignancies in other organs such as lungs, urinary bladder, ovaries or salivary glands^[2-5]. According to the World Health Organization (WHO) classification, invasive micropapillary carcinoma was identified as a distinct histopathological subtype of the breast and urinary tract^[6,7]. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society has considered the micropapillary component to be a new subtype of invasive glandular lung cancer with poor prog-

nosis^[8]. Moreover, this histological lesion has also been documented in cancers of the gastrointestinal system^[9,27]. In the current WHO Classification of the Digestive System Tumors, IMPC is described as a rare histological structure found in papillary-type gastric adenocarcinoma^[28]. Irrespective of location, the micropapillary component is present in high-grade tumors with local lymph node involvement and neoplastic emboli in blood and lymphatic vessels^[2-5,18,21,29,30]. The analysis of literature reports concerning gastrointestinal malignancies, including IMPC in our review, may widen the knowledge of this histological structure in malignant tumors affecting these organs.

MORPHOLOGY

The micropapillary components are clusters of neoplastic cells that closely adhere to each other and are located in distinct empty spaces that resemble small dilated lymphatic vessels^[12,15,19,21]. In order to differentiate cancer cells invading the lymphatic vessels from IMPC, immunohistochemical staining is performed with the use of endothelial cell-specific antibodies. Lack of color reaction of these proteins in the lymphovascular-like pattern helps exclude the presence of the vessels (Table 1). Moreover, IMPC cells are usually small, round or oval, but they are also columnar to polygonal^[24,25,31]. Single cells are characterized by distinct massive acidophilic cytoplasm with numerous fine granules^[21,32]. The nucleus/cytoplasm ratio is found to increase. The nuclei are acinar, with well visible nucleoli and unevenly dispersed chromatin clumps^[16]. They show moderate to high pleomorphism and moderate degree of atypia, as well as diverse mitotic activity^[17,21,24,25]. Clusters of neoplastic cells do not have a fibrous-vascular core^[16,19]. They are separated by bands of fibrous tissue, resembling sponge in structure. Nests can also occur as single focal spaces filled up with flattened fusiform tumor cells^[10,32].

Micropapilla are present on the invasive edges of the tumor, more seldom in its center^[12,29,32,33]. The micropapillary structure may constitute one of the morphological tumor components and occur with other histological types, or it can be the only morphological exponent^[16,19,29]. However, cancers composed only of the micropapillary component are very rare^[29]. Moreover, all the above mentioned morphological features of the micropapillary component are visible in metastases to the lymphatic vessels, lymph nodes and distant organs^[32] (Figure 1).

The IMPC cells show reverse polarity resulting in typical “inside-out” structures, i.e. their basal surface has the properties of the upper part. The electron microscopic examination of this structure has confirmed that the outer surface of the cells is covered with numerous microvilli and shows secretory activity towards the surrounding stroma. Moreover, a slight amount of mucous secretion has been found in the spaces that enclose tumor cell nests^[34,35]. These observations are also supported by immunohistochemical investigations with the use of anti-

MUC1, EMA, CD10, villin antibodies (Table 1). Glycoprotein 1 (MUC-1) is mainly present on the outer surface of epithelial cells in patients with IMPC as compared to the color reaction located in the apical part in normal glandular cells. MUC-1 is responsible for the maintenance of cell integrity in normal glands^[27]. The specific reaction of MUC-1 in the micropapillary component located at the invasion front allows differentiation of these structures from tumor budding^[10]. Hudson *et al.*^[36] observed type I collagen fiber contraction and MUC-1 induced disorders of cytokeratin expression. At the same time, MUC-1 neutralizes the effects of fine intercellular adhesion molecules, *e.g.*, E-cadherin and β -catenin^[37]. Reports on the likely contribution of MUC-1 to IMPC adhesion seem to confirm the investigations in which an increase in the expression of this glycoprotein was related to cell-cell adhesion disorders and cell interactions with the extracellular matrix^[38,39]. E-cadherin is responsible for epithelial cell integrity. A decrease in its expression is associated with a greater potential of tumor cells to metastasize. Deficiency of this protein was noted in a higher percentage of patients with gastric IMPC than in the control^[13,40]. However, positive expression of E-cadherin was found in the cytoplasm of cells of the micropapillary structures as compared to the membranous reaction of this protein in normal ducts and neoplastic glandular ducts^[25]. Moreover, the assessment of β -catenin expression revealed its deficiency in the cytoplasm and/or nucleus in a high percentage of patients with gastric and colon IMPC as compared to the adenocarcinoma groups^[40]. It is suggested that disturbances in β -catenin distribution, its deficiency, may condition IMPC aggressiveness^[40]. It can be assumed that also MUC-2 plays a crucial role in the maintenance of IMPC integrity as it joins tumor cells and acts as a physical protective barrier against their spread^[41]. Lack or low percentage of positive expression of MUC-2 was observed in patients with IMPC in gastric cancer^[17,21,31]. Its deficiency facilitates secretion of metalloproteinases by IMPC that determines cancer spread in the stroma and *via* vessels to local lymph nodes^[27] (Table 1, Figure 2).

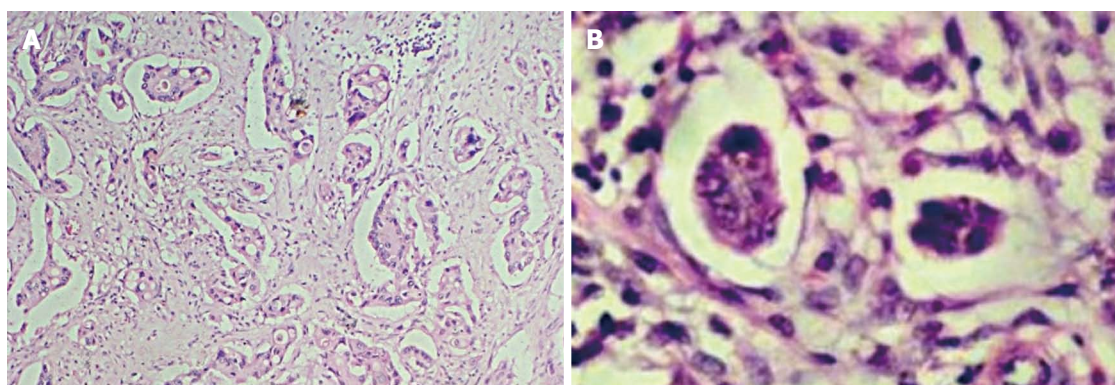
Many data suggest that IMPC polarity reversal determines secretory properties, disturbs adhesion and conditions grade of malignancy. All the above mentioned exponents are responsible for stromal and vascular invasion of tumor cells, resulting in easier spread of cancer cells and lymph node involvement.

INVASIVE MICROPAPILLARY CARCINOMA OF COLON AND RECTUM

Since the description of the micropapillary structure as a separate subtype of breast cancers^[1], this component have been searched for in other organs, including the colon. Up to now, approximately 265 cases of colon cancer with IMPC have been reported, accounting for 9%-19% of all colon cancers. They are most common in males aged 53-72 years^[20,32,10,42,43]. Otsubo *et al.*^[30] observed a single case of IMPC in a 26-year-old woman. Clinical

Table 1 Profile of invasive micropapillary carcinomas in gastrointestinal tract

Immunohistochemical marker	Invasive micropapillary carcinoma	Conventional carcinoma	Ref.
EMA	Outer membranous	Luminal	[10,13,15,19,26,29]
MUC-1	Outer membranous	Luminal	[10,12,19,25,29,30]
CD10	Stroma-facing surface	Membranous	[16,31]
Vilin	Stroma-facing surface	Membranous	[16]
E-catherin	Cytoplasmic	Membranous	[13,25,33,41]
β-catenin	Nuclear/cytoplasmic less frequent	Nuclear/cytoplasmic more frequent	[41]
		Lymphovascular epithelial cells	
D2-40	Negative	Positive	[12,13,15,19,21,22]
CD34	Negative	Positive	[16,21]
CD11	Negative	Positive	[16]

**Figure 1** Characterization of typical invasive micropapillary carcinoma structures. A: Invasive micropapillary carcinoma in the invasive edges of the tumor; B: Morphologically, clusters of small rounded neoplastic cells without fibrous-vascular core. Hematoxylin and eosin stain, × 40, × 400, respectively.

symptoms in patients with IMPC have not been disease-specific and mainly include abdominal pain, anemia, vomiting, diarrhea, constipation and bleeding from the rectum^[10,12-16,19,20,29,43].

These tumors are known to grow as polyp-like forms that narrow the lumen of the respective organ with a tendency to exophytic growth or a lesion with a centrally ulcerating crater^[16,18,19,29]. Unfortunately, macroscopically the lesions do not allow IMPC differentiation from other subtypes. Regardless of the macroscopic picture, all tumors have a characteristic image of the micropapillary structure in light microscopy. IMPC can develop throughout the large intestine, although most lesions are located in the colon and 50 percent of them in the ascending colon^[20,19,32,42]. In the remaining substantial proportion of cases, the rectum is affected^[20,29]. The micropapillary component involves a wide range of 5%-95% of tumor volume and is usually situated in its invasion front^[10,19,32]. Moreover, Verdú *et al.*^[10] described the coexistence of early sigmoid cancer with IMPC in a pedunculated polyp obtained during colonoscopy, in which the component constituted the major tumor morphological exponent. Kondo *et al.*^[15] noted a slight focus of IMPC growing in a tubulovillous adenoma. Lino-Silva *et al.*^[29] observed a single case of pure rectal IMPC, with the micropapillary structure involving > 95% of the whole lesion^[29]. IMPC accompanies neoplastic lesions with clearly defined edges and various differentiation grades. In most cases, IMPC coexisted with moderately differentiated tumors (G2),

with cells lacking mucous secretion^[12,19,20,32]. Many of these cancers infiltrated through the muscle to the subserous layer^[20,32,42].

The presence of IMPC in colorectal tumors is associated with aggressive behavior of the neoplasm. In all the reported cases, tumors invaded blood and lymphatic vessels, whereas the remaining groups showed moderate grade of invasion of these structures^[12,13,18-20,29,30,32]. The involvement of lymph nodes has been estimated at 63%-100% of all cases^[6,5,10,17,43]. Kim *et al.*^[20] showed metastases to local lymph nodes in 2 out of 3 patients with IMPC tumor infiltrating the submucous membrane (pT1). Their findings indicate the importance of adequately early diagnosis of IMPC lesion in biopsy and operative material, which may condition high risk of metastases. In the majority of patients, IMPC invasion involved a considerable proportion of lymph nodes and was the only histological exponent of the metastases formed^[16,19,20]. Moreover, several patients showed metastases of micropapillary structures to the peritoneum and other organs, such as lungs and liver^[10,13,20,29,32]. The multivariate analysis of variance revealed that the presence of the micropapillary component, apart from invasion of the lymphatic/blood vessels and infiltration depth, is an independent prognostic factor determining cancer metastases^[17].

The diagnosis of the micropapillary component is closely associated with worse prognosis^[42,43]. Stage I and II IMPC patients experience shorter survival as compared to the non-IMPC groups and have equally poor

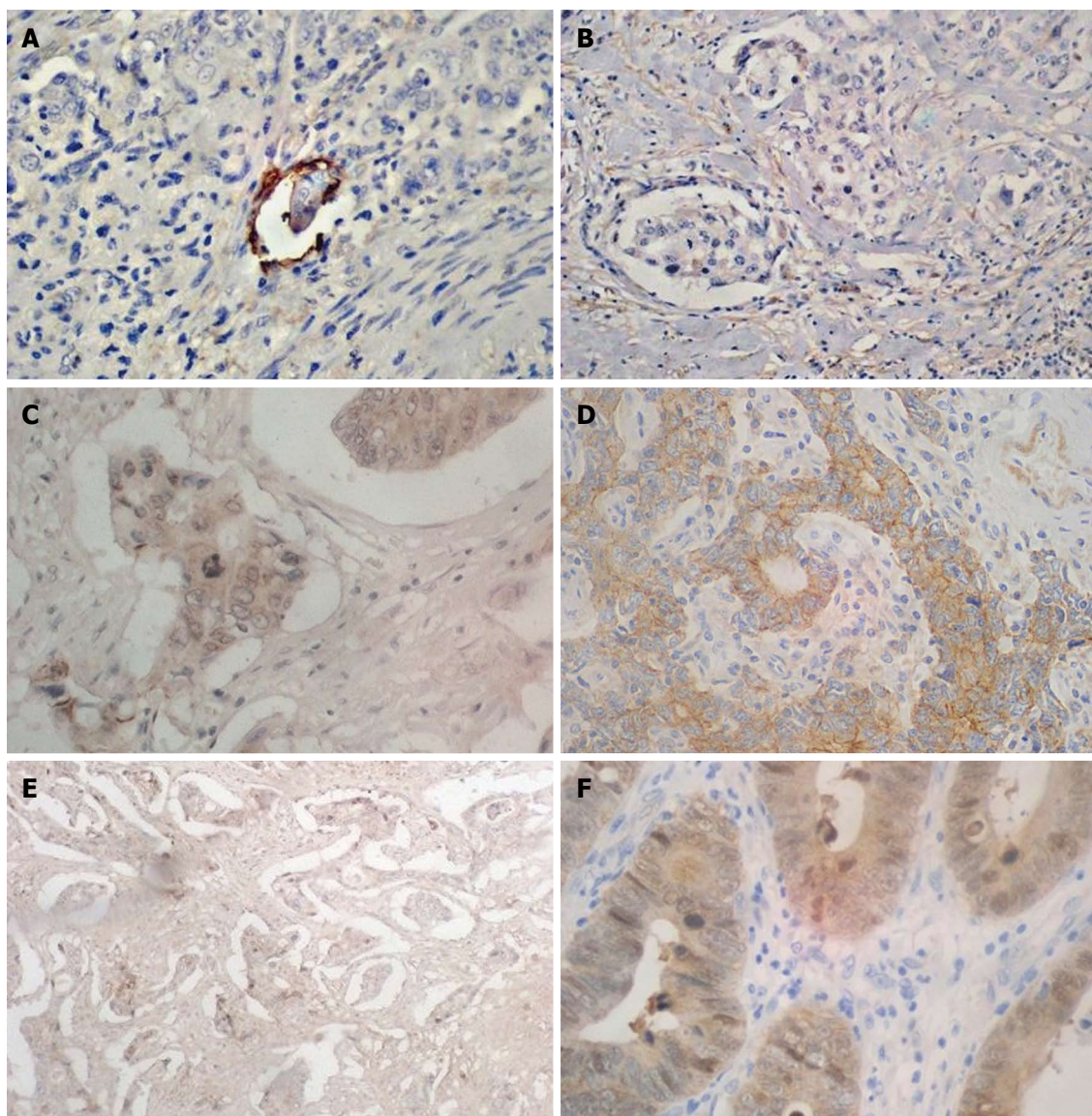


Figure 2 Immunohistochemical characteristics of invasive micropapillary carcinoma structures. Positive immunoreaction of lymphatic endothelial cell performed by D2-40 (A) and lack of reaction in invasive micropapillary carcinoma (IMPC) structures (B). Immunohistochemical expression of E-cadherin in membrane of conventional adenocarcinoma (C) and in cytoplasm of IMPC (D). The cytoplasmic expression of Beta-catenin was observed more frequent in typical adenocarcinoma (F) compare to IMPC (E). Magnification $\times 400$.

prognosis as those in Stage III and IV. The survival rates among these patients after 1, 3, 5 and 10 years were 67%, 53%, 50% and 50%, respectively, as compared to the groups without IMPC (81%, 75%, 73%, 70%, respectively)^[44]. Five-year survival rate was also much lower in patients with IMPC than in those with microsatellite instability-high carcinoma and microsatellite stable one^[43]. The molecular profile of IMPC indicates that these patients have higher proportion of TP53 alterations, and that microsatellite instability is much rarer^[10]. Therefore, it is assumed that like 85% of colorectal cancers, IMPC develops *via* classical chromosomal instability (CIN)^[10]. This confirms that IMPC shows considerable aggressiveness and is associated with shorter survival. The colorectal IMPC group profile is presented in Tables 2 and 3.

INVASIVE MICROPAPILLARY CARCINOMA OF THE STOMACH

Considering a very high incidence of gastric cancer, tumors with IMPC account only for 0.07%-13.40% of gastric cancer cases^[31,45]. The IMPC morbidity peak is observed at the age of 60-70, with the predominance of males being affected. IMPC is most frequently located in the lower-third, then in the middle and upper-third of the stomach. The micropapillary structure coexists with other histological types, including a considerable percentage of papillary and tubular carcinomas^[21,40]. A higher percentage of IMPC are noted in tumors of the intestinal type than diffuse type according to Lauren classification^[40]. The analysis of mucin profile has shown that

Table 2 Characteristics of individual cases of colorectal invasive micropapillary carcinoma

Parameter	Sakamoto <i>et al</i> ^[19] , 2005	Kuroda <i>et al</i> ^[18] , 2007	Kondo <i>et al</i> ^[15] , 2008	Wen <i>et al</i> ^[16] , 2008	Hisanori <i>et al</i> ^[13] , 2009	Sonoo <i>et al</i> ^[12] , 2009	Otsubo <i>et al</i> ^[30] , 2011
Age/ Sex	67/M 68/F 53/F	70/F	70/M	72/F	71/F	64/M	26/F
Location							
Colon	3	1	1	1	1	1	1
Rectum	0	0	0	0	0	0	0
Unspecified	0	0	0	0	0	0	0
Tumor size							
< 5 cm		1	1	1	1	1	1
> 5 cm		0	0	0	0	0	0
Percentage of IMPC	PD	40%	5%	PD	PD	80%	ND
Adenocarcinoma type							
Nonmucinous	2	1	1	1	1	1	1
Mucinous	1	0	0	0	0	0	0
Grade of malignancy (G)	G3-2 G4-1	ND	G1	ND	ND	G2	G4
pT stage (T)	T3-3	T3	T1	T3	T1	T1	T3
Lymphovascular invasion (Yes/No)	Yes-2 No-1	Yes	Yes	ND	Yes	Yes	Yes
Lymph node metastasis (N) (Yes/No)	Yes-3	Yes	No	Yes	Yes	Yes	Yes
Distant metastasis (M) (Yes/No)	No	No	No	No	Yes	No	Yes

PD: Predominant; ND: No data; M: Male; F: Female.

Table 3 Characteristics of study group diagnosed with colorectal invasive micropapillary carcinoma

Parameter	Kim <i>et al</i> ^[20] , 2006	Haupt <i>et al</i> ^[32] , 2007	Xu <i>et al</i> ^[44] , 2009	Verdú <i>et al</i> ^[10] , 2011	Lino-Silva <i>et al</i> ^[42] , 2012
No. of patients	55	34	30	60	15
Mean age, yr	64	66	57	65.8	56
Sex	F-15 M-40	F-15 M-19	F-13 M-17	F- 23 M-37	F-8 M-7
Location					
Colon	33	30	12	24	15
Rectum	22	2	18	36	0
Unspecified	0	2	0	0	0
Percentage of IMPC	5%-80%	5%-60%	5%-75%	5%-30%	10%-80%
Adenocarcinoma type					
Nonmucinous	55	29	29	57	15
Mucinous	0	5	1	3	0
Grade of malignancy (G)					
G1	5	1	13	23	0
G2	43	26	0	30	0
G3	7	7	0	7	6
G4	0	0	17	0	9
pT stage (T)					
T1	2	3	0	3	0
T2	4	5	5	9	0
T3	45	24	25	38	9
T4	4	2	0	10	6
Lymphovascular invasion (LV) (Yes/No)	Yes-25 No-30	Yes-14 No-20	Yes-10 No-20	Yes-28 No- 32	Yes-5 No-10
Lymph node metastasis (N) (Yes/No)	Yes-41 No-14	Yes-25 No-9	Yes-19 No-11	Yes- 38 No-12	Yes-15 No-0
Distant metastasis (M) (Yes/No)	Yes-13 No-42	Yes-4 No-30	Yes- 1 No-29	Yes-10 No-50	Yes-3 No-12
Correlations	N, M, TNM stage	N	G, N, TB	T, TNM stage, N,M, VI, PI	G, N, TNM stage

PD: Predominant; ND: No data; M: Male; F: Female; VI: Venous vessel invasion; PI: Perineural invasion.

the highest percentage of IMPC patients show gastric type (9/17) and null type (6/17) as compared to the other

subtypes^[21]. The IMPC structure most frequently accompanies moderately (G3) and low-differentiated cancers

Table 4 Invasive micropapillary carcinoma of the stomach

Parameter	Shimoda <i>et al</i> ^[17] , 2008	Roh <i>et al</i> ^[31] , 2010	Eom <i>et al</i> ^[40] , 2011	Ushiku <i>et al</i> ^[21] , 2011	Fujita <i>et al</i> ^[22] , 2012	Ninomiya <i>et al</i> ^[46] , 2013	Ohtsuki <i>et al</i> ^[33] , 2013
No. of patients	1	11	72	17	14	1	4
Mean age, yr	74	66	70	67	62	69	69-79
Sex	M	F-3 M-8	F-18 M-54	F-3 M-14	F-4 M-10	M	F-2 M-2
Location							
Upper-third	0	1	8	6	3	1	2
Middle	1	2	20	6	7	0	1
Lower-third	0	8	44	5	4	0	0
Tumor size							
< 5 cm	1	5	40	17	14	1	2
> 5 cm	0	6	32	0	0	0	3
Percentage of IMPC	PD	5%-70%	5%-80%	10%-90%	> 10%	PD	ND
Adenocarcinoma type							
Tubular	Absent	Both	21	16	9	ND	2
Papillary		9/11	43	12	4		2-mixed
Grade of malignancies (G)							
G1	0	ND	11	ND	6	ND	ND
G2	0	2/11	38		7		
G3	1	ND	23		1		
G4	0	ND	0		0		
pT stage (T)							
T1	0	3	20	2	0	0	0
T2	0	4	52	8	6	0	1
T3	1	2		5	8	1	2
T4	0	2		2	0	0	4
Lympho-vascular invasion (LV) (Yes/No)	Yes	Yes-10 No-1	Yes-56 No-16	Yes-17 No-0	Yes-11 No-3	Yes	Yes-1 No-3
Lymph node metastasis (N) (Yes/No)	Yes	Yes- 4 No-7	Yes-62 No-10	Yes-14 No-3	Yes	Yes	Yes-3 No-1
Distant metastasis (M) (Yes/No)	No	ND	ND	ND	No	No	ND

PD: Predominant; ND: No data; M: Male; F: Female.

(G2)^[22,40]. In a study by Fujita *et al*^[22], 1/3 of the cases had papillary adenocarcinoma well-differentiated with IMPC.

The content of micropapillae in the stomach ranges between 5%-90% of tumor tissue, although pure IMPC has never been found in this organ^[21,31,40]. It has been proven that the determination of the ratio of IMPC to the remaining part of the tumor has no effect on the clinicopathological parameters. However, even the smallest IMPC lesion found indicates tumor aggressiveness^[40]. The occurrence of IMPC correlates with the degree of invasion of lymphatic and blood vessels, and with the number of metastases to lymph nodes. The invasion of blood and lymphatic vessels has been found in 78%-91% of cases^[17,21,22,31,40]. Tumors in stage I and II IMPC patients showed higher percentage of vessel invasion than those without the component^[17]. In the majority of cases, IMPC reaches the subserous layer (pT3) or infiltrates by continuity other tissues and organs (pT4)^[22,40]. The proportion of lymph node involvement is very high^[21,22]. Stage I and II patients with the IMPC component showed a considerably higher percentage of metastases to local lymph nodes as compared to the IMPC-free groups^[40]. Statistical significance of infiltration depth and tumor size has been found in the prognostication of lymph node involvement^[40] (Table 4).

Not only is IMPC related to metastasizing but also to

poor outcome of patients. IMPC patients have shorter survival as compared to those without the micropapillary component (59.3% *vs* 80.6%). Significantly shorter 1-, 3-, 5-year overall survival (83%, 55%, 30%) was also noted in comparison with non-IMPC (87%, 70%, 67%). Among stage I and II patients, the likelihood of overall and disease-free 5-year survival was much lower than in the non-IMPC cases^[40]. The IMPC parameter as a potential factor in the prognosis of survival of these patients has also been evaluated^[40]. As revealed by univariate analyses performed by Fujita *et al*^[22], the IMPC component, invasion grade, infiltration of lymphatic vessels and lymph node involvement are the major factors determining survival. These parameters play an especially important role in stage I and II patients, since they are associated with much worse prognosis. On the other hand, according to the multivariate analysis, IMPC is an independent prognostic factor of survival^[22]. These observations, however, have not been confirmed by other researchers^[31,46].

INVASIVE MICROPAPILLARY CARCINOMA OF OTHER LOCATIONS IN GI TRACT

The micropapillary structure in the ampulopancreatobili-

Table 5 Histopathological analysis of invasive micropapillary carcinoma in other sites of the gastrointestinal tract

Parameter	Khayyata <i>et al</i> ^[25] , 2005	Kitagawa <i>et al</i> ^[26] , 2007	Kondo <i>et al</i> ^[23] , 2009	Fujita <i>et al</i> ^[24] , 2010
No. of patients	16	1	1	1
Mean age, yr	69	67	75	53
Sex	F-6 M-10	M	F	M
Location	Ampullo-pancreatobiliary region	Pancreatic head	Bile duct	Ampulla of Vater
Tumor size				
< 5 cm	14	1	1	1
> 5 cm	ND	0	0	0
Percentage of IMPC	> 20%	PD	ND	PD
pT stage (T)				
T1	0	1	0	1
T2	0	0	1	0
T3	0	0	0	0
T4	13	0	0	0
Lymphovascular invasion	Yes-3	Yes	Yes	Yes
(Yes/No)	No-13			
Lymph node metastasis (N)	Yes-11	Yes	Yes	Yes
(Yes/No)	No-4			
Distant metastasis (M)	Yes-4	Yes	ND	Yes
(Yes/No)	No-12			

PD: Predominant; ND: No data; M: Male; F: Female.

ary region of the pancreas was first described by Khayyata *et al*^[25], who found this lesion in 4.1% of all cancers in this location, with the majority of lesions observed in the ampullary region (11%), and the remaining ones in the pancreas (3%). The author classified primary IMPC lesions as focal (20%-50% of the tumor), predominant (51%-80%) or diffuse (> 80%). IMPC above 80% is rare, being present in 3% of periampullary cases and in 1% of pancreatic cases^[25]. Kitagawa *et al*^[26] observed a single case of pure IMPC in the pancreas without typical adenocarcinoma tissue. IMPC was also found in the ampulla of Vater (1.3% of these cancers) and in bile duct^[23,24]. Kondo *et al*^[23] suggested that the adhesion of the mucous membrane invaded by adenocarcinoma with IMPC to bile ducts may condition the occurrence of this lesion type mainly in the region of the pancreatic head. It cannot also be excluded that the location may be determined by the etiologic factors themselves, including the properties of bile content, *e.g.*, reflux^[23]. Therefore, these neoplastic lesions frequently lead to bile duct obstruction, cholestasis and jaundice, and patients complain of general malaise^[23-26].

Macroscopically, the lesions have diverse descriptions, from whitish and greyish nodular tumor exhibiting soft consistency in the pancreas to irregular lesions narrowing the lumen of the bile duct^[24,26]. In some cases, the micropapillary component was found to coexist with classical types of adenocarcinoma^[23,25]. The tumor size found was 0.02-3.2 cm^[23-26]. In two documented cases, tumor infiltrated the submucous membrane (pT1). Kondo *et al*^[23] found a tumor that was limited to the muscular membrane (pT2) of the Oddi's sphincter^[24,26]. However, in 16 patients with IMPC in the pancreas, the cancer was classified as G3 according to the grading scheme of pancreatic adenocarcinoma. In some patients, lymphatic and blood

vessels were invaded by clusters of cancer cells, including vascular microinvasion in the submucous membrane of the duodenum or papilla in 3 patients (< 5% IMPC in tumor)^[25]. In the microscopic picture, the micropapillary structures showed high similarity to those observed in other organs. Contrary to other locations, in the presence of pancreatic micropapillae there was a massive inflammatory infiltrate composed of neutrophilic granulocytes that formed focal intraepithelial microabscesses, and clusters of these cells in the stroma^[23,25]. Moreover, in one case, moderate infiltrate composed of eosinophils was observed to surround the micropapillary structures^[24].

In most cases, local lymph node involvement was found^[23,24,26]. The presence of the predominant part or pure form of IMPC was noted in an early stage, which may indicate metastasizing to numerous lymph nodes and distant organs (liver, intestine, gallbladder) in these cases^[24,26]. Unfortunately, as this conclusion was based on very few observations they have to be verified on a larger group of patients. In the best-described group of patients with pancreatic IMPC there were 11/15 (73%) metastases to local lymph nodes as compared to the conventional carcinoma group (55%). In 4 patients from this group, distant metastases to the liver and lungs were found. Most metastases, both local and distant had micropapillary structures^[25] (Table 5).

All patients underwent surgical treatment. Only in the case of pure pancreatic IMPC, pancreaticoduodenectomy was accompanied by chemotherapy with gemcitabine^[26]. Pharmacotherapy in that case proved to have similar effects to those observed in patients treated for typical pancreatic cancer^[26]. The analysis of survival of patients with pancreatic IMPC revealed a slightly shorter survival than in ordinary ductal carcinoma of the pancreas. The mean survival of patients with pancreatic IMPC was 8 mo and

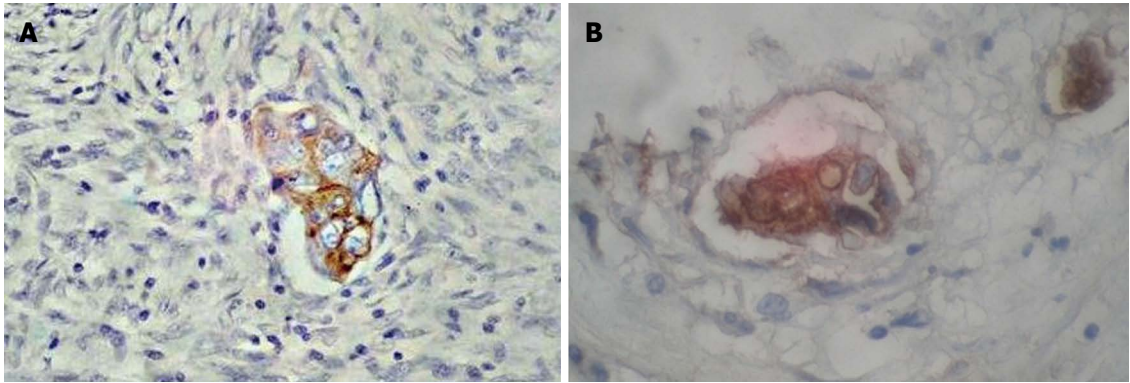


Figure 3 Immunohistochemical characteristics of invasive micropapillary carcinoma of colon. Positive reaction of CK20 (A) and CEA (B). Magnification × 400.

Table 6 Immunohistochemical identification of the primary site of invasive micropapillary carcinoma

Protein/marker	Colon and rectum	Stomach	Pancreas ¹	Breast	Bladder	Ovary	Lung	Salivary glands
CK20	+	-	-	+	+	+	+	+
CK7	-	+	+	-	+	-	-	-
TTF-1	-	-	ND	-	-	-	+	ND
SP-A	-	-	ND	-	-	-	+	ND
ER	-	+/-	ND	+/-	-	+/-	-	-
PgR	-	-	ND	+/-	-	+/-	-	-
CA 125	+/-	ND	ND	-	+	+	ND	ND
CA 19-9	ND	ND	+	ND	ND	ND	ND	ND
CEA	+	ND	-	ND	ND	ND	ND	ND

¹And other sites in gastrointestinal tract. ND: No data.

seems to be comparable to that observed in patients with poorly differentiated carcinoma at the same site^[25]. The follow-up of patients lasted 12, 20, 17 and 42 mo for bile duct, ampulla of Vater, pancreas and pure pancreatic IMPC, respectively^[23-25]. Only 21% of patients treated surgically live without relapse, whereas the remaining percentage of patients died due to advanced cancer or multiorgan failure^[26].

PRIMARY SITE OF IMPC

The micropapillary structure does not belong to the location of any definite organ. Even though in most cases IMPC can be observed in traditional histological types of cancers, being characteristic of a respective location, and may suggest its origin, the presence of pure IMPC in the form of primary site or metastasis does not allow definite lesion localization. Therefore, the primary distribution of this type of cancer has to be confirmed by the whole panel of immunohistochemical investigations.

In immunohistochemical analyses of micropapillae in the digestive system, protein expression was similar to that of conventional adenocarcinoma. Cytokeratin 20 (CK20) (+) CK7 (-) was suggested to be an adequate marker profile for IMPC of the colon^[11,16,18-20,29] (Figure 3). Research also proved a considerable proportion of positive expression of intestinal differentiation marker (CDX2) and carcinoembryonic antigen (CEA) in IMPC as compared to conventional carcinoma^[16,18,20,42]. More-

over, in one case colon IMPC showed positive expression of Cancer Antigen 125 (CA 125), which did not exclude the presence of this structure in the ovary and urinary bladder^[18]. On the other hand, the CK20 (-) CK7(+) variant was found to condition the primary site in the stomach^[17,22,31]. Additionally, positive reactions of proteins with mucins (MUC-5, MUC-6) have been observed in the stomach and colon^[17,21,22,31]. However, MUC-2 has been found to show positive reaction in the colon IMPC, but not in gastric IMPC^[21,22,31,34,42]. IMPC both in the large intestine and in the stomach is characterized by a high proliferative index. IMPC cells exhibit positive expression of Ki-67 and p53 in most cases^[20-22,31,44]. Also DNA mismatch repair protein such as MutL homolog 1, MutS protein homolog 2 and MutS homolog 6 have shown positive expression^[16,20]. In pancreatic IMPC the profile contains CK20(-) CK7(+)^[26]. Moreover, positive expression of carbohydrate antigen 19-9 (CA 19-9) and negative expression for CEA has been found in this organ^[26]. The proposed IMPC profile is based on few reports and requires more detailed analysis on a larger study group.

IMPC differentiation in the digestive system requires the knowledge of immunohistochemical profiles specific to other locations. The CK20(-) CK7(+) thyroid transcription factor-1 (TTF-1) (+) surfactant apoprotein A (SP-A)(+) profile indicates the location of IMPC in the urinary bladder, and CK20(-) CK7(+) estrogen receptor (ER) (+/-) progesteron receptor (PgR) (+/-) is present

in the breast and ovaries. IMPC located in the salivary glands expresses CK20(-) CK7(+)^[16,47]. The immunohistochemical characteristics of IMPC structures with respect to location has been presented in Table 6.

The determination of the primary site of IMPC, due to high metastasizing capacity and advanced clinical status of most cases facilitates proper therapy.

CONCLUSION

In summary, an increasing number of reports confirming the morphological distinction of the micropapillary structure as compared to other histological types indicate an essential impact of this structure on the pathomorphological diagnosis. The morphological properties of IMPC condition the lymphovascular invasion and metastases to regional lymph nodes. This has been confirmed above all by the studies in which numerous metastases were observed in early neoplastic lesions. IMPC can be a prognostic factor for patients with cancers of the stomach, pancreas and with colorectal cancer since it is associated with disease-free and overall survival. Nowadays, IMPC is a great diagnostic challenge, and due to its high aggressiveness, the histology of cancer lesions in the digestive tract requires careful analysis.

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***Helicobacter pylori* infection and diabetes: Is it a myth or fact?**

Cong He, Zhen Yang, Nong-Hua Lu

Cong He, Zhen Yang, Nong-Hua Lu, Department of Gastroenterology, the First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

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Correspondence to: Nong-Hua Lu, MD, Department of Gastroenterology, the First Affiliated Hospital of Nanchang University, 17 Yong Waizheng Street, Donghu District, Nanchang 330006, Jiangxi Province, China. lunonghua@ncu.edu.cn
Telephone: +86-791-88692705 Fax: +86-791-88623153

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Abstract

Helicobacter pylori (*H. pylori*) is one of the most common human bacterial pathogens, and infection causes a wide array of gastric disorders, including simple gastritis, peptic ulcers and gastric malignancies. Gastrointestinal inflammation caused by *H. pylori* can influence the absorption of glucose and lipids, which are also abnormal in diabetes mellitus. Type 2 diabetes mellitus (T2DM), formerly known as non-insulin-dependent diabetes mellitus or adult-onset diabetes, is a metabolic disorder that is characterized by high levels of blood glucose resulting from insulin resistance and relative insulin deficiency. It is an emerging pandemic and is rapidly becoming a serious threat to public health. Emerging data now indicate a strong relationship between *H. pylori* infection and the incidence of T2DM. The mechanisms underlying the pathogenesis of diabetes are complex, involving insulin resistance, chronic inflammation, insulin secretion deficiency as a result of pancreas β -cell dysfunction, glucotoxicity, and lipotoxicity. *H. pylori* infection is known to be involved in

the pathogenesis of insulin resistance, and the growing awareness of its role in diabetes is important for the early detection of glucose dysregulation and prevention of T2DM in high-risk communities. This review probes the possible relationship between *H. pylori* and diabetes according to epidemiological surveys and discusses putative mechanisms underlying this correlation.

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Key words: *Helicobacter pylori*; Type 2 diabetes; Insulin resistance; Inflammation; Cytokines

Core tip: A growing body of evidence suggests that *Helicobacter pylori* (*H. pylori*) infection is associated with diabetes, and may cause insulin resistance and chronic inflammation that contribute to the disease. *H. pylori*-induced gastritis can also potentially affect the secretion of gastric-related hormones and inflammatory cytokines. However, the relationship between *H. pylori* infection and diabetes is still under debate and further studies are warranted to define their association in more detail, and to characterize the corresponding mechanisms and mediators.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral-shaped pathogenic bacterium that specifically colonizes the gastric epithelium causing chronic gastritis, peptic ulcer disease, and/or gastric malignancy^[1,2]. *H. pylori* is mainly acquired in childhood by the fecal-oral, oral-

oral or gastro-oral route^[3], and has been recognized as a worldwide public health problem that is more prevalent in developing countries. The infection induces an acute polymorphonuclear infiltration in the gastric mucosa, which is gradually replaced by an immunologically-mediated, chronic, predominantly mononuclear cellular infiltration^[4]. The mononuclear infiltration is characterized by the local production and systemic diffusion of pro-inflammatory cytokines^[5] that can affect remote tissues and organic systems^[6]. As a result, an increased prevalence of extra-digestive diseases has been reported in those with evidence of *H. pylori* infection in recent years, including ischemic heart disease^[7], autoimmune thyroid diseases^[8], sideropenic anemia^[9], idiopathic thrombocytopenic purpura^[10], neurologic diseases^[11-13], and hepatobiliary diseases^[14-17]. Indeed, this bacterium produces a low-grade inflammatory state, induces molecular mimicry mechanisms, and interferes with the absorbance of nutrients and drugs, possibly influencing the occurrence and/or evolution of many diseases^[18].

Type 2 diabetes mellitus (T2DM) is an emerging pandemic, responsible for an estimated 3.8 million adult deaths worldwide^[19]. The pathogenesis of T2DM is complex, with risk factors associated with lifestyle (*e.g.*, diet, obesity, physical activity), genetic background, and socioeconomic factors^[20,21]. In T2DM, the pancreas can no longer produce enough insulin to overcome the cellular loss of sensitivity, resulting in the accumulation of sugar in the bloodstream^[22]. Identification of treatable causes of this disease will aid in the development of strategies to delay or prevent its onset or slow its progression. Recent evidence implicates the pathological involvement of inflammation in T2DM, which is an important process induced by *H. pylori* infection^[23]. This review focuses on the possible relationship between *H. pylori* and diabetes as well as the potential mechanisms and mediators concerning this correlation.

EPIDEMIOLOGICAL SURVEYS LINKING *H. PYLORI* WITH DIABETES

The link between *H. pylori* infection and diabetes remains controversial, as some studies indicate a higher prevalence of infection in diabetic patients^[24-26], while others report no difference^[27-29]. The relationship between *H. pylori* and diabetes mellitus was first explored in 1989 by Simon *et al.*^[30] who found that the prevalence of *H. pylori* infection in patients with diabetes mellitus was significantly higher than in asymptomatic controls (62% *vs* 21%). However, the test used for detecting *H. pylori* was only a rapid urease test, and their comparison did not adjust for age, which is a major confounding factor. Additional supportive data have come from groups in the Netherlands^[31], Italy^[32], Turkey^[26], and Africa^[33]. Recently, a meta-analysis conducted by Zhou *et al.*^[34] involved 14080 patients from 41 studies with a total *H. pylori* infection rate of 42.29%. The odds ratio (OR) for *H. pylori* infection was increased to 1.33 among patients with diabetes, especially in pa-

tients with T2DM (OR = 1.76). The first demonstration that *H. pylori* infection leads to an increased incidence of diabetes was in a study by Jeon *et al.*^[35] using a prospective cohort of 782 Latino individuals > 60 years of age. Participants, whose diabetic status was not known at the initiation of the study, had serum assayed twice yearly for a decade for antibodies to *H. pylori*, herpes simplex virus 1, varicella zoster virus, cytomegalovirus, and *Toxoplasma gondii*. During the course of the study, 144 individuals developed diabetes (presumably type 2), and individuals who were initially seropositive for *H. pylori* were found to be more than two times more likely to develop diabetes than those who were seronegative, even after adjusting for age, sex, education, and covariates such as smoking, body mass index (BMI), blood pressure, and lipids. In contrast, antibodies to the other infectious agents were not associated with an increased risk for the development of diabetes.

Levels of glycated hemoglobin (HbA1c), which result from the non-enzymatic glycosylation of hemoglobin and reflect the integrated blood glucose levels during the preceding 3-4 mo, can be used to diagnose prediabetes and diabetes and to predict diabetes prevalence and incidence^[36-38]. A study performed by Chen and Blaser has provided new insight into the association between the seroprevalence of *H. pylori* infection and the mean levels of HbA1c in two large national surveys: the National Health and Nutrition Examination Survey (NHANES) III and the NHANES 1999-2000^[39]. Their report showed that *H. pylori* seropositivity, and *H. pylori* *cagA* positivity in particular, was associated with higher mean HbA1c levels, an association that persisted after excluding individuals with a history of diabetes mellitus and controlling for potential confounders. The association was evident mainly in adults over 18 years of age. They also showed a synergistic effect of *H. pylori* and BMI on increased levels of HbA1c, indicating a role of *H. pylori* in impaired glucose tolerance in adults that may be potentiated by a higher BMI level. Similar results were reported in a recent study by Hsieh *et al.*^[40] showing long-term *H. pylori* infection was significantly associated with high levels of HbA1c, decreased insulin secretion, and a higher prevalence of T2DM in Taiwanese patients. Taken together, these results suggest that proper screening of *H. pylori* infection combined with regular monitoring of blood glucose and HbA1c levels may be effective for early detection of glucose dysregulation and prevention of T2DM.

In contrast, other studies have found no association between *H. pylori* infection and diabetes^[27-29,41,42]. In a large, well-designed study by Xia *et al.*^[42], the seroprevalence of *H. pylori* infection was not significantly different in patients with diabetes mellitus compared to nondiabetic controls. In another study conducted in Nigeria, Oluyemi *et al.*^[43] found no significant difference in *H. pylori* prevalence between T2DM patients and controls, which is consistent with the results from various other regions of the world, including Italy^[44], China^[28], Turkey^[45] and Romania^[29]. The discrepancies reported concerning

the association of *H. pylori* and diabetes are likely due to inconsistencies in the methods used to define *H. pylori* positivity and diabetic status, the limited sample sizes, and adjustments for potential confounders such as age and socioeconomic status^[42]. In addition, the accuracy of self-reported data on medical history depends on the subjects' knowledge and understanding of the relevant information, their ability to recall, and their willingness to report^[46], which also may change over time.

PATHOGENETIC MECHANISMS IN *H. PYLORI* AND DIABETES

Although there is no concrete evidence demonstrating that *H. pylori* plays a role in diabetes, the possibility for a causal relationship is an intriguing issue deserving discussion. There are several lines of evidence to implicate increased susceptibility to infection in diabetic patients. Firstly, a diabetes-induced impairment of cellular and humoral immunity may enhance an individual's sensitivity to *H. pylori* infection^[47]. Secondly, diabetes-induced reduction of gastrointestinal motility and acid secretion may promote pathogen colonization and infection rate in the gut^[35]. Thirdly, altered glucose metabolism may produce chemical changes in the gastric mucosa that promote *H. pylori* colonization^[48]. Finally, individuals with diabetes are more frequently exposed to pathogens than their healthy counterparts as they regularly attend hospital settings^[49]. However, there are also indications that *H. pylori* infection may contribute to the development of diabetes. Whereas insulin insensitivity is an early phenomenon, pancreatic β -cell function declines gradually over time before the onset of clinical hyperglycemia, the result of many factors that can be influenced by infection, such as insulin resistance (IR), glucotoxicity, lipotoxicity, β -cell dysfunction, chronic inflammation, and genetic and epigenetic factors^[23,50].

***H. pylori* and IR**

A growing body of evidence has linked *H. pylori* infection to IR^[51-54], which is defined by a state where insulin can no longer effectively induce glucose disposal in skeletal muscle or suppress endogenous glucose production in the liver^[55]. Insulin resistance and abnormal insulin secretion are central to the development of T2DM, and most studies support the view that IR precedes defects in insulin secretion^[56]. The first direct evidence for an association between chronic *H. pylori* infection and IR came from a study by Aydemir *et al.*^[53] showing higher homeostatic model assessment-estimated insulin resistance (HOMA-IR) scores in *H. pylori*-positive individuals. Furthermore, a Japanese study in 2009 that included a large population of 1107 asymptomatic subjects also showed that *H. pylori* significantly and independently contributed to IR^[52].

A recent systematic review of evidence for the association between *H. pylori* infection and quantitative indexes of IR shows a trend toward a positive association between *H. pylori* infection and IR, independent of

several confounders^[51]. However, Gillum *et al.*^[57] maintain that there are no consistent associations between *H. pylori* infection and diabetic prevalence or variables of the IR syndrome in American men 40-74 years of age. Furthermore, Park *et al.*^[58] reported that metabolic and inflammatory parameters, including blood sugar, lipid profiles, IR, white blood cell count, and C-reactive protein (CRP) levels, were not changed after *H. pylori* eradication. It is important to note that *H. pylori* infection was not determined in all studies by histologic detection of organisms in mucosal biopsy specimens, which is considered the diagnostic gold standard. Although the diagnostic utility of serum *H. pylori*-specific IgG antibodies is well established^[59], the inclusion of false-positive or false-negative detections is unavoidable. As anti-*H. pylori* IgGs can be detected even after eradication, it is difficult to determine whether *H. pylori* merely initiates, or chronic active *H. pylori* infection is required to promote, IR^[60]. However, serologic tests are widely available, noninvasive, and inexpensive, and thus suitable for screening and large epidemiologic studies. As IR can develop in the presence of inflammation^[61] or as a result of alterations in counter-regulatory hormones that affect insulin^[62], *H. pylori* may thus promote IR by inducing chronic inflammation and affecting insulin-regulating gastrointestinal hormones^[53].

***H. pylori* and inflammation**

It is commonly believed that the chronic inflammation induced by *H. pylori* infection is strongly linked to the pathogenesis of T2DM, which is associated with a general activation of the innate immune system, and a chronic, cytokine-mediated state of low-grade inflammation. Many tissues are affected by pro-inflammatory cytokines, which cause recognizable features of T2DM^[63]. Inflammation of the adipose tissue is considered a key factor in the pathogenesis of IR, and β -cell autoinflammation mediated by interleukin (IL)-1 β impairs insulin secretion in T2DM. This inflammation is characterized by an increased infiltration of bone marrow-derived macrophages and increased expression of chemokines and cytokines such as IL-1 β ^[64], CRP and IL-6^[65], as well as tumor necrosis factor (TNF)^[66-69]. These and other macrophage-secreted factors exert paracrine effects that result in the activation of serine kinases such as c-jun N-terminal kinases (c-JNK) and the inhibitor of nuclear factor kappa B kinase β , which phosphorylate insulin receptor substrate proteins and create a state of IR in adipose tissue^[70].

Some epidemiological studies have suggested that pathogen burden is a risk factor for the inflammation that leads to IR^[71,72]. Colonization of the gastric epithelium by *H. pylori* brings about active chronic inflammation by infiltrating gastric submucosal neutrophils and monocytes, which can lead to gastric mucosal damage and epithelial remodeling^[73]. The host immune response to *H. pylori* infection is complex and involves upregulation of several proinflammatory cytokines, such as CRP^[74-76], IL-6, and TNF- α ^[77], which are implicated in IR and the develop-

ment of diabetes^[78]. Human CRP is primarily synthesized by hepatocytes and regulated by inflammatory cytokines (mostly TNF- α and IL-6), and levels of high-sensitive CRP (hsCRP) have been the main focus of investigation for diabetes risk. Of 11 prospective studies, seven reported a significant positive association between hsCRP levels and diabetes risk^[65,79-84], and four studies found no association^[85-88]. However, it is not known whether hsCRP itself directly influences IR or diabetes. IL-6 is produced in a variety of tissues, including activated leukocytes, adipocytes, and endothelial cells^[89,90]. Approximately 25% of *in vivo* systemic IL-6 originates from subcutaneous adipose tissue^[89] and is thought to modify adipocyte glucose and lipid metabolism and body weight^[91-93]. Pradhan *et al.*^[65] state that elevated levels of IL-6 predict the development of T2DM and further support a possible role for inflammation in diabetogenesis, an idea also supported by Spranger *et al.*^[69]. Increased production of TNF- α in adipose tissue may be a critical mechanism by which fat cells induce peripheral IR^[94], by the indirect increase in free fatty acid oxidation, stimulation of insulin counter-regulatory hormones or cytokines (*e.g.*, IL-6 and CRP), impairment of endothelial function, or direct inhibitory effects on glucose transporter protein GLUT4, insulin receptor substrates, or glucose-stimulated insulin release by pancreatic β -cells^[84]. Furthermore, *H. pylori* in the gut microbiota leads to increased production of lipopolysaccharide, a constituent of the bacterial cell wall, which also activates innate inflammatory processes^[95]. Concentrations of circulating lipopolysaccharide are higher in obese patients with T2DM than in non-diabetic, lean individuals and correlate with the degree of IR^[96].

Despite the evidence implicating a link between *H. pylori* infection and inflammation that predisposes individuals to T2DM, there are some contradictory data. A study by Jeon *et al.*^[35] failed to find any significant association between levels of inflammatory mediators (CRP and IL-6) and *H. pylori* infection or T2DM. Studies by Danesh *et al.*^[97] and Ridker *et al.*^[98] also found no significant association. Therefore, more investigation is needed to determine whether inflammation triggered by *H. pylori* infection contributes to the development of T2DM.

***H. pylori* and hormones**

H. pylori-induced gastritis can potentially affect the secretion of gastric-related hormones such as leptin and ghrelin^[99,100], as well as gastrin and somatostatin^[101], which may influence a predisposition to diabetes. Gastrin increases food-related and glucose-stimulated insulin release^[102,103], and somatostatin regulates pancreatic insulin secretion and inhibits insulin release^[104,105]. Patients with *H. pylori* infections could therefore have altered insulin release, as they have elevated basal and stimulated serum concentrations of gastrin and decreased somatostatin^[101,106]. The regulation of leptin and ghrelin, which are produced in the stomach and are involved in energy homeostasis^[107,108], affects obesity, insulin sensitivity, and glucose homeostasis^[109,110]. Increasing evidence indicates *H. pylori* can influence the production of leptin and ghrelin, and

thus could promote obesity and the development of diabetes^[100,111-114]. Ghrelin decreases energy expenditure and promotes weight gain^[115], whereas leptin, which is expressed mainly by adipocytes, reduces food intake and increases energy expenditure^[116]. *H. pylori* infection has been shown to impair ghrelin production^[117,118] and enhance the production of leptin^[119]. Low ghrelin levels are associated with elevated fasting insulin concentrations, IR, and T2DM^[120]. Leptin has also been implicated in the development of IR^[121], and elevated levels correlate with IR in lean men^[122] and patients with T2DM^[123]. Elevation of leptin levels is likely deleterious to human islet function, as a clinical study revealed that improved pancreatic β -cell function was independently associated with the decreased leptin and increased adiponectin levels in obese women after standardized weight reduction^[124]. There is evidence that in addition to mitigating the effects of insulin through phosphorylation of Ser-318 of insulin receptor substrate 1^[125], high levels of leptin may also impair glucose-stimulated insulin secretion and induce apoptosis of β cells in human islets *via* activation of c-JNK^[126]. However, a study by Brown *et al.*^[127] indicated that leptin has a protective role on pancreatic β cell function, showing that leptin could prevent apoptosis of pancreatic β cells through modulation of the Bcl protein family.

***H. pylori* and insulin secretion**

Decreased insulin secretion is one of the major pathophysiological defects in T2DM. The progression from normal glucose tolerance to prediabetes and T2DM is characterized by continuing defects in β -cell function^[128]. A study by So *et al.*^[129] found that *H. pylori* titer could independently predict abnormal pancreatic β -cell function in Chinese men. Additionally, Rahman *et al.*^[130] also described a positive association between *H. pylori* infection and impaired insulin secretion. The insulin-producing pancreatic β -cells are especially susceptible to damage by inflammation and oxidative stress^[131], therefore it is plausible that inflammation caused by *H. pylori* infection results in deficits in insulin secretion. Furthermore, it was reported in a study by Hsieh *et al.*^[40] that patients with *H. pylori* infection were more likely to have had impaired insulin secretion at a young age, which may increase the risk for T2DM.

Accumulating evidence indicates that cytokines play important roles in β -cell failure, as chronic exposure to IL-1 β , TNF- α , and IFN- γ inhibits insulin secretion and induces apoptosis of β cells^[132,133]. In addition, *H. pylori* vacuolating cytotoxin stimulates mitochondrial-dependent apoptosis in diabetic patients through downregulation of anti-apoptotic Bcl-2, upregulation of pro-apoptotic Bax, and increased activation of caspase-9 and -3^[134]. Despite these studies, more studies are needed to elucidate the role of *H. pylori* infection in insulin secretion and the incidence of T2DM.

H. PYLORI ERADICATION AND DIABETES

There are limited and conflicting data regarding the effect of *H. pylori* eradication on glucose metabolism and insu-

lin sensitivity^[58,135-137]. However, it may be beneficial for patients at risk of diabetes to be checked for the presence of *H. pylori* infection, as a report by Zojaji *et al*^[136] showed that *H. pylori* treatment can improve the mean HbA1c and the metabolic abnormalities in patients with T2DM. Additionally, Gen *et al*^[137] demonstrated that successful *H. pylori* eradication significantly decreased fasting insulin and HOMA-IR levels. Other studies focused on the effects of eradication on *H. pylori*-stimulated inflammatory cytokines. Some reports indicate that CRP levels are decreased after *H. pylori* eradication, suggesting a beneficial effect on low-grade inflammation^[33,137]. However, there are also reports showing no effect of *H. pylori* eradication on mean HOMA-IR and CRP levels^[58] or HbA1c levels^[135]. Recently, Vafaeimanesh *et al*^[138] found that in patients with T2DM, the mean decrease in HbA1c and fasting plasma glucose levels in eradicated cases was similar to non-eradicated subjects three and six months after treatment.

H. PYLORI-ASSOCIATED FACTORS AND DIABETES

Many additional factors are likely involved in the relationship between *H. pylori* infection and diabetes. For example, lifestyle is a critical factor affecting both chronic *H. pylori* infection and T2DM, as it has been shown that older subjects with a low-risk lifestyle are less likely to develop T2DM^[139]. Gastroduodenal conditions resulting from *H. pylori* infection could delay gastric emptying, which has been postulated to cause mismatch between the onset of insulin action and the absorption of carbohydrates in insulin-dependent children with diabetes^[140,141]. However, it has also been suggested that delayed gastric emptying is a potential advantage, rather than a disadvantage, in relation to glycemic control in T2DM patients not treated with insulin^[142], and others maintain that *H. pylori* infection does not affect the rate of gastric emptying in diabetic patients^[143]. *H. pylori* infection has also been implicated in platelet activation and aggregation, increases in pro-atherogenic factors such as homocysteine, production of reactive oxygen species, and increases in lipid peroxides^[144].

Obesity

There is now solid evidence that obesity is the main etiological cause of T2DM, with new, controlled, clinical trials showing that a weight loss of as little as 5% is sufficient to prevent most obese subjects with impaired glucose tolerance from developing the disease^[145]. However, there is no clear evidence linking obesity and *H. pylori* infection. According to some studies, obesity^[146] and/or a high BMI^[112] may be associated with an increased incidence of *H. pylori* colonization, likely resulting from reduced gastric motility. A study by Cohen *et al*^[147] demonstrated that adults infected with *H. pylori* had higher BMI levels, even if asymptomatic, and further suggested that *H. pylori* therapy may lead to weight loss and improve diabetic control. In contrast, other studies

showed no association between *H. pylori* seropositivity or CagA antibody status and BMI^[148,149], or even an inverse relationship between morbid obesity and *H. pylori* seropositivity^[150]. Nevertheless, there are data demonstrating that *H. pylori* eradication significantly increases the incidence of obesity in patients with peptic ulcer disease, as it increases BMI^[151,152], and/or enhances the appetite of asymptomatic patients by elevating plasma ghrelin^[113] and reducing leptin^[153] levels.

Dyslipidemia

Disturbances in the production and clearance of plasma lipoproteins are among the metabolic abnormalities that commonly accompany diabetes. Moreover, dyslipidemia may foster the development of diabetes^[154]. High concentrations of plasma triglyceride and low-density lipoprotein cholesterol (LDL-c), along with low concentration of high-density lipoprotein cholesterol (HDL-c), are attributed mostly to IR and insulin deficiency^[155,156]. *H. pylori* infection may induce dyslipidemia, as it leads to elevated plasma levels of total cholesterol^[157,158], LDL-c^[158] and triglyceride concentrations^[159] and decreased levels of HDL-c^[160,161]. It was postulated that chronic *H. pylori* infection may promote atherogenic lipid profiles through the action of pro-inflammatory cytokines, such as IL-6, interferon- α and TNF- α , which activate adipose tissue lipoprotein lipase, stimulate hepatic fatty acid synthesis and influence lipolysis^[158,162]. However, as not all studies found significant changes in plasma levels of total cholesterol, triglycerides, and LDL-c with *H. pylori* infection^[163,164], further studies are needed to verify this association.

Age

Although there have been indications that T2DM may predispose an individual to *H. pylori* infection^[35,47-49], this seems unlikely, considering the age at which the disease is typically acquired. A model of age-related pleiotropy, or life-course perspective, with respect to *H. pylori* colonization has been proposed by Atherton *et al*^[165]. The potential benefits of *H. pylori* occur predominantly earlier in life, including reduced risks for asthma^[166,167], tuberculosis reactivation^[168], childhood diarrhea^[169], and gastroesophageal reflux disease^[170-172]. However, among older individuals, *H. pylori* can promote adverse health effects, such as peptic ulcer disease, gastric cancer, and perhaps increased glucose intolerance.

CONCLUSION

Since the discovery of *H. pylori*, a variety of epidemiological studies, therapeutic trials, and case reports have evaluated the direct or indirect involvement of this bacterium in the pathogenesis of various extragastric disorders. Although no current data provide concrete evidence that *H. pylori* plays a role in diabetes mellitus, the possibility cannot be ruled out. The evidence concerning an association between *H. pylori* infection and IR, chronic inflammation, the secretion of gastric-related hormones, and insulin

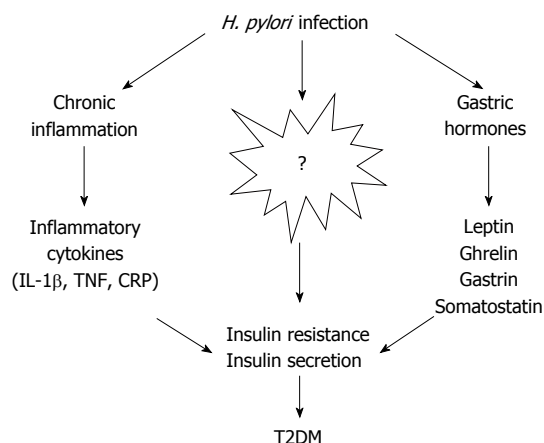


Figure 1 Potential mechanisms for contribution of *Helicobacter pylori* to type 2 diabetes mellitus. Insulin resistance and abnormal insulin secretion are central to the development of type 2 diabetes mellitus (T2DM). On the one hand, *Helicobacter pylori* (*H. pylori*) infection brings about chronic low-grade inflammation with upregulation of several cytokines such as C-reactive protein (CRP), tumor necrosis factor (TNF) and interleukin (IL)-1 β , which may influence insulin action and pancreatic β cell secretion. On the other hand, *H. pylori*-induced gastritis can potentially affect the secretion of gastric hormones, including leptin, ghrelin, gastrin, and somatostatin, which could affect insulin sensitivity and glucose homeostasis. In addition, other mechanisms and mediators may be involved in the possible causative relationship between *H. pylori* infection and T2DM.

secretion deficiency implicate *H. pylori* in a predisposition to diabetes (Figure 1). However, the pathophysiology of T2DM is complex, and many other factors could contribute to this process after *H. pylori* infection, such as lifestyle, changes in gastric emptying, dyslipidemia and so on. Diabetes mellitus is a multifaceted and multistep disease that is unlikely to result from a single cause, though risk factors that deserve attention include gastrointestinal infections and the composition of intestinal microbiota. Larger prospective studies investigating the impact of *H. pylori* infection on diabetes and corresponding mediating factors are warranted. Meanwhile, large interventional studies are urgently needed to evaluate the long-term benefit of *H. pylori* eradication for prevention and progression of diabetes. Evidence supporting an etiological role of *H. pylori* in the development of T2DM would indicate that preventive measures, such as increased hygiene and treatments using antibiotics and proton pump inhibitor combinations, should be explored as targets of intervention in high-risk communities.

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Role of pomegranate and citrus fruit juices in colon cancer prevention

Saravana Kumar Jaganathan, Muthu Vignesh Vellayappan, Gayathri Narasimhan, Eko Supriyanto

Saravana Kumar Jaganathan, Eko Supriyanto, IJN-UTM Cardiovascular Engineering Centre, Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia, Johor Bahru 81310, Malaysia

Saravana Kumar Jaganathan, Muthu Vignesh Vellayappan, Gayathri Narasimhan, Department of Research and Development, PSNA College of Engineering and Technology, Dindigul 624 622, India

Author contributions: Jaganathan SK designed the research; Vellayappan MV, Narasimhan G and Supriyanto E participated in the research design; Jaganathan SK, Vellayappan MV and Narasimhan G collected the literature; Vellayappan MV and Narasimhan G wrote the review in consultation with Jaganathan SK; Supriyanto E participated in writing and editing of manuscript by providing suggestions; all the authors verified the final manuscript. Correspondence to: Saravana Kumar Jaganathan, MSc, PhD, Senior Lecturer, IJN-UTM Cardiovascular Engineering Centre, Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia, Johor Bahru 81310, Malaysia. jaganathaniitkgp@gmail.com

Telephone: +60-7-5558548 Fax: +60-7-5558553

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Abstract

Colorectal cancer is the second leading cause of cancer-related deaths in the United States. Recent studies prove that though chemotherapeutic agents are being used for the treatment of colon cancer, they become non-effective when the cancer progresses to an invasive stage. Since consumption of certain dietary agents has been linked with various cancers, fruit juices have been investigated for their consistently protective effect against colon cancer. The unique biochemical composition of fruit juices is responsible for their anticancer properties. In this review, the chemo-preventive effect of fruit juices such as pomegranate and citrus juices against colon cancer are discussed. For this purpose, the bioavailability, *in vitro* and *in vivo* effects of these fruit juices on colorectal cancer are highlighted. More-

over, there is a scarcity of studies involving human trials to estimate the preventive nature of these juices against colon cancer. This review will support the need for more preclinical tests with these crude juices and their constituents in different colorectal cancer cell lines and also some epidemiological studies in order to have a better understanding and promote pomegranate and citrus juices as crusaders against colon cancer.

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Key words: Colon cancer; Fruit juices; Pomegranate; Citrus fruits; Chemoprevention

Core tip: Recent studies prove that though chemotherapeutic agents are being used for the treatment of colon cancer, they become non-effective when the cancer progresses to an invasive stage. This problem can be minimized by the regular intake of fruit juices. The unique biochemical composition of fruit juices is responsible for their chemo-preventive properties. In this review, the chemo-preventive effects of fruit juices such as pomegranate and citrus juices against colon cancer are discussed.

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INTRODUCTION

Colorectal cancer is the third leading cause of cancer-related deaths in the United States when men and women are considered separately, and the second leading cause when both sexes are combined. It is expected to cause

about 51690 deaths during 2012^[1]. The American Cancer Society's most recent estimates for the number of colorectal cancer cases in the United States for 2012 are: 103170 new cases of colon cancer and 40290 new cases of rectal cancer. Duke's classification helps to identify the severity of the disease in different stages of colon cancer. Such a classification enables us to understand the degree of disease progression and the best treatment that is possible. Even after spending decades of years in studies related to treatment and cure for colon cancer, conventional cancer treatments offer little promise to patients. The main drawback lies in the fact that even after various cancer treatments, the disease is found to recur and this time will exacerbate the previous symptoms. A conventional treatment does not aspire to treat the root cause but only its symptoms. The use of chemotherapeutic agents and radiation exhausts the anti-oxidants available and induces oxidative stress, which increases with disease progression. Hence, it is high time to look at alternative yet completely curative measures for treating colon cancers.

In this scenario, various epidemiological studies have shown that a diet which is rich in fiber can minimize the risk of developing colon cancer^[2-4]. Similar studies also proved that a phytochemical-rich diet which is absorbed by the body from fruit and vegetable sources can decrease the risk of developing colon cancer^[5,6]. Further reports have shown the inhibition of colon carcinogenesis by dietary supplements^[7]. Moreover, other reports have also shown that colon cancer is one of the most preventable forms of cancer and have depicted the importance of dietary modification for preventing colon carcinogenesis^[8]. Fruits, nuts, vegetables and grains contain major non-nutrient components called polyphenols which have chemo-preventive properties against colon cancer^[9]. The major mechanisms through which they exert this activity are through the combination of properties such as anti-proliferative, pro-apoptotic and antioxidant properties of the polyphenolics^[10].

Consumption of fruit juices by various ethnic groups is prevalent and there is a good market-share between real fruits and the fruit juices. Intake of fruits as juices has gained wider acceptance among the young population because it is easier to consume, and also the intake amount of juices can be increased significantly compared to fruits itself. Further, the availability of 100% fruit juices in the retail market and also the functional claims of such juices further motivate people to consume fruit juices. Since fruit juices contain polyphenolics which help in reducing the growth of colon cancer, they can be consumed as dietary intake regularly to reduce the incidence of colon cancer. Furthermore, there are no side-effects as seen in the conventional treatments as the treatment is aimed at the molecular level. Moreover, since fruit juices alkalize the body and provide an abundance of enzymes, vitamins, minerals, phytochemicals and other nutrients, they prove to be a better alternative for preventing the colon cancer. A review article summarizing the effect of pomegranate on various cancers was recently pub-

lished^[11]. However, till now the effect of pomegranate juice against colon cancer has not been reviewed extensively. Hence, we are discussing the effects of fruit juices such as pomegranate and citrus against colon cancer in this article. For this purpose, we summarize the effect of these fruit juices on colon cancer cell lines and animal models along with their bioavailability studies.

POMEGRANATE JUICE

The botanical name of pomegranate is *Punica granatum*. The native source of this fruit is Iran and now it has been cultivated in Asian areas such as the Caucasus and the Himalayas in Northern India. The number of seeds present in a pomegranate can vary from 200 to 1400, but some believe that all pomegranates have an equal number of seeds. The pomegranate juice is obtained by crushing the seeds of the pomegranate. This pomegranate juice contains different types of polyphenols such as gallo, ellagitannin and flavonoid classes.

Bioavailability and metabolism of pomegranate juice in relation to colon cancer

As mentioned above, pomegranate juice is rich in polyphenol compounds such as gallo, ellagitannin and flavonoid classes. The commercially available pomegranate juice which is obtained by hydrostatic pressing of whole fruit contains cyanidin 3,5-diglucoside, pelargonidin-3,5-diglucoside, flavonols such as kaempferol and quercetin, flavones such as luteolin, anthocyanins such as cyanidin-3-glucoside, delphinidin-3-glucoside, ellagitannins such as the punicalagins and punicalins, which exist as β -anomers and R- and acyclic hydroxylaldehyde^[12]. A significant portion of the pomegranate juice contains the pomegranate polyphenols called ellagitannins and they often coexist with ellagic acid, the main product obtained through hydrolysis of the class tannins. Besides ellagitannins, pomegranate juice also contains variable amounts of the polyphenol called gallic acid. This ellagic acid is obtained by the metabolism of the ellagitanins by the intestinal bacteria. Ellagic acid is found to be analogous to urolithins. The urolithins are reported to be systematically bioavailable where they accumulate in organs such as colon, prostate and intestine.

The modulation of chemical carcinogenesis induced by dietary carcinogens can be achieved using drug-metabolizing enzymes, through cytochrome P450 (CYP) enzyme inhibition and/or by induction of phase-2 conjugating enzymes. It was found that ellagic acid prevents cancer initiation and inhibits the CYP1 activation of procarcinogens^[13]. Moreover, the ellagic acid also induces phase-2 enzymes like glutathione S-transferase. However, the urolithins and ellagitannins were not tested regarding whether they have anti-carcinogenic activity through inhibition of induction of phase II conjugating enzymes and/or inhibition of CYP1. Thus, the above-mentioned mechanisms are some of the potential mechanisms by which pomegranate juice consumption might inhibit co-

lon cancer formation.

The pomegranate (*Punica granatum* L.) is consumed in various forms such as pomegranate juice, wine and jam. Pomegranate juice exhibits some atherosclerotic as well as antioxidant properties due to its high content of polyphenols such as ellagic acid, ellagitannins, and other flavonoids (luteolin glycosides, quercetin, and kaempferol)^[14]. Among these polyphenols, punicalagin is present in a great amount and is responsible for greater than 50% of the juice's potential antioxidant activity.

***In vitro* effect of pomegranate juice on colon cancer**

Kasimsetty *et al.*^[15] investigated the action of ellagitannins and urolithins against HT-29 human colon cancer cells. It was found that urolithins A and C inhibited 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin)-induced CYP1-mediated ethoxyresorufin-O-deethylase activity *in vitro* with IC₅₀ values ranging from 56.7 to 74.8 μ mol/L. Both of these compounds inhibited the HT-29 cell proliferation in a time- and dose-dependent manner by inducing apoptosis. Hence, they concluded that drinking pomegranate juice in considerable amounts may hinder the colon cancer progression.

Studies done by Seeram *et al.*^[16] on the effect of pomegranate juice and purified ellagitannins on colon cancer have shown that they inhibit the induction and proliferation of colon cancer cell lines. It was also found that their results are in accordance with the reported anti-proliferative activity of pomegranate polyphenols in breast and prostate cancers^[17]. Moreover, this recent study depicts proliferation inhibition by treatment of HT-29 cancer cells with a cyclooxygenase-2 (COX-2) specific inhibitor and NS398. Other studies also show the correlation between increased cell proliferation and enhanced COX-2 expression. Hence, it is hypothesized that COX-2 expression modulation by pomegranate juice might be an important mechanism for the colon cancer anti-proliferative activity of the pomegranate juice. The COX-2 expression in HT-29 cells is found to be decreased by pre-treatment with the pomegranate juice and punicalagin in a dose-dependent manner. Besides, it was proven that pomegranate juice has better potential in decreasing the COX-2 expression. This is mainly because of the important interactions with other bioactive polyphenols in pomegranate juice such as flavonols and anthocyanins. Thus, this result has led to a conclusion that when the individual polyphenols are separated from the pomegranate juice it can decrease the overall activity due to the requirement of other components. Signaling pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/nuclear factor-kappa B (NF κ B) mediate COX-2 expression. The modulation of NF κ B activity is mediated by PI3K *via* AKT. In the case of mesangial cells, PI3K activation resulted in increased cell proliferation as well as COX-2 expression^[18]. Hence, this has illustrated the specific relationship between COX-2 and PI3K. In accordance with the above observations, other works have depicted that pretreatment with

pomegranate juice inhibits NF κ B activation, AKT activity and expression of COX-2 in HT-29 cells^[19].

Even though some studies have concluded that the COX-2 expression in HT-29 cells depends on NF κ B activity, studies done by Jobin *et al.*^[20] in 1998 have demonstrated that NF κ B inhibition by wortmannin decreased the COX-2 expression only partially. Thus, this leads to a conclusion that other signaling pathways may influence the modulation of COX-2 expression in HT-29 cells in collaboration with the NF κ B activity. MAPK pathways (SAPK, p38 and ERK1/2) are potential candidates for this role. This is because MAPK was found to be mediating COX-2 expression in a large number of studies^[21]. Besides, *in vitro* studies have shown that p28 and ERK modulate the NF κ B activity^[22]. Moreover, other studies also have shown that both MAPK and NF κ B may mediate COX-2 expression, but the inter-relationship between these protein signaling pathways is yet to be determined. The application of pomegranate extracts 30 minutes before TPA treatment of mice resulted in JNK1/2 activity, p38, ERK1/2 and COX-2 expression inhibition.

The availability of the flavonoids in various food materials is a relatively unexplored field. On the other hand, various studies show that they are poorly absorbed in the upper gastrointestinal tract. The rate of absorption of this component in the small intestine ranges from 0% to 60% of the ingested dose, which relies on the food source^[23]. Hence, the flavonoids reach the colon in an unabsorbed form or they are secreted as absorbed conjugates where ultimately they are secreted in the bile. However, the inhibition of NF κ B, AKT and COX-2 provides us with greater knowledge about the anticancer mechanism actions of the pomegranate juice in colon cancer. These works have also presented us with direction for future studies on the role of pomegranate juice in prevention and treatment of colon cancer.

It was found that COX-2 expression, indicative of an inflammatory signaling process leading to the initiation and progression of colon cancer in HT-29 colon cancer cells, was inhibited by the pomegranate juice. Moreover, the whole juice was found to be more powerful in the inhibition process (79% suppression) than its individual components^[24]. Larrosa *et al.*^[25] have shown the induction of apoptosis of colon cancer cells by punicalagin and ellagic acid from pomegranate juice. The intrinsic pathway of apoptosis occurred when mitochondrial cytochrome c leakage in the cytosol was caused by punicalagin and ellagic acid. A downregulation of the anti-apoptotic Bcl-2 protein was achieved with 30 μ mol/L ellagic acid and 100 μ mol/L punicalagin. It was found that procaspase 3 and caspase 9, which are members of the caspase family of proteases, were induced by punicalagin and ellagic acid. However, caspase 8, which is related to extrinsic pathways (*e.g.*, induced by cytokines) of apoptosis, was not activated by ellagic acid and punicalagin. Likewise, the incubation of ellagic acid or punicalagin with anti-Fas ZB4 antibody resulted in no inhibitory effect on apoptosis. Hence, this antibody was utilized for inhibition of



Figure 1 Pomegranate and Citrus juices are depicted in this figure.

interaction of ellagic acid or punnicalagin with the Fas receptor. Thus, all this data supports the intrinsic mechanism of a pomegranate juice-induced apoptotic effect in colon carcinogenesis.

A positive effect on COX-2 expression has been observed due to action of the PI3K/AKT/NF κ B pathway. Initially, the P13K activates the AKT. Later, this AKT will phosphorylate and activate I κ B kinase, ultimately leading to the activation of NF κ B. It was found that P13K is associated with colon cancer. Here, phosphatase and tensin homologue gene (PTEN) mutations occur where PTEN inhibits P13K^[26]. Moreover, an increased level of P13K activity is observed in adenocarcinoma cell lines and colon cancer cell lines^[27]. Inhibition of P13K activity in ovarian cancer cell lines as well as colon cancer lines leads to inhibition of cell proliferation^[28].

In vivo effect of pomegranate juice on colon cancer

For the purpose of analyzing the changes associated with colon cancer, Boateng *et al.*^[29] conducted a study on the effect of pomegranate juice on aberrant cryptic foci (ACF). This study revealed that the pomegranate fruit juice reduced the number of ACF of the colon by 91% in male F-344 rats. The animals utilized in this study were given 20% pomegranate juice before and after treatment of the rats with a specific colon carcinogen called azoxymethane. Later, histopathology of the rat colon was studied after the 17th week of treatment. It was found that there was a significant decrease in the number of large crypts in pomegranate juice-fed rats. Moreover, the observed number of crypts/ACF was also few in these animals. Compared to fruit juices such as cranberry and water melon, the pomegranate juice showed better inhibition of ACF in rat colon. The pomegranate juice-fed rats' food intake and weight gain increase indicates the possibility of the protective effect of the pomegranate juice against cancer cachexia. This is because the activity of hepatic glutathione S transferase (GST) was found to be three times higher in the case of rats being fed with pomegranate juice. GST is well known for the scavenging of free radicals that are produced from oxidative stress. When this enzyme activity is induced, it supports the mechanism of pomegranate anti-oxidative actions in

other experimental models^[30].

The intake of pomegranate seed oil in the diet was found to cease the multiplicity of colonic adenocarcinomas, but dose-dependent variation was not observed. The tumor incidence was found to be coupled with enhanced expression of peroxisome proliferator-activated receptor gamma protein in the normal non-tumor mucosa^[7]. Hence, all these results depict the useful effects of pomegranate, which acts against the growth of colonic tumors in mice.

CITRUS JUICES

The botanical name of orange is *Satsuma mandarin*. It is generally seedless with thin skin (Figure 1). The fruit is grown in cool subtropical regions of Japan, Spain, and central China, Korea, Russia, Turkey, southern South Africa, South America, central California and northern Florida. The pulp and juice of the citrus fruit contain flavonoids such as apigenin, naringenin, hesperidin, nobiletin and limonoids, and cryptoxanthin, a carotenoid. Also, the peel of citrus fruits contains a phytochemical called tangeritin. All these components act as chemo-preventive agents. Bio-availability and metabolism of citrus juice and its effect on colon cancer cells and animal models are discussed below.

Bio-availability and metabolism of citrus juice in colon cancer

Satsuma mandarin (Citrus unshiu Mar.) juice contains β -cryptoxanthin, a carotenoid, and hesperidin, a flavonoid, which are potential chemo-protective compounds. A pulp (CHRP) containing high amounts of β -cryptoxanthin and hesperidin made from *Satsuma mandarin* inhibited chemically induced colon carcinogenesis in rats^[31]. CHRP and citrus juices suppress the expression of pro-inflammatory cytokines and inflammatory enzymes in colon. β -Cryptoxanthin with non-substituted β -ionone cycles and pro-vitamin A possesses several biological activities including scavenging of free radicals, enhancement of gap junctions, immune-modulation, and regulation of enzyme activity involved in carcinogenesis and inhibition of tumorigenesis^[32]. Hesperidin is found in various vegetables and fruits, and it is shown to exhibit antioxidant activity, anti-inflammatory effect and an inhibiting effect on prostaglandin biosynthesis. This flavonoid inhibits chemically induced carcinogenesis in several organs^[33].

In response to CHRP treatment in rats, GST and quinone reductase (QR) levels are elevated by limonin in the colon. CHRP and citrus juices also exhibit suppressing effects on hyper-cell proliferation activity induced by carcinogens in the colon, thereby inhibiting carcinogenesis^[34]. They also suppress mRNA expression of several cytokines [tumour necrosis factor-alpha, interleukin (IL)-1 β , IL-6] and inflammatory enzymes [COX-2 and inducible nitric oxide synthase (iNOS)] and enhance mRNA expression of Nrf2 in colon that received a carcinogen.

Nrf2 is a transcription factor and a key regulator of the inducible expression of enzymes such as GST and QR. GST and QR are involved in catalyzing the detoxification of reactive electrophiles and oxidants that contribute to the formation of mutations leading to cancers. Nrf2 also regulates the cytoprotective transcriptional response leading to prevention of damage to DNA, proteins and lipids, as well as recognition, repair, and removal of macromolecular damage and tissue renewal following toxicity. With cancer development in tissues there is an association of chronic inflammation regulated and mediated by cytokines. Any imbalance in their levels of production results in tumor invasion and metastasis. In addition, inflammatory bowel disease is an important risk factor for the development of colorectal cancer (CRC). Inflammation is also likely to be involved with other forms of sporadic as well as heritable CRC. Thus, Nrf2 is one of the targets for cancer chemoprevention in the colon, and the positive effects of CHRP and citrus juices are attractive for reducing tumor formation when considering the relationship between inflammation and cancer development^[35].

In vitro studies based on the effect of citrus juice in colon cancer cell lines

The anti-proliferative effects of naringenin have also been demonstrated in HT-29 colon cancer cells^[36]. Cell culture experiments have reported anti-proliferative effects for hesperetin, the aglycone form of hesperidin, nobiletin, apigenin, and a limonoid glucoside mixture^[37]. Citrus flavonoids mainly interact with cyclooxygenase and protein tyrosine kinases. Tangeritin, containing five methoxy groups, is a more potent phytochemical than flavonoids with free hydroxyl groups. Tangeritin is shown to inhibit cancer cell growth by increasing the gap junctional intracellular communication. A study by Pan *et al*^[38] on human colon cancer cell lines was performed to determine the effects of flavonoids like tangeritin, nobiletin, quercetin, apigenin, luteolin and rutin on the growth of colon cancer cells. Levels of cyclin, p53 protein levels, the activities of some kinases and phosphorylation state of Rb were measured. It was found that growth of colon cancer cells was inhibited mainly by tangeritin, but luteolin and nobiletin also contributed to the inhibition. The mechanism underlying the inhibition of growth of colon cancer by tangeritin is the blockade of the cell cycle in the G₀/G₁ phase, reduced levels of cyclins (A, D1 and E) and the decreased phosphorylation of Rb. Production of p53, p27 and p21 was increased further by tangeritin. Thus, these results indicate that tangeritin inhibits growth of colon cancer by increasing levels of cyclin-dependent kinase inhibitors (p21, p27 and p53) and decreasing the activity of some cdk.

In vivo studies related to effect of citrus juice on colon cancer

Ornithine decarboxylase activity and ACF numbers were reduced by apigenin, and it reduced tumor formation in azoxymethane-induced CF-1 mice^[39]. ACF numbers

in 1,2-dimethylhydrazine-treated Wistar rats were also reduced by diets containing hesperitin (the aglycone of hesperidin)^[40]. A mixture of apigenin and epigallocatechin gallate suppressed colon neoplasia recurrence in human subjects with resected colon cancers^[41]. Isolated limonin and naringin suppressed the high multiplicity aberrant crypt foci (HMACF) because of lower levels of proliferation and enhanced apoptosis. Lower levels of iNOS and COX-2 in response to limonin in the diet, and a lower level of iNOS in response to naringin in the diet, suggest that changes in nitric oxide and/or prostaglandin synthesis may be mediating the benefits derived from these dietary interventions^[42]. Kohno *et al*^[43] found that nobiletin decreased prostaglandin E₂ (PGE₂) production in rats. This strengthens the hypothesis that citrus flavonoids (hesperidin, nobiletin, apigenin, naringenin) and limonoids (a limonin glucoside/obacunone glucoside mixture) could act as chemo-preventive agents at the promotion stage of colon carcinogenesis.

Rats treated with naringenin showed a reduced proportion of proliferating colon cells and smaller expansion of the proliferative zone. Hanske *et al*^[44] recently demonstrated that apigenin-7-glucoside is metabolized to not only the aglycone form of apigenin, but also to low levels of naringenin (and other compounds) in *in vivo* studies. Therefore, apigenin, which is involved in reducing proliferation *in vitro*, possibly may not show the same *in vivo* effect due to its metabolism within the intestinal tract. Surface cell apoptosis of colon cells was enhanced in rats provided with naringenin and apigenin. Since naringenin and apigenin up-regulated apoptosis, they could inhibit HMACF^[45].

The pro-inflammatory enzymes, COX-2 and iNOS, are expressed in high levels in human colorectal adenomas and adenocarcinomas. A positive correlation was shown between COX-2 level and proliferative zone in rats provided with naringenin; this was expected based on the literature linking PGE₂ and cell proliferation^[46]. Naringenin and apigenin thus prove to be naturally occurring chemo-preventive agents against colon carcinogenesis.

CONCLUSION

The main purpose of our work is to consolidate the various chemo-preventive effects of two different types of juices - pomegranate juice and citrus juice - on colon cancer. This review article mainly discusses the *in vitro* and *in vivo* effect of these juice varieties on colon cancer, as well as bioavailability and metabolism of these juices which is relevant to colon cancer. Tables 1 and 2 summarize the *in vitro* and *in vivo* effects of the above juices against colon cancer.

The motive of our work is to address the need for more preclinical tests to be carried out on different colon cancer cell lines other than the commonly used type of cell lines such as HT-29 and Caco-2. In addition to that, in most of the work done on animal studies, normal rats and mice were utilized as a subject instead of transgenic

Table 1 *In vitro* summary of action against colon cancer by fruit juices and their components

Juice and its components	Cell line tested	Observation/result	Ref.
Whole/crude pomegranate juice	HT-29 human colon cancer cells	Inhibition of NFκB activation, AKT activity and COX-2 expression Inhibition of COX-2 expression leading to the prevention of initiation and progression of colon cancer	[18,19] [24]
Ellagitannins of pomegranate juice	Caco-2 cells	Apoptosis of Caco-2 cells through the mitochondrial pathway	[16]
Punicalagin of pomegranate juice	HT-29 colon cancer cells	Down regulation of the anti-apoptotic Bcl-XL protein was achieved with 30 μmol/L ellagic acid and 100 μmol/L punicalagin Induction of intrinsic pathway of apoptosis in colon cancer cells	[25]
Urolithins A and C of pomegranate juice	HT-29 human colon cancer cells	Inhibition of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced CYP1-mediated ethoxyresorufin-O-deethylase activity IC50 values range: 56.7-74.8 μmol/L Induction of apoptosis in a time- and dose-dependent manner	[15]
Naringenin of citrus juice	HT-29 colon cancer cells	Inhibition of cell proliferation at doses greater than 0.71 mmol/L demonstrated using colorimetric assay	[36]
Limonoids of citrus juice	Human colon cancer cell lines	Induction of apoptosis and cytotoxic effects in MCF-7 and SKOV-3 cells at high concentrations	[37]
Flavonoids of citrus juice	Human colorectal carcinoma-COLO 205 cells	Cell cycle blockade in G ₀ /G ₁ phase	[38]
Tangeritin		Reduced levels of cyclins (A, D1 and E)	
		Decreased phosphorylation of Rb	
		Increased production of cyclin-dependent kinase inhibitors, p53, p21, p27	
Luteolin		Inhibition of growth of colon cancer cells	[39]
Nobiletin		Inhibition of growth of colon cancer cells	

NFκB: Nuclear factor-kappa B; AKT: Protein kinase B; COX-2: Cyclooxygenase 2; CYP: Cytochrome P450; MCF: Human breast cancer cell line; SKOV-3: Human colorectal cancer cell line.

Table 2 *In vivo* summary of action against colon cancer by fruit juices and their components

Juice and its components	Animal model used	Observation/result	Ref.
Whole/crude pomegranate juice	Male F-344 rats	Histopathological studies of azoxymethane-induced rat colon Significant decrease in number of large cryptic foci Increase in feed intake and weight gain Protective effects against cancer cachexia Three times higher activity of GST	[29]
	Mice	Anti oxidative actions by scavenging free radicals	[30] [39]
Apigenin of citrus juice	Azoxymethane-induced CF-1 mice	Inhibition of JNK1/2 activity, p38, ERK1/2 and COX-2 expression Reduced ODC activity and ACF numbers Reduced tumor formation	
Mixture of apigenin and epigallocatechin-gallate	Patients with colorectal neoplasia	Suppressed colon neoplasia recurrence rates	[41]
Hesperitin of citrus juice	DMH-treated male Wistar rats	Reduced number of ACF at a dose of 20 mg/kg	[40]
Nobiletin of citrus juice	Azoxymethane-treated male F344 rats	Decreased PGE2 production in rats Chemo-preventive agents at the promotion stage of colon carcinogenesis	[43]
Naringenin of citrus juice	Azoxymethane-injected Sprague Dawley rats	Reduced levels of COX-2 and iNOS Decrease in proliferation of colon cancer cells	[45,46]

GST: Glutathione S transferase; JNK: c-Jun N-terminal kinase; COX-2: Cyclooxygenase2; ODC: Ornithine decarboxylase; ACF: Aberrant crypt foci; PGE2: Prostaglandin E2; iNOS: Inducible nitric oxide synthase; DMH: 1,2-dimethylhydrazine.

animals. Hence, the transgenic animals have to be utilized for animal studies involving the efficacy determination of citrus and pomegranate juices against colon cancer to improve the reliability of the results. It would be appropriate for testing the efficacy of the above juices to use the Apc^{Min/+} mouse (colon cancer model with a dominant germ-line mutation at codon 850 of the homolog of the human adenomatous polyposis coli gene) in order to confirm their colon cancer prevention potential.

Besides that, our work is also aimed at throwing light on the importance of carrying out more clinical trials in human beings with the pomegranate and citrus juices.

To assess whether these juices have preventive effects against colon cancer, a study could be initiated with 25 healthy participants or 25 participants with increased risk for colon cancer to assess its predictive efficiency. However, phase II and phase III clinical trials involving larger groups of participants who are at high risk for colon cancer may validate the effect of these fruit juices and provide information whether these agents have protective effects against the colon cancer biomarkers. However, these research proposals demand large research grants which makes the study a costly and impracticable thing. Moreover, cancer prevention using dietary agents is still

a promising field of oncology where scientists in both basic and clinical sciences face great challenges.

In the current scenario, there are no human clinical trials that have been done to study the effect of pomegranate and citrus juices on colon cancer. However, some recent human clinical trials evaluated the effect of pomegranate juice against prostate cancer. In one of these trials, it was found that regular pomegranate juice consumption by prostate cancer patients decreased the disease progression by increasing prostate specific antigen doubling time from 15 to 54 mo. The researchers demonstrated that post-treatment serum analysis showed a decrease in cell proliferation and increase in cancer cell death^[47]. Hence, there is supporting evidence for the chemo-preventive potential of fruit juices which may be extended positively against colon cancer.

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Gastric nNOS reduction accompanied by natriuretic peptides signaling pathway upregulation in diabetic mice

Hong-Li Lu, Xu Huang, Yi-Song Wu, Chun-Mei Zhang, Xiang-Min Meng, Dong-Hai Liu, Young-chul Kim, Wen-Xie Xu

Hong-Li Lu, Xu Huang, Yi-Song Wu, Chun-Mei Zhang, Xiang-Min Meng, Dong-Hai Liu, Wen-Xie Xu, Department of Physiology, Shanghai Jiaotong University School of Medicine, Shanghai 200240, China

Young-chul Kim, Department of Physiology, Chungbuk National University College of Medicine, Chungbuk 361-763, South Korea

Author contributions: Lu HL, Huang X and Wu YS performed the majority of the experiments; Zhang CM, Meng XM and Liu DH performed the intracellular recording; Kim Y was involved in editing the manuscript; Xu WX designed the study and wrote the manuscript.

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Correspondence to: Wen-Xie Xu, Professor, Department of Physiology, Shanghai Jiaotong University School of Medicine, 800 Dongchuan Road, 328 Wenxuan Medical Building, Shanghai 200240, China. wenxiexu@sjtu.edu.cn

Telephone: +86-21-34205639 Fax: +86-21-34205639

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Abstract

AIM: To investigate the relationship between neuronal nitric oxide synthase (nNOS) expression and the natriuretic peptide signaling pathway in the gastric fundus of streptozotocin (STZ)-induced diabetic mice.

METHODS: Diabetic mice were induced by injection of STZ solution. Immunofluorescence labeling of HuC/D, nNOS and natriuretic peptide receptor-A, B, C (NPRs) in the gastric fundus (GF) was used to observe nNOS expression and whether NPRs exist on enteric neurons. The expression levels of nNOS and NPRs in the diabetic GF were examined by western blotting. An isometric force transducer recorded the electric field stimulation (EFS)-induced relaxation and contraction in the diabetic GF. An intracellular recording method assessed EFS-

induced inhibitory junction potentials (IJP) on the GF. GF smooth muscles acquired from normal mice were incubated with different concentrations of the NPRs agonist C-type natriuretic peptide (CNP) for 24 h, after which their nNOS expressions were detected by western blotting.

RESULTS: Eight weeks after injection, 43 diabetic mice were obtained from mouse models injected with STZ. Immunofluorescence indicated that the number of NOS neurons was significantly decreased and that nNOS expression was significantly downregulated in the diabetic GF. The results of physiological and electrophysiological assays showed that the EFS-induced relaxation that mainly caused by NO was significantly reduced, while the contraction was enhanced in the diabetic GF. EFS-induced IJP showed that L-NAME sensitive IJP in the diabetic GF was significantly reduced compared with control mice. However, both NPR-A and NPR-B were detected on enteric neurons, and their expression levels were upregulated in the diabetic GF. The nNOS expression level was downregulated dose-dependently in GF smooth muscle tissues exposed to CNP.

CONCLUSION: These findings suggested that upregulation of the NPs signaling pathway may be involved in GF neuropathy caused by diabetes by decreasing nNOS expression.

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Key words: Diabetic gastroparesis; Natriuretic peptides; Nitric oxide synthase; Enteric neuron

Core tip: The results demonstrated that the expressions of neuronal nitric oxide synthase (nNOS) and numbers NOS neurons were significantly downregulated while natriuretic peptides (NPs) and the natriuretic peptide receptor-A, B, C (NPRs) signaling pathway were upregulated. C-type natriuretic peptide, an NPRs agonist, inhibited

ited nNOS expression in cultured gastric fundus tissue. These findings suggested that upregulation of the NPs signaling pathway may be involved in gastric fundus neuropathy caused by diabetes, by decreasing nNOS expression. The results are interesting and may represent a molecular mechanism of diabetic gastroparesis.

Lu HL, Huang X, Wu YS, Zhang CM, Meng XM, Liu DH, Kim Y, Xu WX. Gastric nNOS reduction accompanied by natriuretic peptides signaling pathway upregulation in diabetic mice. *World J Gastroenterol* 2014; 20(16): 4626-4635 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4626.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4626>

INTRODUCTION

Diabetic gastroparesis is a representative diabetic dysmotility, and is associated with dysphagia, heartburn, nausea, vomiting and delayed gastric emptying^[1,2]. It occurs in up to 50% of patients with type 1 diabetes and in 30% of patients with type 2 diabetes^[3,4]. Gastroparesis seriously affects patients' quality of life and makes the control of blood glucose more difficult. Although diabetic gastroparesis is a significant health problem, the pathogenesis of this gastric dysfunction and its mechanisms are still not well understood. The gastric motility dysfunction may be caused by several factors, such as hyperglycemia, neuropathy, myopathy and depletion of interstitial cells of Cajal (ICC)^[5,6]. The enteric nervous system (ENS), composed of excitatory and inhibitory neurons, exists throughout the entire gastrointestinal (GI) tract, which plays an important role in controlling and coordinating the GI tract motility. The ENS has a vital regulatory role in gastrointestinal motility; therefore, it has attracted more attention in recent years. It is generally considered that enteric neuropathy is one of the causative factors of diabetic gastroparesis. Numerous studies have shown that neurons expressing NOS in myenteric plexus are damaged and the number of nNOS immunoreactive positive neurons are significantly reduced^[7]; both mRNA and protein expression levels of nNOS are downregulated and accompanied by attenuation of NO-induced relaxation in diabetic gastroparesis mice^[8,9]. To date, the mechanisms of diabetes-induced enteric neuropathy remain unclear, and many investigators have reported that neuronal apoptosis, oxidative stress, advanced glycation end product (AGEs), changes of nerve growth factors and impaired brain-gut interactions may be involved^[9]. However, whether the effects of important intracellular signaling pathways participate in diabetes-induced enteric neuropathy has not been widely examined.

Atrial natriuretic peptide was isolated from the atrium by de Bold *et al.*^[10] in 1981. From then on, brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis natriuretic peptide, micrurus natriuretic

peptide (MNP), and ventricular natriuretic peptide have been discovered. These natriuretic peptides are distributed all over the body and exert a variety of biological effects, such as natriuretic-diuretic, vasorelaxation, and other functions designed to decrease blood pressure and to control electrolyte homeostasis. Three types of single-transmembrane natriuretic peptide receptors (NPRs) for natriuretic peptides (NPs) have been identified^[11-13]: natriuretic peptide receptor A (NPR-A), NPR-B and NPR-C. They are divided into two major categories. NPR-A and NPR-B are membrane-bound guanylyl cyclase receptors that activate guanylyl cyclase, which catalyzes the formation of cGMP from GTP^[14-16]. NPR-C, primarily controls NPs concentrations *via* receptor-mediated internalization and degradation, and has been reported in many signaling pathways in the GI tract^[17].

Nitric oxide (NO), identified as a biological signaling molecule in the 1980s, is a major nor-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter, which mediates smooth muscle relaxation. It is synthesized by NO synthase (NOS) and its three isoforms (eNOS, nNOS and iNOS) are expressed in many tissues, including endothelium, vascular smooth muscle, specific segments of the nephron and the heart^[18,19]. It has been reported that nNOS is expressed on inhibitory neurons and plays an important role in regulation of NO production in the GI tract. NO binds to soluble GC and increases cGMP levels. Many studies have reported the relationships between NPs and NOS. CNP interacts with the NPR-C receptor coupled *via* G proteins leading to the activation Ca²⁺-calmodulin dependent endothelial NOS (eNOS), and subsequent increasing in NO production would induce the reduction in cardiac myocyte contractility^[20]. By contrast, it has been demonstrated that BNP can increase iNOS and eNOS expression in the rat myocardium and cultures of cardiomyocytes, respectively^[21-22].

Our previous studies demonstrated that both NPR-A and NPR-B were distributed in the rat gastric smooth muscle layers and that CNP caused relaxation of the gastric circular and longitudinal smooth muscle tissues in stomachs of humans, rats and guinea pigs^[23-25]. Recently, we also reported that the NPs/NPRs signaling pathway is upregulated in the gastric antrum and corpus smooth muscle layers, which may be involved in diabetes-induced loss of gastric ICC *via* decreasing the production of mSCF indirectly^[26]. However, it is not clear whether natriuretic peptides play a role in diabetes-induced neuropathy. In this study, we investigated whether the NOS neurons are damaged and the relationships between CNP/NPRs signaling pathways and nNOS expression in the GF of STZ-induced diabetic mice.

MATERIALS AND METHODS

STZ-induced diabetic mouse model

Male imprinting control region (ICR) mice (5-wk-old) used for this study were purchased from the Experimen-

tal Animal Center of Shanghai Jiaotong University School of Medicine. One hundred mice were randomly divided into two groups: the control group and the diabetic model group. Mice in the diabetic model group were fasted overnight and intraperitoneally injected with STZ (Sigma-Aldrich, Saint Louis, MO, United States) solution. STZ was prepared freshly in 0.1 mol/L ice-cold citrate buffer (pH = 4.0) and used at a dose of 200 mg/kg body weight. Mice in the control group were intraperitoneally injected with the same volume of 0.1 mol/L citrate buffer. The animals had free access to food and water, and were maintained under standard housing conditions (room temperature 24–27 °C; humidity 60%–65%) with a 12 h light and dark cycle. After two months, blood glucose and body weight of each mouse were measured. Blood withdrawn from mouse tail vein after fasting for 8 h and the blood glucose concentration was measured with One-touch blood glucose monitoring system (Johnson and Johnson Medical Company, New Brunswick, NJ, United States). A mouse was declared diabetic when its blood glucose concentration was above 16 mmol/L.

Tissue preparation

Whole stomachs were quickly excised from the mice and placed in a Sylgard base dish with pre-oxygenated Krebs solution (containing in 118.1 mmol/L NaCl, 4.7 mmol/L KCl, 1.0 mmol/L KH₂PO₄, 1.0 mmol/L MgSO₄, 25.0 mmol/L NaHCO₃, 2.5 mmol/L CaCl₂, and 11.1 mmol/L glucose), which was equilibrated with 95% oxygen and 5% CO₂. The mesenteric fat was removed, and the stomach was cut along the greater curvature and pinned to the Sylgard base with the mucosa facing upward. Mucosal and submucosal layers were carefully removed under a dissecting microscope and the smooth muscle layers in the GF were used for immunohistochemistry and other experiments.

Immunohistochemistry

Smooth muscle tissues (10 mm × 10 mm) from the GF were fixed with ice-cold paraformaldehyde (4% w/v) for 25 min. These tissues were then washed in 0.1 mol/L phosphate buffered saline (PBS) overnight at 4 °C. To reduce non-specific antibody binding, they were preincubated in 5% bovine serum albumin (Sigma) for 1 h at room temperature before incubation with the rabbit anti-nNOS antibody (1:1000; Cell Signaling Technology, Danvers, MA, United States) and mouse anti-HuC/HuD antibody (A-21271, Abcam, Burlingame, CA, United States). To achieve greater penetration during labeling, incubation solutions with the primary antibody were mixed with Triton-X 100 (0.5%; Sigma). Tissues were incubated in the primary antibodies for 48 h at 4 °C. Following washing in 0.1 mol/L PBS overnight at 4 °C, tissues were incubated with the corresponding secondary antibody (DyLight 488 conjugated anti-rabbit IgG and DyLight 549 conjugated anti-mouse IgG, 1:400, CoWin Biotech, China) for 1 h at 25 °C. Tissues were washed in 0.1M PBS for 4 h before being mounted on a slide glass with an anti-fading agent

(Molecular Probes, Mississauga, Ontario, Canada) and examined using a confocal microscope (TCS-SP2; Leica Microsystems, Wetzlar, Germany).

We also used frozen tissue sections to verify whether NPRs were expressed on the myenteric plexus. Small GF tissues were collected after the mice were killed by cervical dislocation. The samples were fixed with 4% paraformaldehyde overnight at 4 °C and sectioned at 5 µm thickness. The sections were then blocked with 10% goat serum in PBS for 1 h at room temperature. The blocking solution was removed and primary antibody solution added (NPR-A, ab70848, 1:100; NPR-B, ab14357, 1:200; HuC/D, A-21271, Abcam), before being incubated overnight at 4 °C. The sections were washed three times in PBS (10 min per time), followed by 1 h incubation in secondary antibody solution (DyLight 488 conjugated anti-mouse IgG and DyLight 549 conjugated anti-rabbit IgG, 1:400, CoWin Biotech, China). The negative control group was created by incubating sections without primary antibodies. Sections were then washed in 0.1 mol/L PBS for 15 min three times before being mounted on a slide glass. These slices were visualized and photographed under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

Isometric tension measurement

Fresh GF smooth muscle strips (approximately 2 mm × 8 mm) were acquired by cutting along the circular axis from the gastric smooth muscle tissue. After a silk thread (USP 5/0) was attached to both ends of the muscle strips, the strips were mounted along the circular axis in 10 mL organ baths containing warmed (37 °C) and oxygenated (95% O₂: 5% CO₂) Krebs solution. An isometric force transducer (RM6240C, Chengdu Instrument Factory, China) that was connected to an amplifier recorded the isometric contraction measurements. The muscle strips were incubated at the appropriate tension for 40 min before the experiment. To observe the excitatory and inhibitory signals in the GF, electric field stimulation (EFS) was applied. Muscle strips were subjected to EFS at 1, 5, 10, 15, 20 and 25 Hz under a constant voltage was 50 V. The pulse width was 0.5 ms, and the duration of stimulation was 10 s. At every interval, 4 min were allowed for recovery of spontaneous activity. After each series of stimulations, the bath solution was exchanged. At the conclusion of each experiment, 50 mmol/L KCl was used to normalize the differences among the readings for each sample.

Intracellular microelectrode recording

Muscle strips (approximately 5 mm × 10 mm) dissected from the GF were isolated and pinned onto the base of a Sylgard-coated chamber, circular muscle side up, and continuously perfused with warmed (37 °C) and oxygenated Krebs solution. Strips were allowed to equilibrate for approximately 2 h before the recording commenced. Experiments were carried out in the presence of nifedipine (1 µmol/L) to minimize the movement of

muscles. Glass microelectrodes filled with 3 mol/L KCl (30–60 M Ω of resistance) were inserted into the cells. Membrane potentials were recorded using a standard electrometer (Duo 773, WPI Inc., Sarasota, FL, United States). EFS was applied in this experiment under a constant voltage of 50 V. The pulse width was 0.5 ms, the duration of stimulation was 20 ms and the slow inhibitory junction potentials (IJP) of circular smooth muscle in normal and diabetic GF were recorded.

Tissue incubation

Smooth muscle tissues in the GF were obtained from normal mice as mentioned above. They were washed using sterile PBS three times for five minutes each. To study the relationship between natriuretic peptides (NPs) and nNOS expression in the GF, these tissues were exposed to DMEM containing 0.5% FCS and different concentrations of CNP (10^{-8} , 10^{-7} , 10^{-6} mol/L) for 24 h. These tissues were then cleaved into protein sample solutions and detected by western blotting.

Western blotting analysis

Protein samples were extracted from the smooth muscle tissues in the GF as recommended by the manufacturer of RIPA buffer (Beyotime chemical Co., Jiangsu, China). They were mixed with $2 \times$ loading buffer and in a 100 °C water bath for 10 min before a protein assay (Bio-Rad, Hercules, California, United States) was used to determine the protein content. Equivalent amounts of protein (normally 40 μ g per lane) and pre-stained markers were separated by 10% SDS-PAGE and electro transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, United States). Membranes were then blocked in Tris buffered saline-Tween 20 (TBS-T) with 5% (w/v) non-fat dry milk for 2 h at room temperature. The membranes were incubated overnight at 4 °C with rabbit anti-nNOS polyclonal antibody (1:1000; Cell Signaling Technology, Boston, MA, United States), rabbit anti-NPR-A antibody (1:400; sc-25485, Santa Cruz Biotechnology, Dallas, Texas, United States), rabbit anti-NPR-B antibody (1:300; sc-25486, Santa Cruz Biotechnology, United States), rabbit anti-NPR-C antibody (1:400; sc-25487, Santa Cruz Biotechnology) or rabbit anti-GAPDH monoclonal antibody (1:1000; Cell Signaling Technology). After washing three times (five minutes each) with TBS-T, the membranes were incubated with the alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (1:1000; CoWin Biotech, Beijing, China) for 1 h at room temperature. Following removal of the secondary antibody, membranes were washed three times and BCIP/NBT Phosphatase Substrate System (KPL Inc., Gaithersburg, MD, United States) was used to detect the signals on the blots. The image from each western blotting was quantitatively analyzed by using Quantity One software (Bio-Rad) and normalized by the GAPDH signal.

Ethics

This study was carried out in strict accordance with the

recommendations in the Guide for the Care and Use of Laboratory Animals of the Science and Technology Commission of PRC (STCC Publication No. 2, revised 1988). The Committee on the Ethics of Animal Experiments of Shanghai Jiaotong University School of Medicine approved the protocol (permit number: Hu 686-2009).

Statistical analysis

The data were expressed as the mean \pm SE. Analysis of differences between multiple groups of data was performed with one-way ANOVA, followed by a post hoc Bonferroni test. For comparison between two data sets, a paired or unpaired Student's *t*-test was used. Differences were considered to be significant at a *P* value less than 0.05.

RESULTS

Changes in blood glucose concentration and body weight

Two months after injection of STZ, the majority mice exhibited hyperglycemia. Plasma glucose concentrations of 43 of the STZ-treated mice were above 16 mmol/L and were thus defined as diabetic. Their mean blood glucose concentration was 23.6 ± 1.9 mmol/L ($n = 43$), which was significantly higher than the control group (5.9 ± 0.6 mmol/L, $n = 43$, $P < 0.01$). Their average body weight was 20.5 ± 0.6 mg, which was significantly lower than the control group (31.7 ± 0.6 mg, $n = 43$, $P < 0.01$).

Distribution of NOS neurons and nNOS expression in gastric fundus smooth muscle tissues

To determine whether diabetes-induced neuropathy had occurred, myenteric neurons were observed in the control and STZ-induced diabetic mice. According to other reports, specific anti-nNOS antibodies and anti-HuC/D antibodies were used to detect NOS neurons and all myenteric neurons in gastric fundus smooth muscle, respectively^[27–29]. Green fluorescence showed nNOS and red fluorescence showed HuC/D. Fewer nNOS immunopositive cells were detected in the diabetic mice (Figure 1B) compared with the control mice (Figure 1A) and the NOS neurons were significantly damaged in the STZ-induced diabetic mice (Figure 1B), as observed from the typical merged images (Figure 1).

The protein expression level of nNOS in gastric fundus smooth muscle tissue was further analyzed by western blotting. The results showed that the nNOS expression level in STZ-induced diabetes was 0.17 ± 0.03 , which was significantly lower than that of control mice (0.33 ± 0.02 , Figure 2, $n = 7$, $P < 0.05$).

Change of NOS neuron function in diabetic GF

To further confirm whether NOS neurons were damaged and to observe the functional changes in the GF caused by diabetes, physiological and electrophysiological methods were applied. EFS (50 V, 0.5 ms pulse width,

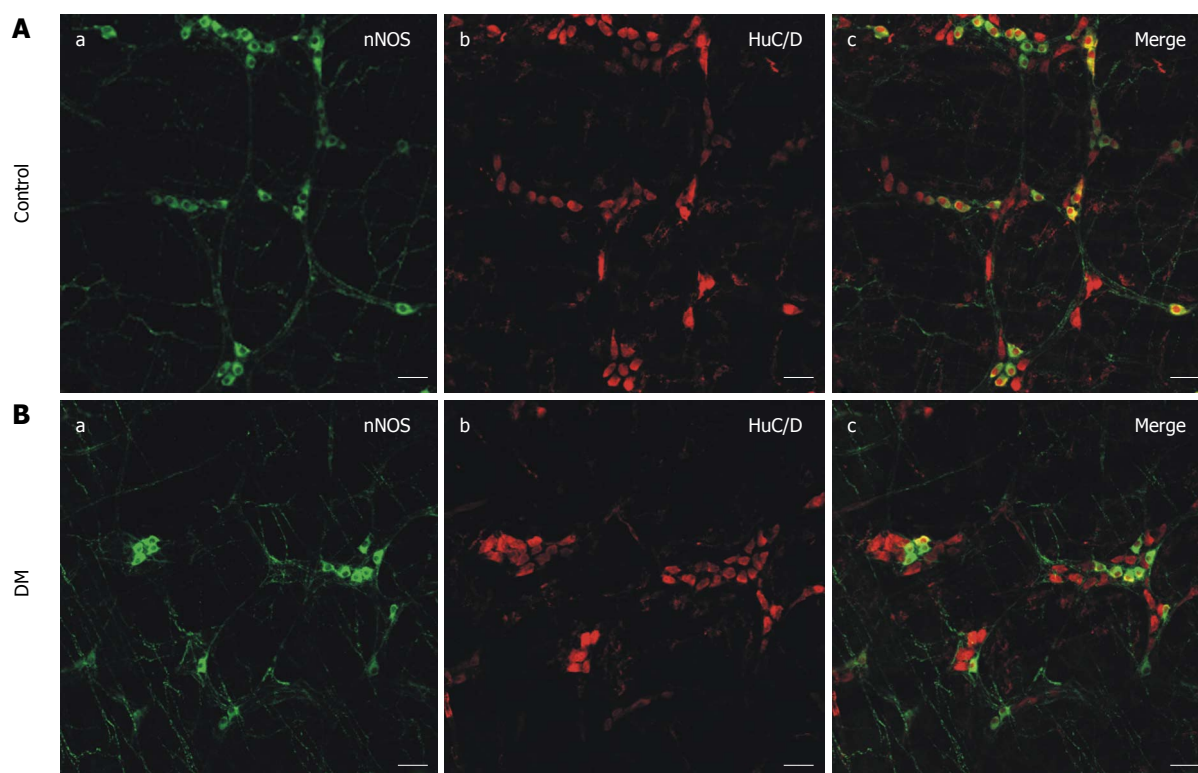


Figure 1 Whole mount immunostaining of nitric oxide synthase and HuC/D in gastric fundus smooth muscle tissue. Neuronal nitric oxide synthase (nNOS) staining is used to show NOS neurons, while HuC/D staining is used to label all enteric neurons in the control and diabetic mice (DM) groups. Bar = 50 μ m.

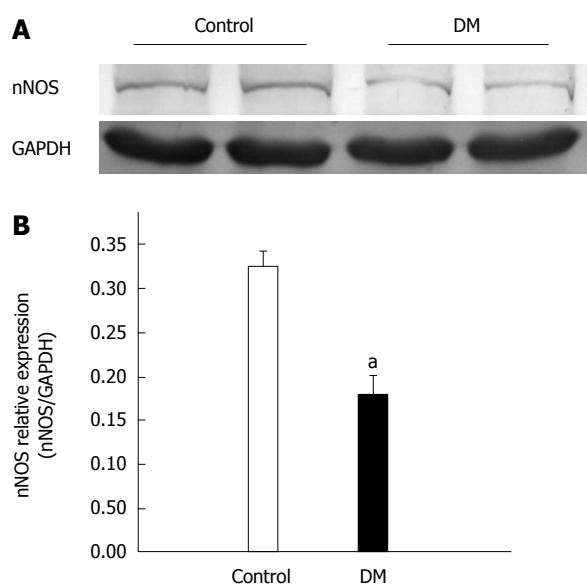


Figure 2 Expression of nitric oxide synthase in gastric fundus smooth muscle tissues. A: Representative bands of nitric oxide synthase (nNOS) protein expression in control and diabetic mice (DM) groups; B: The nNOS expression level in gastric fundus smooth muscle tissue was significantly decreased in the DM group ($n = 7$, $^aP < 0.05$ vs control).

10 s duration, 15 Hz) induced a relaxation and following contraction of gastric fundus smooth muscle strips in normal mice. However, the EFS-induced relaxation disappeared in the presence of 200 μ mol/L L-NAME, an NOS inhibitor (Figure 3, $n = 5$). The results showed that EFS-induced relaxation in the GF was caused by NO

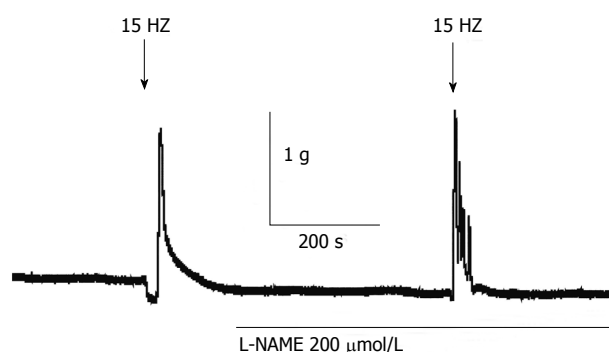


Figure 3 Electric field stimulation-induced relaxation and contraction in gastric fundus smooth muscle of normal mice. A typical raw trace showing that electric field stimulation EFS (15 Hz)-induced relaxation was completely blocked by L-NAME ($n = 5$).

synthesized by NOS neurons.

Different frequencies of EFS (1, 5, 10, 15, 20, 25 Hz) were applied in the GF, respectively (Figure 4A). In the diabetic mice, the EFS-induced relaxation was almost completely inhibited while the excitability contraction amplitude was significantly enhanced compared with control mice (0.18 ± 0.02 , 0.28 ± 0.04 , 0.55 ± 0.03 , 0.73 ± 0.05 , 0.86 ± 0.05 and 1.1 ± 0.07 g in diabetic mice, and 0 , 0.18 ± 0.04 , 0.45 ± 0.03 , 0.63 ± 0.03 , 0.77 ± 0.03 and 0.83 ± 0.04 g in controls, respectively, Figure 4B, $n = 8$, $P < 0.05$).

An intracellular recording technique was used to determine the EFS-induced IJP on gastric fundus smooth muscle tissues (Figure 5A and B). The duration of the

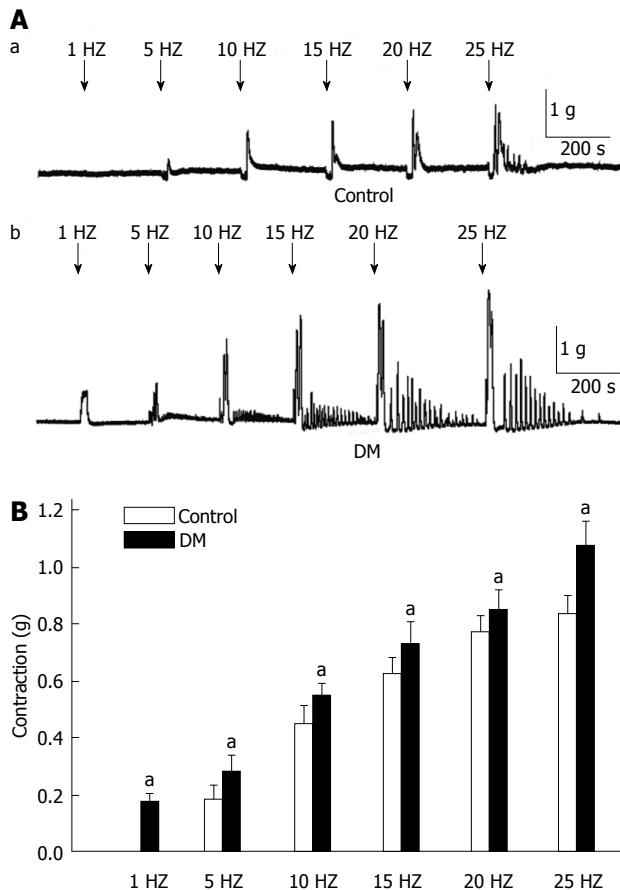


Figure 4 Electric field stimulation-induced contraction of gastric fundus smooth muscle was recorded in control and diabetic mice. A: Typical raw traces of fundus smooth contractions induced by electric field stimulation (EFS) at different frequencies in control and diabetic groups; B: The amplitude of contraction from two groups and the smooth muscle contraction was more sensitive to EFS in the diabetic mice (DM) group ($n = 8$, $^aP < 0.05$ vs control).

L-NAME sensitive, NO-mediated IJP in diabetes was 2.3 ± 0.07 s, which was significantly reduced compared with the control (3.4 ± 0.08 s, Figure 5C, $n = 9$, $P < 0.05$).

Expressions of NPRs in gastric fundus smooth muscle tissues

To examine whether there were significant changes in NPRs expression in the diabetic GF, total homogenate of GF tissues was used and analyzed by western blotting. Figure 6A shows that NPRs were detected in the GF. The expression levels of NPR-A, NPR-B, NPR-C in diabetic and control mice were 0.68 ± 0.03 , 0.94 ± 0.03 and 0.43 ± 0.03 , and 0.54 ± 0.03 , 0.7 ± 0.02 and 0.20 ± 0.02 , respectively. The expression levels of NPRs were all upregulated in STZ-induced diabetic mice (Figure 6B, $n = 8$, $P < 0.05$).

Effect of NPRs on nNOS expression in cultured tissue

NPRs were overexpressed in diabetic GF smooth muscle; therefore, the role of NPRs in diabetes-induced neuropathy should be investigated. Firstly, we tried to evaluate whether NPRs were expressed on myenteric neurons. Cryosection staining results revealed that both NPR-A

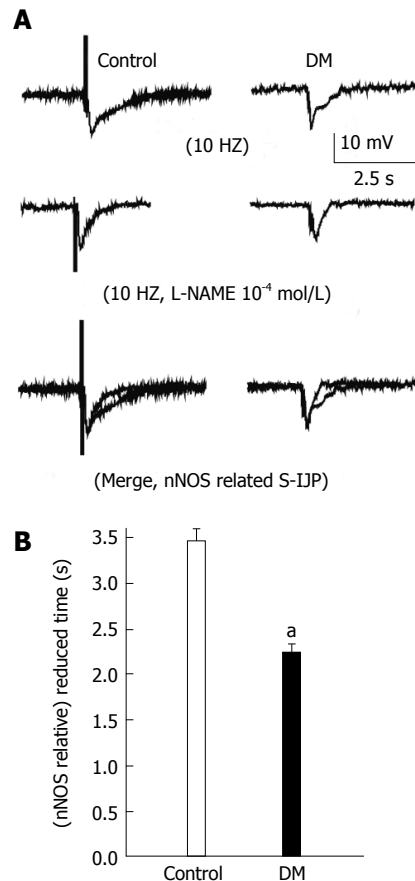


Figure 5 Slow inhibitory junction potentials of gastric fundus smooth muscle were recorded in normal and diabetic mice. A: Representative raw traces of slow inhibitory junction potentials (slJP) elicited by electric field stimulation in control and diabetic mice (DM) groups; B: Summarized data showing that the NO-mediated duration of slJP was significantly decreased in the DM group ($n = 9$, $^aP < 0.05$ vs control).

and NPR-B were detected on myenteric neurons (red fluorescence, Figure 7). To further investigate the relationship between upregulation of NPRs and nNOS expression, GF smooth muscles were exposed to different concentrations of CNP, a NPRs agonist and the nNOS expression levels were detected. The nNOS expression levels were 0.49 ± 0.02 in the control and 0.47 ± 0.02 , 0.35 ± 0.04 , 0.28 ± 0.03 in the presence of 10^{-8} , 10^{-7} , 10^{-6} mol/L CNP, respectively. CNP significantly reduced nNOS expression in cultured GF tissues in a concentration-dependent manner (Figure 8B, $n = 7$, $P < 0.05$).

DISCUSSION

Gastroparesis is a syndrome characterized by delayed gastric emptying in the absence of mechanical obstruction of stomach. It is a well-recognized chronic complication of long-standing diabetes and affects patients' digestion and absorption functions seriously. Although diabetic gastroparesis (DGP) is a significant health problem, the pathogenesis of gastric dysfunction is still not well understood. The mutual cooperation and coordination between ENS, ICC, and smooth muscle play an

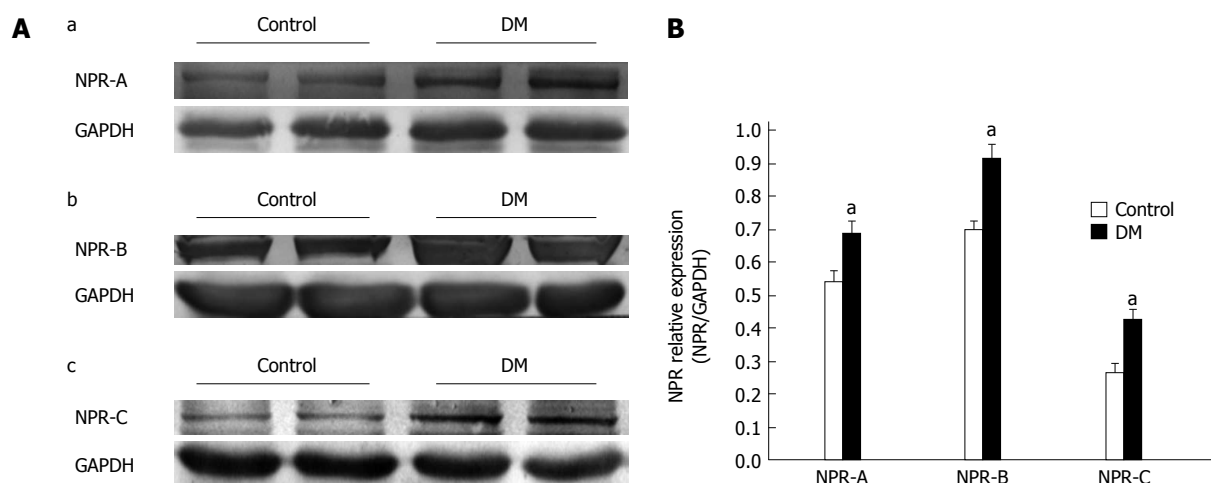


Figure 6 Natriuretic peptide receptors expression in gastric fundus smooth muscle tissues in control and diabetic mice. A: Representative bands of natriuretic peptide receptors (NPRs) protein expression in control and diabetic mice (DM) groups; B: Summarized data showing that the levels of NPRs expression in the DM group were significantly decreased ($n = 8$, $^aP < 0.05$ vs control).

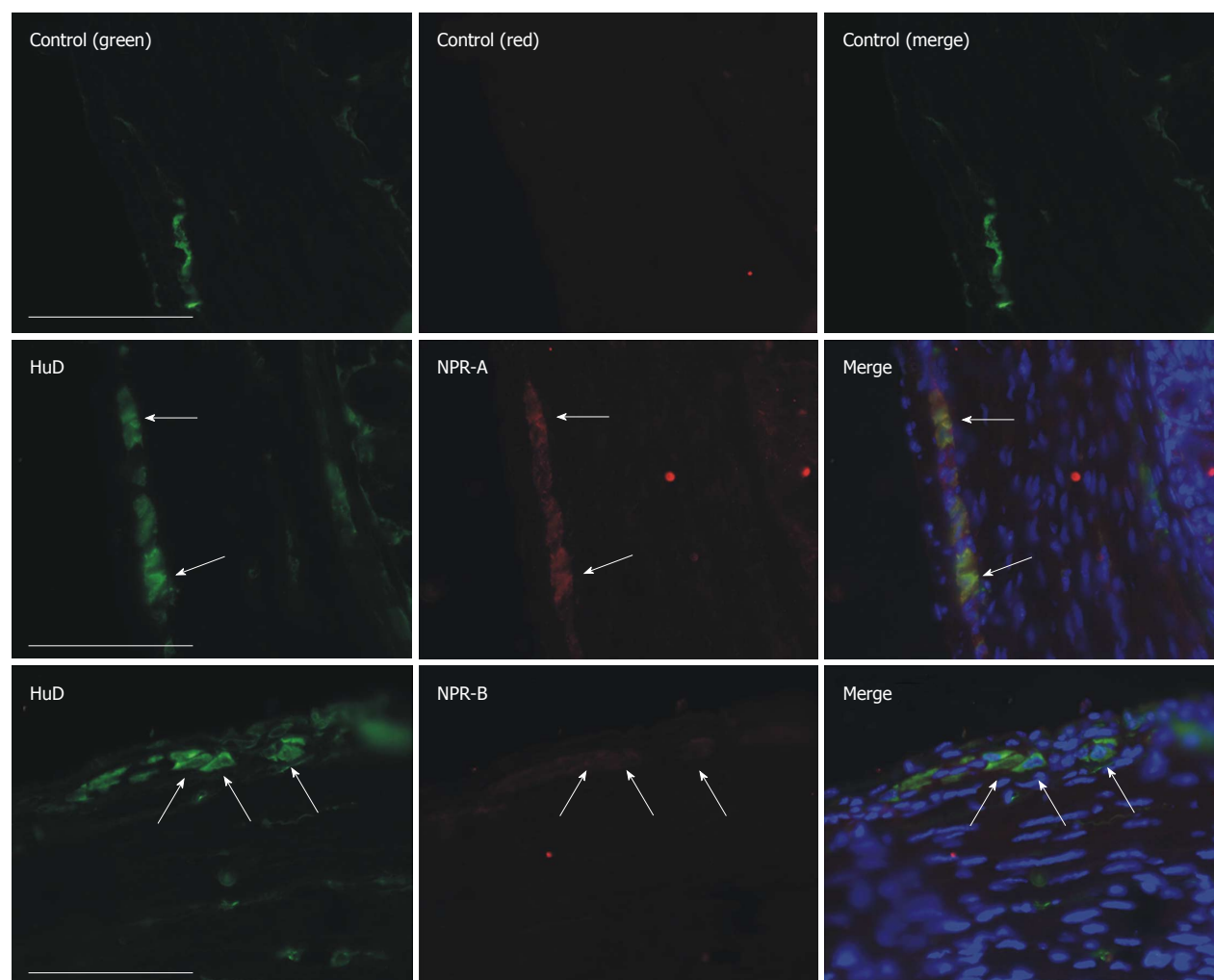


Figure 7 Natriuretic peptide receptors-A and B immunofluorescence staining in gastric fundus smooth muscle tissue of normal mice. Myenteric neurons were labeled by an anti-HuD antibody and natriuretic peptide receptors (NPRs) were labeled by an anti-NPR-A, B antibody. Bar = 100 μ m.

important role in maintaining normal gastrointestinal motility. Several studies have reported that DGP may be caused by many factors, such as the depletion of ICC,

diabetes-induced neuropathy and damage to NOS neurons^[30-33]. High-density NOS neurons are primarily involved in gastric receptive relaxation and pyloric sphinc-

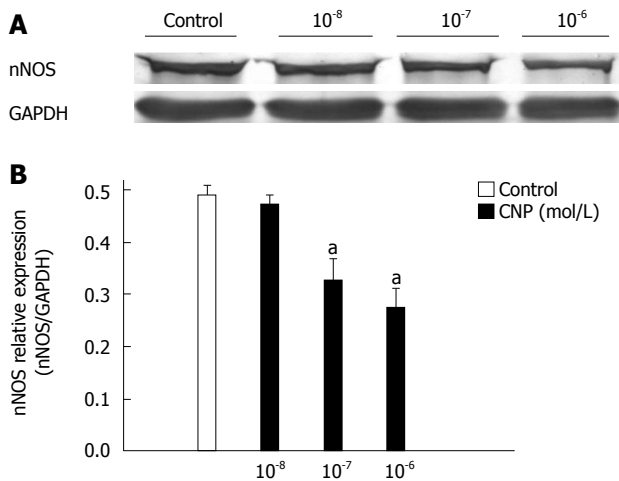


Figure 8 Effects of C-type natriuretic peptide on the nitric oxide synthase expression in gastric fundus smooth muscle tissues of normal mice. A: Representative bands of nitric oxide synthase (nNOS) protein expression in control and C-type natriuretic peptide (CNP) groups; B: Summarized data shows that CNP significantly inhibited nNOS expression in a dose-dependent manner in cultured gastric fundus smooth muscle tissue ($n = 7$, $^aP < 0.05$ vs control).

ter relaxation, which is extremely important for normal gastric emptying^[34-37]. The first part of this study was focused on whether the NOS neurons were destroyed in STZ-induced diabetic mice. Our results indicated that the relative fluorescence intensity of nNOS was much weaker in diabetic GF smooth muscle compared with that of the control (Figure 1). The nNOS expression detected by western blotting revealed a consistent decrease in the protein level (Figure 2). The results suggested that the number of NOS neurons was significantly decreased and further detection showed nNOS expression levels to be significantly downregulated in diabetic mice compared with the control.

There is no spontaneous rhythmic contraction in the GF, therefore, electric field stimulation (EFS) was used to induce relaxation and contraction. Firstly, we tried to study whether NO is involved in EFS-induced response in GF smooth muscle tissues (Figure 3). Different frequencies of EFS were applied on the GF of diabetic and control mice. The results showed that the EFS-induced relaxation was significantly reduced while contraction was enhanced in the diabetic GF (Figure 4). Junction potentials (JPs) occur spontaneously and can be evoked by EFS. Output from the enteric nervous system to the gastric smooth muscle can be detected as neuromuscular excitatory and inhibitory junction potentials (EJPs and IJPs)^[38]. The IJP has both rapid and slow components. The rapid component of the IJP is mediated by P2Y₁ receptors and is widely considered to be transmitted by ATP^[39,40]. The slow component of the IJP is nitrogen, and can be blocked by NOS inhibitors^[41]. In this experiment, IJPs were evoked by EFS on GF smooth muscles and the slow component (NO component) duration was significantly reduced in diabetic mice compared with the control (Figure 5). This result might imply that diabetes

causes serious neuropathy, especially NOS neurons damage, resulting in reduced NO production, further inducing abnormal excitability contraction and reduced sIJP duration time.

The NPs system is a local endocrine system in the gastrointestinal tract. It plays an important role in regulation of motility, secretion and absorption. Our previous studies showed that NPs can induce smooth muscle relaxation and the NPs signaling pathway participates in diabetes-induced ICC damage^[23-26]. Many studies have reported the relationships between NPs and NOS^[20-22]. In this study, we tried to evaluate whether NPs are involved in NOS neuron damage. Firstly, the protein expression levels of NPRs in diabetic GF smooth muscle were detected by western blotting. The results showed that the expression levels of three types of natriuretic peptide receptor (NPR-A, B, C) in diabetes were much higher than in control mice (Figure 6). Secondly, we observed the distribution of NPRs on GF enteric nerve system in frozen sections by immunohistochemistry. The results showed that there were many NPR-A, B proteins expressed on myenteric neurons (Figure 7). To investigate the relationship between upregulated NPRs and nNOS expression, GF smooth muscles were incubated with different concentrations of CNP and then the nNOS expression level was detected. The results showed that CNP decreased nNOS expression in a concentration-dependent manner (Figure 8). We can conclude that NPs may be involved in diabetes-induced neuropathy *via* decreasing nNOS expression.

In summary, we found that the number of NOS neurons was reduced and nNOS expression was down-regulated, while the NPRs expressions were upregulated in GF smooth muscle of STZ-induced diabetic mice. Diabetes-induced NOS neuron damage resulted in poor production of NO, which eventually caused abnormal excitability contraction and damaged relaxation in diabetic GF. Diabetes-induced upregulation of the NPs signaling pathway may be involved in NOS neurons injury.

COMMENTS

Background

Diabetic gastroparesis is a common complication of diabetic dysmotility. It is generally considered that enteric neuropathy is one of the causes of diabetic gastroparesis. Numerous studies have shown that neurons that synthesize the nitric oxide synthase (NOS) in the myenteric plexus were damaged and the number of nNOS immunoreactive neurons were significantly reduced; however, the mechanism of diabetes-induced enteric neuropathy remain unclear.

Research frontiers

The natriuretic peptides (NPs) system is a local endocrine system in the gastrointestinal tract. It plays an important role in regulation of motility, secretion and absorption. Previous studies have reported that NPs can induce smooth muscle relaxation and the NPs signaling pathway participates in diabetes-induced interstitial cells of Cajal damage. In this study, the authors demonstrated that upregulation of the NPs signaling pathway might be involved in gastric fundus neuropathy caused by diabetes *via* decreasing nNOS expression.

Innovations and breakthroughs

Recent reports have highlighted the importance of damage to NOS neurons accompanied by upregulation of NPs/NPRs/cGMP signaling pathway in the diabetic gastric fundus. C-type natriuretic peptide (CNP), a NPRs agonist, inhib-

ited nNOS expression in cultured gastric fundus tissue. This is the first study to report the relationship between NPs/NPRs signaling pathway and NOS neuron damage in STZ-induced diabetic gastric fundus tissues.

Applications

By understanding the mechanism by which NOS neurons are damaged in diabetic gastroparesis, this study may represent a future strategy for therapeutic intervention in the treatment of patients with diabetic gastroparesis.

Terminology

The NPs are a family of three polypeptide hormones termed atrial natriuretic peptide, brain natriuretic peptide, and CNP. In gastrointestinal tract NPs are involved in gastrointestinal motility, absorption and secretion.

Peer review

The authors examined numbers of NOS neurons, and the expressions of nNOS and natriuretic peptide receptor-A, B, C (NPRs) in diabetic gastric fundus. The results demonstrated that the numbers of NOS neurons and the expression of nNOS were significantly downregulated while the NPs/NPRs signaling pathway was upregulated. CNP, a NPRs agonist, inhibited nNOS expression in cultured gastric fundus tissue. The results are interesting and may represent a molecular mechanism of diabetic gastroparesis.

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Combined probiotic bacteria promotes intestinal epithelial barrier function in interleukin-10-gene-deficient mice

Chen-Zhang Shi, Hong-Qi Chen, Yong Liang, Yang Xia, Yong-Zhi Yang, Jun Yang, Jun-Dong Zhang, Shu-Hai Wang, Jing Liu, Huan-Long Qin

Chen-Zhang Shi, Hong-Qi Chen, Yong Liang, Yang Xia, Yong-Zhi Yang, Jun Yang, Huan-Long Qin, Department of Surgery, Affiliated Sixth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Jiao Tong University, Shanghai 200233, China

Jun-Dong Zhang, Shu-Hai Wang, Jing Liu, Sine institute of Materia Medica, Shanghai Sine Pharmaceutical Co., Ltd, Shanghai 201206, China

Author contributions: Shi CZ, Chen HQ, Qin HL conceived and designed the study; Shi CZ, Chen HQ, Liang Y, Xia Y and Yang YZ performed the experiments; Shi CZ, Chen HQ, Liang Y and Qin HL analyzed the data; Shi CZ, Chen HQ, Liang Y and Qin HL, drafted the article or revised it critically for important intellectual content; Shi CZ and Qin HL wrote the paper; and all authors have read and approved the final version to be published.

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Correspondence to: Huan-Long Qin, MD, Professor, Department of Surgery, Affiliated Sixth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Jiao Tong University, 600 Yishan Road, Shanghai 200233, China. hlqin10@163.com

Telephone: +86-21-64361349 Fax: +86-21-64368920

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colon epithelial cell line, Caco-2, was used to test the benefit of Bifico *in vitro*. Enteroinvasive *Escherichia coli* (EIEC) and the probiotic mixture Bifico, or single probiotic strains, were applied to cultured Caco-2 monolayers. Barrier function was determined by measuring transepithelial electrical resistance and tight junction protein expression.

RESULTS: Treatment of IL-10 KO mice with Bifico partially restored body weight, colon length, and epithelial barrier integrity to wild-type levels. In addition, IL-10 KO mice receiving Bifico treatment had reduced mucosal secretion of tumor necrosis factor- α and interferon- γ , and attenuated colonic disease. Moreover, treatment of Caco-2 monolayers with Bifico or single-strain probiotics *in vitro* inhibited EIEC invasion and reduced the secretion of proinflammatory cytokines.

CONCLUSION: Bifico reduced colon inflammation in IL-10 KO mice, and promoted and improved epithelial-barrier function, enhanced resistance to EIEC invasion, and decreased proinflammatory cytokine secretion.

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Key words: Probiotic bacteria; Intestinal barrier function; Tight junction proteins; Interleukin-10 gene-deficient mice; Caco-2 monolayers

Abstract

AIM: To investigate the protective effects of combinations of probiotic (Bifico) on interleukin (IL)-10-gene-deficient (IL-10 KO) mice and Caco-2 cell monolayers.

METHODS: IL-10 KO mice were used to assess the benefits of Bifico *in vivo*. IL-10 KO and control mice received approximately 1.5×10^8 cfu/d of Bifico for 4 wk. Colons were then removed and analyzed for epithelial barrier function by Ussing Chamber, while an ELISA was used to evaluate proinflammatory cytokines. The

Core tip: We investigated the protective effects of combinations of probiotic bacteria (Bifico) on interleukin (IL)-10 gene-deficient (IL-10 KO) mice and Caco-2 cell monolayers. Treatment of IL-10 KO mice with Bifico partially restored body weight, colon length, and epithelial barrier integrity to wild-type levels. Treatment of Caco-2 monolayers with Bifico or single-strain probiotics inhibited enteroinvasive *Escherichia coli* (EIEC) invasion and reduced secretion of proinflammatory cytokines. Oral administration of Bifico reduced colon inflamma-

tion, and directly promoted epithelial barrier function. In addition, Bifico improved epithelial barrier function, and enhanced resistance to EIEC invasion *in vitro*.

Shi CZ, Chen HQ, Liang Y, Xia Y, Yang YZ, Yang J, Zhang JD, Wang SH, Liu J, Qin HL. Combined probiotic bacteria promotes intestinal epithelial barrier function in interleukin-10-gene-deficient mice. *World J Gastroenterol* 2014; 20(16): 4636-4647 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4636.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4636>

INTRODUCTION

The human gut is colonized with a wide variety of microorganisms, including pathogenic, probiotic and commensal bacteria. *Bifidobacterium*, *Lactobacillus* and *Enterococcus faecalis* are probiotics with beneficial effects on maintenance therapy of human intestinal diseases^[1-3]. For example, oral treatment with specific probiotic bacteria can ameliorate inflammatory bowel disease (IBD). In addition, multiple studies have demonstrated that colonization strategies using defined commensals or exogenous specific probiotic treatment may prevent host intestinal inflammation and ameliorate intestinal epithelial barrier function^[3-9].

The intestinal barrier prevents microbial contamination of interstitial tissues. Tight junctions play an important role in modulating intestinal epithelial paracellular permeability^[10] and promoting defenses against harmful molecules and microorganisms. Tight junctions are common targets of enteric pathogens, and their disruption occurs with IBD and/or diarrhea^[11]. For example, many virulence genes of bacteria encode toxins and other proteins that either directly disassemble tight junction proteins^[12,13] or modulate intracellular pathways that lead to tight junction redistribution^[14,15]. Enteroinvasive *Escherichia coli* (EIEC)^[12,13], *Salmonella typhimurium*^[16], *Shigella flexneri*^[17,18], and *Campylobacter jejuni*^[14,15] all infect host cells by targeting the paracellular pathway.

Past studies have demonstrated that IBD patients have reduced bifidobacteria and lactobacilli in their gut microbiota^[19,20] suggesting these patients may benefit from probiotic treatment. Indeed, recent clinical trials have confirmed the therapeutic effects of probiotics in virus-, bacterium-induced intestinal infections and antibiotic-induced diarrhea^[21-23]. Among the most distinctive benefits of probiotic bacteria are modulation of host defense responses, and protection against infectious diseases^[24,25]. However, the molecular mechanisms underlying these effects have not fully been elucidated.

The probiotic compound, Bifico (Bifico Pharmaceuticals, Sine, Shanghai, China), contains about 1.0×10^9 cfu/g of viable lyophilized bifidobacteria (*Bifidobacterium longum*), 1.0×10^9 cfu/g lactobacilli (*Lactobacillus acidophilus*), and 1.0×10^9 cfu/g *Ent. faecalis*. This probiotic combination has been effective in the maintenance therapy

of diarrhea induced by intestinal flora disturbance or enteritis. However, the use of Bifico as a primary therapy for IBD has not yet been investigated. To address this issue, we treated interleukin (IL)-10-gene-deficient (IL-10 KO) with Bifico and monitored the presence of IBD, which spontaneously develops in these mice. In addition, Caco-2 cells were cultured *in vitro* with EIEC with or without Bifico pretreatment to monitor EIEC invasion. Bifico had a direct effect on epithelial barrier function *in vivo* by reducing mucosal secretion of tumor necrosis factor (TNF)- α and interferon (IFN)- γ , and altered the expression and distribution of tight junction proteins. Bifico exposure *in vitro* reduced bacterial invasion. Moreover, the effects of combined probiotics were more pronounced than single-strain probiotics.

MATERIALS AND METHODS

Animals

Homozygous IL-10 KO mice, generated on a 129 Sv/Ev background, and normal 129 Sv/Ev controls (Jackson Laboratory, Bar Harbor, ME, United States) were housed under specific-pathogen-free conditions in Shanghai Jiao Tong University Medical School. Mice were fed a standard sterile diet and filtered water *ad libitum* under a 12-h light-dark cycle. Animal studies were approved by the Ethical Committee of the Affiliated Sixth People's Hospital of Shanghai Jiao Tong University.

In vivo Bifico therapy

Ten-week-old female IL-10 KO mice ($n = 12$) and wild-type (WT) controls ($n = 12$) were randomized and divided into two groups each. Mice then either received a daily oral gavage of Bifico dissolved in 0.5 mL physiological saline at 3.0×10^8 cfu/mL or saline alone for 4 wk.

Ussing Chamber assay

Mice were sacrificed following Bifico therapy, and a segment of the colon was removed for mucosa isolation from the muscular layer. Mucosal cells were mounted in Lucite Chambers, exposing mucosal and submucosal surfaces to 10 mL oxygenated Krebs Buffer (EasyMount-CSYS-8 Using Chamber Systems; San Diego, CA, United States). The buffers were maintained at 37 °C by a heated water jacket and circulated in CO₂/O₂^[26]. The nonabsorbable tracer molecule inulin-FITC (2000-5000 kDa, Sigma-Aldrich, St Louis, MO, United States) (1.0 mg/mL) was then added to the mucosal side of the Lucite Chambers. At 0, 30, 60, 90 and 120 min following addition of inulin-FITC, 100 μ L buffer from the submucosal side of the Lucite Chambers was collected and analyzed for fluorescence in black-walled 96-well plates (Costar, Corning, NY, United States) using a Spectral Scanning Multimode Reader (Thermo Scientific Varioskan Flash, Vantaa, Finland) at an excitation wavelength of 485 nm and emission at 530 nm^[27,28]. Standard curves were obtained by diluting inulin-FITC in Krebs Buffer. The barrier function of the intestinal epithelium was also determined by measuring

transepithelial electrical resistance (TER) (multi-channel voltage current clamp, VCC MC8, San Diego, CA, United States).

Histological injury grading

Mice were sacrificed after 4 wk Bifico treatment. Colons were harvested and fixed in 10% phosphate-buffered formalin. The samples were paraffin-embedded, sectioned at 5 μ m, and stained with hematoxylin and eosin (HE) for microscopic examination and imaging (Nikon Eclipse 80i, Tokyo, Japan). The slides were reviewed in a blinded fashion by two pathologists and were assigned a histological score for intestinal inflammation based on previously described criteria^[29,30]. No inflammation was scored as 0; modest numbers of infiltrating cells in the lamina propria as 1; infiltration of mononuclear cells leading to separation of crypts and mild mucosal hyperplasia as 2; massive infiltration with inflammatory cells accompanied by disrupted mucosal architecture, loss of goblet cells, and marked mucosal hyperplasia as 3; these issues plus crypt abscesses or ulceration as 4; with a total score from 0 to 15.

Transmission electron microscopy

To observe the ultrastructural changes of tight junctions, colonic segments were prepared for transmission electron microscopy (JEM1230 Electro-microscope; JEOL, Tokyo, Japan) as previously described^[8].

ELISA

The serum levels of TNF- α and IFN- γ were measured using ELISA kits (BD Pharmingen, Oxford, United Kingdom) as previously described^[8]. The levels of the cytokines TNF- α , macrophage inflammatory protein (MIP)-1 α , IL-6 and IL-8 in the supernatant of cultured Caco-2 monolayers were measured using Sandwich ELISA kits (R&D, Minneapolis, MN, United States) according to manufacturer's instructions.

Caco-2 monolayers

Caco-2 cells (Shanghai Institute of Cell Biology, Chinese Academy of Science) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, United States) supplemented with 100 mL/L heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cells were cultured in 25-cm² flat-bottom culture flasks (Corning, Corning, NY, United States) and seeded onto Transwell semipermeable filters (filter grown; 1.12 cm² polyester membranes, 3.0 μ m pore size, 12 well) in Transwell units (Costar; Corning). The barrier function of tight junctions in Caco-2 monolayers was determined by measuring TER.

Bacterial cultivation

B. longum, *L. acidophilus*, and *Ent. faecalis* were obtained from Shanghai Sine Pharmaceutical Co. Ltd. EIEC (O124: NM, ATCC 43893) was obtained from the Shanghai Municipal Center for Disease Control and Prevention.

B. longum was cultured in tryptone polypeptone yeast extract broth agar (Shanghai Sine Pharmaceutical Co. Ltd) at 37 °C. *Ent. faecalis* was cultured in Slanetz and Bartley agar (Shanghai Sine Pharmaceutical Co. Ltd.) at 37 °C. *L. acidophilus* was cultured in MRS agar (Merck, Darmstadt, Germany) at 37 °C. EIEC was cultured in LB agar (Oxoid, Hampshire, United Kingdom) at 37 °C. Bacteria were added to DMEM and then subjected to photoelectric colorimeter to measure consistency.

Infection of Caco-2 monolayers with EIEC

Caco-2 cells were washed three times in Hank's Solution to remove antibiotics. The inoculation ratio of EIEC to Caco-2 cells was approximately 100:1. Prior to EIEC infection, four groups of Caco-2 monolayers were incubated with *B. longum* (B), *Ent. faecalis* (F), *L. acidophilus* (L) or the triple bacteria (BFL) for 30 min. The ratio of probiotics to EIEC was 10:1. EIEC was allowed to infect Caco-2 monolayers for 24 h. TER of Caco-2 monolayers was measured with a voltmeter (Millicell-ERS; Millipore, CA, United States) for 6 h. Untreated Caco-2 monolayers served as a control (C). Caco-2 cells infected with EIEC alone served as the EIEC (E) group. Incubation medium was collected and processed for cytokine analysis using Sandwich ELISA kits (R&D).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), western blotting and immunofluorescence

Tissue total RNA (SLNco, Shanghai, China) was extracted and analyzed by qRT-PCR (Funglynn, Ontario, Canada) as previously described^[8,13]. Rabbit polyclonal antibodies against zonula occludentes 1 (ZO-1, Invitrogen), claudin-1 (Invitrogen) and occludin (Invitrogen) were used in western blotting and immunofluorescence assays according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using the GraphPad Prism 5 software (San Diego, CA, United States) and expressed as mean \pm SEM. Differences in parametric data were evaluated by Student's two-tailed unpaired *t* test. Differences with *P* < 0.05 were considered statistically significant.

RESULTS

Bifico reduced clinical disease and prevented colonic epithelial permeability in IL-10 KO mice

Bifico therapy in IL-10 KO mice was used to assess the effects of probiotics in reducing IBD. As seen in the representative photomicrographs of Figure 1, the morbidity in IL-10 KO mice was greater than in IL-10 KO mice treated with Bifico (Figure 1A). During the 4-wk observation, IL-10 KO mice displayed a significant reduction in body weight compared to WT mice. However, Bifico treatment restored the body weight in IL-10 KO mice (Figure 1B). In addition, the presence of diarrhea was greater in IL-10 KO mice than in mice receiving Bifico

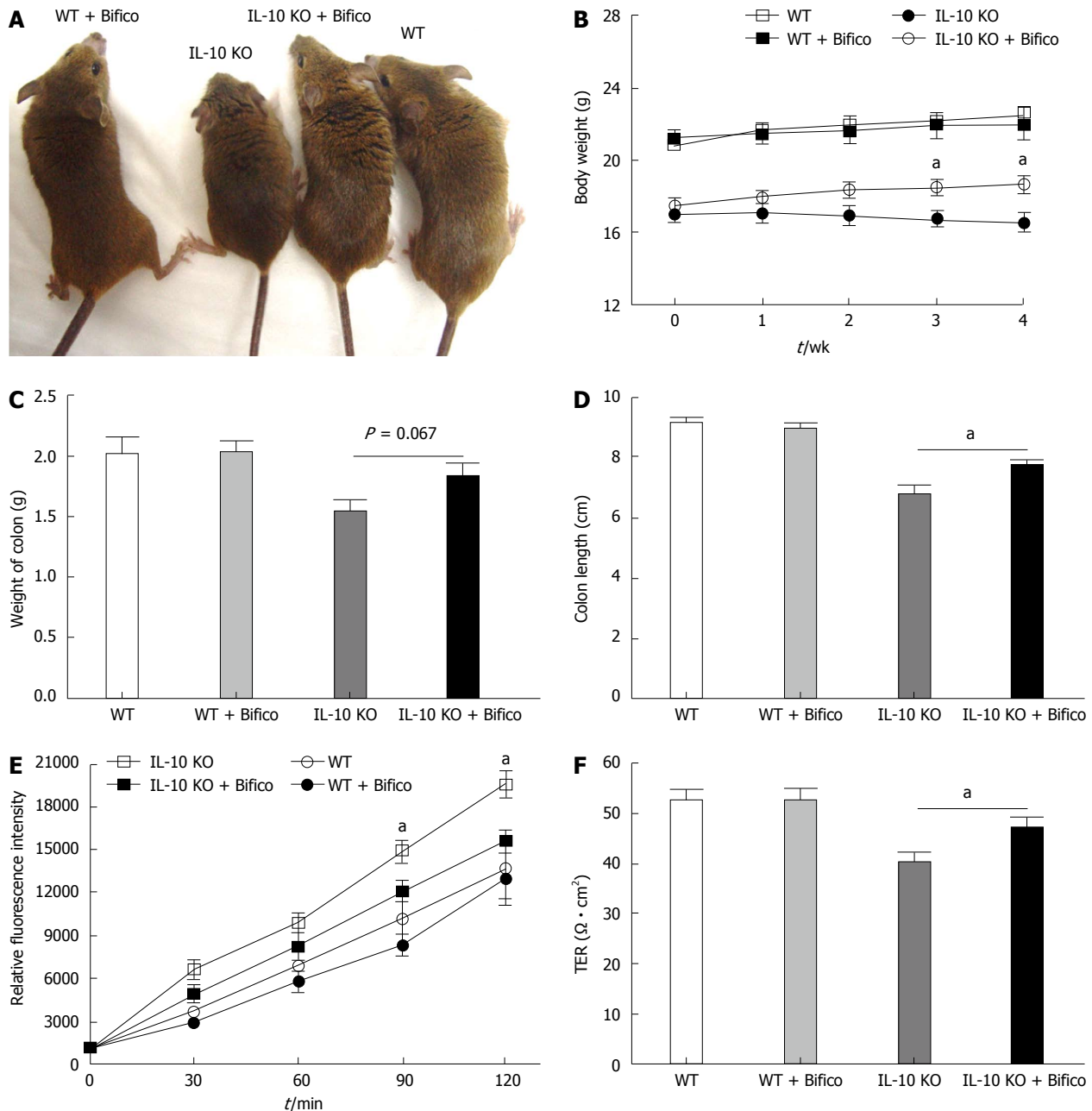


Figure 1 Bifido reduced clinical disease activity and prevented colonic epithelial permeability in interleukin-10 gene-deficient mice. A: Representative photographs from the indicated mice for 4 wk following Bifido treatment; B: Changes in body weight. The IL-10 KO group was significantly lighter than the IL-10 KO + Bifido group (mean \pm SEM, $n = 6$ per time point per group, $^aP < 0.05$, Student's t test); C: Colon weight were measured at 28 d following Bifido treatment; D: Colon length was measured at 28 d following Bifido treatment. IL-10 KO mice had shorter colons than IL-10 KO mice treated with Bifido. No difference in colon weight was observed (mean \pm SEM, $n = 6$ per group, $^aP < 0.05$, Student's t test); E: Colonic paracellular permeability measured by cumulative permeability of nonabsorbable tracer molecule inulin-FITC; F: Colonic paracellular permeability measured by TER. IL-10 KO mice presented higher permeability than IL-10 KO mice treated with Bifido (mean \pm SEM, $n = 5$ per time point per group, $^aP < 0.05$, Student's t test).

treatment. The colonic weight did not differ significantly between Bifido-treated or untreated IL-10 KO mice (Figure 1C); however, the colons of IL-10 KO mice were significantly shorter than those of IL-10 KO receiving Bifido treatment (Figure 1D).

To assess the effects of Bifido on colon function, we measured colonic permeability by Ussing Chamber. IL-10 KO mice exhibited a significant increase in the cumulative permeation of inulin-FITC through the colonic mucosa compared with WT and Bifido-treated WT mice

(Figure 1E). In accordance with increased inulin-FITC permeability, a significant decrease of TER was observed in IL-10 KO mice (Figure 1F). However, Bifido treatment restored colon function in IL-10 KO mice (Figure 1E and F).

Bifido therapy ameliorated inflammation and reduced production of proinflammatory cytokines

To evaluate further the effects of Bifido *in vivo*, mice were sacrificed following Bifido therapy and mucosal cells

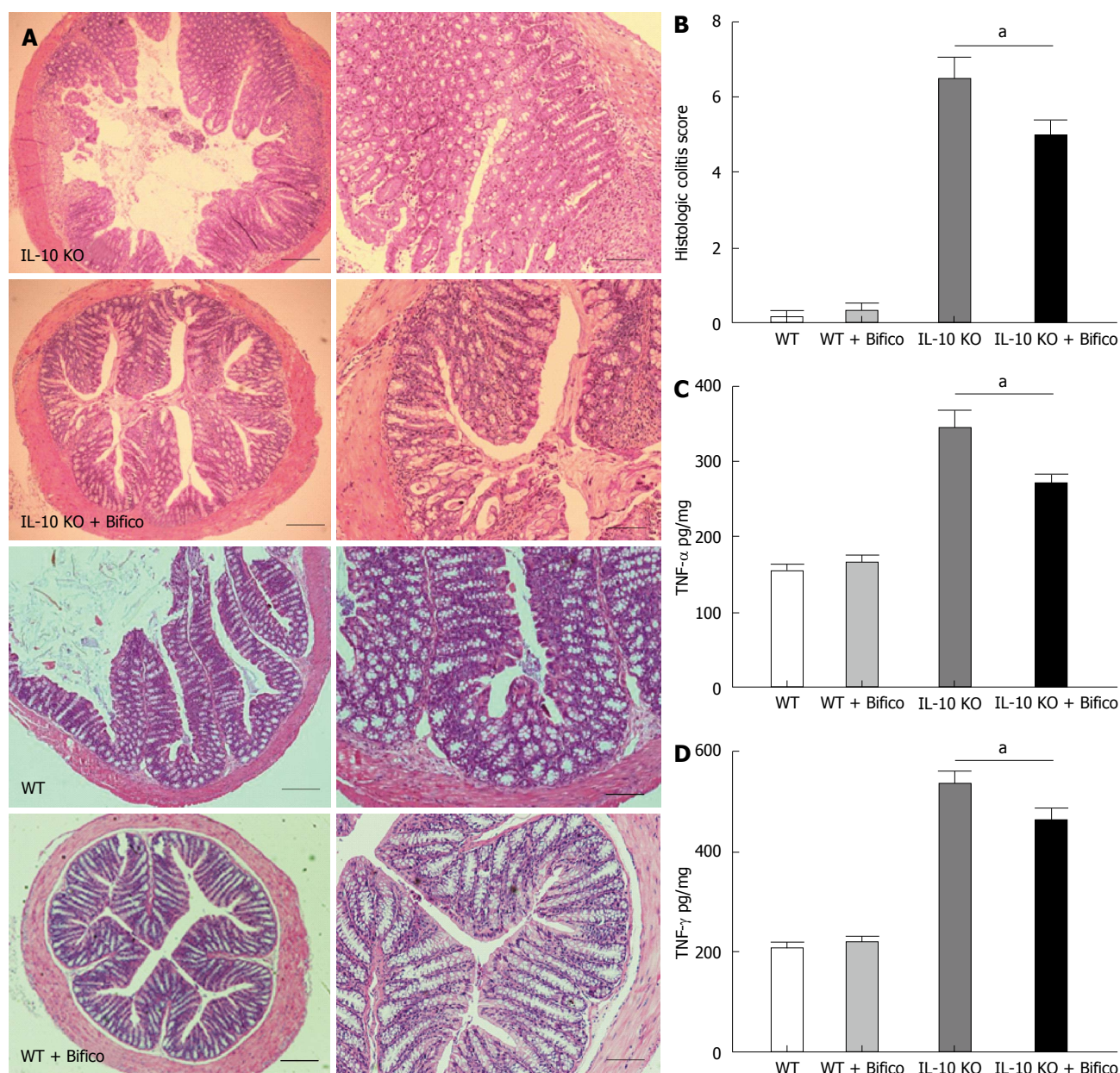


Figure 2 Bifido attenuated inflammation and production of proinflammatory cytokines. A: Representative photomicrographs showing colonic inflammation and damage (HE stain, $\times 40$ magnification, higher magnification photomicrographs on the right $\times 100$); B: Histological colitis scores (mean \pm SEM, $n = 6$ per group, $^aP < 0.05$, Student's *t* test); C and D: TNF- α and IFN- γ were measured by ELISA in colon after 4 wk Bifido treatment (mean \pm SEM, $n = 6$ per group, $^aP < 0.05$, Student's *t* test).

were evaluated by histology. HE staining revealed a large amount of mucosal damage and inflammatory cell infiltration in the lamina propria of IL-10 KO mice (Figure 2A). In addition, all IL-10 KO mice developed colitis, and the inflammatory score was significantly greater in IL-10 KO than WT mice. However, IL-10 KO mice receiving Bifido treatment had reduced inflammatory scores with only mild cell infiltration and mucosal damage (Figure 2B).

We next evaluated the secretion of proinflammatory cytokines from colonic tissue *ex vivo*. Expression of TNF- α and IFN- γ in the colonic mucosa was significantly increased in IL-10 KO mice (Figure 2C and D). However, 4 wk Bifido treatment significantly reduced the levels of TNF- α and IFN- γ (Figure 2C and D). These

data suggest that Bifido treatment reduces the induction of IBD in IL-10 KO mice.

Bifido therapy altered apical junction protein expression and distribution

To identify whether there were changes in apical junction protein expression and distribution following Bifido treatment, immunofluorescence, western blotting and real-time qRT-PCR assays of ZO-1, claudin-1 and occludin were performed on the colonic tissue. There were significantly decreased in the expression of protein and mRNA of ZO-1, claudin-1 and occludin. As expected, the network of ZO-1, claudin-1 and occludin was predominantly intact and localized along the apical cellular border in WT mice. In IL-10 KO mice, the expression of ZO-1, claudin-1

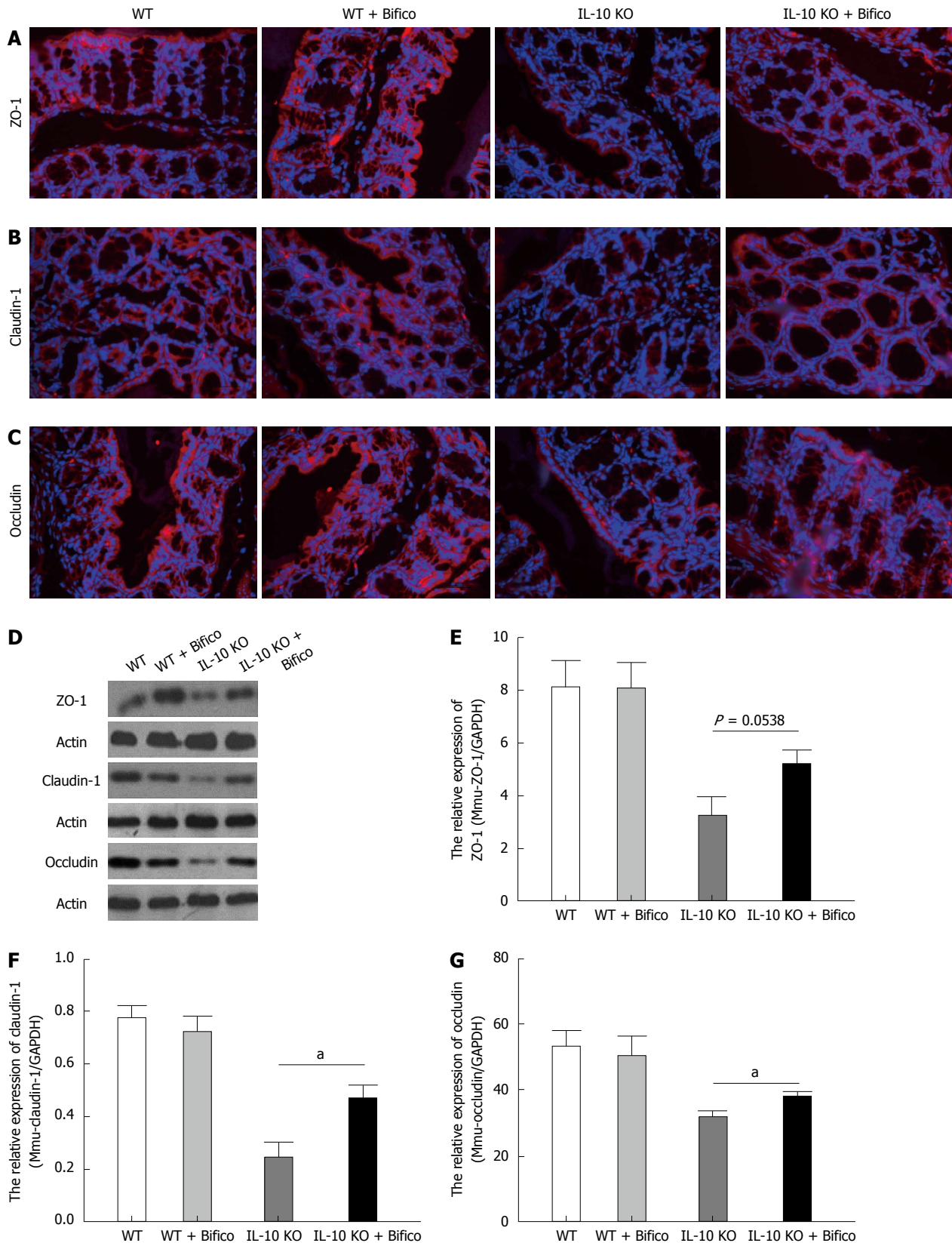


Figure 3 Bifido increased expression of tight junction proteins in interleukin-10 gene-deficient mice. A: Representative immunofluorescence photomicrographs for tight junction proteins zonula occludentes (ZO)-1, in the colonic epithelium ($n = 3$ for each group); B: Representative immunofluorescence photomicrographs for tight junction proteins claudin-1 ($n = 3$ for each group); C: Representative immunofluorescence photomicrographs for tight junction proteins occludin ($n = 3$ for each group). The tight junction proteins were stained red ($\times 400$ magnification); D: Western blotting of tight junction proteins in the colonic tissues; E: mRNA expression of ZO-1 in the colon (mean \pm SEM, $n = 4-6$ per group, $^aP < 0.05$, Student's t test); F: mRNA expression of claudin-1 in the colon (mean \pm SEM, $n = 4-6$ per group, $^aP < 0.05$, Student's t test); G: mRNA expression of occludin in the colon (mean \pm SEM, $n = 4-6$ per group, $^aP < 0.05$, Student's t test).

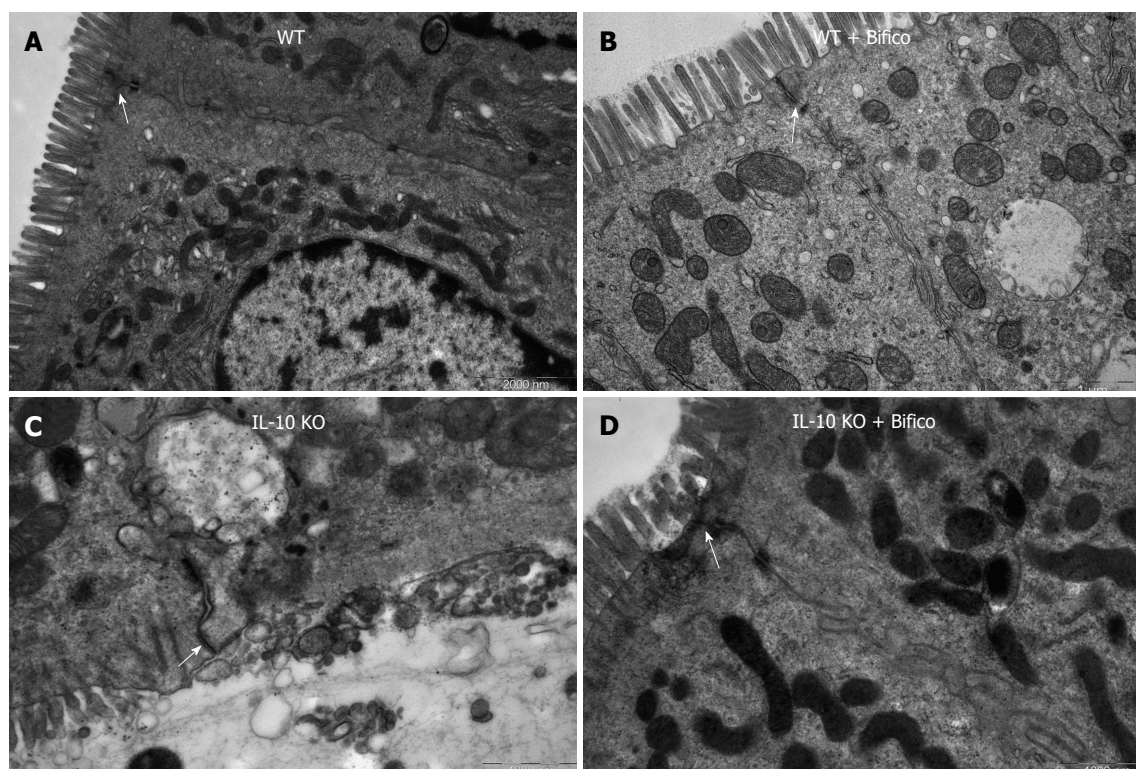


Figure 4 Bifico ameliorated the ultrastructure of colonic epithelium in interleukin-10 gene-deficient mice. A: Intercellular tight junctions (indicated by the white arrows) and normal ultrastructure in colonic epithelium of WT (scale bar = 2000 nm); B: Intercellular tight junctions (indicated by the white arrows) and normal ultrastructure in colonic epithelium of Bifico treated WT mice (scale bar = 1000 nm); C: Tight junctions (indicated by the white arrows) and microvilli, increased intercellular gap, and abnormal ultrastructure of epithelial cells in the colonic epithelium of IL-10 KO mice (scale bar = 1000 nm); D: Partly recovered intercellular tight junctions (indicated by the white arrows) and microvilli in colonic epithelium of Bifico treated IL-10 KO mice (scale bar = 1000 nm).

and occludin at the apical cellular border was decreased, discontinuous, and redistributed. Bifico treatment ameliorated these changes in IL-10 KO mice (Figure 3A-C).

Western blotting and RT-PCR showed that the protein and mRNA levels of ZO-1, claudin-1 and occludin were reduced in IL-10 KO mice when compared with WT mice (Figure 3D). Four weeks of Bifico therapy restored the protein and mRNA levels of ZO-1, claudin-1 and occludin in IL-10 KO mice (Figure 3E-G). These data suggest that Bifico treatment promotes proper tight junction protein expression and distribution.

Bifico therapy normalized ultrastructure in colonic epithelium of IL-10 KO mice

Colonic epithelial paracellular permeability is controlled mainly by tight junction proteins. Given that Bifico treatment restored tight junction distribution, we examined the ultrastructural changes of intercellular tight junction proteins by transmission electron microscopy. Compared with WT controls (Figure 4A and B), the colonic epithelium of IL-10 KO mice displayed significant ultrastructural changes in tight junction proteins and microvilli, along with marked increases in intercellular gaps and intracellular vacuolization (Figure 4C). However, Bifico therapy increased intact intercellular tight junctions and microvilli and decreased intercellular gaps and intracellular

vacuolization (Figure 4D).

Probiotic treatment increased expression of tight junction proteins in EIEC-treated Caco-2 monolayers

Caco-2 monolayers were incubated with EIEC alone, or were pretreated with Bifico or single-strain probiotics for 24 h. Real-time qRT-PCR was then performed to analyze the expression of mRNA of ZO-1, claudin-1 and occludin in treated and untreated Caco-2 monolayers. Expression of ZO-1, claudin-1 and occludin mRNA was significantly decreased after treatment with EIEC in comparison to untreated controls. The decrease in tight junction proteins was inhibited by pretreatment with probiotics, with Bifico being the most effective (Figure 5A-C).

We next used western blotting to determine the relative proteins levels of ZO-1, claudin-1 and occludin in control and EIEC-treated Caco-2 monolayers incubated with or without various probiotics. Levels of tight junction proteins were markedly decreased after treatment with EIEC compared to untreated controls (Figure 5D-G). However, preincubation of Caco-2 cells with Bifico or single-strain probiotics significantly inhibited the reduction of tight junction proteins after EIEC infection, with Bifico being the most effective (Figure 5D-G). These data suggest that Bifico therapy can prevent EIEC

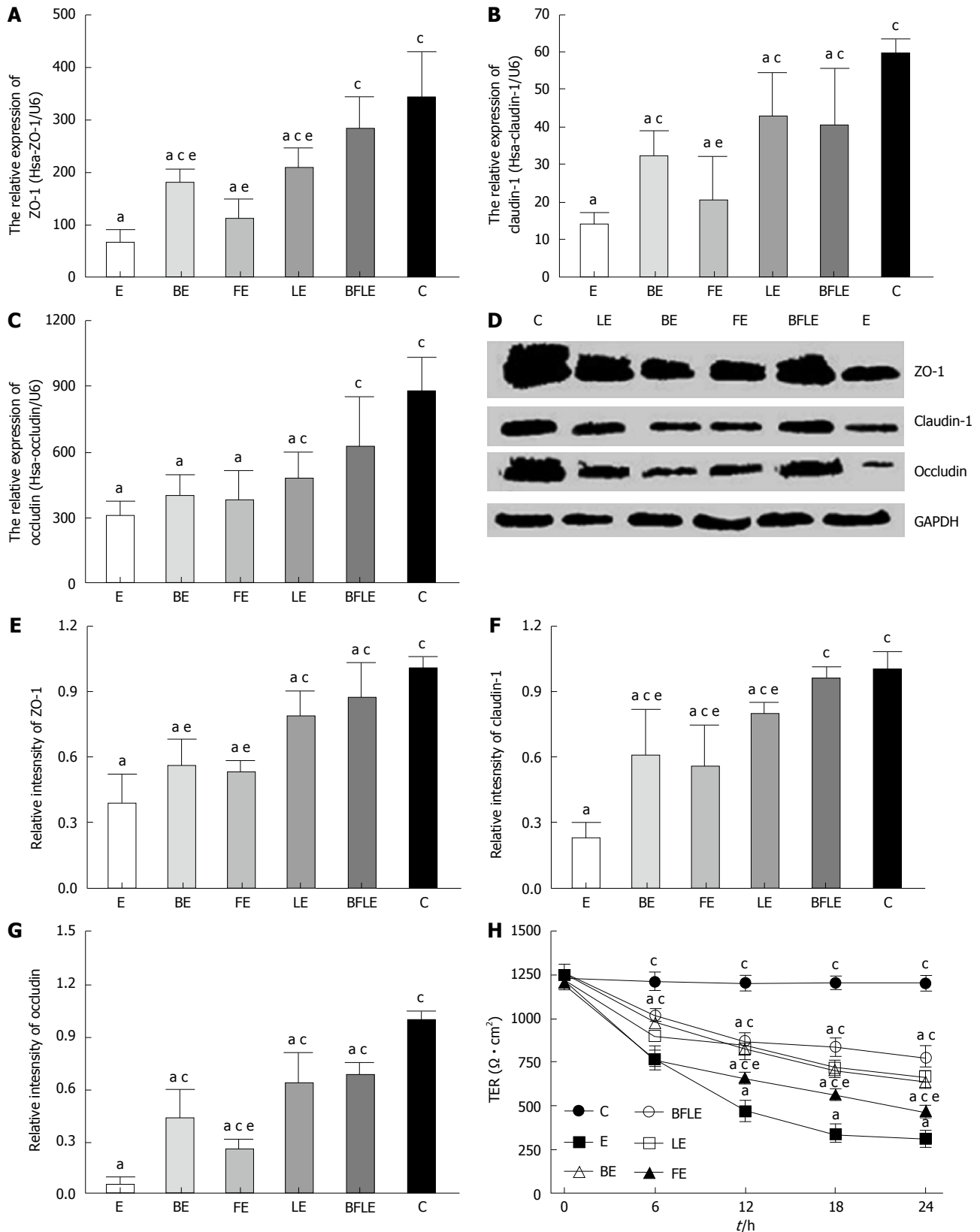


Figure 5 Bifico rescued expression of tight junction-associated proteins in following Enteroinvasive *Escherichia coli* infection. A: mRNA levels of tight junction proteins zonula occludentes (ZO)-1 were significantly decreased in Caco-2 monolayers after treatment with Enteroinvasive *Escherichia coli* (EIEC) in comparison to control untreated cells; B: mRNA levels of tight junction proteins claudin-1 were significantly decreased in Caco-2 monolayers; C: mRNA levels of tight junction proteins occludin were significantly decreased in Caco-2 monolayers. This decrease was reversed by pretreatment with Bifico or single strain probiotics; D: Representative experiment showing Western blot (WB) analysis of ZO-1, claudin-1 and occludin expression; E: Expression of ZO-1 was quantified by densitometry for three independent experiments; F: Expression of claudin-1 was quantified by densitometry for three independent experiments; G: Expression of occludin was quantified by densitometry for three independent experiments; H: Bifico or single-strain probiotics increased the EIEC-treated TER of Caco-2 monolayers (means \pm SEM, $n = 3$ per group in WB, $n = 5$ per group in qRT-PCR, $n = 5$ per time point per group in TER. * $P < 0.05$ compared with control group, * $P < 0.05$ compared with EIEC group, * $P < 0.05$ compared with BFLE group, Student's t test). B. longum (B), E. faecalis (F), L. acidophilus (L), triple bacteria (BFL), control group (C), EIEC group (E). Preincubated with probiotics or/and commensal bacteria prior to EIEC infection created four groups (BE, FE, LE and BFLE).

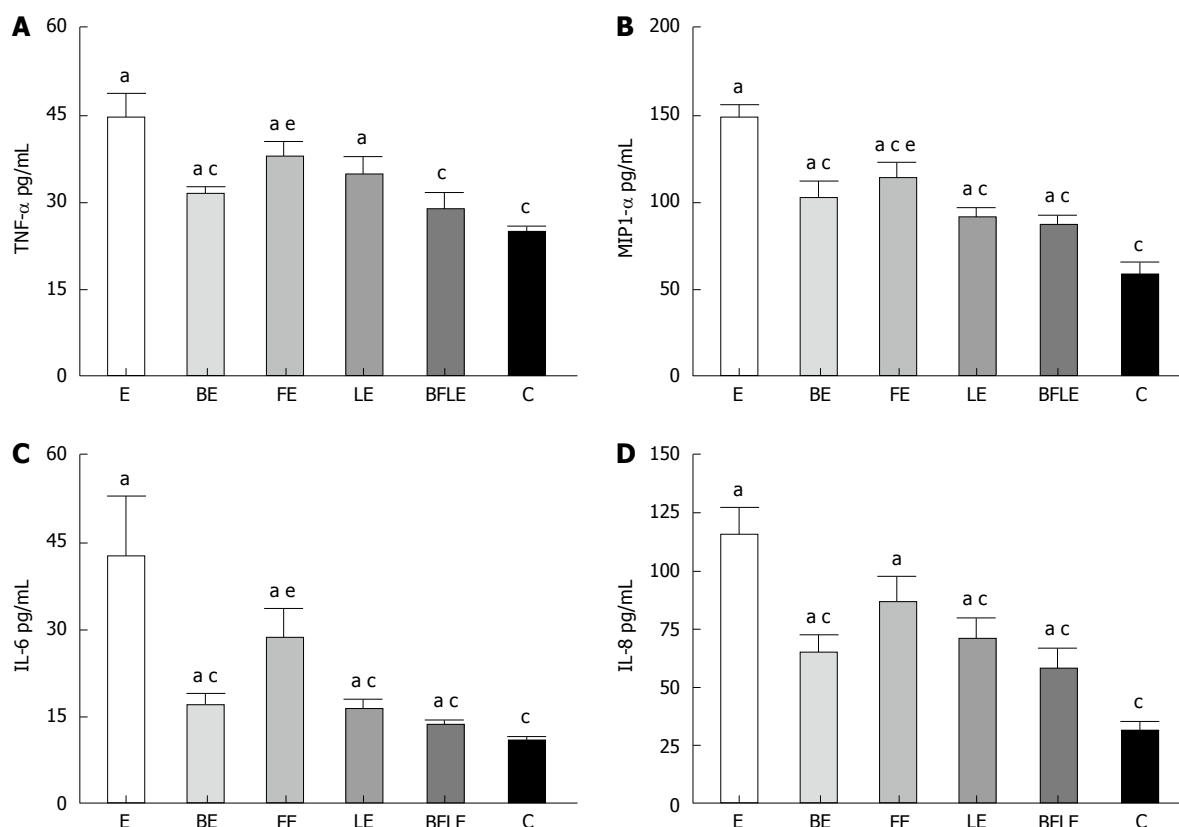


Figure 6 Bifico inhibited Enteroinvasive *Escherichia coli*-induced proinflammatory cytokines secretion. A: The level of TNF- α was significantly inhibited by Bifico or single-strain probiotic pre-treatment of Caco-2 monolayers prior to Enteroinvasive *Escherichia coli* (EIEC) infection; B: The level of macrophage inflammatory protein (MIP)-1 α was significantly inhibited by Bifico or single-strain probiotic pre-treatment of Caco-2 monolayers prior to EIEC infection; C: The level of IL-6 was significantly inhibited by Bifico or single-strain probiotic pre-treatment of Caco-2 monolayers prior to EIEC infection; D: The level of IL-8 was significantly inhibited by Bifico or single-strain probiotic pre-treatment of Caco-2 monolayers prior to EIEC infection (means \pm SEM, $n = 6$ per group, $^aP < 0.05$ compared with control group, $^cP < 0.05$ compared with EIEC group, $^{a,c}P < 0.05$ compared with BFLE group, Student's *t* test).

infection by promoting tight junction protein expression.

Probiotic treatment increased TER in EIEC-treated Caco-2 monolayers

To investigate the effect of probiotics on EIEC-treated Caco-2 monolayers, we measured the TER of untreated and treated Caco-2 monolayers. Caco-2 monolayers were incubated with EIEC alone, or were pretreated with Bifico or single-strain probiotics for 30 min and then incubated with EIEC for 24 h. TER was measured for 6 h. Infection with EIEC resulted in an about 80% decrease in TER when compared with the untreated group. However, preincubation of Caco-2 monolayers with Bifico or single-strain probiotics attenuated TER reduction, with Bifico treatment being the most effective (Figure 5H).

Probiotics altered cytokine response in EIEC-treated Caco-2 monolayers

To address further the effects of probiotics on EIEC invasion, we measured the cytokine response in EIEC-treated Caco-2 monolayers. Caco-2 monolayers were incubated as described above, and the cytokines in the cell culture supernatant were evaluated by ELISA. The proinflammatory cytokine release from EIEC-treated Caco-2 monolayers was significantly inhibited by pretreatment

with Bifico or single-strain probiotics (Figure 6). In addition, Bifico treatment was the most effective.

DISCUSSION

In the present study, we demonstrated that treating IL-10 KO mice with the probiotic mixture, Bifico, partly recovered colonic barrier integrity, reduced mucosal secretion of proinflammatory cytokines, and attenuated histopathological changes. Furthermore, *in vitro* studies revealed that epithelial barrier function and resistance to EIEC invasion was enhanced following exposure to Bifico.

The intestinal luminal microflora and their products are important initiating and modulating factors in the pathogenesis of IBD and some diarrhea diseases in humans and animals^[31-34]. IL-10 KO mice spontaneously develop IBD, and this is associated with altered colonic microflora colonization^[35]. These data suggest that probiotic therapy could reduce IBD in these mice. Indeed, inoculation of *Lactobacillus* sp. reduced IBD in IL-10 KO mice. In the present study, we demonstrated that IBD was reduced in IL-10 KO mice by the probiotic mixture Bifico, and this reduction was greater than with the use of single-strain probiotics. These data are consistent with other *in vivo* experiments using VSL#3 (VSL Pharma-

ceuticals, Gaithersburg, MD, contains 9×10^{10} cfu/g of viable, lyophilized bifidobacteria (*B. longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*), 8×10^{10} lactobacilli (*L. acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *L. bulgaricus*, and *Lactobacillus plantarum*), and 20×10^{10} *Streptococcus salivarius* subsp. *Thermophilus*) compound strains, which are more effective than the use of a single *Lactobacillus* in improving colitis and normalizing epithelial function^[3]. These data suggest that combinations of adherent probiotic strains can influence the adhesion and activity of other strains in the human intestinal tract^[36]. Our study is believed to be the first to show that Bifico therapy is effective in ameliorating epithelial damage and restoring epithelial function in both *in vivo* and *in vitro* models.

In IL-10 KO mice, colonic inflammation is related to high levels of mucosal TNF- α and IFN- γ ^[32]. Recently, multiple studies have characterized the ability of various strains of probiotics to alter the activity and cytokine expression in the colons of IL-10 KO mice^[3,8,37,38]. TNF- α and IFN- γ produced by colonic mucosa are both normalized when IL-10 KO mice are raised under germ-free conditions^[32], suggesting that colitis in IL-10 KO mice occurs as a consequence of a Th1-predominant intestinal inflammation in the presence of the gut flora^[3,8,37,38]. In present study, treating IL-10 KO mice with the probiotic mixture Bifico attenuated TNF- α and IFN- γ secretion. In addition, pretreatment of Caco-2 cells with Bifico significantly inhibited the secretion of TNF- α , MIP-1 α , IL-6 and IL-8 following EIEC infection. These results indicate that the gut is able to discriminate and define selective responses to different bacteria.

To reduce inflammation, the normal intestinal barrier is impermeable to bacteria, bacterial products, and dietary antigens that are present within the lumen. Thus, probiotic bacteria may exert protection through enhancing intestinal barrier function, including decreasing intestinal permeability and maintaining normal expression and distribution of tight junction proteins^[6,8]. IL-10 KO mice demonstrated increased colonic permeability that is absent in mice raised under germ-free conditions^[32]. Bifico treatment, however, reduced epithelial permeability in IL-10 KO mice, and increased expression of tight junction proteins in both colonic epithelia *in vivo* and in EIEC-treated Caco-2 monolayers. Moreover, the use of the Bifico therapy was more effective than the use of a single-strain probiotics in preventing disruption and normalizing function of Caco-2 monolayers following EIEC infection.

Although Bifico therapy was the most effective, single-strain probiotics were also effective in restoring Caco-2 cells function following EIEC infection. These data suggest that epithelial cells may respond directly to certain probiotic bacteria. Certain strains of *Lactobacillus*, for example, release surface-active components, which inhibit adhesion of pathogenic bacteria^[38]. Thus, probiotic bacteria may protect epithelium by receptor competition, whereby probiotics compete with microbial pathogens

for a limited number of receptors present on the surface epithelium.

In conclusion, the probiotic mixture, Bifico, is highly effective in reducing colitis in IL-10 KO mice. Furthermore, the present study revealed that Bifico treatment reduced colonic epithelial permeability, recovered normal expression and distribution of tight junction proteins, and protected against pathogenic bacterial invasion both *in vivo* and *in vitro*.

COMMENTS

Background

The human gut is colonized with a wide variety of microorganisms, including pathogenic, probiotic and commensal bacteria. *Bifidobacterium*, *Lactobacillus* and *Enterococcus faecalis* are probiotics with beneficial effects on maintenance therapy of human intestinal diseases. For example, oral treatment with specific probiotic bacteria can ameliorate inflammatory bowel disease. The colonization strategies using defined commensals or exogenous specific probiotic treatment may prevent host intestinal inflammation and ameliorate intestinal epithelial barrier function.

Research frontiers

The probiotic mixture, Bifico, is highly effective in the reducing colitis in interleukin (IL)-10 KO mice. Furthermore, Bifico treatment reduced colonic epithelial permeability, recovered normal expression and distribution of tight junction proteins, and protected against pathogenic bacterial invasion both *in vivo* and *in vitro*.

Innovations and breakthroughs

The probiotic mixture, Bifico had a direct effect on epithelial barrier function *in vivo* by reducing mucosal secretion of tumor necrosis factor- α and interferon- γ , and altering expression and distribution of tight junction proteins. Bifico exposure *in vitro* reduced bacterial invasion. Moreover, the effects of combined probiotics were more pronounced than those with single-strain probiotics.

Applications

By understanding the mechanism and effects of Bifico on the intestinal mucosal barrier, this study may represent a future strategy in the treatment of patients with colitis and increased intestinal permeability, such as ulcerative colitis, Crohn's disease and irritable bowel syndrome.

Peer review

The authors have performed a very good study of improving gastrointestinal mucosal biology in a colitis-like model and evaluating the effect of Bifico on the intestinal mucosal barrier, the expression and distribution of epithelial tight junction proteins, and secretion of inflammatory cytokines *in vivo* and *in vitro*.

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Resveratrol inhibits collagen I synthesis by suppressing IGF-1R activation in intestinal fibroblasts

Ping Li, Mei-Lan Liang, Ying Zhu, Yao-Yao Gong, Yun Wang, Ding Heng, Lin Lin

Ping Li, Ying Zhu, Yao-Yao Gong, Yun Wang, Ding Heng, Lin Lin, Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Mei-Lan Liang, Nanping First Hospital Affiliated to Fujian Medical University, Nanping 353000, Fujian Province, China

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Correspondence to: Lin Lin, MD, PhD, Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Rd, Nanjing 210029, Jiangsu Province, China. lin9100@aliyun.com

Telephone: +86-25-83718836-6920 Fax: +86-25-83674636

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Abstract

AIM: To investigate whether resveratrol (3,4,5-trihydroxy-trans-stilbene) inhibits collagen I synthesis induced by insulin growth factor-1 (IGF-1) in intestinal fibroblasts, and to explore the possible molecular mechanisms.

METHODS: Male Sprague-Dawley rats were randomly divided into two groups: a control group and a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis group. After 21 d of TNBS administration, the degree of inflammation and fibrosis in colon was measured by HE staining and Masson's trichrome staining. Western blotting was used to examine collagen I, IGF-1 and silent information regulator 1 (SIRT1) protein expression in colitis tissues. Western blotting and quantitative real-time polymerase chain reaction were used to characterize collagen I protein and col1a2 mRNA expression in

mouse intestinal fibroblasts and CCD-¹⁸Co cells treated with IGF-1. A MEK inhibitor (U0126) was used to determine whether IGF-1-induced collagen I expression was mediated by extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent mechanism. Effects of resveratrol on collagen I protein level, insulin growth factor-1 receptor (IGF-1R) and ERK1/2 phosphorylation levels were also examined after IGF-1 treatment in fibroblasts. To evaluate whether SIRT1 was necessary for the anti-fibrosis effect of resveratrol, cells were transfected with SIRT1-specific small interfering RNAs, wild-type SIRT1, and deacetylase-inactive mutant SIRT1.

RESULTS: Collagen I and IGF-1 expression was increased, and SIRT1 expression was decreased (0.67 ± 0.04 vs 1.05 ± 0.07 , $P < 0.001$) in TNBS-induced colitis compared with the control group. *In vitro*, IGF-1 could induce collagen I expression, mainly through the ERK 1/2 signal pathway. Resveratrol reduced basal and IGF-1-induced collagen I gene and protein expression in intestinal fibroblasts. Overexpression of wild-type SIRT1, not deacetylase-inactive mutant SIRT1, decreased expression of collagen I induced by IGF-1. Moreover, silencing SIRT1 restored collagen I expression in fibroblasts challenged with resveratrol. However, disruption of SIRT1 did not influence the anti-fibrotic effects of resveratrol and IGF-1-induced collagen I expression. Further analysis revealed that resveratrol significantly decreased phosphorylation of IGF-1R and its downstream signaling molecules by inhibiting IGF-1 binding to its receptor.

CONCLUSION: Our data suggest that resveratrol effectively inhibits collagen I synthesis in IGF-1-stimulated fibroblasts, partly by inhibiting IGF-1R activation, and SIRT1 is also responsible for the process.

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Key words: Intestinal fibrosis; Insulin-like growth fac-

tor-1; Resveratrol; Silent information regulator 1; Fibroblasts

Core tip: This study showed that the expression of silent information regulator 1 (SIRT1) was decreased in 2,4,6-trinitrobenzenesulfonic acid-induced colitis tissues, and resveratrol down-regulated insulin growth factor (IGF)-1-induced collagen I synthesis by inhibiting IGF-1 receptor (IGF-1R) phosphorylation and its downstream extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway in intestinal fibroblasts. Resveratrol alone suppressed collagen I synthesis through up-regulating activity of SIRT1. Our data highlight a previously unknown function of resveratrol on IGF-1R activation and provide novel insight of resveratrol as a therapeutic agent for intestinal fibrosis.

Li P, Liang ML, Zhu Y, Gong YY, Wang Y, Heng D, Lin L. Resveratrol inhibits collagen I synthesis by suppressing IGF-1R activation in intestinal fibroblasts. *World J Gastroenterol* 2014; 20(16): 4648-4661 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4648.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4648>

INTRODUCTION

Intestinal fibrosis is a common and serious complication of Crohn's disease (CD) and can increase the risk of intestinal stenosis or obstruction, and ultimately lead to surgical intervention at substantial personal and economic cost^[1-3]. Until now, no effective therapy exists for averting such fibrogenic events, because the fibrotic process becomes autopropagative and fails to respond to antiinflammatory interventions^[4,5].

The pathological process of intestinal fibrosis is characterized by mesenchymal cell proliferation and extracellular matrix (ECM) accumulation in interstitial space^[6,7]. Collagens I is one of the major matrix molecules involved in intestinal fibrogenesis. The activation of extracellular signal-regulated kinase 1/2 (ERK1/2) is one of the major downstream signaling events that participate in collagen I synthesis^[8,9]. Insulin-like growth factor 1 (IGF-1) is a potent profibrogenic agent involved in intestinal remodeling^[10,11]. Laboratory- and population-based studies have shown that expression of IGF-1 in Crohn's disease patients is significantly increased and IGF-1 receptor (IGF-1R) is also altered in the intestines of ulcerative colitis and Crohn's disease patients^[12-14]. IGF-1 binding to IGF-1R allows the β -subunits of IGF-1R to display intrinsic tyrosine kinase activity and activates downstream signals *via* phosphorylation of key proteins including phosphatidylinositol 3-kinase and mitogen-activated protein (MAP) kinase (MAPK)^[15]. IGF-1 not only stimulates proliferation and inhibits apoptosis of fibroblasts and myofibroblasts, but also increases collagen expression and production in intestinal smooth

muscle cells^[16,17]. Collagen-producing fibroblasts and myofibroblasts are central cell types in intestinal fibrogenesis. There are no data showing the effect of IGF-1 on collagen I in intestinal fibroblasts.

Resveratrol (3,4,5-trihydroxy-trans-stilbene) is a polyphenol naturally occurring in grapes and red wine that exhibits beneficial health effects such as extending the life span and regulating tumor growth and oxidation. Resveratrol activates silent information regulator-1 (SIRT1), a nicotinamide adenine dinucleotide-dependent deacetylase, which has many biological functions by deacetylating a number of key transcription factors, including p53, nuclear factor- κ B, and peroxisome proliferator-activated receptor gamma co-activator-1 α ^[18]. In addition, resveratrol also has a dramatic antifibrotic effect in rodent models of renal fibrosis^[19,20], cardiac fibrosis^[21], and hepatic fibrosis^[22,23], but the molecular mechanism(s) are currently unknown.

The protective role of resveratrol in colitis has been demonstrated in models of colitis induced by dextran sulfate sodium (DSS) and trinitrobenzene sulphonic acid (TNBS)^[24-26]. Resveratrol may protect against colitis through up-regulation of SIRT1 in immune cells, which functions as an inverse regulator of NF- κ B activation and inflammation in the colon^[25]. Mounting evidence suggests that resveratrol also has an antifibrotic effect in the peptidoglycan-polysaccharide rat model of Crohn's disease and can diminish IGF-1-stimulated collagen production in intestinal smooth muscle cells^[27,28]. However, the mechanism for resveratrol to inhibit collagen synthesis and IGF-1-induced collagen production has not been established, and it is unclear whether resveratrol inhibits collagen expression through augmentation of SIRT1 activity in intestinal fibroblasts. The aim of this study was to investigate the effect of resveratrol on collagen I synthesis in intestinal fibroblasts and to explore the possible molecular mechanisms.

MATERIALS AND METHODS

Animals and induction of colitis

The technique for induction of colitis with TNBS (Sigma) was as described previously^[29]. Male Sprague-Dawley rats (200-250 g) were purchased from and maintained in the Animal Center of Nanjing Medical University (Nanjing, China). To induce chronic fibrotic colitis, rats were fasted for 24 h, lightly anesthetized with diethyl ether, and TNBS solution [2.5% in 50 % ethanol (v/v)] was injected *via* a catheter advanced to 8 cm proximal to the anus. In order to distribute the TNBS within the colon, the rat was kept in a vertical position with the head downwards for 3 min after the injection. All rats were checked daily for loss of body weight, stool consistency, and the presence of gross bleeding. The disease activity index (DAI) was calculated as a sum of the scores of the three parameters according to the scoring system described by Murthy *et al.*^[30]. Animals were sacrificed after 21 d and body weight, colon weight, and colon length were recorded.

HE staining and Masson's trichrome staining were used to measure the degree of inflammation and fibrosis in the colon by microscopy.

Fibroblast isolation and culture

Mouse intestinal fibroblasts (MIFs) were isolated and cultured as described previously^[31,32]. The intestine tissue isolated from Balb/c mice (7 d) was cut into 1-mm pieces. Epithelial cells were removed in Hank's Balanced Salt Solution without Ca²⁺ and Mg²⁺ with 2 mmol/L EDTA. The remaining tissue was rinsed and then digested for 30 minutes at 37 °C with 1 mg/mL collagenase II and 0.3 mg/mL DNase I in PBS. The isolated cells were cultured in 25-cm² culture flasks (Corning) with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS, Hyclone) and 10 µg penicillin/streptomycin (Gibco). Nonadherent cells were removed by subsequent changes of medium after 2 h. The remaining cells were characterized by immunocytochemistry staining for vimentin (1:200) and α -smooth muscle action (1:200) as previously described^[32]. For all experiments, fibroblasts were used between passages 3 and 8, and were treated with recombinant mouse IGF-1 (100 ng/mL, RD) or Resveratrol (50, 100 µmol/L, sigma).

Cell culture and treatment

CCD-18Co cells (CRL 1459) at passage 6 were obtained from American Type Culture Collection and used between passages 8 and 15. Cells were grown in DMEM supplemented with 10% FBS and 10 µg penicillin/streptomycin. Cells were maintained at 37 °C in a 5% CO₂ incubator. Cells were treated with recombinant human IGF-1 (100 ng/mL, RD) or resveratrol (50, 100 µmol/L, Sigma) for 24 h as indicated.

Plasmids and transient transfection

WT and deacetylase-inactive mutant SIRT1 (H363Y) constructs were a gift from Dr. Yong Xu (Nanjing Medical University, Nanjing, China) and have been described previously^[33]. Silencing of SIRT1 was mediated by small interfering RNAs (siRNAs) using the following sequences: for human SIRT1, 1: 5'-CGGGAAUCCAAAGG AUAAUTT-3', 2: 5'-CCAUCUCUCUGUCACAAAUUTT-3' and 3: 5'-CCAAGCAGCUA AGAGUAAUUTT-3'. CCD-18Co cells were transfected at 30%-40% confluency using either Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen). At 24 h post-transfection, cells were treated with IGF-1 followed by treatment with serum-free medium for 12 h. For siSIRT1 experiments, cells at 48 h post-transfection were treated for an additional 24 h with either 100 µmol/L resveratrol or 100 ng/mL IGF-1.

Whole cell protein extraction and Western blotting

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mmol/L Tris pH = 7.4, 150 mmol/L NaCl, 1% Triton X-100) with freshly added protease inhibitor and phosphatase inhibitor tablet (Roche). Cells lysates were subjected to SDS-PAGE and trans-

ferred onto PVDF membranes (Millipore) using a Semi-Phor system (Bio-Rad). After blocking in PBS containing 5% non-fat dry milk, blots were incubated with primary antisera for overnight at 4 °C, washed in PBS containing 0.05% Tween, and then incubated with peroxidase conjugated secondary antibodies for 30 min at RT. Immunoreactive proteins were identified using the ECL detection system. Antibodies against collagen type I were obtained from Rockland. IGF-1R, phosphorylated IGF-1R, ERK1/2, phosphorylated ERK1/2 and MEK inhibitor (U0126) were purchased from Cell Signaling Technology. β -actin (1:1000) and GAPDH were purchased from Bioworld, and SIRT1 monoclonal antibody (1:1000) was from Abcam.

RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from cells grown in 60-mm tissue culture dishes (Corning) using TRIzol Reagent (Gibco), according to the manufacturer's instructions. Reverse transcription reactions were performed using PrimeScript RT Master Mix and (Takara). Real-time PCR reactions were performed using SYBR-Green PREMIX EX TAQ (Takara) on an ABI Prism 7500 system. The primers used for real-time reactions were: (1) mouse col1a2, forward primer (5'-GGAGGGAACGGTCCACGAT-3') and reverse primer (5'-GAGTCCGCGTATCCACAA-3'); (2) mouse col1a1, forward primer (5'-CCGGCTCCTGCTCCTCTTA-3') and reverse primer (5'-CCATTGTGTATGCAGCTG ACTTC-3'); (3) mouse β -actin, forward primer (5'-CATCGTGGGCGGCTCTA-3') and reverse primer (5'-CACCCACACATAGGAGTCCTTCTG-3'); (4) human col1a2, forward primer (5'-GCCCCCAGGCAGAGA-3') and reverse primer (5'-CCAACTCCTTTTCCAT CATACTGA-3'); (5) human col1a1, forward primer (5'-ACGAAGACATCCCACCA ATC-3') and reverse primer (5'-GCACATCCAAACCAC TGA-3'); (6) human sirt1, forward primer (5'-TGAGGCACTTCATGGGGTATGG-3') and reverse primer (5'-TCCTAGGTTGCCAGCTGATGAA-3'); and (7) human GAPDH, forward primer (5'-GAAATCCCATCACCATCTTCCAGG-3') and reverse primer (5'-GAGCCCCAGCCT TCTCCATG-3').

Statistical analysis

The results are expressed as mean \pm SD. The SPSS statistical package (version 14.0; SPSS Inc, Chicago, IL, United States) was used for statistical analyses. The differences between the two groups were analyzed using Student's *t* test. Unless otherwise specified, *P* values smaller than 0.05 were considered statistically significant.

RESULTS

SIRT1 expression is decreased in TNBS-induced colitis

We initially tested the expression of collagen I and SIRT1 in colitis induced with TNBS. Compared with control normal rats, the DAI, body weight and colon weight significantly increased, and colon length decreased

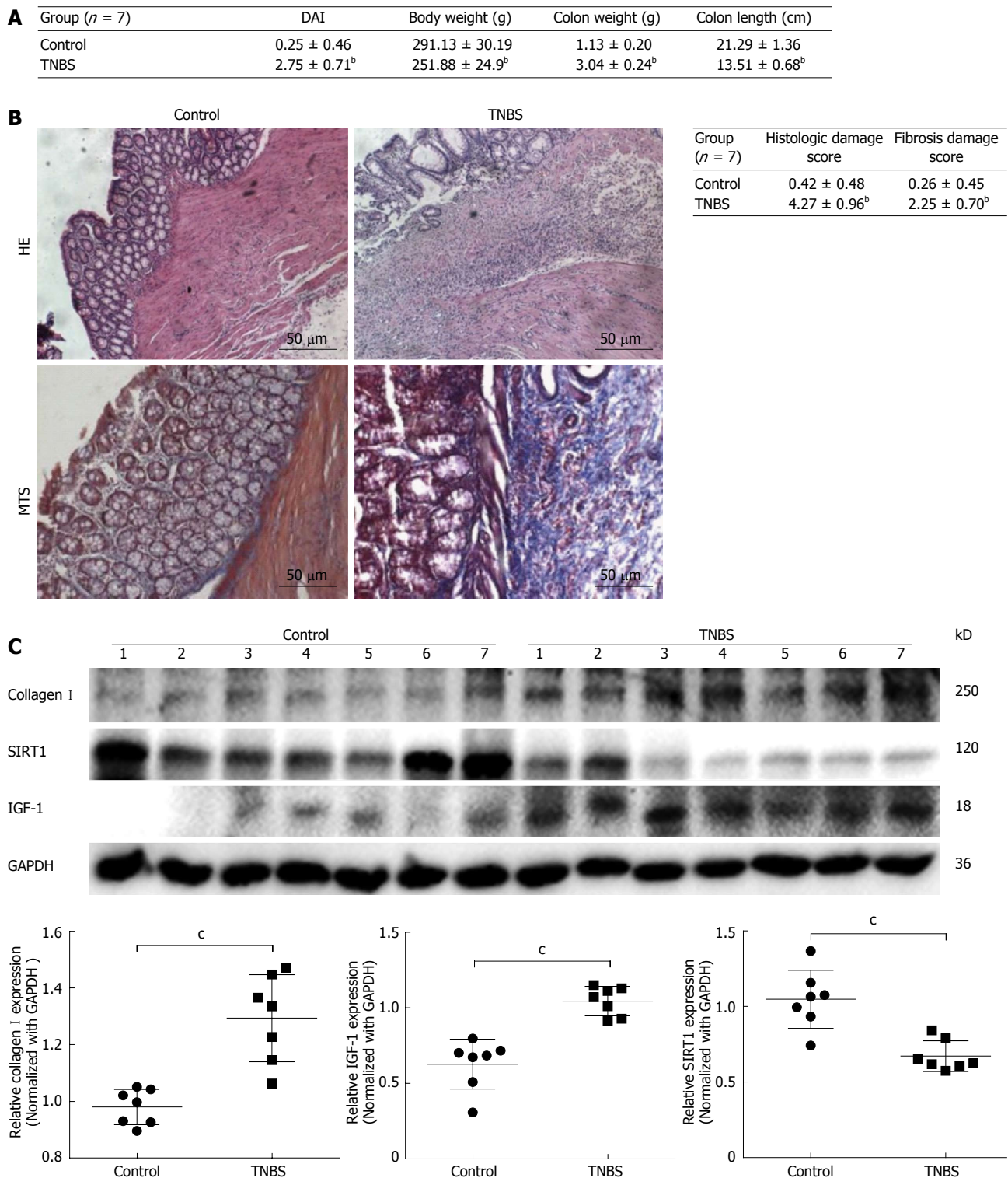


Figure 1 Silent information regulator 1 expression is decreased in 2,4,6-trinitrobenzenesulfonic acid-induced colitis. A, B: Change of clinical symptoms shown by disease activity index (DAI) and histological characterization of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis (original magnification × 200); C: The proteins collagen I, insulin growth factor-1 (IGF-1) and silent information regulator 1 (SIRT1) levels were measured by Western blotting. Data are expressed as mean ± SD (*n* = 7 in each group). ^b*P* < 0.01 vs those of normal rats. MTS: Masson's trichrome staining; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

in TNBS-treated rats (Figure 1A). Paraffin-embedded colonic tissue samples from TNBS-treated and control rats were assessed for inflammation and fibrosis after HE and Masson's trichrome staining (MTS). TNBS-treated rats showed significant colitis marked by submucosa thickening, epithelial layer destruction, and lymphocyte infiltration

(Figure 1B). The sections stained with MTS displayed diffuse extracellular matrix deposition and fibrosis in the mucosa and submucosa (Figure 1B). Histologic scores for both inflammation and fibrosis were greater in TNBS-induced colitis rats than in normal rats (Figure 1B).

Furthermore, Western blotting analysis showed that collagen I protein level was increased in colitis rats compared to normal rats (Figure 1C). IGF-1 protein expression was also increased in colitis rats, whereas SIRT1 was decreased (Figure 1C). These data indicate that resveratrol may protect against intestinal fibrosis through stimulating SIRT1 expression.

IGF-1-induced expression of collagen I is dependent on MAPK in intestinal fibroblasts

Since earlier studies documented that IGF-1 attenuates inflammation and exacerbates intestinal fibrosis, we chose to investigate the possible mechanism underlying IGF-1 induced collagen I synthesis in intestinal fibroblasts.

We first examined the expression of collagen I in both MIFs and CCD-¹⁸Co cells treated with increasing concentrations (50, 100 or 150 ng/mL) of IGF-1 for 24 h. IGF-1 potently increased both col1a2 mRNA levels (Figure 2A) and protein levels (Figure 2B) of collagen I in a dose-dependent manner. IGF-1 treatment for 24 h showed a maximal effect on collagen I expression. In addition, IGF-1 also induced col1a1 mRNA expression (Figure 2A and B).

To investigate the molecular mechanism underlying the induction of collagen I expression by IGF-1, we measured the phosphorylation of IGF-1R and ERK1/2 in response to IGF-1 treatment. IGF-1 significantly increased levels of phospho-IGF-1R and ERK1/2 in a time-dependent manner in both MIFs and CCD-¹⁸Co cells, and the most prominent effect was seen at 30 min (Figure 2C). Fibroblasts were pretreated with U0126 (50 μ mol/L) for 1 h to block MEK1/2 phosphorylation and then coincubated with IGF-1 (100 ng/mL) for another 24 h. The ability of IGF-1 to increase collagen I expression was significantly inhibited by the MAP-kinase inhibitor (Figure 2D). Taken together, these data suggest that IGF-1 inhibited collagen I synthesis in fibroblasts through the IGF-1/IGF-1R/MAP-kinase pathway.

Resveratrol treatment abrogated collagen I synthesis partly through SIRT1

Since we observed that SIRT1 expression was decreased in colonic tissues of colitis rats, we evaluated the effect of the SIRT1 activator, resveratrol, on collagen I synthesis in intestinal fibroblasts and elucidated the underlying molecular mechanisms.

As shown in Figure 3A and B, resveratrol markedly decreased collagen I protein and mRNA levels induced by IGF-1 at a concentration of 100 μ mol/L. In addition, resveratrol alone also inhibited collagen I synthesis (Figure 3B).

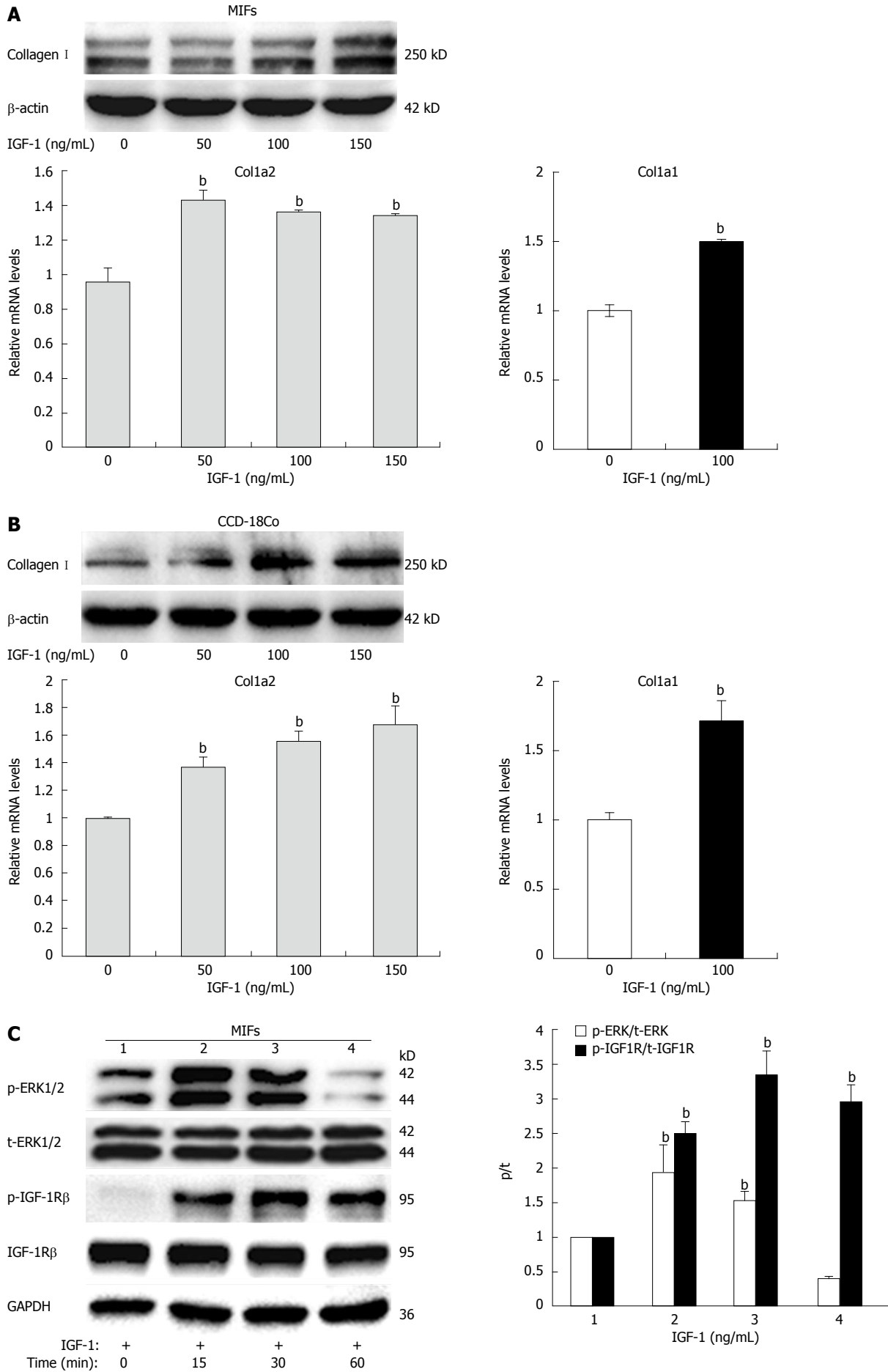
Next, to verify whether SIRT1 is required for resveratrol induced repression of collagen I, we performed the following experiments. First, fibroblasts were transfected with SIRT1 expression constructs (WT or HY) followed by IGF-1 treatment, and collagen I protein level was assessed. Overexpression of WT, but not enzyme defi-

cient (HY) SIRT1, markedly decreased IGF-1-induced collagen I synthesis (Figure 3C). Overexpression of WT SIRT1 also led to the reduction of collagen I (Figure 3D). Second, we transfected specific siRNAs targeting SIRT1 into fibroblasts. To confirm the efficiency of siRNA-mediated SIRT1 knockdown, 293T cells were transiently transfected with the SIRT1 siRNA (siSIRT1 1, 2, 3) or scrambled siRNA with transfection reagents, and total cell lysates were prepared 48 h after transfection and used for Western blotting using anti-SIRT1 antibody or control anti-GAPDH antibody (Figure 3E). As shown in Figure 3F, depletion of SIRT1 by siRNA (1 and 3) in CCD-¹⁸Co cells blocked the reduction of collagen I expression induced by resveratrol. However, there was no effect of resveratrol on collagen I induced by IGF-1 (Figure 3F). Collectively, these results clearly documented that SIRT1 was partly involved in the resveratrol-dependent repression of collagen I.

Resveratrol inhibits IGF-1-induced phosphorylation of IGF-1R and ERK1/2 independent of activating SIRT1

Our data so far demonstrate that IGF-1 promotes collagen I synthesis through the MAPK pathway, and resveratrol down-regulates collagen I expression induced by IGF-1. To investigate whether resveratrol suppressed the IGF-1/IGF-1R/ERK1/2 pathway, we next probed the effect of resveratrol on IGF-1R expression and phosphorylation with or without IGF-1 in both MIFs and CCD-¹⁸Co cells.

Resveratrol remarkably inhibited the phosphorylation of IGF-1R and ERK1/2 stimulated by IGF-1 for 30 min, and had no effect on the expression of total IGF-1R and ERK1/2 (Figure 4A), suggesting that resveratrol reduces IGF-1R activity and the intracellular ERK signaling cascade and thereby enhances the expression of collagen I. In addition, overexpression of WT or HY SIRT1 had no effect on phosphorylation of IGF1R (Figure 4B), and we did not observe any significant difference between SIRT1 WT and HY-transfected cells (Figure 4B). Since binding of IGF-1 to the IGF-1 receptor results in autophosphorylation of the receptor β subunits, and increased receptor tyrosine kinase activity, we further examined whether resveratrol affects the binding of IGF-1 to the IGF-1R. CCD-¹⁸Co cells were pretreated with resveratrol (100 μ mol/L) for 24 h to induce SIRT1 expression, then removed and incubated with IGF-1 alone for another 30 min (Figure 4C, column 4) or IGF-1 and resveratrol for 30 min (Figure 4C, column 5), and the phosphorylation of IGF-1R and ERK1/2 was tested by immunoblot analysis. The results showed that phosphorylation levels of IGF-1R were down-regulated only after treatment with IGF-1 plus resveratrol for 30 min (column 5), compared with treatment with IGF-1 alone. In other words, resveratrol inhibited IGF-1R phosphorylation only when resveratrol was incubated together with IGF-1. Collectively, these data suggest that the repression of collagen I synthesis mediated by resveratrol can be partly attributed to the inhibition of IGF-1R/ERK1/2



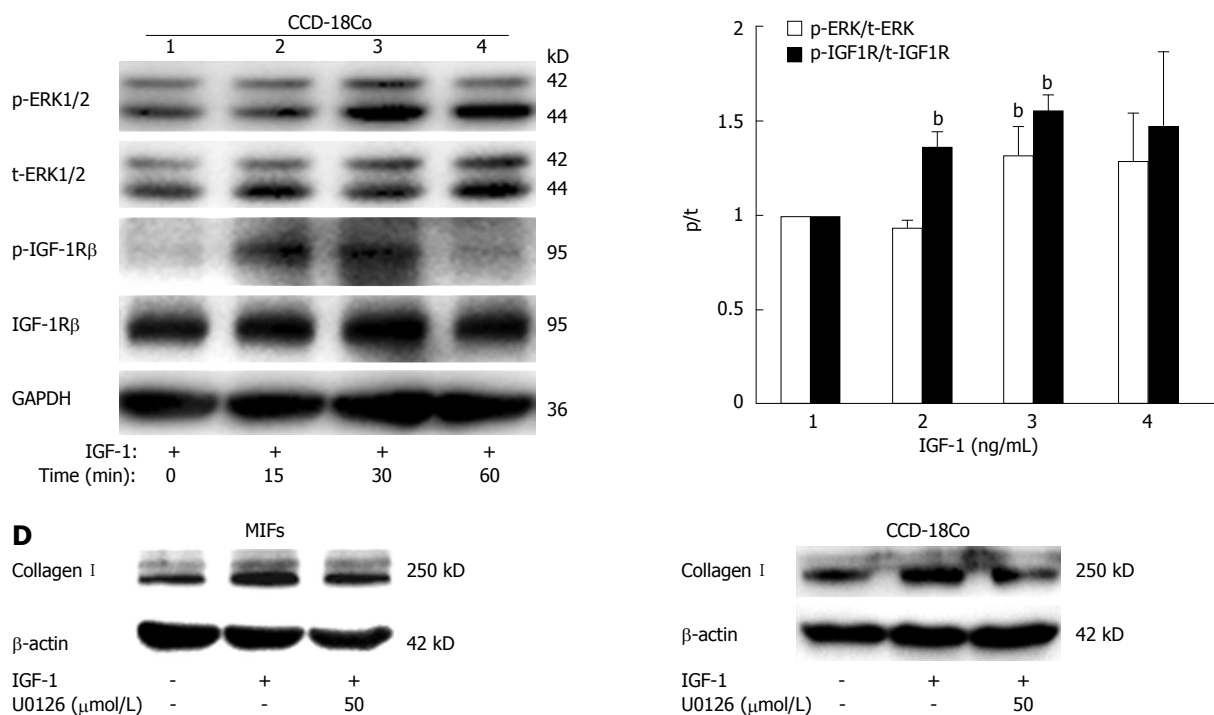


Figure 2 Insulin growth factor-1 induces collagen I expression via mitogen-activated protein-kinase pathway. A, B: Collagen I protein and col1a2, col1a1 mRNA expression in mouse intestinal fibroblasts (MIFs) and CCD-18Co cells stimulated with increasing concentrations of insulin growth factor-1 (IGF-1) for 24 h; C: Phosphorylation of IGF-1 receptor (IGF-1R) (p-IGF-1R) and extracellular signal-regulated kinase 1/2 (ERK1/2) (p-ERK1/2) in MIFs and CCD-18Co exposed to 100 ng/mL IGF-1 for 0, 15, 30, or 60 min. The bar graph represents the quantitation of the Western blotting normalized to the control; D: Cells were pretreated with 50 μmol/L mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/ERK inhibitor U0126 or vehicle for 1 h and then coincubated with 100 ng/mL IGF-1 for 24 h. Collagen I protein level was measured. The experiment was repeated three times and obtained similar results. Values represent mean ± SD. ^b*P* < 0.01 vs no treatment group. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

signaling in a SIRT1 independent mechanism, and resveratrol may inhibited IGF-1 binding to its receptor.

DISCUSSION

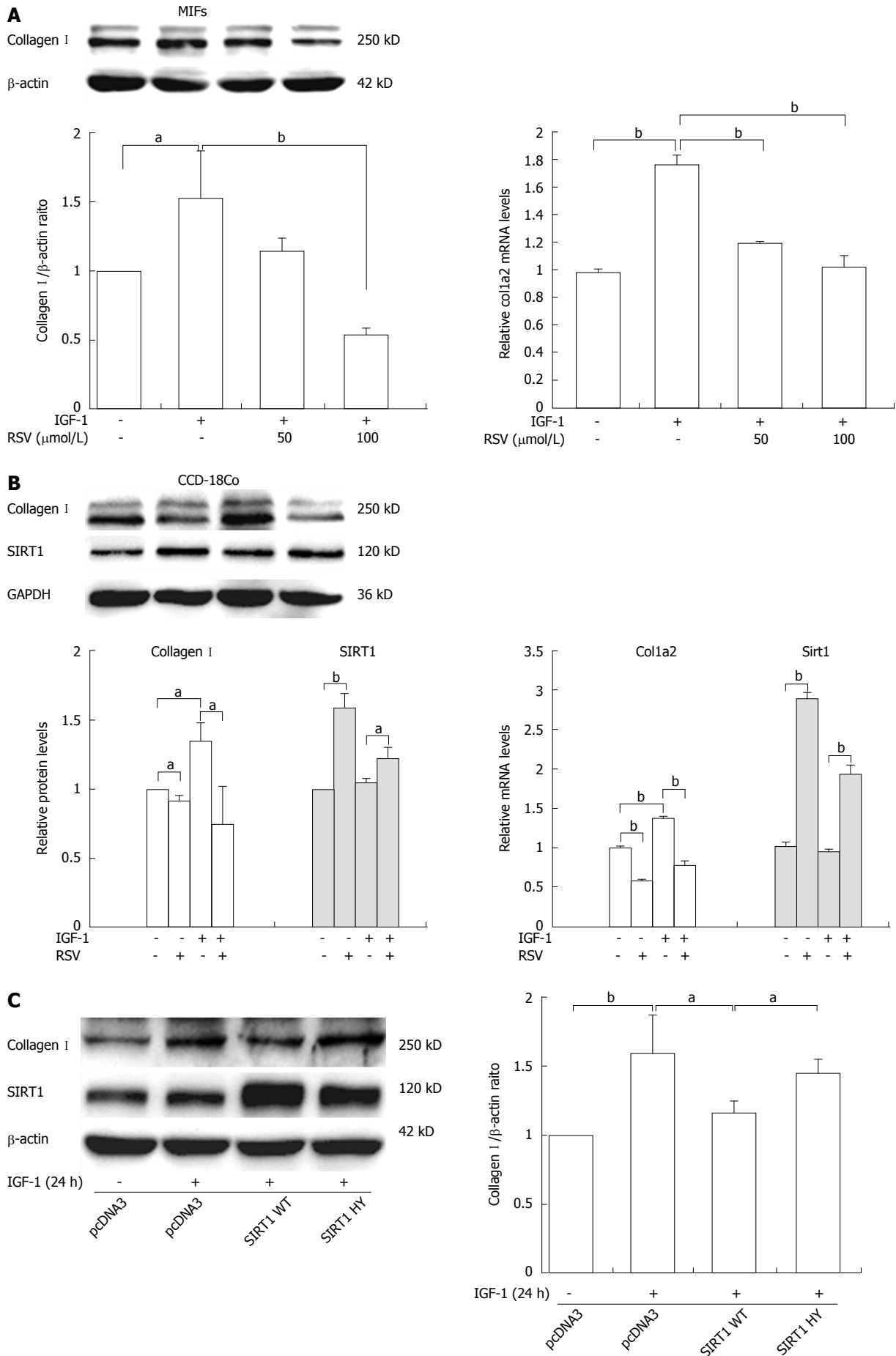
Intestinal fibrosis in the form of fibrotic strictures is a well described complication of longstanding Crohn's disease^[34]. Currently, there are no inflammatory bowel disease therapies that have been shown to effectively decrease fibrosis, leaving surgery as the only treatment for symptomatic strictures^[4]. It has long been documented that the accumulation of ECM in the intestinal wall contributes to intestinal remodeling and stricture formation. Since collagen I is one of the major matrix molecules involved in intestinal fibrogenesis, reduction and degradation of collagen I are a prominent treatment for CD intestinal fibrosis.

Resveratrol, a naturally occurring phytochemical, also known as SIRT1 activator, possesses anti-inflammatory and antioxidative effects^[35,36]. *In vivo* studies have demonstrated the antifibrotic role of resveratrol in experimental colitis^[28]. Increasing evidence has implicated the role of resveratrol in the regulation of inflammatory cytokines, profibrotic factors and procollagen^[28]. These facts suggest that resveratrol could be utilized as a therapeutic against intestinal fibrosis. Results obtained by Susana Sánchez-Fidalgo *et al*^[37] showed that dietary supplementation of resveratrol exerted a significant beneficial effect

in chronic DSS-induced colitis. Larrosa *et al*^[38] found that resveratrol pro-prodrugs prevented the rapid metabolism of resveratrol and delivered higher quantities of resveratrol to the colon in DSS-induced colitis. However, there are no preclinical and clinical studies that have shown that resveratrol can be used clinically in patients with intestinal fibrosis. Moreover, the rapid metabolism of resveratrol diminishes its effectiveness in the colon. According to previous studies, long-term epidemiologic studies and controlled clinical trials are also necessary for developing resveratrol to become a standard clinical agent.

According to previous studies, detailed investigations of the underlying mechanisms are limited. In this study, we reported several new findings that reveal the regulatory effect of resveratrol on collagen I synthesis and the mechanisms in intestinal fibroblasts. As a preliminary test, we initially induced intestinal fibrosis in rats with TNBS, and identified that collagen I and IGF-1 expression was significantly increased, but protein level of SIRT1 was decreased in colitis tissues. These data suggest that low expression of SIRT1 is related to intestinal fibrosis, and may explain the anti-fibrotic effect of resveratrol.

Based on these findings *in vivo*, we further researched the functions of resveratrol *in vitro*. We used intestinal fibroblasts for our subsequent experiments. Evidence suggests that the IGF system, including IGF-1, IGF-2, IGF-1R, and the IGF-binding proteins (IGFBPs, IGFBP1-6) play a crucial role in the gastrointestinal



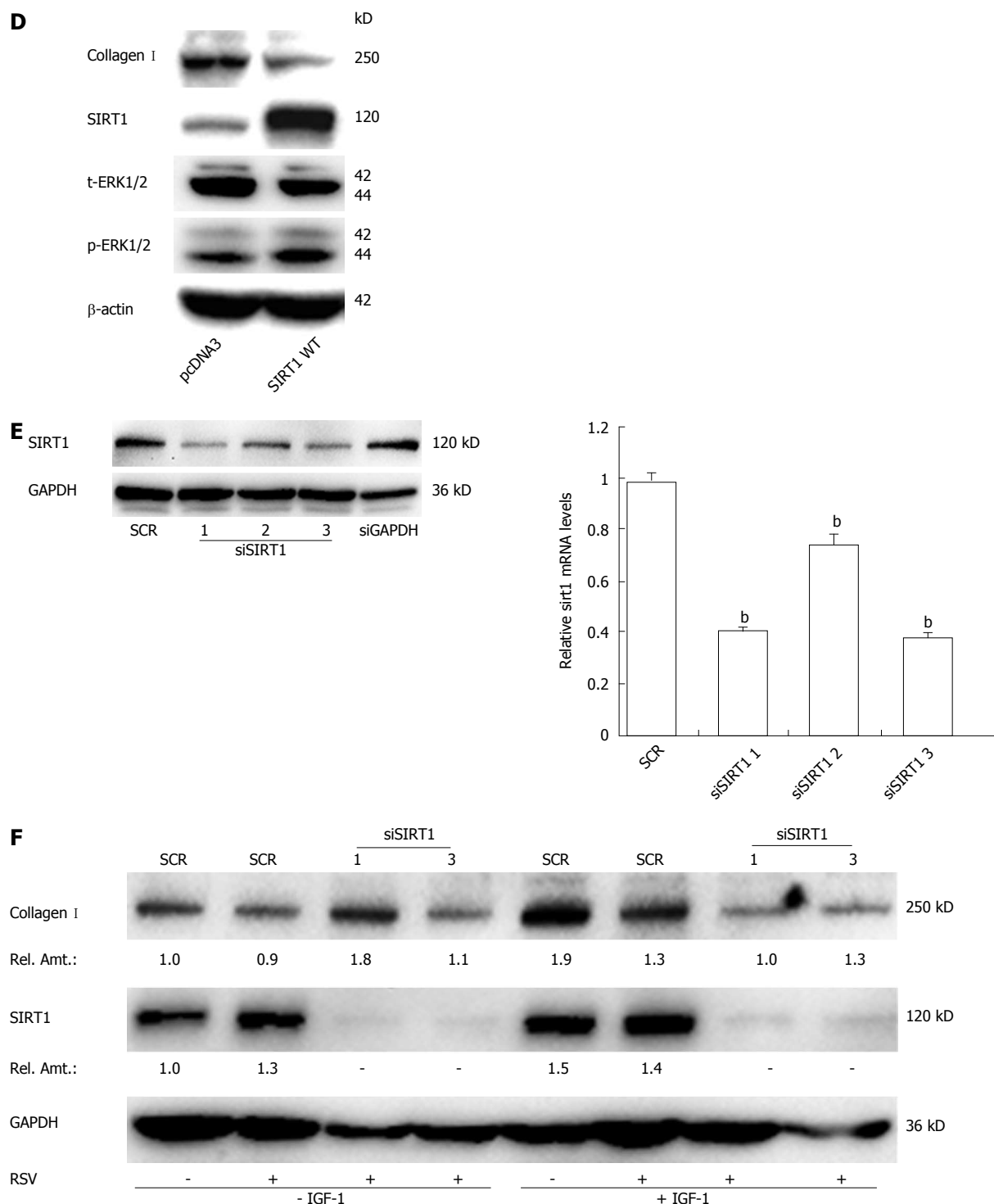
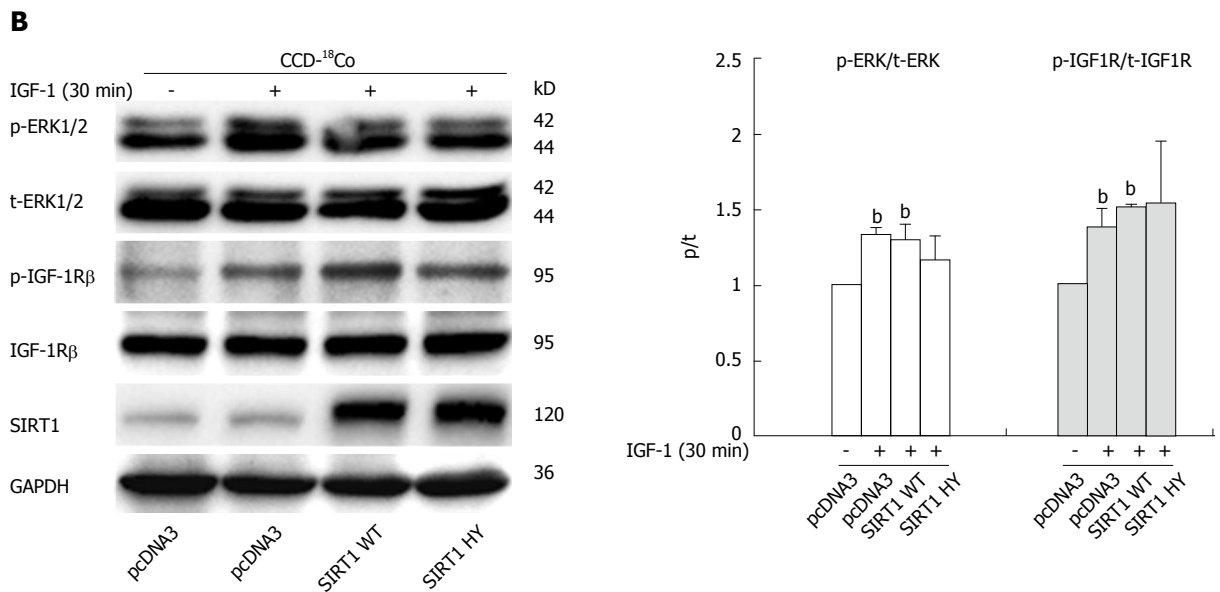


Figure 3 Resveratrol inhibits insulin growth factor-1-induced collagen I expression partly through silent information regulator 1. A, B: Collagen I protein and col1a2 mRNA expression were measured in fibroblasts stimulated with insulin growth factor-1 (IGF-1) (100 ng/mL) or IGF-1 plus resveratrol (RSV 50, 100 μ mol/L) for 24 h; C: Wild type silent information regulator 1 (SIRT1) (WT) or enzyme deficient SIRT1 (HY) constructs were transfected into CCD-¹⁸Co cells followed by treatment with IGF-1. Collagen I protein was measured by Western blotting; D: Cells were transfected with the empty vector (pcDNA3.0) or SIRT1 WT, then collagen I, SIRT1, phosphorylation of extracellular signal-regulated kinase (ERK) (p-ERK) or total ERK1/2 (t-ERK1/2) were examined by Western blotting; E: 293T cells were transiently transfected with SIRT1-specific small interfering RNAs (siRNAs) (1, 2, 3) or scrambled siRNA (SCR) for 48 h, then SIRT1 protein and mRNA expression was measured by Western blotting and quantitative real-time polymerase chain reaction; F: CCD-¹⁸Co cells were transfected with SIRT1 siRNAs (1, 3) or SCR followed by treatment with resveratrol. The collagen I /glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ratio shows the quantification of the expression levels of collagen I (Relative Amount). The experiment was repeated three times and obtained similar results. Values represent mean \pm SD. ^a $P < 0.05$, ^b $P < 0.01$ vs control.

tract^[15,39]. Since IGF-1 is regarded as a principal mediator of intestinal fibrosis, we investigated the effect of IGF-1 on collagen I synthesis in intestinal fibroblasts.

Here, we reported that IGF-1 increased the protein and mRNA expression of collagen I in intestinal fibroblasts and that this upregulation was inhibited by pre-treatment



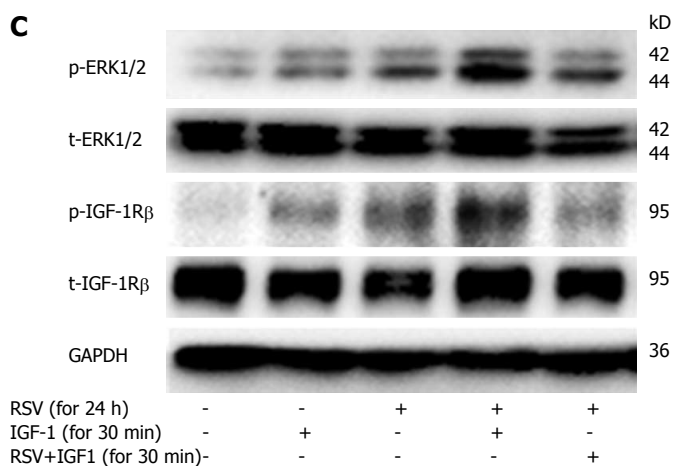


Figure 4 Resveratrol attenuates insulin growth factor-1 receptor and extracellular signal-regulated kinase 1/2 phosphorylation induced by insulin growth factor-1 via a silent information regulator 1-independent pathway. A: Mouse intestinal fibroblasts (MIFs) and CCD-¹⁸Co cells were treated with 100 ng/mL insulin growth factor-1 (IGF-1) in the absence or presence of resveratrol (100 μ mol/L) for 30 min; B: CCD-¹⁸Co cells were transfected with silent information regulator 1 (SIRT1) expression constructs [wild type SIRT1 (WT) or enzyme deficient SIRT1 (HY)] followed by exposure to 100 ng/mL IGF-1 for 30 min. The phosphorylated levels of IGF-1R and extracellular signal-regulated kinase (ERK)1/2 were measured; C: Serum-starved quiescent CCD-¹⁸Co cells were pretreated with 100 μ mol/L of resveratrol for 24 h, then removed and stimulated with IGF-1 alone (column 4) or IGF-1 plus resveratrol (column 5) for 30 min. The bar graph represents the quantitation of the Western blotting normalized to the control. The experiment was repeated three times and obtained similar results. Values represent mean \pm SD. * P < 0.01 vs control.

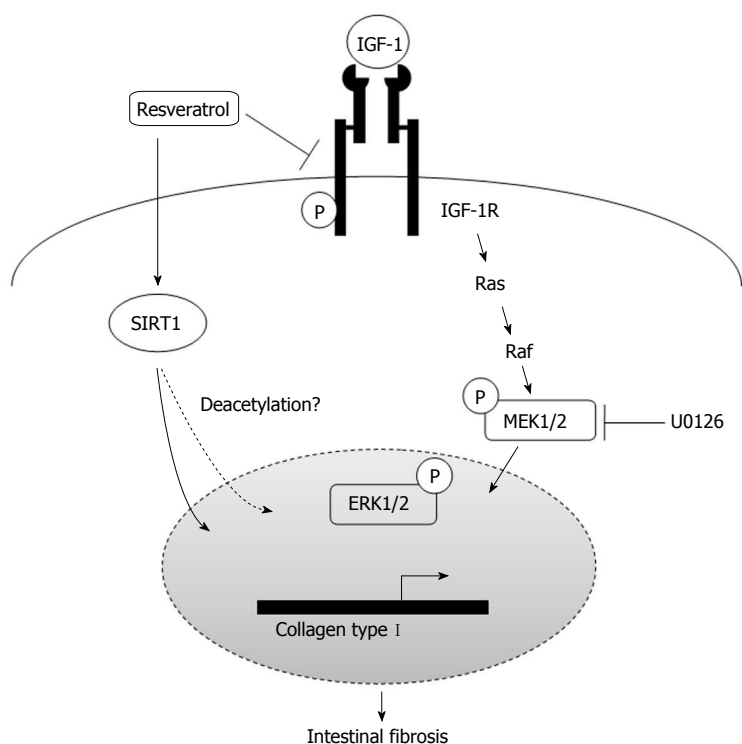


Figure 5 Proposed mechanism by which resveratrol inhibits insulin growth factor-1-induced collagen I synthesis in intestinal fibroblasts. SIRT1: Silent information regulator 1; IGF-1: Insulin growth factor-1; ERK: Extracellular signal-regulated kinase; MEK: Mitogen-activated protein/extracellular signal-regulated kinase; IGF-1R: IGF-1 receptor.

with MEK1/2 inhibitor U0126. The phosphorylation of IGF-1R and ERK1/2 then gradually increased in a time-dependent manner after the incubation with IGF-1. Thus, these data suggest that IGF-1 stimulates collagen I synthesis in intestinal fibroblasts and that its action mechanism could be attributed to the IGF-1/IGF-1R/ERK1/2 pathway.

Previous *in vitro* studies have shown that resveratrol

causes cell cycle arrest, decreased collagen synthesis, and apoptosis in rat intestinal smooth muscle cells^[27]. We next explored the molecular mechanisms of anti-fibrosis effect of resveratrol in intestinal fibroblasts. Our experiments suggested that resveratrol significantly decreased collagen I expression induced by IGF-1 and resveratrol alone also inhibited collagen I synthesis. Resveratrol is known to activate deacetylase SIRT1, and this com-

pound can also inhibit a number of other signaling pathways^[40-42]. Several lines of evidence indicate that SIRT1 may play an important role in organ fibrosis. SIRT1 has been documented to inhibit tumor necrosis factor- α -induced inflammation in NIH/3T3 fibroblast cell line^[43]. SIRT1 deacetylates smad3 and suppresses the transforming growth factor- β 1-driven renal fibrosis^[19]. Therefore, we used several approaches to examine whether the ability of resveratrol to inhibit collagen I operated *via* SIRT1. First, overexpression of WT but not HY SIRT1 provided a protective effect against IGF-1-induced collagen I synthesis in CCD-18Co. Second, knockdown of SIRT1 protein reversed the effect of resveratrol. These results indicate that resveratrol may protect against fibrosis through up-regulation of SIRT1 and enzymatic activity of SIRT1 may be responsible for its inhibitory effect on collagen I synthesis. Whether the mechanism underlying the effects is deacetylase activity of SIRT1 remains to be determined.

Since the IGF-1/IGF-1R/ERK1/2 signaling pathway has been previously described to be necessary and sufficient for the induction of collagen by IGF-1, we focused our investigation on the effect of resveratrol on IGF-1 signaling. Whether the expression and/or activity of IGF-1R were influenced by resveratrol? Our data demonstrated that treatment with resveratrol plus IGF-1 for 30 min inhibited the activation of IGF-1R and its downstream signaling molecules such as MAP-kinase (ERK1/2) in intestinal fibroblasts. Treatment with resveratrol for 24 h or overexpression of SIRT1 WT resulted in the down-regulation of collagen I expression in fibroblasts. However, overexpression of SIRT1 WT or HY had no effect on activation of IGF-1R. Thus, our findings suggest that resveratrol exerts its negative effect on the phosphorylation of IGF-1R independent of activating SIRT1, and resveratrol may regulate IGF-1R activity by directly inhibiting IGF-1 binding to its receptor. Further studies are needed to confirm these findings and elucidate the exact molecular mechanism underlying resveratrol-inhibited activation of IGF-1R.

In summary, our findings (Figure 5) allude to a scheme wherein upon challenge with IGF-1, collagen I expression is increased in intestinal fibroblasts, which can be repressed by resveratrol through either activating SIRT1 or inhibiting activation of IGF-1R. Collectively, these observations provide a new mechanistic framework to better understand the effects of SIRT1 activators on intestinal fibrosis, and will allow us to examine the possibility that dysregulation of the IGF-1/IGF-1R/ERK1/2 axis is involved in collagen synthesis in fibroblasts.

COMMENTS

Background

Intestinal fibrosis is an incurable complication of Crohn's disease involving mesenchymal cell proliferation and extracellular matrix deposition. Until now, no effective therapy exists for averting such fibrogenic events. Insulin-like growth factor-1 (IGF-1), a potent profibrotic mediator, has been reported to be involved in gastrointestinal tract growth and tissue repair. Resveratrol is a polyphenol

naturally occurring in grapes and its putative antifibrotic actions have been demonstrated in models of colitis.

Research frontiers

Mounting evidence suggests that resveratrol has anti-inflammatory and antifibrotic effects in the animal models of colitis. Resveratrol diminishes IGF-1-stimulated collagen production in intestinal smooth muscle cells. However, the mechanism of resveratrol on collagen I synthesis in intestinal fibroblasts remains unclear.

Innovations and breakthroughs

In this study, the authors found that resveratrol down-regulated IGF-1-induced collagen I synthesis in intestinal fibroblasts by inhibiting IGF-1 receptor (IGF-1R) phosphorylation and its downstream extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase signaling pathway. In addition, resveratrol alone suppressed collagen I synthesis through up-regulating activity of silent information regulator 1 (SIRT1).

Applications

These findings highlight a previously unknown function of resveratrol on IGF-1R activation and provide novel insight of resveratrol as a therapeutic agent for intestinal fibrosis.

Terminology

SIRT1 is a nicotinamide adenine dinucleotide-dependent deacetylase which modulates metabolic homeostasis, stress resistance, cellular survival, cellular senescence/aging, inflammation-immune function, endothelial functions by deacetylating a number of key transcription factors.

Peer review

The authors investigated the effect of resveratrol on collagen I synthesis in intestinal fibroblasts and explored the mechanism. Resveratrol effectively decreased collagen I expression in IGF-1-stimulated fibroblasts by inhibiting IGF-1R/ERK1/2 signaling in a SIRT1 independent manner. However, resveratrol alone inhibited collagen I synthesis by activating SIRT1. Overall, this manuscript is highly relevant and interesting.

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Protective effect of glutamine on intestinal injury and bacterial community in rats exposed to hypobaric hypoxia environment

Chun-Lan Xu, Rui Sun, Xiang-Jin Qiao, Cui-Cui Xu, Xiao-Ya Shang, Wei-Ning Niu

Chun-Lan Xu, Rui Sun, Xiang-Jin Qiao, Cui-Cui Xu, Xiao-Ya Shang, Wei-Ning Niu, The Key Laboratory for Space Bioscience and Biotechnology, School of Life Sciences, Northwestern Polytechnical University, Xi'an 710072, Shaanxi Province, China
Author contributions: Xu CL conceived and designed the study; Sun R, Qiao XJ and Xu CC performed the experiments; Sun R analyzed the data; Shang XY and Niu WN contributed reagents/materials/analysis tools; Xu CL wrote the manuscript.

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Correspondence to: Chun-Lan Xu, Associate Professor, The Key Laboratory for Space Bioscience and Biotechnology, School of Life Sciences, Northwestern Polytechnical University, 127 Youyixi Road, Xi'an 710072, Shaanxi province, China. clxu@nwpu.edu.cn

Telephone: +86-29-88460543 Fax: +86-29-88460332

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Abstract

AIM: To investigate the protective effect of glutamine (Gln) on intestinal injury and the bacterial community in rats exposed to hypobaric hypoxia environment.

METHODS: Sprague-Dawley rats were divided into control, hypobaric hypoxia (HH), and hypobaric hypoxia + Gln (5.0 g/kg BW·d) (HG) groups. On the first 3 d, all rats were placed in a normal environment. After the third day, the HH and HG groups were transferred into a hypobaric chamber at a simulated elevation of 7000 m for 5 d. The rats in the HG group were given Gln by gavage daily for 8 d. The rats in the control and HH groups were treated with the same volume of saline. The intestinal morphology, serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), interleukin-6

(IL-6), tumor necrosis factor- α (TNF- α), interferon-gamma (IFN- γ) and diamino oxidase (DAO) were examined. We also evaluated the expression levels of occludin, toll-like receptor 4 (TLR4), nuclear factor- κ B p65 (NF- κ B p65) and myeloid differentiation factor 88 (MyD88), and examined the bacterial community in caecal contents.

RESULTS: Hypobaric hypoxia induced the enlargement of the heart, liver, lung and kidney, and caused spleen atrophy. Intestinal villi damage was also observed in the HH group. Supplementation with Gln significantly alleviated hypobaric-induced damage to main organs including the intestine, increased serum SOD (1.14 ± 0.03 vs 0.88 ± 0.04 , $P < 0.05$) and MDA (8.35 ± 1.60 , $P < 0.01$) levels and decreased serum IL-6 (1172.13 ± 30.49 vs 1407.05 ± 34.36 , $P < 0.05$), TNF- α (77.46 ± 0.78 vs 123.70 ± 3.03 , $P < 0.001$), IFN- γ (1355.42 ± 72.80 vs 1830.16 ± 42.07 , $P < 0.01$) and DAO (629.30 ± 9.15 vs 524.10 ± 13.34 , $P < 0.001$) levels. Moreover, Gln significantly increased occludin (0.72 ± 0.05 vs 0.09 ± 0.01 , $P < 0.001$), TLR4 (0.15 ± 0.05 vs 0.30 ± 0.09 , $P < 0.05$), MyD88 (0.32 ± 0.08 vs 0.71 ± 0.06 , $P < 0.01$), and NF- κ B p65 (0.16 ± 0.04 vs 0.44 ± 0.03 , $P < 0.01$) expression levels and improved the intestinal bacterial community.

CONCLUSION: Gln treatment protects from intestinal injury and regulates the gut flora imbalance in hypoxia environment. These effects may be related to the TLR4/MyD88/NF- κ B signaling pathway.

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Key words: Hypobaric hypoxia; Glutamine; Intestinal mucosa; Immunomodulation; Bacterial community

Core tip: Gastrointestinal problems at high altitudes are common. Gut microbes may also play an important role in host health. Glutamine has been demonstrated

to be an important source of fuel for the gut. In the study, we investigated the protective effect of glutamine on intestinal barrier damage induced by hypobaric hypoxia. The research provides a basic understanding of possible mechanism of hypobaric hypoxia-induced damage of intestinal barrier function and bacterial community imbalance. The altered bacterial communities in the intestine and the toll-like receptor 4/myeloid differentiation factor 88/nuclear factor- κ B signal pathway may represent the significant therapeutic targets for the prevention/treatment of intestinal barrier dysfunction and consequent intestinal diseases.

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INTRODUCTION

High altitudes create a special type of environment because the atmospheric pressure is lower than it is at sea level. However, more than 140 million people permanently live at a high altitude (> 2500 m) in North America, Central America, South America, East Africa, and Asia. Furthermore, every year several hundred thousand people from lowland areas move to higher altitudes for work or travel. High altitude hypoxia is a challenge for people residing in or visiting high altitudes. The exposure to high altitude causes severe damage to different organs, especially the intestinal tract. The incidence of digestive system disease is reported to be higher among high-altitude residents and immigrants^[1,2]. The primary function of the intestinal tract is to regulate water, electrolyte and nutrient transport. To perform these functions, the epithelium lining the intestinal tract is in close contact with the gastrointestinal lumen. Because the lumen is connected to the external environment and may have a high bacterial and antigen load, the epithelium must also prevent pathogenic agents within the gastrointestinal lumen from gaining access to internal tissues^[3]. Hypoxia may induce severe primary intestinal barrier dysfunction, promote bacterial and endotoxic translocation, and cause systemic inflammatory response; it is the major factor causing high-altitude multiple organ dysfunction syndrome^[4]. During studies of the complex physiological function of the intestine, we noted that the intestine is not only an important organ of digestion and nutrient absorption, but also has immunomodulatory, endocrine, and mucosal barrier functions. Intestinal mucosal barrier function is an important part of the barrier system of the body and has been studied by many researchers. It is composed of a mechanical barrier, an immune barrier, a chemical barrier, and a biological barrier. The different structures, molecular mechanisms,

and biological functions of each barrier allow them to collectively defend against the invasion of foreign antigens through combined signaling pathways^[5]. Although acute hypobaric hypoxia is the most common pattern, studies on this issue are limited.

Glutamine (Gln) has not traditionally been used as a nutritional supplement because it is synthesized endogenously and is considered a “non-essential amino acid”^[6]. However, some studies have found that the effect of Gln is far more than that of a “non-essential amino acid”. Gln consumed as a “conditionally essential amino acid” is a special nutrient under physiological conditions and maintains normal immunological function under stress or pathological conditions. Gln plays a significant role in adjusting the cellular metabolism and cellular immune function^[7]. Gln is a major source of energy for enterocytes and supports nucleotide biosynthesis. Additionally, Gln may protect epithelial cells against endotoxin/oxidant-related injury and enhance the expression of heat stress proteins following stress in gastrointestinal tract therapy^[8]. Long-term treatment with Gln that was started before advanced age prevented the loss of body weight without limiting sarcopenia and had a beneficial effect on enterocytes in very old rats^[9]. Currently, the protective effect of Gln on intestinal mucosal barrier function is still unknown under hypobaric hypoxia.

Gut microbes may also play an important role in host health^[10]. In the absence of the gut microbiota, normal immune development and function are impaired. Understanding the influence of hypoxia on the composition of the microbial community in the intestine is crucial for regulating the microflora, and will improve gut health. Therefore, the present study was conducted to investigate the unique role of glutamine in the preservation of epithelial barrier function in the gastrointestinal tract of rats exposed to a hypobaric hypoxia environment. We observed the ultrastructure of the duodenum, jejunum, and ileum, evaluated changes in the expression of occludin in the ileum and detected several serum inflammatory mediators. In addition, we investigated the role of the toll-like receptors (TLRs)/myeloid differentiation factor 88 (MyD88)/nuclear factor- κ B (NF- κ B) signaling pathway in the protective effect of Gln on intestinal barrier damage induced by hypobaric hypoxia. We also analyzed the bacterial community in the intestinal contents.

MATERIALS AND METHODS

Reagents

The main reagents used in this study were the following: L-glutamine (Xi'an Guoan, China), MDA kit (Nanjing Jiancheng, China), SOD kit (Nanjing Jiancheng, China), Total protein kit (Nanjing Jiancheng, China), 4% paraformaldehyde (Beijing Dingguo Changsheng Biotechnology Co., Ltd.), rat IL-6, TNF- α , IFN- γ , and DAO enzyme linked immunosorbent assay (ELISA) kits (RD systems, United States), NF- κ B p65 and TLR4 rabbit polyclonal antibody, occludin rabbit polyclonal antibody, β -actin mouse polyclonal antibody (Santa Cruz, United States), and MyD88

rabbit polyclonal antibody (Abcam, United States).

Animals

This study was approved by the Institutional Animal Care and Use Committee of the Northwestern Polytechnical University and was conducted in accordance with the National Institutes of Health guidelines for the care and use of experimental animals. Thirty adult male Sprague-Dawley (SD) rats (200 ± 20 g) were purchased from the Experimental Animal Center of College of Medicine, Xi'an Jiaotong University.

Experimental regimen

Thirty male rats were randomly divided into three groups of ten rats each as follows: a normal control group (Control), a hypobaric hypoxia group (HH), and a hypobaric hypoxia plus Gln group (HG). For the first 3 d, all of the rats were placed in a normal environment. During this period, the rats in the HG group were given 5.0 g/kg BW/d Gln by gavage daily. The rats in the control and HH groups received intragastric administration of an equal volume of saline. The rats in HH and HG groups were transferred to a hypobaric chamber (Guizhou Fenglei Aviation Ordnance Co., Ltd, China) simulating an elevation of 7000 m for 5 d. During the hypoxia treatment, all of the rats were treated with saline or Gln as previously described. The chamber altitude was returned to sea level daily for 30 min to clean the cages, replenish the food and water and give drugs. All of the animals had free access to food and water and were weighed daily.

Animal observation and sample collection

The rats were weighed before gavage every day, and their mental state, spontaneous activity, and eating status were monitored.

Five days after exposure to hypobaric hypoxia, the rats were anesthetized with ether, and the abdomen was opened to collect 5 mL of blood from the abdominal aorta. The blood was centrifuged at 1000 *g* for 10 min at 4 °C, and the serum was separated and stored in Eppendorf tubes. The general conditions of the rats and overall changes in the abdominal cavity were observed. Additionally, the heart, liver, spleen, lungs and kidney were removed from the rats and weighed. Approximately 5 cm of the duodenum, jejunum and ileum were collected into RNAase-free tubes. Caecal contents were collected and stored in freezing tubes. All of the samples were frozen by immersion in liquid nitrogen and stored at -80 °C until needed for analysis.

Body, heart, liver, lung, kidney, and spleen weight

The rats were sacrificed after completing the hypoxia. The body weight and weights of the heart, liver, lung, kidney and spleen were determined for each animal. The organ index was calculated as percentage of body weight.

Light microscopy for observation of intestinal morphology

Approximately 2 cm of the duodenum, jejunum and

ileum were obtained and cut open longitudinally and transversely. Then each collected intestinal segment was washed with normal saline immediately, fixed in 4% formaldehyde at 4 °C for 24 h, rinsed with phosphate buffered solution (PBS) and embedded in paraffin. The tissue was consecutively cut into 4-μm thick sections that were stained with hematoxylin and eosin (HE). The intestinal morphology was observed using a fluorescence microscope (Nikon, Japan), and the length and area of the intestinal villi were measured and calculated according to the following equation: $\text{Area} = 2\pi rh$, where *r* represents the radius of the villus and *h* is the villus height.

Measurement of serum SOD and MDA levels

Approximately 0.1 mL serum was used to detect the SOD activity and measure the MDA content. The SOD activity was measured using the hydroxylamine method. The MDA content was measured by the thiobarbituric acid colorimetric method. The kits were used according to the manufacturer's instructions. The absorbance was measured at 550 and 532 nm, respectively, using an ultraviolet spectrophotometer (HITACHI, Japan). The activity of SOD was expressed as units per mL. The MDA content was calculated using the following formula: (nmol/mL) = [A(sample) - A(sample blank)]/[A(standard) - A(standard blank)], where A represents the absorbance value.

Detection of serum IL-6, TNF-α, IFN-γ, and DAO levels

The levels of IL-6, TNF-α, IFN-γ, and DAO in the serum were detected using commercially available ELISA kits according to the manufacturer's recommended protocol. A Synergy HT Multi-Detection Microplate Reader (Bio-Tek) was used to read the optical density at 450 nm. The concentrations of IL-6, TNF-α, IFN-γ, and DAO in the samples were determined using a standard curve.

Western blotting for detection of NF-κB p65, TLR4, MyD88 and occludin expression

Ileum mucosal tissues (100 mg) were homogenized in 1 mL lysis buffer (Sangon Biotech, China). The total protein was extracted with a Protein Extract Kit (Beyotime, China) according to the manufacturer's instructions. The protein concentration was measured *via* a bicinchoninic acid assay using a BioRad protein microassay (BioRad, Hercules, CA). An aliquot containing 30 μg of protein was diluted in loading buffer (loading buffer: sample = 5:1, v/v) and heated to 98 °C for 10 min. The protein sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred onto a 0.45 μm-pore polyvinylidene difluoride membrane (PVDF, Immuno-Blot, BioRad) at 100 V for 1 h. The membranes were blocked at room temperature for 2 h with 5% fat-free milk in PBS-T (PBS with 0.1% Tween-20). The following primary antibodies were used: NF-κB p65 (1:500), TLR4 (1:1000), MyD88 (1:500), occludin (1:500), and β-actin (1:1000). The primary antibodies were incubated at 4 °C overnight. After washing with PBS-T (0.1% BSA), the membranes were

incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (diluted 1:3000; Boster Co., Wuhan, China) for 2 h at room temperature. After additional washing, bound conjugates were detected with ECL SuperSignalTM West Pico substrate (Pierce, Rockford, IL, United States). The proteins were visualized by exposing the blot to an X-ray film and were photographed with a digital camera. The net intensities of individual bands were measured using Quantity One (version 4.6.2). The relative expression levels of the proteins were expressed as the gray value of the target band over the gray value of β -actin in the same sample.

Composition and diversity of bacterial community through 454 pyrosequencing analysis

Genomic DNA in caecal contents was extracted using the E.N.Z.A.® DNA Kit (Omega Bio-Tek) according to the manufacturer's protocol with a slight modification, then identified by 1% agarose gel electrophoresis. DNA purity and concentration were analyzed using the ultraviolet spectrophotometer (HITACHI, Japan). According to the specific sequence region (533R-27F) in the 16S rRNA gene that covering the V1-V3 region, the bar-coded primers 27F and 533R containing the A and B sequencing adaptors were synthesized and used to amplify this region. The forward primer (B-27F) was 5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAGTTTGATCCTGGCTCAG*-3', where the sequence of the B adaptor is shown in italics and underlined. The reverse primer (A-533R) was 5'-*CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNNTTACCGCGGCTGCTGGCAC*-3', where the sequence of the A adaptor is shown in italics and underlined and the series of Ns represent an eight-base sample specific barcode sequence. The identified DNA was subjected to polymerase chain reaction (PCR) using TranStartFastpfu DNA Polymerase (MBI. Fermentas, United States) in a 20 μ L volume containing 5 mmol each of the primer, 10 ng of template DNA, 5 \times FastPfu Buffer, and 1 U of FastPfu DNA Polymerase. PCR was performed in a thermocycler (Gene Amp® PCR System 9700, ABI, United States). The PCR profile included denaturation at 95 °C for 2 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Triplicate PCR products of the same sample were mixed, and then detected by 2% agarose gels electrophoresis containing ethidium bromide. PCR products were recycled and purified with an AxyPreDNA gel extraction kit (Axygen, China) according to the manufacturer's instructions. The recycled PCR products were visualized on agarose gels. Furthermore, the PCR products were quantitatively determined using QuantiFluorTM-ST Fluoremeter (Promega, United States) and PicoGreen® dsDNA Quantitation Reagent (Invitrogen, Germany). Following quantitation, the amplification products from each reaction mixture were pooled in equimolar ratios based on their concentrations and were sub-

jected to emulsion PCR (emPCR) using RocheGS FLX Titanium emPCR kits to generate amplification libraries. Amplification pyrosequencing was performed from the A-end using a 454/Roche A sequencing primer kit on a Roche Genome Sequencer GS FLX Titanium platform at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

Statistical analysis

Most of the data except for the bacterial community analysis are presented as mean \pm SEM. The data were analyzed by one-way analysis of variance and Student's *t*-test (version 9.1, SAS, NC, United States). Differences were considered to be statistically significant at $P < 0.05$. The pyrosequencing data were subjected to bioinformatic analysis. Prior to analysis, the original pyrosequencing data must be filtered and optimized to obtain the valid and trimmed sequences through Seqcln and Mothur (http://sourceforge.net/projects/seqclean/http://www.mothur.org/wiki/Main_Page). Then, these trimmed sequences were analyzed from two aspects: operational taxonomic units (OTUs) cluster (97% similarity) and taxonomy which were mainly performed on Mothur (<http://www.mothur.org>) and compared with the Bacterial SILVA database (<http://www.arb-silva.de/>), by methods of kmer searching (<http://www.mothur.org/wiki/Align.seqs>) and UCHIME (<http://drive5.com/uchime>). Rarefaction analysis and Good's coverage for the nine libraries were determined. Community figure was generated using R tools according to the data from document "tax.phylum.xls". Heatmap figure was generated using Vegan-package (distance measure with Bray-Curtis; cluster analysis with complete).

RESULTS

General conditions including body, heart, liver, lung, kidney, and spleen weights

There were no animal deaths during the experiment. The rats in the control group were active and energetic and had no pathological reaction in the abdominal cavity that was visible to the naked eye. The rats in the HH and HG groups were less active and were notably more tired. Prominent swelling was observed in their intestinal canals, and the intestinal mucosa was congested. While in the hypobaric chamber, the food intake was significantly lower in the HH and HG groups than in the control group. Thus, reduced body weight was observed in all rats in the groups exposed to hypobaric hypoxia (Figure 1). On the fifth day of exposure to the hypobaric chamber, the body weights of the rats in group HG increased and were higher than those in the HH group. The eyes of the rats from the HH and HG groups were reddish brown. Table 1 showed that the heart, liver, kidney, and lung indices in the HH group were significantly increased compared to the control group ($P < 0.01$; $P < 0.05$; $P < 0.05$; $P < 0.001$, respectively). However, the spleen index in the HH group decreased compared to the control

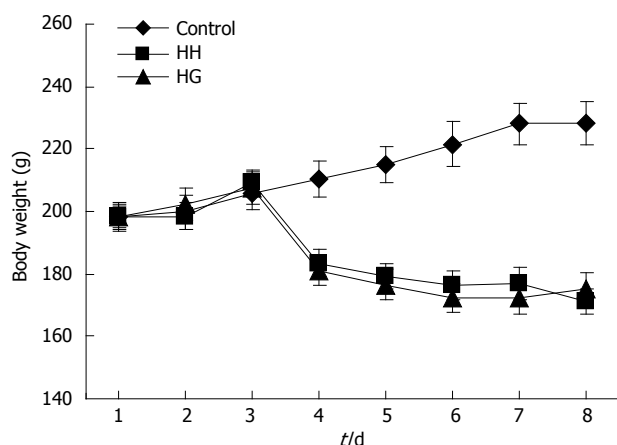


Figure 1 Effects of glutamine treatment on body weight in rats of different groups. Gln: Glutamine; Control: Control group; HH: Hypobaric hypoxia group; HG: Hypobaric hypoxia plus Gln group.

group ($P < 0.05$).

Observation of morphology of the intestine using light microscopy

The intestinal villi with intact epithelia were dense and long and showed an ordered arrangement in the control group. Additionally, the intestinal mucosa was smooth and thick. There were no detected defects in the intestinal mucosa and villi using a light microscope (Figure 2). The intestinal villi in the HH group were sparse, short, and defective and had a scattered arrangement. In addition, the lodged and exfoliated villi became thinner than those in the control group. The tight junctions between the intestinal epithelial cells were widened. The intestinal mucosa was exfoliated and showed signs of atrophy. Compared with the HH group, the intestinal villi in the HG group were relatively intact, long, and dense and showed an orderly arrangement. A statistical analysis showed that the height of the intestinal villi ($P < 0.001$), the thickness of the mucosa ($P < 0.001$), and the villi area ($P < 0.001$) were significantly decreased in the HH group as compared to the control group (Table 2). Treatment with Gln alleviated the damage to the intestine morphology and structure in rats exposed to hypobaric hypoxia.

Serum total SOD activity and MDA concentration

The total serum total SOD activity and MDA concentration in the HH group were significantly lower than those in the control group ($P < 0.001$) (Figure 3). However, supplementation with Gln significantly increased ($P < 0.05$) the total SOD activity and decreased ($P < 0.01$) the MDA concentration in serum compared to the HH group. The HH group values were significantly increased compared to the control group.

Serum IL-6, TNF- α , IFN- γ , and DAO levels

As shown in Figure 4, serum levels of IL-6 ($P < 0.05$), TNF- α ($P < 0.001$), and IFN- γ ($P < 0.01$) in the HH group were significantly higher than those in the control

group. This result suggests the presence of hypoxia-induced inflammatory response. The levels of IL-6 ($P < 0.05$), TNF- α ($P < 0.001$), and IFN- γ ($P < 0.01$) in the serum of rats from the HG group were lower than those from the HH group. Moreover, serum DAO levels in the HH and HG groups were lower than those in the control group ($P < 0.05$). The administration of Gln significantly increased serum DAO levels compared to the HH group ($P < 0.001$). These results suggested that supplementation with Gln reversed hypoxia-induced increases of inflammatory mediators.

Protein expression of occludin, TLR4, MyD88 and NF- κ B p65 in ileum tissues

Western blotting analysis (Figure 5) showed that the expression levels of NF- κ B p65 ($P < 0.01$), MyD88 ($P < 0.01$) and TLR4 ($P < 0.05$) in the HH group were higher than those in the control group. Supplementation with Gln significantly decreased the expression levels of NF- κ B p65 ($P < 0.01$), MyD88 ($P < 0.01$), and TLR4 ($P < 0.05$) compared with the HH group. However, the influence of hypobaric hypoxia on occludin expression was contrary to the expression of NF- κ B p65, MyD88 and TLR4. The expression level of occludin in the HH group was decreased significantly as compared to the control group. The supplementation with Gln rescued the hypoxia-induced reduction of occludin, and the HG group showed increased occludin expression.

Bacterial composition and diversity in caecal contents

A total of 80521 valid reads and 9679 OTUs were obtained from the nine samples through 454 pyrosequencing analysis. The rarefaction curves tended to approach the saturation plateau (data not shown). Good's coverage estimations revealed that 92%-95% of the species were obtained in all of the samples. All of the sequences were classified from phylum to genus according to the program Mothur using the default setting, and 14 different genus groups were identified from these samples. The nine libraries showed very dissimilar 16S rRNA profiles at the genus level distribution (Figure 6). The HG libraries included the maximum number of genera and included the following: No_Rank, *Prevotellaceae_uncultured*, *Prevotella*, *Lactobacillus*, *Lachnospiraceae_uncultured*, *Ruminococcaceae_uncultured*, *Bacteroides*, *Peptostreptococcaceae_Incertae_Sedis*, *Alistipes*, *Lachnospiraceae_Incertae_Sedis*, *Treponema*, *Ruminococcus*, and *Wohlfabritiimonas*. These were the most important groups and accounted for 96% of the reads. The HH libraries showed relatively simple diversity and contained the lowest number of *Lactobacillus*, *Peptostreptococcaceae_Incertae_Sedis* and *Treponema*, and the highest number of *Prevotellaceae_uncultured* and *Prevotella*. Compared with the other two groups, the numbers of *Acinetobacter*, *Comamonas*, *Enterobacter*, and *Enterococcus Wohlfabritiimonas* in the HG libraries were high. A hierarchically clustered heatmap analysis based on the bacterial community profiles at the family level indicated that the HH samples clustered with the control and HG samples

Table 1 Effects of glutamine treatment on visceral indices (mg/g) in experimental rats

Group	Heart	Liver	Kidney	Lung	Spleen
Control	3.900 ± 0.173	28.330 ± 0.513	3.933 ± 0.240	5.433 ± 0.267	2.933 ± 0.117
HH	4.800 ± 0.000 ^b	34.290 ± 0.751 ^b	8.200 ± 0.307 ^b	9.257 ± 0.281 ^d	2.583 ± 0.175 ^a
HG	4.729 ± 0.000	32.960 ± 0.909	6.600 ± 0.860 ^c	8.814 ± 0.451	2.650 ± 0.140

The cardiac, hepatic, renal, and lung indices in SD rats in the HH group were increased compared to the control group, but the spleen index in the HH group decreased. The visceral indices in the HG group had no obvious differences compared to the HH group. ^a*P* < 0.05, ^b*P* < 0.01, ^d*P* < 0.001 *vs* Control; ^c*P* < 0.05 *vs* HH. Values are expressed as mean ± SEM (*n* = 7, each). Gln: Glutamine; Control: Control group; HH: Hypobaric hypoxia group; HG: Hypobaric hypoxia plus Gln group.

in order (Figure 7).

DISCUSSION

Gastrointestinal problems at high altitudes are common. Special geological and climatic environments might cause the decrease of body resistance and the increase of susceptibility to intestinal diseases observed for humans or animals exposed to high altitudes. There were currently no effective measures to prevent or treat intestinal diseases. In the current study, the body weight of rats decreased after exposure to hypobaric hypoxia at a stimulated elevation of 7000 m for 5 d. However, if we supplemented the rats with Gln daily for eight consecutive days, including three days before entry into hypobaric chamber, the body weight of the Gln-treated rats recovered on the eighth day. Moreover, hypobaric hypoxia induced the enlargement of the heart, liver, kidney, and lung, and caused spleen atrophy. However, supplementation with exogenous glutamine effectively alleviated the occurrence of the above pathological phenomenon. We know that Gln as a conditionally essential amino acid, has many roles in the human body. Gln may enhance immune function in individuals who are critically ill and immune suppressed, prevent infection in postsurgical patients, and support the integrity of the gut mucosa after intestinal damage. In the presence of critical illness and catabolic stress, the body's glutamine consumption exceeds the normal supply. Thus, Gln becomes an "essential" amino acid^[11,12].

The intestinal mucosa actively participates in host defense by engaging the mucosal immune system^[3]. However, the intestinal mucosa and villi of the rats were seriously injured by the hypobaric hypoxia environment. The villi height and crypt depth of the small intestine were significantly decreased. In addition, the intestinal villi had bizarre shape changes in the form of partial loss, sloughing and vacuolization because of the hypobaric hypoxia environment. The insufficient energy synthesis caused by hypoxia decreases the frequency of cilia swing, slows peristalsis, and inhibits self-cleaning in the intestinal tract. In addition, the blood and oxygen supplies required for the normal functioning of the intestinal mucosa vary greatly under different conditions. The special anatomical structures of the intestinal microvilli were extremely sensitive to hypoxia^[13,14]. Hypobaric hypoxic environments can also aggravate damage to the intestinal villi^[15]. However, the intestinal mucosa and villi of the rats

supplemented with Gln (5.0 g/kg•d) were normal and intact. Gln is a key factor in maintaining mucosal structure and may have special effects on the maintenance of tight junction and permeability of the intestinal mucosa. Oral glutamine may be effective in protecting the human intestinal mucosa^[16], enhancing the villi height of the jejunum and ileum^[17], preventing jejunal atrophy and mitigating the overall disruption^[18].

High altitude is characterized by hypobaric hypoxia, which is considered an acute physiological stress leading to oxidative stress^[19]. Oxidative stress describes the steady state level of oxidative damage in a cell, tissue, or organ, caused by the reactive oxygen species (ROS). ROS also triggers lipid peroxidation that is a chain reaction that provides a continuous supply of free radicals by oxidizing the polyunsaturated fatty acids in membranes and causing oxidative cell damage. MDA is formed as an end product of lipid peroxidation and acts as a main marker of endogenous lipid peroxidation^[20,21]. In the current study, supplementation with Gln significantly decreased MDA levels in serum. This result indicated that Gln could protect rats from hypoxia-induced lipid peroxidation. Although a variety of mechanisms contribute to protection against ROS-mediated cell and tissue injury, intracellular AOE, including SOD, are considered to play a major role. SOD catalyzes the conversion of superoxide radical to hydrogen peroxide. In the present study, SOD activities in the serum were substantially increased in rats treated with Gln. Therefore, antioxidant enzymes can alleviate the toxic effects of ROS and limit the effects of oxidant molecules on tissues. The antioxidant enzymes are active in the defense against oxidative cell injury because they are free radical scavengers^[22].

Studies have shown that the function of the intestinal barrier may be regulated by a network of multiple cytokines including ILs, IFNs, and TNF- α ^[23]. An imbalance of pro-inflammatory and anti-inflammatory cytokines is another important mechanism of intestinal mucosal injury. It is well established that hypobaric hypoxia causes mucosal hyperpermeability *in vivo*. Subsequently, the activity of innate immune cells is increased, and this is associated with the activation of the mucosal immune system^[24]. TNF- α , IL-6, and IFN- γ are important inflammatory factors that play important roles in various inflammatory reactions and are highly correlated with the severity of inflammation^[25]. INF- γ is a cytokine that is critical for innate and adaptive immunity against viral and

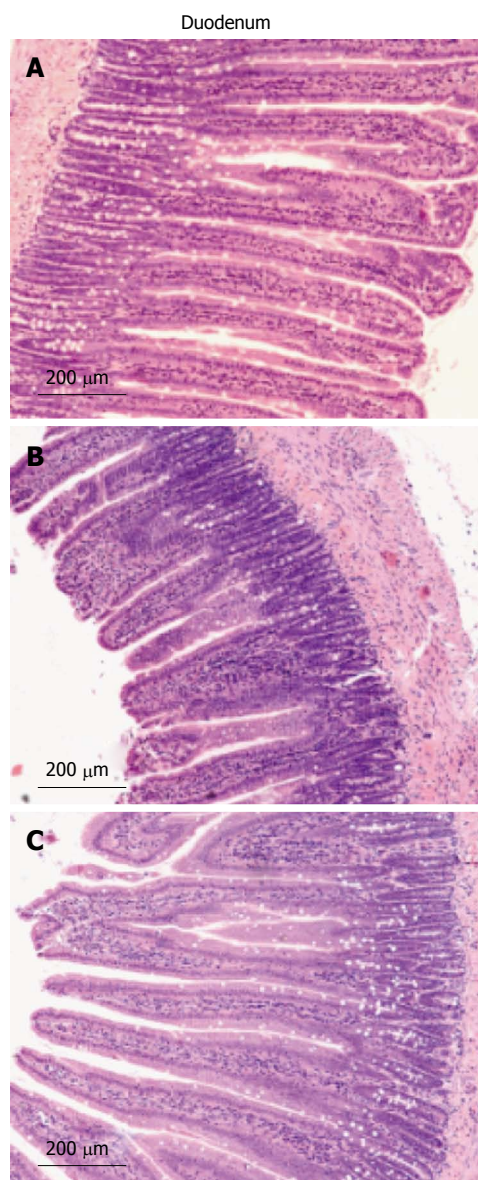


Figure 2 Effects of glutamine on intestinal morphology in rats. Smooth intestinal mucosa with intact epithelia and ordered villi in the control group, atrophic and thinned villi with a loose and disordered arrangement, and exfoliated and incomplete intestinal mucosa in the hypobaric hypoxia (HH) group, and relatively intact intestinal villi with an ordered and tight arrangement in the hypobaric hypoxia + Gln (5.0 g/kg BW·d) (HG) group (hematoxylin and eosin, $\times 200$). A: Control; B: HH; C: HG.

bacterial infections^[26]. Increases in serum TNF- α , IL-6, and INF- γ after hypobaric hypoxia stimulation were observed in the study. However, treatment with Gln significantly decreased the levels of TNF- α , IL-6, and INF- γ . This result suggests that Gln may improve the permeability of the intestinal mucosa and protect the intestine.

DAO belongs to the class of copper-containing amine oxidases that convert primary amines to corresponding aldehydes, hydrogen peroxide, and ammonia. Human DAO may play an important role in histamine metabolism. Histamine is a potent pharmacological agent with profound biological effects^[27]. The levels of serum DAO is a useful marker of intestinal mucosal integrity that in-

Table 2 Effect of hypoxia and glutamine on intestinal villi and mucosa

Group	Height of villi (μm)	Thickness of mucosa (μm)	Villous area (μm^2)
Control	750.800 \pm 13.530	283.900 \pm 6.096	813.900 \pm 56.500
HH	378.300 \pm 20.310 ^b	203.700 \pm 2.758 ^b	342.200 \pm 39.550 ^b
HG	736.300 \pm 20.640 ^d	233.700 \pm 3.989 ^d	827.400 \pm 35.160 ^d

The height of the intestinal villi ($P < 0.001$) and the thickness of the mucosa ($P < 0.001$) and villous area ($P < 0.001$) were decreased in the HH group as compared to the control group and were increased in the HG group as compared to the HH group. ^b $P < 0.001$ vs Control, ^d $P < 0.001$ vs HH. Values are expressed as mean \pm SEM ($n = 7$, each). Gln: Glutamine; Control: Control; HH: Hypobaric hypoxia group; HG: Hypobaric hypoxia plus Gln group.

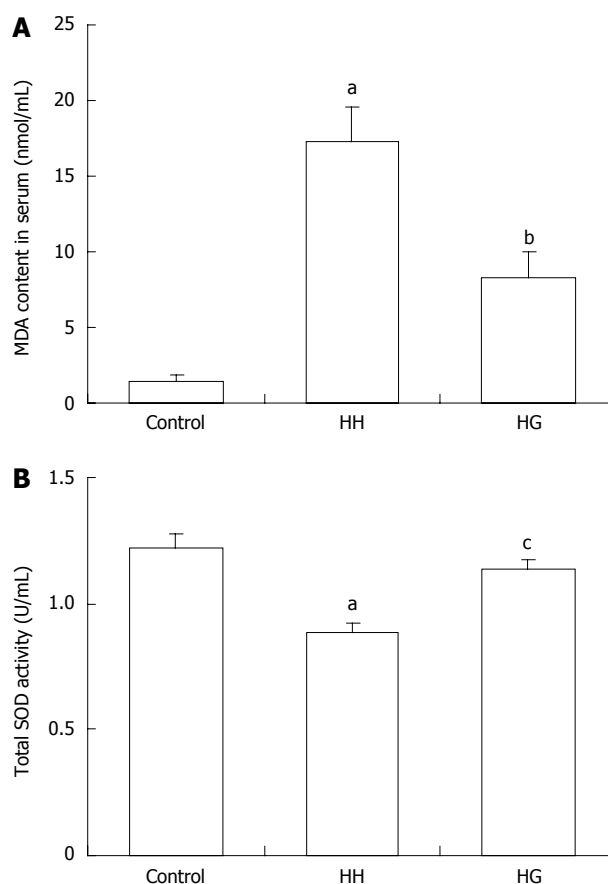


Figure 3 Effects of glutamine treatment on malondialdehyde contents in serum (A) and superoxide dismutase (B). ^a $P < 0.001$ vs Control; ^b $P < 0.05$, ^c $P < 0.01$ vs HH. Values are expressed as mean \pm SEM ($n = 7$, each). Gln: Glutamine; SOD: superoxide dismutase, MDA: malondialdehyde; Control: Control group; HH: Hypobaric hypoxia group; HG: Hypobaric hypoxia plus Gln treatment group.

dicates the function and structure of the intestine^[28]. Gln treatment significantly increased serum DAO concentration. Tight junction (TJ) proteins, including occludin, claudins, and cytoskeleton proteins play a critical role in maintaining the intestinal barrier integrity. Occludin was the first transmembrane protein discovered in the tight junction. Occludin plays a crucial role in the assembly or maintenance of epithelial tight junctions^[29]. The absence of occludin increases the ion permeability of TJs^[30]. In

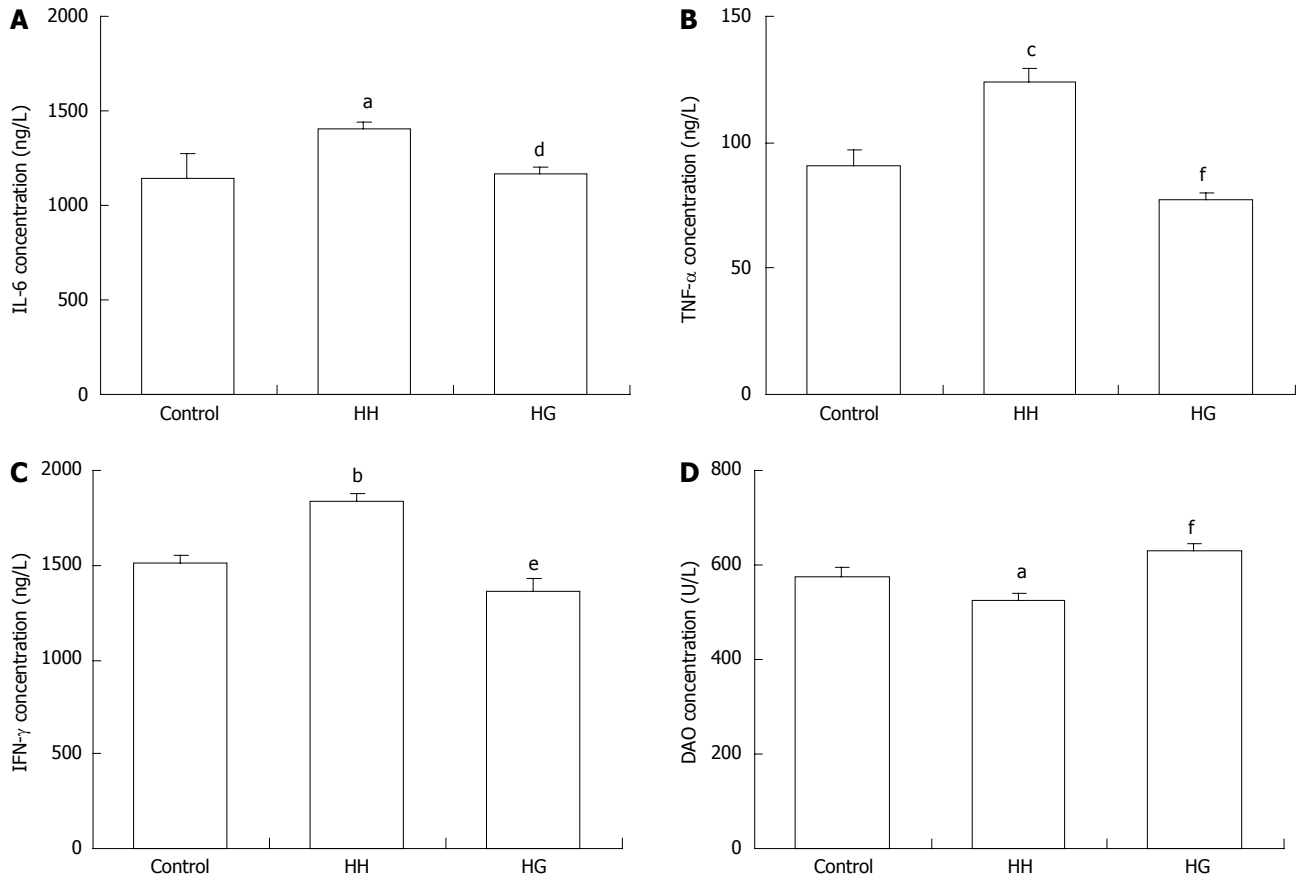


Figure 4 Effects of glutamine treatment on interleukin-6 (A), tumor necrosis factor- α (B), interferon- γ (C) and diamine oxidase contents in serum (D). ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ vs Control; ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$ vs HH. Values are expressed as mean \pm SEM ($n = 7$, each). Gln: Glutamine; IL-6: Interleukin-6; TNF- α : Tumor necrosis factor- α ; IFN- γ : Interferon- γ ; DAO: Diamine oxidase; Control: Control group; HH: Hypobaric hypoxia group; HG: Hypobaric hypoxia plus Gln treatment group.

the present study, hypobaric hypoxia caused a significant decrease in the expression of occludin. This result indicates that hypobaric hypoxia exposure leads to intestinal barrier dysfunction and increased intestinal permeability. However, supplementation with Gln effectively alleviated the decrease of occludin expression levels in rats living in a hypobaric hypoxia environment. Under stress conditions, such as hypobaric hypoxia, the physiological level of Gln is inadequate, and Gln can repair intestinal mucosa damage. Therefore, Gln must be supplemented. Gln is best known for its ability to serve as a source of fuel for cells, such as enterocytes, renal epithelial cells, hepatocytes, neurons, and immune cells^[31]. The enteral administration of glutamine stimulates intestinal mucosal protein synthesis, protects enterocytes from apoptosis, and promotes many functional activities of immune cells^[32]. Therefore, after Gln treatment lymphocytes can secrete cytokines in the hypobaric hypoxia environment. The cytokines protect intestinal immunity. The synthesis of glutathione, a major endogenous antioxidant in mammalian cells, requires glutamine as a precursor^[33]. Gln in combination with N-acetyl cysteine and zinc partially restores the tight junction integrity^[33,34], decreases the intestinal mucosal permeability, and maintains the intestinal integrity, similar to occludin. In short, the protective effect of Gln on intestinal mucosa barrier function may be

exerted *via* a variety of mechanisms.

Complex intestinal microbial communities are believed to provide some benefits to their host^[35] and are now the focus of many research efforts. There are currently few published reports examining the effect of hypobaric hypoxia on the microbiome. In the present study, there were significant decreases in *Lactobacillus*, *Treponema*, and *Peptostreptococcaceae*_Incertainae_Sedis and obvious increases in *Prevotellaceae*_uncultured and *Prevotella* in the caecal contents of the HH group. These results suggest that hypoxia may influence the composition of the microbial community in the intestine. The potential pathogens and probiotics are important members of the intestinal microbiota. The numbers of *Lactobacillus*, *Comamonas*, *Enterobacter*, *Peptostreptococcaceae*_Incertainae_Sedis, *Acinetobacter*, *Enterococcus* and *Wohlfahrtiimonas* in the HG libraries were higher than those in the HH library. The data suggest that supplementation with Gln for animals in a hypobaric hypoxia environment improved the microbial community. Changes in the composition and diversity of the bacterial community in the intestine may occur following a breach of the intestinal microfloral barrier, which results from deficiencies in the host immune defense system or damage to the intestinal mucosal barrier. Recent studies have shown that hypoxia alone can damage the function of the gastrointestinal barrier and cause flora imbalance

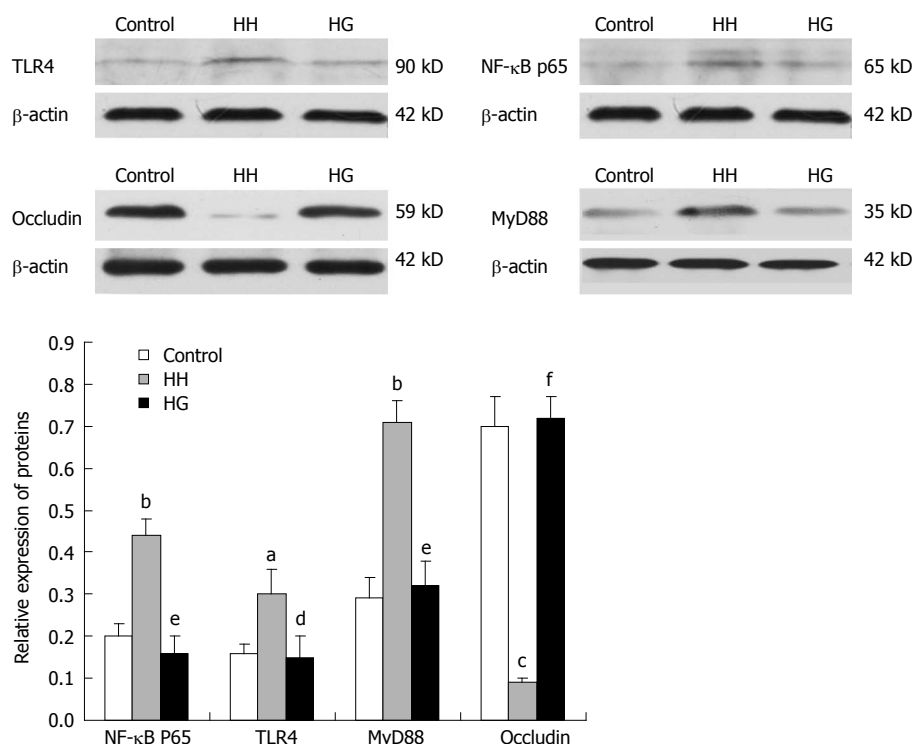


Figure 5 Toll-like receptor 4, nuclear factor-κB, myeloid differentiation factor 88, and occludin protein expression in the ileum of rats of different groups determined by Western blotting. The occludin expression level in the HH group was lower than that in the Control group. After Gln treatment, the occludin expression level in the HG group was significantly higher than that in the HH group. ^aP < 0.05, ^bP < 0.01, ^cP < 0.001 vs Control; ^dP < 0.05, ^eP < 0.01, ^fP < 0.001 vs HH. Values presented as means ± SEM (n = 4, each). Gln: Glutamine; Control: Control group; HH: Hypobaric hypoxia group; HG: Hypobaric hypoxia plus Gln group.

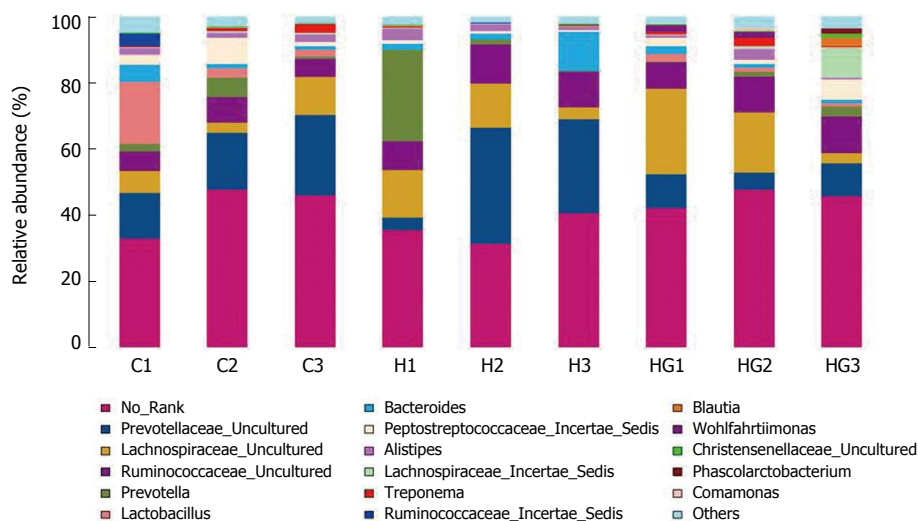


Figure 6 Bacterial composition differences. Relative read abundance of different bacterial genera within the different communities. Sequences that could not be classified into any known group were assigned as "No_Rank".

in rats^[36]. Supplementation with Gln alleviated intestinal mucosal injury and increased bacterial translocation in rats exposed to high-altitude hypoxia^[4,36,37].

Toll-like receptors (TLRs) are a family of pattern-recognition receptors that play a key role in the innate immune system. As a key transmembrane protein closely related to bacterial recognition, TLR4 is thought to be involved in the first immune barrier of the gastrointestinal tract. NF-κB is the final effector molecule of the TLR4 signaling pathway. TLRs trigger a complex signal-

ing cascade involving different adaptor proteins, kinases and transcriptional factors. Thus, various TLRs have been shown to activate both NF-κB and the mitogen-activated protein kinase pathway *via* MyD88. MyD88 is a common adaptor molecule that is recruited towards the Toll/IL-1 receptor domain of TLRs. NF-κB induces the transcription and translation of inflammatory cytokines and leads to the massive release of inflammatory mediators^[38-40]. Locally, these molecules can lead to the apoptosis of intestinal mucosal epithelial cells and damage the

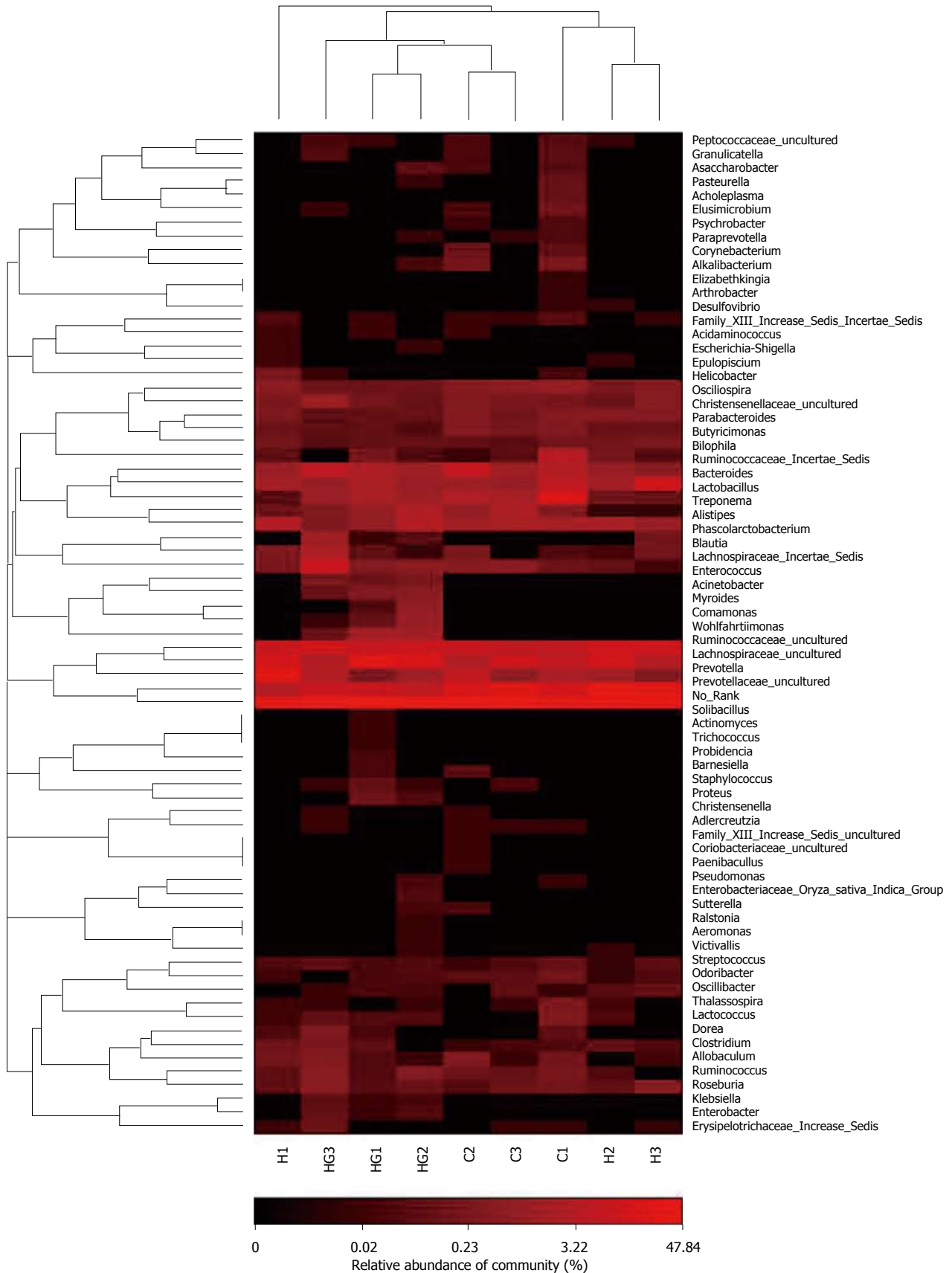


Figure 7 Bacterial distribution among the nine groups. Double hierarchical dendrogram showing the bacterial distribution among the nine groups. The bacterial phylogenetic tree was calculated using the neighbor-joining method and the relationship among samples was determined by Bray distance and the complete clustering method. The heatmap plot depicts the relative percentage of each bacterial family shown in the legend indicated at the bottom of the figure. Clusters based on the distance of the seven samples along the X-axis and the bacterial families along the Y-axis are indicated in the upper and left portions of the figure, respectively.

tissues and organs of the intestinal tract. TLR4 promotes the proliferation of epithelial cells and inhibits intestinal bacterial translocation^[41]. TLR4 might play an important role in recruiting granulocytes after intestinal damage^[42]. Our result suggests that hypobaric hypoxia upregulated TLR4 expression and activated the TLR4/MyD88/NF- κ B signaling pathway. Moreover, the activation of TLR4/MyD88/NF- κ B signaling was consistent with changes in serum levels of TNF- α , IL-6, and IFN- γ and damage to the morphology and structure of the intestinal mucosa. Furthermore, we found that the expression of TLR4/MyD88/NF- κ B signaling proteins was inversely correlated with the expression level of occludin under hypobaric hypoxic conditions. Thermal damage to the intestinal permeability increased distant organ injury that was associated with significantly reduced occludin expression and TLR4 activation, but this injury was attenuated in TLR4-deficient mice^[43]. In addition, the activation of TLR4 can also alter the cellular localization of occludin^[44]. This redistribution of occludin might damage barrier function. Thus, hypobaric hypoxia-induced activation of TLR4/MyD88/NF- κ B may influence TJ complexes and eventually cause damage to the intestinal barrier that results in bacterial translocation. Luo *et al.*^[37] also found that TLR4 and NF- κ B expression was increased in rat intestinal tissues after acute hypoxia exposure. Pyrrolidinedithiocarbamic acid treatment reversed TLR4 and NF- κ B upregulation and alleviated the damage to the intestinal tract and bacterial translocation. These results suggest that the TLR4/MyD88/NF- κ B signaling pathway may be related to the mechanism of damage in intestinal barrier function and changes in the bacterial community caused by a hypobaric hypoxic environment.

CONCLUSION

In summary, our results showed that a hypobaric hypoxia environment causes pathological changes in many rat organs including damage to the intestinal villi, increased expression of cytokines, and activation of the TLR4/MyD88/NF- κ B signaling pathway. Gln can protect the intestinal mucosal barrier and regulate the diversity and the composition of the intestinal bacterial community. The altered bacterial communities in the intestine and TLR4/MyD88/NF- κ B signal pathway may represent significant therapeutic targets for the prevention/treatment of intestinal barrier dysfunction and consequent intestinal diseases.

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COMMENTS

Background

High altitude hypoxia can cause severe damage to different organs including the intestine. Up to now, there are no effective measures to prevent and treat

intestinal diseases. The main reason may be that the mechanism of hypobaric hypoxia-induced intestinal barrier function damage even intestinal diseases is unclear. Many studies indicate that glutamine supplementation preserves the gut barrier function and prevents permeability to toxins and pathogens from the gut lumen into mucosal tissue and circulation. However, the molecular mechanism regulating the effects of glutamine on intestinal barrier function is poorly understood, especially under hypobaric hypoxia environment.

Research frontiers

Glutamine can become situationally essential amino acids such as gastrointestinal disease. Supplementation of glutamine can bring many benefits, including facilitating nitrogen metabolism, fueling the cells that line the intestine, supporting protein synthesis, and serving as a critical substrate for the cellular immune response. In the area of protection of gastrointestinal health with glutamine, the research hotspot is to elucidate the mechanism underlying the effect of glutamine on intestinal barrier function in physiological and pathological conditions.

Innovations and breakthroughs

Glutamine is the most abundant amino acid in the body. However, under the condition of extreme stress, the body may need more glutamine than it can make. Previous studies focused on the application effect of glutamine in food, medicine and feed. Its underlying mechanisms are still unknown. Hypobaric hypoxia is one major kind of environmental stress at high altitudes for humans, which usually causes damage to organs including the intestine and even induces intestinal diseases. Up to now, there are no effective measures to prevent and treat it. However, many studies pay more attention to the effect of hypobaric hypoxia on the nervous system and respiratory system. Little is known about the mechanism of intestine barrier dysfunction caused by hypobaric hypoxia and the protective effect of glutamine on intestine barrier dysfunction. The present study was conducted to explore the role of glutamine in the preservation of intestinal barrier function and maintaining the flora balance in rats exposed to hypobaric hypoxia environment, and investigate the role of the TLRs/MyD88/NF- κ B signal pathway and tight junction protein occludin in the protective effect of Gln against intestinal barrier damage induced by hypobaric hypoxia.

Applications

The results indicate that glutamine can play a role to protect the intestinal barrier function damage and regulate the diversity and composition of intestinal bacterial community under hypobaric hypoxia environment. The altered bacterial communities in the intestine and TLR4/MyD88/NF- κ B signal pathway may represent the significant therapeutic targets for the prevention/treatment of intestinal barrier dysfunction and consequent intestinal diseases.

Terminology

Hypobaric hypoxia: hypobaric hypoxia that is particularly more likely to happen for humans at high altitude areas and for pilots in flight is a condition where the body is deprived of a sufficient supply of oxygen from the air to supply for body tissues whether in quantity or molecular concentration; intestinal barrier function: intestinal barrier function regulates transport and host defense mechanisms at the mucosal interface with the outside world, and the barrier consists of an intrinsic layer, including epithelial cells and tight junctions, and an extrinsic layer, which is comprised of bacteria and a coating of mucus with high concentrations of secretory IgA. Glutamine: glutamine with many functions in the body is an amino acid that is used as a nutritional supplement in the treatment of a variety of diseases.

Peer review

This is a well-designed study aimed to investigate the beneficial effects of glutamine on intestinal damage in an animal model exposed to hypobaric hypoxic environment. The key findings presented here are of interest. The results are quite convincing and it will be interesting to reproduce them in humans.

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Role of observation of live cases done by Japanese experts in the acquisition of ESD skills by a western endoscopist

Peter V Draganov, Myron Chang, Roxana M Coman, Mihir S Wagh, Qi An, Takuji Gotoda

Peter V Draganov, Roxana M Coman, Mihir S Wagh, Division of Gastroenterology, Hepatology and Nutrition, University of Florida, Gainesville, FL 32608, United States

Myron Chang, Qi An, Department of Biostatistics, University of Florida, Gainesville, FL 32608, United States

Takuji Gotoda, Department of Gastroenterology and Hepatology, Tokyo Medical University, Tokyo 160-0022, Japan

Author contributions: Draganov PV, Chang M, Coman RM, Wagh MS, An Q and Gotoda T contributed to the manuscript.

Correspondence to: Peter V Draganov, MD, Division of Gastroenterology, Hepatology and Nutrition, University of Florida, 1329 SE 16th Street, Suite 5251, Gainesville, FL 32608, United States. peter.draganov@medicine.ufl.edu

Telephone: +1-352-2739472 Fax: +1-352-392-9002

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Abstract

AIM: To evaluate the role of observation of experts performing endoscopic submucosal dissection (ESD) in the acquisition of ESD skills.

METHODS: This prospective study is documenting the learning curve of one Western endoscopist. The study consisted of three periods. In the first period (pre-observation), the trainee performed ESDs in animal models in his home institution in the United States. The second period (observation) consisted of visit to Japan and observation of live ESD cases done by experts. The observation of cases occurred over a 5-wk period. During the third period (post-observation), the trainee performed ESD in animal models in a similar fashion as in the first period. Three animal models were used: live 40-50 kg Yorkshire pig, explanted pig stomach model, and explanted pig rectum model. The outcomes from the ESDs done in the animal models before and after observation of live human cases (main study intervention) were compared. Statistical analysis of the data

included: Fisher's exact test to compare distributions of a categorical variable, Wilcoxon rank sum test to compare distributions of a continuous variable between the two groups (pre-observation and post-observation), and Kruskal-Wallis test to evaluate the impact of lesion location and type of model (*ex-vivo* vs live pig) on lesion removal time.

RESULTS: The trainee performed 38 ESDs in animal model (29 pre-observation/9 post-observation). The removal times post-observation were significantly shorter than those pre-observation (32.7 ± 15.0 min vs 63.5 ± 9.8 min, $P < 0.001$). To minimize the impact of improving physician skill, the 9 lesions post-observation were compared to the last 9 lesions pre-observation and the removal times remained significantly shorter (32.7 ± 15.0 min vs 61.0 ± 7.4 min, $P = 0.0011$). Regression analysis showed that ESD observation significantly reduced removal time when controlling for the sequence of lesion removal ($P = 0.025$). Furthermore, it was also noted a trend towards decrease in failure to remove lesions and decrease in complications after the period of observation. This study did not find a significant difference in the time needed to remove lesions in different animal models. This finding could have important implications in designing training programs due to the substantial difference in cost between live animal and explanted organ models. The main limitation of this study is that it reflects the experience of a single endoscopist.

CONCLUSION: Observation of experts performing ESD over short period of time can significantly contribute to the acquisition of ESD skills.

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Key words: Endoscopic submucosal dissection; Training; Animal models; Early gastric cancer; Learning curve

Core tip: Endoscopic submucosal dissection is complex procedure which and requires intense and lengthy training. There is a consensus that an essential component of the training is observation of experts performing endoscopic submucosal dissection (ESD). In this study, we prospectively evaluated the impact of observation of experts performing ESD on the acquisition of ESD skills. Our data show a decrease in time needed to remove the lesion and a decrease in complication rate after the period of observation, which confirm that observing experts while performing ESD has significant impact in acquiring ESD expertise. Interestingly, there was no significant difference in the time needed to remove lesions in different animal models, which has implications in designing training courses or programs due to the substantial difference in cost between live animals and explanted organs.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) was developed in Japan in the late 1990s as an advanced, minimally invasive technique for endoscopic removal of early gastric cancers^[1]. In Japan, ESD quickly gained popularity and has become the preferred modality for the management of superficial lesions containing early cancer or high grade dysplasia throughout the gastrointestinal tract^[2-5]. The use of ESD instead of endoscopic mucosal resection (EMR) is supported by the well-documented higher *en-bloc* and curative resection rates as well as decreased local recurrence^[4,6]. As a result, the technique of ESD has been disseminated to other Asian countries but has not been widely accepted in the United States, where superficial neoplastic lesions are still largely managed by EMR or laparoscopic resection. There is a number of reasons for this slow dissemination of ESD in the United States including the complexity of the procedure, long procedure times, higher complication rates and the lack of dedicated reimbursement code. However, the main obstacle to the wide availability of ESD in the West has been and remains the very flat learning curve and lack of training resources^[7,8].

A number of investigators have evaluated the learning curve of acquiring ESD skills but no definitive conclusions could be reached due to the differences among studies as far as the type of lesions included, degree of trainee supervision, type of training system, trainee exposure to animal models, definition of outcomes and, in the case of colonic ESD, the degree of prior experience with

gastric ESD^[9-24]. Although in Japan, training approaches vary among institutions, typically ESD skills are acquired over the course of few years in the traditional time honored apprenticeship model. This approach is not applicable to Western endoscopists trying to learn ESD and, as a result, even greater variability in training pathways exists. Despite the significant variations in ESD training models around the world, there is a consensus that observation of experts performing ESD is an essential component of training^[7,25,26]. Thus, observation of ESD cases is routinely recommended as part of any training algorithm yet its role has never been formally evaluated^[7,25]. Therefore, we prospectively evaluated the impact of observation of Japanese experts performing ESD on the acquisition of ESD skills.

MATERIALS AND METHODS

Study design

This is a prospective study documenting the learning curve of one Western physician training in ESD. The trainee is an experienced endoscopist with background in advanced therapeutic endoscopy including endoscopic retrograde cholangiopancreatography (ERCP), endoscopic ultrasound (EUS) and EMR but no prior experience with ESD.

The study consisted of three time periods. In the first period (pre-observation), the trainee performed ESDs in animal models in his home institution in the United States. The second period (observation) consisted of visit to Japan and observation of live ESD cases done by experts. The observation of cases occurred over a 5-wk period in 3 Japanese major referral centers. During the observation period, the trainee observed live ESD cases performed by expert Japanese endoscopist. The trainee observation involved the pre-procedure evaluation, the room set-up, the diagnostic and therapeutic portions of the procedure. During the third period (post-observation), the trainee performed ESD in animal models in a similar fashion as in the first period. The outcomes from the ESDs done in the animal models before and after observation of live human cases (main study intervention) were compared. The study protocol was approved by the Institutional Animal Care and Use Committee.

ESD equipment and procedure

One of three animal models were used: (1) live 40-50 kg Yorkshire pig, (2) explanted pig stomach model; and (3) explanted pig rectum model. ESDs in the animal models were done using the Dual (KD-650L) and IT 2 (KD-612L) knives (Olympus America Inc., Center Valley, PA, United States). A "lesion" was first created by placing marks on the mucosa using the tip of the Dual knife. Injection of the submucosal space with mixture of normal saline and indigo carmine was then carried out to lift the lesion using a 25 g injection needle (NM-200U-0525, Olympus America Inc., Center Valley, PA, United States). That was followed by circumferential incision and sub-

Table 1 Lesion characteristics

	Pre-observation	Post-observation	P value
Location	(n = 29)	(n = 9)	
Antrum/body	14	1	
Incisura/lesser curve	10	2	
Cardia	5	3	
Rectum	0	3	
Type of Model	(n = 29)	(n = 9)	0.08 ¹
Ex-vivo	20	9	
Live pig	9	0	
Size (mm ²)	(n = 25)	(n = 9)	0.86 ²
mean ± SD	1143.2 ± 515.8	1280.9 ± 882.4	
Median	1044.6 (452.4, 2277.7)	804.2 (510.5, 2777.2)	
(min, max)			

¹Fisher's exact test; ²Wilcoxon rank sum test.

mucosal dissection. Injection of saline was repeated as needed to maintain adequate submucosal cushion.

Study outcomes

The main study outcome was lesion removal time in the animal model. The time for lesion removal was measured from the time the first mucosal mark was placed until the completion of the submucosal dissection. Secondary outcomes included successful lesion removal, *en-bloc* resection rate and complications.

Statistical analysis

We performed Fisher's exact test to compare distributions of a categorical variable and the Wilcoxon rank sum test to compare distributions of a continuous variable between the two groups (pre-observation and post-observation). We used the Spearman coefficient to evaluate the correlation between the two continuous variables. We performed the Kruskal-Wallis test (a non-parametric alternative to one-way analysis of variance) to evaluate the impact of lesion location and type of model (*ex-vivo* vs live pig) on lesion removal time. Since physician's skill is expected to improve with cumulative experience, we used a regression model to assess the impact of training on lesion removal time while controlling for the sequence of lesion removal in addition to the comparison of lesion removal times without adjustment. All data analyses were performed using the SAS software version 9.3. (SAS Institute Inc., Cary, NC, United States).

RESULTS

Observation of live human cases

The trainee observed a total of 43 human ESD cases done by Japanese experts in three large volume Japanese academic centers over a period of 5 wk. The lesion location included 10 in the esophagus, 21 in the stomach and 12 in the colorectum (7 rectal and 5 colonic).

ESD in animal models

The trainee attempted ESD in 38 lesions in animal

model. Twenty nine of the lesions were done in the pre-observation and nine in the post-observation period. Lesion characteristics and the type of animal model used are summarized in Table 1. We found that there was no significant difference in lesion sizes between pre- and post-observation cases (mean 1143.2 ± 515.8 mm² vs 1280.9 ± 882.4 mm², $P = 0.86$). Furthermore, lesion location and the type of model did not significantly affect lesion removal time ($P = 0.31$ and $P = 0.17$, respectively by Kruskal-Wallis test). There was a significant negative correlation between removal time and the sequence of lesion removal (Spearman coefficient = -0.67 , $P < 0.01$), indicating that the physician's skill was improving with cumulative experience.

The removal times post-observation were significantly shorter than those pre-observation (mean 32.7 ± 15.0 min vs 63.5 ± 9.8 min, $P < 0.001$). To minimize the impact of improving physician skill, we compared removal times in the 9 lesions post-observation to the last 9 lesions pre-observation, and found that the removal times post-observation remained significantly shorter than those in pre-training (mean: 32.7 ± 15.0 min vs 61.0 ± 7.4 min, $P = 0.0011$). Furthermore, we performed regression analysis with removal time as the response variable and sequence of lesion removal and observation (pre- vs post-observation) as explanatory variables. We found that the ESD observation significantly reduced the lesion removal time when controlling for the sequence of lesion removal ($P = 0.025$). For all successful ESDs the lesion was removed *en-bloc*. There was a trend towards decreased rate of failure of lesion removal (4/29 pre-observation versus 0/9 post-observation) and decreased rate of complications (4/29 pre-observation versus 0/9 post-observation), although the difference did not reach statistical significance ($P = 0.55$). All complications consisted of perforation.

DISCUSSION

ESD is a technically demanding procedure requiring a high level of endoscopic skill. The ESD learning curve is very flat and, in the typical Japanese training program, the trainees will acquire the background knowledge and the manual skill to perform ESD over a period of 3-4 years^[7,8,25]. Although in Japan there is no universally accepted training algorithm, most programs follow the traditional apprentice/mentor model^[25]. Trainees will progress in stepwise fashion starting with the accumulation of basic knowledge for lesion evaluation and procedure indications. That is followed by a lengthy period of observation of experts in action. The trainees will then assist in procedures and finally start performing ESD under supervision^[25]. Unfortunately, the extensive Japanese experience in ESD training cannot be directly applied in the West due to a number of substantial differences. Importantly, in the West, there is only a handful of highly qualified experts in ESD. Therefore, observing large number of cases over long period of time is not feasible

in the home country^[8,9,25]. As such, the typical Western approach to ESD training follows the steps outlined in our study: (1) self-study and hands-on training in animal models to consolidate the theoretical knowledge and augment the acquisition of technical skills; (2) brief visit to expert Japanese to observe experts performing ESD. At this time, it is well accepted that most highly experienced endoscopists performing ESD are located in Japan. In the West, there are very few experts and high-volume centers, which limit the opportunities to pursue ESD training. Our trainee spent 5 wk in Japan, observing a total of 43 live ESD procedures. Based on this experience, we encourage at least 4-5-wk visit to a high volume ESD center in Japan. We recommend this visit to occur after the endoscopist has already trained on animal models and acquired basic ESD skills; (3) further hands-on training in models to continue practice of newly acquired skills; and (4) start performing “easier” ESD cases in humans and then gradually expanding the degree of difficulty^[7,8].

Despite outlined differences, observation of experts in action is routinely recommended in both Japanese and Western training environments^[7,25]. The belief that observation of live cases would be beneficial in ESD training is purely based on the time honored approach of learning procedures in Medicine: “see one, do one, teach one”. Our data, for the first time confirm that observing live cases done by experts, indeed, significantly contributes to the acquisition of ESD technical skills. Furthermore, we noted a trend towards decrease in failure to remove lesions and decrease in complications after the period of observation.

Importantly, there was no significant difference in the time needed to remove lesions in different animal models. This finding can have important implications in designing training programs due to the substantial difference in cost between live animal and explanted organ models. The main perceived advantage of the live models is that they may provide more realistic environment and may allow the trainee to treat ESD related bleeding which significantly contributes to the procedure complexity in humans^[11]. It is our subjective impression, confirmed by our objective measurements (*e.g.*, time needed to remove the lesion), that the use of live pigs does not significantly enhance the training experience because bleeding is a rare occurrence and tends to be of a small magnitude compared with that in humans. Finally, we demonstrate that short period of observation of live cases can have significant impact on ESD technical skills. That makes the training algorithm described by us feasible because it requires a relatively short time period to be spent away from the trainee home institution thus minimizing problems related to clinical workload coverage and financial constraints related to an extended stay in Japan.

Our study is not without limitations. It is based on a single trainee experience and the findings may not be applicable to all trainees, specifically to trainees in the East. The technical expertise and background of endoscopists embarking on ESD in the West differ significantly than

those of their Eastern counterparts. At present, in Japan, the typical trainee learning ESD is a GI fellow. On the other hand, in the West, physicians embarking on ESD tend to be otherwise more mature and well experienced therapeutic endoscopists typically with background in ERCP and EUS. From that perspective, our Western trainee matches well with the prototypical Western endoscopist pursuing ESD training. In our study, we used animal models to evaluate pre- and post-observation evaluation of technical skills. This brings some limitations including the different magnitude of procedure related bleeding and, most importantly, the need to create virtual lesions in the animal model. Nevertheless, training in models is an essential part of the Western training experience and since the mentor/apprentice approach will not be feasible in the foreseeable future, the use of models will remain a key component in ESD training in the West. Finally, our post-observation sample size is relatively small which did not allow us to apply a multivariate regression model. After completing the 9 post-observation cases, our trainee started performing ESD cases in humans. Since the performance of ESD in humans can directly impact on the acquisition of ESD skills we could not enroll additional post-observation animal cases. Nevertheless, we believe that our experience is representative of the number of animal model ESDs that most Western endoscopists will perform as part of their training.

In summary, we found that observation of Japanese experts performing ESD over relatively short period of time can significantly contribute to the acquisition of ESD skills. A trend toward decreased rate of complications and decreased failure to remove lesions was also noted. Performing ESDs in explanted organ model appears to provide adequate environment for training which can decrease the cost related to the use of live animal models.

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COMMENTS

Background

Endoscopic submucosal dissection (ESD) represents an important advancement in the therapy of early GI cancers, offering the potential for *en-bloc* removal of mucosal and submucosal tumors, with high curative rates. Despite these advantages, ESD is a challenging procedure, requiring advanced endoscopic skills and focused training to acquire these skills. Thus, there has been a recognized need for a structured training system for ESD to enhance trainee experience and to reduce the risks of complications and inadequate treatment. A number of training algorithms has been proposed in Japan with the goal to standardize ESD training. These algorithms however cannot be directly applied in the West due to substantial differences including the availability of highly qualified mentors, the trainee's background and the type of pathology seen.

Research frontiers

An important part of the training is watching and learning from experts performing live ESD cases. This is easily accomplished in Asian country where ESD

is performed in many hospitals and ESD experts are readily available. In the West, where ESD has had a slower dissemination and acceptance, only very few medical centers perform ESD cases routinely. Thus, at this time, as the most highly experienced endoscopists performing ESD are in Japan, a visit to a specialized Japanese center will most likely remain, for some time, an essential component of ESD training in the West. The use of animal models in ESD training is a highly debated topic and, while not a requirement in Japan, these models can be a valuable resource when training in the West. Studies showed that practicing on animal models can augment the acquisitions of skills in low-volume centers.

Innovations and breakthroughs

The study made two important observations which can have significant consequences for the Western endoscopist embarking in ESD training. First, as expected, there was a significant improvement in reducing operative time and complications after observing ESD experts performing live cases. The novelty of this finding consists in the significant improvement in ESD skill even after only a short observation period of several weeks. In addition, this study showed that there is no difference in the time needed to remove lesions when using live animal models versus harvested organs. This finding has implications in designing training courses or programs due to the substantial difference in cost between live animals and explanted organs.

Applications

Following the traditional Japanese algorithm, the ESD training spans over the course of 3-4 years. This experience cannot be directly applied in the West, as the gastroenterologist embarking on learning ESD is usually a well-established advanced endoscopist. The algorithm proposed in this manuscript, which includes a 4-6-wk visit to Japan, appears to be a more feasible and realistic approach to ESD training in the West because it requires a relatively short time period to be spent away from the trainee home institution thus minimizing problems related to clinical workload coverage and financial constraints related to an extended stay in Japan.

Terminology

Early gastric neoplasms are malignant tumors which involve only the mucosa and submucosal space, thus being amenable to endoscopic removal. An animal model is a living animal or harvested organ from an animal used during the research and investigation of human disease, for the purpose of better understanding the disease process without the added risk of harming an actual human.

Peer review

The manuscript entitled "The Role of Observation of Live Cases Done by Japanese Experts in the Acquisition of Endoscopic Submucosal Dissection Skills by a Western Endoscopist" by Draganov *et al* reported the usefulness of hospital visit to learn the skill of ESD.

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Safety and efficacy of *Hansenula*-derived PEGylated-interferon alpha-2a and ribavirin combination in chronic hepatitis C Egyptian children

Suzan El Naghi, Tawhida Y Abdel-Ghaffar, Hanaa El-Karakasy, Elham F Abdel-Aty, Mona S El-Raziky, Aleef A Allam, Heba Helmy, Hanaa A El-Araby, Behairy E Behairy, Mohamed A El-Guindi, Hatem El-Sebaie, Aisha Y Abdel-Ghaffar, Nermin A Ehsan, Ahmed M El-Hennawy, Mostafa M Sira

Suzan El Naghi, Pediatric Department, National Hepatology and Tropical Medicine Research Institute, 11441 Cairo, Egypt
Suzan El Naghi, Tawhida Y Abdel-Ghaffar, Yassin Abdel Ghaffar Charity Center for Liver Disease and Research, 2851 Cairo, Egypt

Tawhida Y Abdel-Ghaffar, Pediatric Department, Ain Shams University, 11566 Cairo, Egypt

Hanaa El-Karakasy, Mona S El-Raziky, Heba Helmy, Department of Pediatrics, Cairo University Pediatric Hospital, 11562 Cairo, Egypt

Elham F Abdel-Aty, Aleef A Allam, Hanaa A El-Araby, Behairy E Behairy, Mohamed A El-Guindi, Mostafa M Sira, Pediatric Hepatology Department, National Liver Institute, Menofiya University, Shebin El-koom, 32511 Menofiya, Egypt
Hatem El-Sebaie, Biochemistry Department, National Liver Institute, 32511 Menofiya, Egypt

Aisha Y Abdel-Ghaffar, Clinical Pathology Department, Ain Shams University, 11566 Cairo, Egypt

Nermin A Ehsan, Pathology Department, National Liver Institute, Menofiya University, Shebin El-koom, 32511 Menofiya, Egypt

Ahmed M El-Hennawy, Pathology Department, Cairo University, Faculty of Medicine, Kasr El-Aini, 11562 Cairo, Egypt

Author contributions: El Naghi S, Abdel-Ghaffar TY, El-Karakasy H, El-Raziky MS, El-Araby HA, Behairy BE, El-Guindi MA and Sira MM were involved in the study concept and design; El Naghi S, Abdel-Ghaffar TY, El-Karakasy H, Abdel-Aty EF, El-Raziky MS, Allam AA, Helmy H, El-Araby HA, Behairy BE, El-Guindi MA and Sira MM were involved in recruitment of patients, clinical management, follow up and contributed to data acquisition; Sira MM performed the statistical analysis and designed the figures; El Naghi S, Abdel-Ghaffar TY, El-Karakasy H and Sira MM performed the data interpretation; El Naghi S, Abdel-Ghaffar TY, El-Raziky MS and Sira MM wrote the manuscript; Abdel-Ghaffar AY and El-Sebaie H performed laboratory tests and genotyping; Ehsan NA and El-Hennawy AM performed histopathological examinations; all the authors reviewed the manuscript.

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Correspondence to: Mostafa M Sira, MD, Department of Pediatric Hepatology, National Liver Institute, Menofiya University, Gamal Abd El-Nasir, Shebin El-koom, 32511 Menofiya, Egypt. msira@liver-eg.org

Telephone: +20-48-2222740 Fax: +20-48-2234586

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Abstract

AIM: To investigate the safety and efficacy of a *Hansenula*-derived PEGylated (polyethylene glycol) interferon (IFN)-alpha-2a (Reiferon Retard) plus ribavirin customized regimen in treatment-naïve and previously treated (non-responders and relapsers) Egyptian children with chronic hepatitis C infection.

METHODS: Forty-six children with chronic hepatitis C virus (HCV) infection were selected from three tertiary pediatric hepatology centers. Clinical and laboratory evaluations were undertaken. Quantitative polymerase chain reaction (PCR) for HCV-RNA was performed before starting treatment, and again at 4, 12, 24, 48, 72 wk during treatment and 6 mo after treatment cessation. All patients were assigned to receive a weekly subcutaneous injection of PEG-IFN-alpha-2a plus daily oral ribavirin for 12 wk. Thirty-four patients were treatment-naïve and 12 had a previous treatment trial. Patients were then divided according to PCR results into two groups. Group I included patients who contin-

ued treatment on a weekly basis (7-d schedule), while group II included patients who continued treatment on a 5-d schedule. Patients from either group who were PCR-negative at week 48, but had at least one PCR-positive test during therapy, were assigned to have an extended treatment course up to 72 wk. The occurrence of adverse effects was assessed during treatment and follow up. The study was registered at www.ClinicalTrials.gov (NCT02027493).

RESULTS: Only 11 out of 46 (23.9%) patients showed a sustained virological response (SVR), two patients were responders at the end of treatment; however, they were lost to follow up at 6 mo post treatment. Breakthrough was seen in 18 (39.1%) patients, one patient (2.17%) showed relapse and 14 (30.4%) were non-responders. Male gender, short duration of infection, low viral load, mild activity, and mild fibrosis were the factors related to a better response. On the other hand, patients with high viral load and absence of fibrosis failed to respond to treatment. Before treatment, liver transaminases were elevated. After commencing treatment, they were normalized in all patients at week 4 and were maintained normal in responders till the end of treatment, while they increased again significantly in non-responders ($P = 0.007$ and 0.003 at week 24 and 72 respectively). The 5-d schedule did not affect the response rate (1/17 had SVR). Treatment duration (whether 48 wk or extended course to 72 wk) gave similar response rates (9/36 *vs* 2/8 respectively; $P = 0.49$). Type of previous treatment (short acting IFN *vs* PEG-IFN) did not affect the response to retreatment. On the other hand, SVR was significantly higher in previous relapsers than in previous non-responders ($P = 0.039$). Only mild reversible adverse effects were observed and children tolerated the treatment well.

CONCLUSION: Reiferon Retard plus ribavirin combined therapy was safe. Our customized regimen did not influence SVR rates. Further trials on larger numbers of patients are warranted.

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Key words: Children; Chronic hepatitis C; *Hansenula polymorpha*; PEGylated interferon; Response rate; Ribavirin; Treatment

Core tip: Egypt has the highest prevalence of hepatitis C virus (HCV) infection in the world (15%-25%) and the main (90%) genotype is type 4. Prevalence in Egyptian children was found to be 3% in upper Egypt and 9% in lower Egypt. PEG-IFN-alpha-2a or -2b and ribavirin have been used in small numbers of HCV-infected children, whose SVRs are higher in genotypes 2/3 than in genotypes 1/4. A novel 20-kDa PEG-IFN-alpha-2a (Reiferon Retard) derived from the *Hansenula polymorpha* expression system has been used in adults with chronic HCV, achieving an SVR ranging from 56% to 60.7%, while no studies have been reported in chil-

dren before.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a serious health problem worldwide that establishes a chronic infection in up to 85% of cases^[1]. Estimates of prevalence range from less than 1.0% in northern Europe to more than 2.9% in northern Africa^[2]. Egypt has the highest prevalence of adult HCV infection in the world (15%-25%), especially in rural communities^[3,4] and the main (90%) HCV genotype is type 4^[5]. Studies of the magnitude of HCV infection in Egyptian children revealed a prevalence of 3% in upper Egypt and 9% in lower Egypt^[6].

Blood transfusion was a major risk factor for HCV transmission, but has been virtually eliminated in countries where screening of blood donors has been implemented^[7]. Vertical transmission of HCV infection is the most common route of acquiring HCV in infants and children^[8]. It affects 4%-10% of children born to infected mothers, with the highest risk in mothers having a high viral load or co-infected with human immunodeficiency virus^[9]. In a large prospective cohort from Egypt, HCV infection was determined in 10% of infants born to infected mothers: 5.47% cleared the virus by 1 year of age and 2.1% cleared the virus by 2-3 years. Persistent infection was detected in 2.43%^[10]. HCV infection seems to progress more slowly to fibrosis and cirrhosis in childhood-acquired disease than in adults^[11], even in those who were vertically infected, the infection has been reported as mild^[9].

Treatment of chronic HCV aims at slowing disease progression, preventing complications of cirrhosis, reducing the risk of hepatocellular carcinoma and treating extrahepatic complications of the virus^[12].

Currently, standard antiviral treatment for chronic HCV involves once weekly PEGylated interferon (PEG-IFN)-alpha injections and daily oral ribavirin. Some reports showed that among adult genotype 4 patients treated with PEG-IFN-alpha and ribavirin, 63% had a sustained virological response (SVR)^[13,14].

Treatment individualization has been adopted recently as a therapeutic strategy to improve the SVR rate^[15]. On treatment, virological response appears to be crucial in both tailoring the length of treatment and influencing treatment outcome. It has been reported that early viro-

logical response (EVR) at week 12 has a positive predictive value (PPV) of 65%-72% for subsequent SVR, while patients with no EVR have no possibility of SVR, with a negative predictive value (NPV) of 98%-100%^[16,17]. Whether EVR can be used in children, as in adults, to stop therapy early in patients destined to be non-responders, is not clear^[18]. Drusano and Preston^[19] hypothesized that the longer HCV-RNA remained undetectable after initial clearance, the higher the chance in attaining an SVR. Thus, it might be expected that extended treatment duration in patients with slow virological response may improve their SVRs.

Reiferon retard, a *Hansenula*-derived novel patented PEG-IFN, available in the Egyptian market for 6 years, is a 20 kDa PEG-IFN-alpha-2a. As stated by the manufacturer, *Hansenula polymorpha* represents a stable, robust and safe expression system, which is capable of reaching the highest productivity of recombinant proteins ever described. Reiferon retard has been used in adult Egyptians with safety and efficacy comparable to other PEGylated interferons^[20-22].

In the current study, our aims were as follows: firstly, to investigate the efficacy and safety of Reiferon retard in attaining an SVR in treatment-naïve and previously treated (non-responders and relapsers) chronic HCV infected children; secondly, to assess the effect of tailoring treatment on the SVR [by decreasing the interval between injections (5 d *vs* 7 d) and prolonging duration of therapy (72 wk *vs* 48 wk)] based on the on-treatment virological response; thirdly to assess predictors of an SVR.

MATERIALS AND METHODS

Study population

This study included 46 children with compensated chronic hepatitis C infection recruited from three pediatric hepatology tertiary centers: the Pediatric department in Yassin Abdel Ghaffar Charity Center for Liver Disease and Research; Cairo University Pediatric Hospital; and the Pediatric Hepatology department, National Liver Institute, between February 2009 and July 2009. The study was completed on August 2011. Diagnosis was based on serological and virological tests; HCV-antibody (Ab) by a third generation enzyme linked immunosorbent assay (ELISA), and qualitative and quantitative PCR for HCV-RNA.

Criteria for inclusion were children aged 3-19 years with compensated chronic HCV infection (HCV-RNA positive by PCR for more than 6 mo), whose hemoglobin (Hb) was ≥ 10 g/dL, absolute neutrophil count (ANC) $> 1500/\text{mm}^3$, platelet count $> 75000/\text{mm}^3$, and who had normal random blood sugar, serum creatinine, serum ferritin, thyroid function tests and lipid profile and no other associated liver disease [autoimmune hepatitis, Wilson disease, alpha-1 antitrypsin deficiency, hepatitis B virus (HBV) infection]. Liver biopsy was mandatory for enrollment.

Patients with decompensated cirrhosis, any other cause of liver disease associating HCV infection, body

mass index (BMI) ≥ 95 percentile, severe psychiatric conditions, uncontrolled seizure disorder, decompensated cardiovascular disease, renal insufficiency, evidence of retinopathy, decompensated thyroid disease, hemoglobinopathy, immunologically mediated diseases or any other chronic illness requiring long term immunosuppressive drugs or previous IFN therapy within one year of enrollment, were excluded from the study. A signed informed consent was obtained from the guardians of all the patients before enrollment in the study. The Research Ethics Committee in the three participating centers approved this study.

Treatment regimens and follow up protocol

All patients were assigned to receive a weekly subcutaneous injection of PEG-IFN-alpha-2a (Reiferon Retard; Minapharm, Rhein-Biotech, Germany) in a dose of $100 \mu\text{g}/\text{m}^2$ per week plus ribavirin 15 mg/kg daily in two divided doses for a total of 12 wk. Patients were then divided into two groups according to HCV-RNA results at week 12.

Group I comprised patients who continued treatment on a weekly basis (7-d schedule). This group included patients who were HCV-RNA negative at week 12 and those who had < 1 log decrease in HCV-RNA viremia. Group II comprised patients who continued treatment on a 5-d schedule. This group included patients who had ≥ 1 log decrease in viremia (compared to pre-treatment level) at week 12.

At week 48, patients who were PCR-positive stopped treatment. Patients who were persistently HCV-RNA negative by PCR (at weeks 4, 12, 24 and 48) also stopped treatment and their SVR was checked 6 mo after stopping treatment (SVR 1). Patients who were PCR-negative at week 48, but had at least one PCR-positive test during therapy at weeks 4, 12, or 24 (delayed response or breakthrough) were assigned to have an extended treatment course of 6 mo duration. PCR was performed at 72 wk in those patients to detect end of treatment response and those who were HCV-RNA negative, were tested after a further 6 mo for an SVR (SVR 2).

All patients had their full medical history taken and received a thorough clinical examination before starting treatment, with stress laid on the duration and possible cause of infection, previous trial of antiviral therapy, psychiatric history and fundus examination. The occurrence of adverse effects was assessed during the treatment and follow up periods.

Laboratory investigations

Laboratory investigations, including complete blood count (CBC), albumin, alanine transaminase (ALT) and aspartate transaminase (AST), gamma-glutamyl transpeptidase, alkaline phosphatase, prothrombin time (PT), kidney function tests, alpha-fetoprotein, thyroid function tests (T3, T4, TSH), lipid profile (triglycerides, cholesterol and low and high density lipoproteins), serum autoantibodies (anti-nuclear antibodies, anti-smooth muscle

antibodies and liver-kidney microsomal antibodies) and PT were performed for every patient before starting treatment. CBC, ALT and AST were done weekly for the first month, every two weeks for 2 mo and monthly thereafter. PT was performed at the third month and at the end of treatment. Viral markers [HCV-Ab (Inno-genetics, Ghent, Belgium), HBV surface antigen, HBV core immunoglobulin (Ig)M and IgG Abs (all from Dia Sorin, Saluggia, Italy)] were performed using ELISA, according to the manufacturer instructions. Real-time PCR for HCV-RNA was performed using COBAS® Ampli-rep/COBAS® TaqMan®, Roche Molecular Systems, Inc., Branchburg, NJ, 08876 United States (the detection limit was 15 IU/mL). According to the viral load, viremia was classified arbitrarily for descriptive purpose into low ($\leq 2 \times 10^5$ IU/mL), moderate ($> 2 \times 10^5$ - 2×10^6 IU/mL) and high viremia ($> 2 \times 10^6$ IU/mL). HCV genotyping/subtyping was done by restriction fragment length polymorphism analysis, using restriction enzymes *Hae*III, *Rsa* I, *Mva* I and *Hinf* I on PCR-amplified 5'-untranslated region.

Dose modification regimen

The doses of PEG-IFN and ribavirin were modified according to ANC, Hb and platelets. If Hb dropped to < 10 g/dL, ribavirin dose was reduced by 25% and if to < 7.5 mg/dL, erythropoietin was administered. If ANC was 500-800/mm³ and/or platelet count < 80000 /mm³, the IFN dose was reduced by 25%. If ANC was 300-500/mm³, the IFN dose was reduced by 50%. If ANC < 300 /mm³ and/or platelet count ≤ 50000 /mm³, the IFN dose was skipped and resumed later after the count reached safe levels.

Definitions of virological response

Virological responses during therapy were defined as reported by Ghany *et al*^[23].

Liver biopsy and histopathological evaluation

Liver biopsy was performed for all patients except one who had hemophilia. Hepatic necroinflammatory activity and liver fibrosis were evaluated according to Ishak staging and grading scores^[24]. Necroinflammatory activity was classified into mild (score 1-5), moderate (score 6-8), and severe (score 9-18). Fibrosis was classified into mild (stage 1), moderate (stages 2-3), and severe fibrosis or cirrhosis (stages 4-6)^[25]. Steatosis was graded semi-quantitatively by determining the percentage of affected hepatocytes and the following scoring system was employed: grade 0: $< 5\%$, grade 1: 5%-33%, grade 2: 34%-66%, grade 3: $> 66\%$ ^[26].

Statistical analysis

Descriptive results were expressed as mean \pm SD or number (percentage) of individuals with a condition. Statistical significance between groups was tested either by a non-parametric test (Mann-Whitney *U* test), Pearson's χ^2 test or Fisher's exact test. Sensitivity, specificity, PPV

and NPV were expressed as percentages. Results were considered significant if $P \leq 0.05$. Statistical analysis was performed using SPSS software version 13 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patient population characteristics

Forty-six children were enrolled in the study. They were 33 boys and 13 girls, aged between 4 and 19 years (mean 10.32 ± 3.46 years). Forty-four patients completed the full course of treatment and follow up regimen, while two patients did not show up after completing 48 wk treatment; therefore, they could not be evaluated for SVR. Two patients had glucose-6-phosphate dehydrogenase deficiency, one had hemophilia and one had situs inversus. The demographic and epidemiologic characteristics of the studied population are summarized in Table 1. Blood transfusion was considered a possible risk factor in 34.8% of patients, while mother to child transmission was considered a possible one in 17.4% of them. Eighteen patients (39.1%) had an HCV infected family member and most of the patients (43 out of 46) had more than one possible risk factor of infection, while in three patients no possible source of infection was identified.

The mean of expected duration of infection was 5.29 ± 3.97 years and the mean BMI was 18.20 ± 2.77 . Low, moderate and high viremic loads were found in 41.3%, 54.3% and 4.3% of patients, respectively. HCV genotype was detected in 38 out of 46 patients. All were genotype 4; 30 (65.2%) were 4a and 8 (17.4%) were 4b. The genotype could not be determined in eight patients. The majority of patients had mild fibrosis (66.7%) and mild activity (97.8%) in their liver biopsy. Fibrosis was absent in 28.9% of patients, while only 4.4% had moderate fibrosis.

Response to treatment in the group as a whole

In the group as a whole, only 11 out of 46 (23.9%) showed an SVR. There were 14 (30.4%) non-responders, where HCV-RNA was detectable throughout treatment. Breakthrough was seen in 18 (39.1%) patients and delayed response in eight (17%) patients. Relapse occurred in one (2.17%) patient. Two patients had an end of treatment response (ETR) but were lost to follow up and dropped out of the evaluation of SVR (Table 2). Figure 1 shows the treatment algorithm according to PCR results for all cases during treatment and follow up periods.

Predictors of response

There was no significant statistical difference in the response rate of the three centers. Responders were nine males (9/31 = 29.1%) and two females (2/13 = 15.4%). Twelve patients had history of a previous treatment trial; two (18.2%) of them achieved SVR while nine (81.8%) were non-responders. The last one achieved ETR but was lost to follow up. The type of previous treatment (short acting IFN *vs* PEG-IFN) did not affect the re-

Table 1 Demographic, laboratory and histopathological parameters in all patients *n* (%)

Parameter	All patients (<i>n</i> = 46)
Age (yr)	10.32 ± 3.46
Male	33 (71.7)
Duration of infection (yr)	5.29 ± 3.97
BMI	18.20 ± 2.77
Possible risk of infection	
Surgery	14 (30.4)
Blood transfusion	16 (34.8)
Tonsillectomy	5 (10.9)
Circumcision	33 (71.7)
Minor procedures ¹	30 (65.2)
Vertical transmission	8 (17.4)
Family contact	18 (39.1)
More than one possible risk	43 (93.5)
Unknown risk factor	3 (6.5)
Hemoglobin (g/dL)	12.5 ± 1.1
ANC (× 10 ³ /μL)	3.13 ± 1.7
Platelets (× 10 ³ /μL)	280.7 ± 82.4
Albumin (g/dL)	4.1 ± 0.37
Alanine transaminase (U/L)	56.6 ± 55.03
Aspartate transaminase (U/L)	49.2 ± 31.65
Gamma-glutamyl transpeptidase (U/L)	32.1 ± 26.6
Alkaline phosphatase (U/L)	212.5 ± 96.1
Prothrombin time (sec)	12.9 ± 0.62
Hepatomegaly (US)	3 (15.9)
Splenomegaly (US)	3 (15.9)
Viremia (IU/mL)	
Low (≤ 2 × 10 ⁵ IU/mL)	19 (41.3)
Moderate (> 2 × 10 ⁵ - 2 × 10 ⁶ IU/mL)	25 (54.3)
High (> 2 × 10 ⁶ IU/mL)	2 (4.3)
Genotype:	
4a	30 (65.2)
4b	8 (17.4)
Not determined	8 (17.4)
Fibrosis stage	
Absent	13 (28.9)
Mild	30 (66.7)
Moderate	2 (4.4)
Activity grade	
Mild	44 (97.8)
Moderate	1 (2.2)

¹Minor procedures were: Sutures, abscess drainage, ICU hospitalization, endoscopy, ear piercing, tattooing, prolonged hospitalization and dental care. US: Ultrasound; BMI: Body mass index.

sponse to retreatment. On the other hand, the SVR was significantly higher in previous relapsers than in previous non-responders ($P = 0.039$). Seven out of the 11 (64%) responders had low viremia. Patients infected with HCV subtypes, whether 4a or 4b, had similar response rates (4/28 and 1/8 respectively). The majority of patients (44) had mild activity; 11 out of them had an SVR (13 achieved ETR). Mild fibrosis was seen in 30 patients; 10 (33%) out of them achieved an SVR (12 achieved ETR), while among 13 patients with absent fibrosis, only one (7.7%) achieved an SVR. All patients with steatosis (4 patients) did not achieve an SVR (Table 3).

Effect of tailoring treatment according to on-treatment response

Of the 17 patients who followed the 5-d schedule, one

Table 2 Response outcome

Response type	<i>n</i> = 46
End of treatment response	13 (28.2)
SVR	11 (23.9)
Dropped out SVR	2 (4.3)
Non-responder	14 (30.4)
Breakthrough	18 (39.1)
Delayed responders ended by breakthrough	8 (17.4)
Breakthrough ended at 48 wk as non-responders	8 (17.4)
Breakthrough ended at 72 wk by relapse	2 (4.3)
Relapse	1 (2.17)

Two patients who had end of treatment response (ETR) but were lost to follow up. SVR: Sustained virological response.

patient (1/17=6%) achieved an SVR. Extended treatment (72 wk) was given to eight patients. Whatever the duration of treatment, a quarter of the cases in each group achieved an SVR (Table 3).

SVR according to rapid virological response and EVR

Patients who achieved an SVR (11/44) had 81.8% rapid virological response (RVR), 90.9% EVR, 100% negative PCR at 24 wk of treatment, and 100% ETR. These data are highly significant (Table 4). RVR and EVR showed high sensitivity (81.8% and 90.9% respectively), but low specificity (60.6% and 63.6% respectively) in predicting an SVR. They had a very good NPVs of 90.9% and 95.45% respectively (Table 5).

Effect of treatment on liver enzymes

During treatment, ALT and AST normalized in both responder and non-responder groups at week 4 and were maintained normal in responders till the end of the treatment, whereas they increased again significantly in non-SVR group from week 12 onwards (Figure 2).

Treatment safety

Regarding the safety of combined therapy, all side effects were temporary and mild (Table 6). Fever was seen in the first few weeks of treatment in 27 (58.7%) patients and flu-like symptoms appeared in 15 (32.6%) patients. Both anemia and neutropenia were treated by reduction or skipping of doses.

DISCUSSION

Since the introduction of IFN, attempts have been made to introduce novel IFNs with the aim of increasing therapeutic efficacy, reducing adverse events and/or reducing the cost of therapy.

The current study used the *Hansenula*-derived PEG-INF-alpha-2a (a 20 KDa Reiferon Retard) plus ribavirin customized regimen for treatment of chronic HCV infected children.

To date, only four Egyptian studies investigating the efficacy and safety profile of the *Hansenula*-derived PEG-INF-alpha-2a plus ribavirin for the treatment of adult

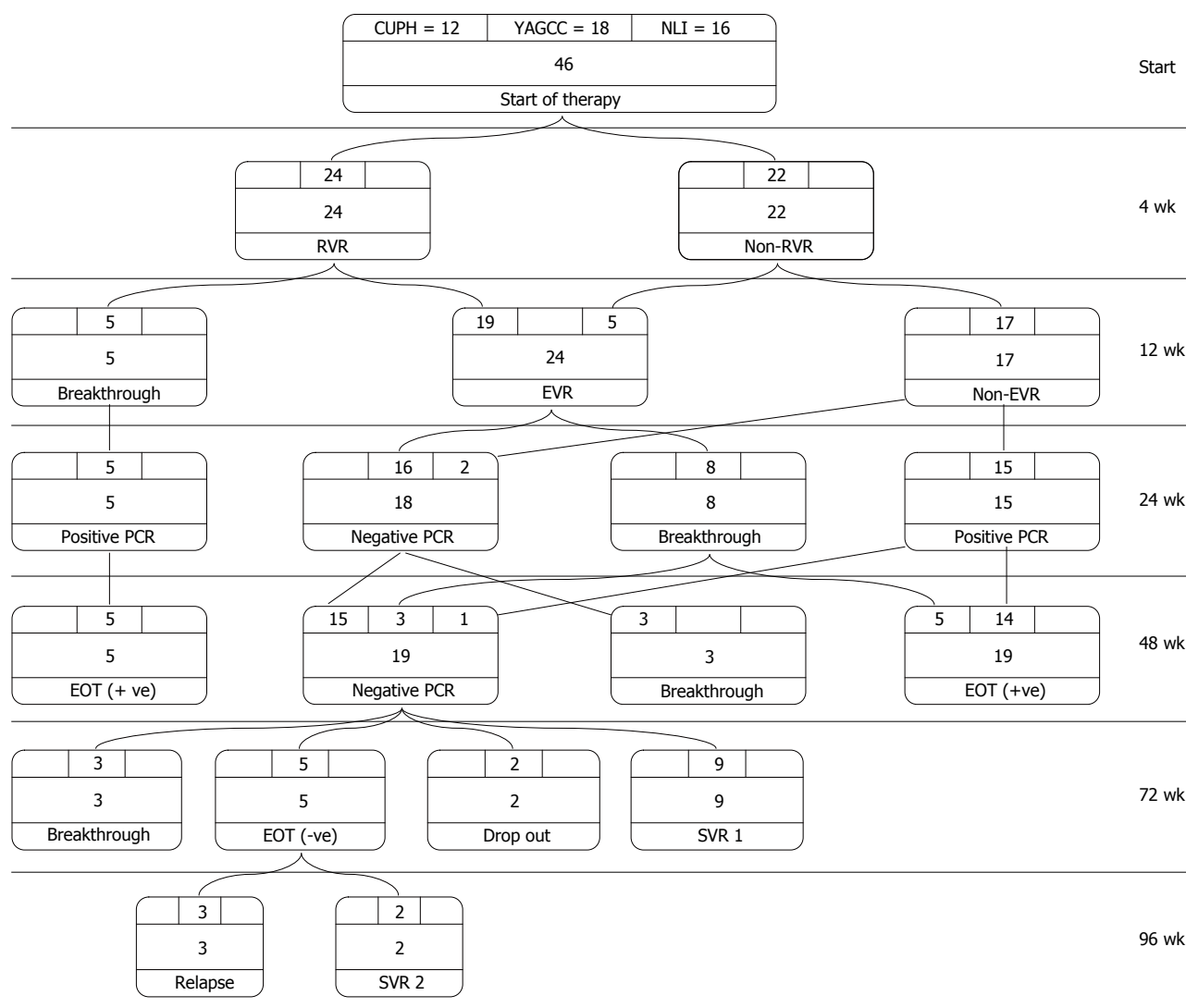


Figure 1 Treatment algorithm and response to therapy. At weeks 4, 24 (52.17%) patients attained a rapid virological response (RVR) and 22 (47.82%) had no RVR. At week 12, five patients (10.86%) had breakthrough, while the remaining 19 patients had an early virological response (EVR). Those patients, in addition to five patients who turned negative from ones with no RVR formed a total of 24 (52.17%) patients with EVR. At week 24, those who had breakthrough continued to be polymerase chain reaction (PCR)-positive and eight patients from those with EVR had breakthrough. The remaining 16 patients, in addition to two patients from those with no EVR, turned PCR-negative, making a total of 18 (39.13%) patients with negative PCR. At week 48, three of those 18 patients had breakthrough, while the remaining 15 patients and one patient from those positive at week 24, in addition to three patients who had breakthrough at week 24 (making a total of 19 patients), had negative PCR. Of the 19 negative-PCR patients, nine had an SVR at week 72 and two patients dropped out. The remaining eight patients had an extended 6-month therapy (three patients had breakthrough and five patients were PCR-negative at their ETR). At week 96, three out of five patients with extended course had relapse, while the other two patients attained an SVR, making the total SVR 11 out of 44 (25%). EOT: End of treatment.

Egyptian patients with genotype 4 chronic hepatitis C are available. The SVRs ranged from 56% to 60.7% following 48 wk of combination therapy^[20,27-29]. These results are comparable to those obtained using the existing two PEG-IFN- α -2a and α -2b agents for treatment of genotype 4 in adults^[30-33], with the exception of a single report that demonstrated an SVR rate of 33.3% after treatment with PEG-IFN- α -2b^[34].

In children, Wirth *et al.*^[35] evaluated the efficacy and safety of PEG-IFN- α -2b and ribavirin, disclosing a high SVR of 90% in genotypes 2 and 3, and 53% for children with genotype 1. Another large trial concluded that children with HCV genotype 1 had a 47% response rate with PEG-IFN- α -2a/ribavirin^[36].

The results of a meta-analysis of eight trials^[35-42] performed in 2013 by Druyts *et al.*^[43], indicated that EVR and SVR were both higher for genotypes 2/3 (87% and 89%, respectively) than for genotypes 1/4 (61% and 52%, respectively). The sensitivity analysis comparing PEG-IFN- α -2a and PEG-IFN- α -2b indicated that these two treatments were comparable in terms of efficacy and safety.

In the present study, the ETR was 28.2% (two cases were lost to follow up) and 23.9% showed an SVR. Breakthrough was seen in 39.1%. One patient showed relapse and 14 (30.4%) were non-responders.

The lower proportion of SVRs in genotype 4 infected children in this trial compared with other trials in adults

Table 3 Comparison between patients with sustained virological response and non-sustained virological response according to different variables *n* (%)

Parameter	SVR (<i>n</i> = 11)	Non-SVR (<i>n</i> = 33)	<i>P</i> value
Center:			
CUPH	1 (9)	9 (27.3)	0.330
YAGCC	5 (45.5)	13 (39.4)	
NLI	5 (45.5)	11 (33.3)	
Age (yr)	9.9 ± 3.76	10.39 ± 3.45	0.385
Male	9 (81.8)	22 (66.7)	0.340
Expected duration of infection (yr)	3.77 ± 3.28	5.61 ± 4.02	0.341
Previous treatment trial	2 (18.2)	9 (27.3)	0.546
Previous treatment type ²			
Short-acting IFN + RBV	2 (100)	8 (88.9)	1.000
PEG-IFN + RBV	0 (0)	1 (11.1)	
Previous response type ²			
Non-responder	0 (0)	7 (77.8)	0.039 ¹
Relapser	2 (100)	2 (22.2)	
Possible cause of infection			
Surgery	4 (36.4)	10 (30.3)	0.709
Blood transfusion	4 (36.4)	11 (33.3)	0.854
Tonsillectomy	1 (9.1)	4 (12.1)	0.784
Circumcision	9 (81.8)	22 (66.7)	0.340
Minor procedures	8 (72.7)	22 (66.7)	0.709
Vertical transmission	2 (18.2)	6 (18.2)	1.000
Family contact	4 (36.4)	14 (42.4)	0.723
Injection interval:			
5 d	1 (9)	16 (48.5)	0.020 ¹
7 d	10 (91)	17 (51.5)	
Treatment duration:			
48 wk	9 (81.8)	27 (81.8)	0.940
72 wk	2 (18.2)	6 (18.2)	
Genotype:			
4a	4 (80)	24 (77.4)	0.890
4b	1 (20)	7 (22.6)	
Viral load:			
Low	7 (64)	11 (33.4)	0.180
Moderate	4 (36)	20 (60.6)	
High	0 (0)	2 (6.0)	
Histological Activity:			
Mild	11 (100)	31 (96.9)	0.550
Moderate	0 (0)	1 (3.1)	
Fibrosis stage:			
No	1 (9.1)	12 (37.5)	0.112
Mild	10 (90.9)	18 (56.3)	
Moderate	0 (0)	2 (6.3)	
Steatosis:			
No	11 (100)	28 (87.5)	0.460
Mild	0 (0)	3 (9.4)	
Moderate	0 (0)	1 (3.1)	

¹Significant; ²Percentages were calculated for those with previous treatment trial. SVR: Sustained virological response.

using the same IFN type and in children using other IFN types might be partially explained by the high percentage of previous non-responders (9/12) and relapsers (2/12) included in the study (12/46). Of those 12, only two showed an SVR (they were the previous relapsers).

Retreatment of children who do not demonstrate an SVR may be beneficial in patients who relapse or show viral breakthrough during treatment, but is not helpful in non-responders^[39,44]. Of our patients, 39.1% had breakthrough and 2.17% showed relapse giving an opportunity for retreatment.

Table 4 Rapid virological response, early virological response, polymerase chain reaction at week 24, and end of treatment response in sustained virological response *vs* non-sustained virological response *n* (%)

Parameter	RVR	EVR	PCR at week 24	ETR
SVR (<i>n</i> = 11)	9 (81.8)	10 (90.9)	11 (100)	11 (100)
Non-SVR (<i>n</i> = 33)	13 (39.4)	12 (36.4)	0 (0)	0 (0)
<i>P</i> value	0.015 ¹	0.002 ¹	< 0.001 ¹	< 0.001 ¹

¹Significant. SVR: Sustained virological response; ETR: End of treatment response; PCR: Polymerase chain reaction; EVR: Early virological response.

Table 5 Predictive value of rapid virological response and early virological response to sustained virological response

	Sensitivity	Specificity	PPV	NPV
RVR	81.8	60.6	40.9	90.9
EVR	90.9	63.6	45.45	95.45

PPV: Positive predictive value; RVR: Rapid virological response; NPV: Negative predictive value; EVR: Early virological response.

In this study, although the relapse rate was comparable to other studies^[39,43,45], there was a very high rate of breakthrough, which may also explain our low response rate, as the EVR was 24/46 (52%); however, this dropped to an ETR of 13/46 (28.2%) because of the high rate of breakthrough (39.1%).

The cause of viral breakthrough is not well understood. Some have postulated that it occurs as a result of neutralizing antibodies to IFN, downregulation of IFN receptors or development of IFN resistance and emergence of quasispecies that are less sensitive to IFN^[46]. Also, the overall adherence to ribavirin significantly influences the SVR. Notably only one (2%) patient had ribavirin dose reduction in this study.

The 5-d schedule did not affect the response rate. Treatment duration (whether 48 wk or extended course to 72 wk) gave the same response rate. In the extended treatment group, four patients received this extended treatment because of breakthrough (three at week 24 and one at week 12). None benefited from the 72 wk treatment. On the other hand, two out of the four patients who received the extended course because of delayed response achieved an SVR.

In the study of Druyts *et al*^[43], only 4% of patients discontinued treatment because of breakthrough.

In our study, 10 out of 11 patients (90.9%) who had achieved SVR had EVR. EVR had a PPV of 45.45% and an NPV of 95.45% for SVR. Nine of the SVR patients (81.8%) had RVR. RVR had a PPV of 40.9% and an NPV of 90.9% for SVR. Thus, EVR is slightly better than RVR for the negative and positive prediction of SVR. According to Druyts *et al*^[43], most of the patients who achieved an EVR (70%) also achieved an SVR (58%). This emphasizes that the EVR is crucial and cost-effective in selecting those who can discontinue treatment at week 12 if they remain positive.

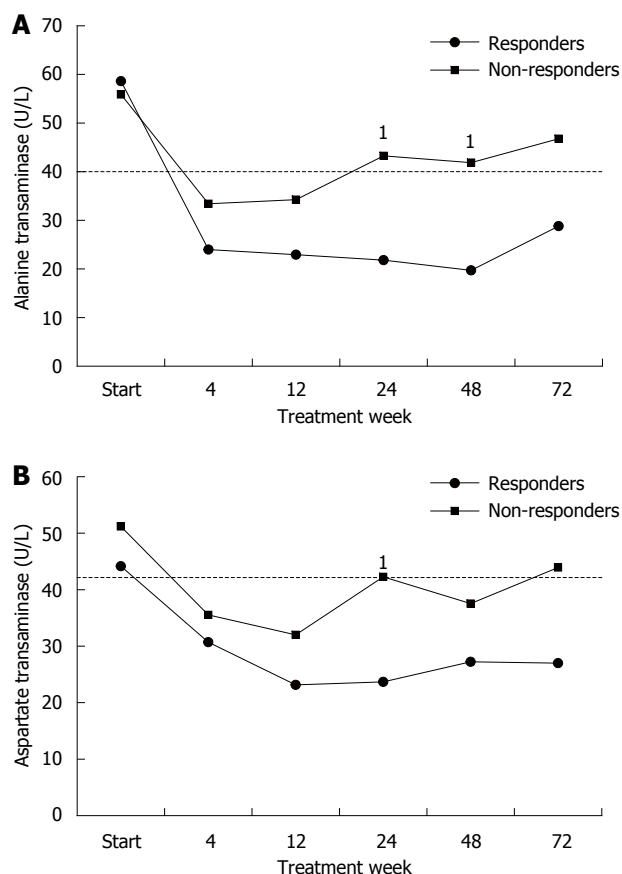


Figure 2 Alanine aminotransferase and aspartate aminotransferase mean levels during treatment in responders and non-responders. A: Alanine aminotransferase (ALT) mean levels remained normal in responders all through the follow up period, while in non-responders, after starting as normal, they increased again to significantly higher levels than in responders from week 12 onwards, especially at weeks 24 and 48 ($P = 0.007$, 0.003 respectively); B: Aspartate aminotransferase (AST) mean levels remained normal in responders all through the follow up period, while in non-responders, after starting as normal, they significantly increased again in week 24 ($P = 0.007$) and once more in week 72. The dashed line represents the upper limit of normal.

According to the present study, factors related to better response were male gender, short duration of infection, low viral load, mild activity and mild fibrosis. Patients with high viral load and absence of fibrosis showed failure to respond to treatment. In addition, all patients with steatosis (four patients) failed to achieve an SVR. Furthermore, those with previous treatment trials, namely the previous non-responders, showed failure of retreatment with IFN/ribavirin. Novel direct acting antiviral drugs (DAAs), which target specific hepatitis C virus enzymes, showed encouraging results in adults when used alone or in combination with IFN/ribavirin. DAAs have been studied in relapsers, partial responders and null responders to prior PEG-IFN/ribavirin therapy. The SVR rates were approximately 85%, 57%, and 31%, respectively^[47]. A Japanese study of 10 prior null responders with HCV genotype 1b found 90% achieved an SVR using a combination of two DAAs^[48]. Such regimens may be of benefit and worth future clinical trials in children to ensure safe and appropriate use of these new agents, especially in those with treatment failure to IFN-based

Table 6 Treatment side effects *n* (%)

Side effect	<i>n</i> = 46
Flu like symptoms	15 (32.6)
Headache	15 (32.6)
Fever	27 (58.7)
Injection site reaction	7 (15.2)
Itching	1 (2.2)
Fainting	1 (2.2)
Vomiting	6 (13)
Nervousness	1 (2.2)
Loss of appetite	10 (21.7)
Sleeplessness	1 (2.2)
Rigors	3 (6.5)
Neutropenia	6 (13)
Anemia (Hb 8.5-10 g/dL)	8 (17.4)
Anemia (Hb \leq 8.5 g/dL)	2 (4.3)
Abdominal pain	2 (4.3)
Arthralgia	5 (10.9)
Diarrhea	4 (8.7)

therapy.

In other studies^[36,39,49], predictors of response were early viral response, lower baseline HCV-RNA levels, female sex, non-maternal route of transmission of HCV, absence of steatosis on liver histology, and moderate inflammation on liver biopsy.

Constitutional symptoms are almost universal in children undergoing IFN therapy. Bone marrow suppression induced by IFN constitutes the next most common toxicity after constitutional symptoms, occurring in approximately one third of treatment recipients^[35,36]. In the current study, only mild reversible adverse effects were observed. Fever was seen in the first few weeks of treatment in 58.7% of patients, and both flu-like symptoms and headaches appeared in 32.6% of patients. Anemia and neutropenia were found at a rate of 21.7% and 13%, respectively, and were treated by reduction or skipping of doses; none of the patients developed thrombocytopenia. According to a meta-analysis reported in 2013^[43], neutropenia was the most common hematological adverse event evaluated (32%), whereas anemia and thrombocytopenia were less frequent (11% and 5%, respectively). Dose reductions for neutropenia occurred in 38% of patients in the North American study and 12% in the European study respectively^[35,36]. Drug cessation because of neutropenia did not occur in either study. In the North American study^[36], there was no significant thrombocytopenia, and in the European study, one patient discontinued therapy at week 42 because of thrombocytopenia (platelet count 45000 cells/mm^3)^[35].

The strength of this study is that it is a multicenter one; including 46 children with chronic HCV, using the *Hansenula*-derived PEG-IFN-alpha-2a (a 20 KDa Reiferon Retard) plus ribavirin, using customized treatment and reporting the response to treatment in children. The limitation of the study is the relatively small sample size.

In conclusion, combined therapy in the form of Reiferon retard plus ribavirin was safe. Children tolerated the treatment well, with only mild reversible adverse effects.

The end of treatment response was 28.2% and the factors related to better response were male gender, short duration of infection, low viral load, mild activity and mild fibrosis. Our customized regimen did not influence the SVR rate, and future clinical trials with novel antiviral drugs may be of benefit to those with treatment failure.

ACKNOWLEDGMENTS

The study was registered at www.ClinicalTrials.gov (NCT02027493).

COMMENTS

Background

Despite recent success after the introduction of combination therapy with interferon (IFN)-alpha and ribavirin, genotype 4 is considered difficult-to-treat. Approximately 60% of patients fail to respond. Resistance to antiviral therapy remains a serious problem in the management of chronic hepatitis C. Establishing novel therapeutic agents, treatment customization, and determining the factors associated with better response rates remain the targets of many researchers.

Research frontiers

Reiferon Retard is a novel 20-kDa PEGylated (PEG)-IFN-alpha-2a derived from the *Hansenula polymorpha* expression system. It has been used in adults with chronic hepatitis C virus to achieve a sustained virological response (SVR) ranging from 56% to 60.7%, while no studies have been reported in the pediatric population.

Innovations and breakthroughs

The current study is the first to use the novel *Hansenula*-derived PEG-IFN-alpha-2a in children. Treatment customization regarding duration (72 wk vs extended course of 48 wk) and IFN injection frequency (5-d schedule vs 7-d schedule) demonstrated safety and tolerability in children, yet did not improve response rates. This may be, in part, explained by the high percentage of previous non-responders included in the study.

Applications

The study results suggest that combined therapy in the form of Reiferon Retard plus ribavirin was safe. Children tolerated the treatment well, with only mild reversible adverse effects. Treatment duration extension and/or shortening the injection interval did not improve the SVR rates. Male gender, short duration of infection, low viral load, mild activity, and mild fibrosis are associated with favorable response.

Terminology

The *Hansenula polymorpha* expression system is known for its superior characteristics. Increasing numbers of products and protein candidates have been derived from this expression system; therefore, it has been gaining greater popularity in recent years. *Hansenula polymorpha* represents an absolute mitotic stable, robust and safe expression system, which boasts one of the highest productivities ever described for a recombinant protein, with maximum purity and high biological activity. In addition, production processes based on *Hansenula polymorpha* technology are very cost effective. The cost effectiveness is strongly related to very short fermentation times and to a significantly reduced number of downstream steps, resulting in a higher purity, with no forms of oxidized interferon being detected.

Peer review

This is a well done study, presented in a detailed fashion. Though it is already known that extending treatment beyond 48 wk achieves little extra benefit, your paper convincingly proves the case for genotype 4 infected children (including prior non-responders), which is a not so widely studied sub-group.

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Localization and vasopressin regulation of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter in the distal colonic epithelium

Hong Xue, Zi-Juan Zhang, Xiao-Shuang Li, Hai-Mei Sun, Qian Kang, Bo Wu, Ya-Xi Wang, Wan-Jing Zou, De-Shan Zhou

Hong Xue, Xiao-Shuang Li, Hai-Mei Sun, Qian Kang, Bo Wu, Ya-Xi Wang, Wan-Jing Zou, De-Shan Zhou, Department of Histology and Embryology, School of Basic Medical Science, Capital Medical University, Beijing 100069, China
Zi-Juan Zhang, Experiment Teaching Center, School of Basic Medical Science, Henan University of Traditional Chinese Medicine, Zhengzhou 450008, China

Author contributions: Xue H, Wang YX, Kang Q and Li XS performed the experiments; Xue H, Zhang ZJ and Sun HM contributed to the reagents; Xue H, Wu B and Zou WJ collected the data; Zhou DS and Xue H designed the research and wrote the paper.

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Correspondence to: De-Shan Zhou, MD, Department of Histology and Embryology, School of Basic Medical Sciences, Capital Medical University, Xitoutiao 10, Beijing 100069, China. zhouds08@ccmu.edu.cn

Telephone: +86-10-83950079 Fax: +86-10-83911449

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Abstract

AIM: To investigate whether $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (NKCC2) is expressed in the mouse distal colonic epithelia and whether it is regulated by vasopressin in the colon.

METHODS: The mRNA expression of NKCC2 in the mouse colonic mucosa was examined by reverse transcription-polymerase chain reaction. NKCC trafficking in the colon stimulated by 1-D-amino(8-D-arginine)-vasopressin (dDAVP) infusion (10 ng/mouse, intraperitoneal injection) within 15 min, 30 min and 1h was investigated by laser confocal scanning microscopy. Total and

membrane NKCC2 expression in the colonic mucosa from control and dDAVP-treated mice was detected by Western blotting. Short circuit current method was performed to determine regulation of NKCC2 by vasopressin in the colon.

RESULTS: NKCC2 was predominantly located in the apical region of the surface of the distal colonic epithelia; by comparison, a large amount of NKCC1 was distributed in the basolateral membrane of the lower crypt epithelia of the mouse distal colon. Short-term treatment with dDAVP, a V2-type receptor-specific vasopressin analog, induced NKCC2 re-distribution, *i.e.*, NKCC2 traffics to the apical membrane after dDAVP stimulation. In contrast, no obvious NKCC1 membrane translocation was observed. Western blotting results confirmed that membrane NKCC2 had significantly higher abundance in the dDAVP-treated mouse colonic mucosa relative to that in the untreated control, which is consistent with our immunostaining data. Moreover, the short-circuit current method combined with a NKCC2 inhibitor demonstrated that NKCC2 was also activated by serosal vasopressin in isolated distal colonic mucosa.

CONCLUSION: Our results provide direct evidence that vasopressin also plays an important role in the colonic epithelia by stimulating NKCC2 trafficking to the apical membrane and inducing NKCC2-mediated ion transport.

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Key words: $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter; Apical membrane; Vasopressin; Distal colonic epithelia; Trafficking

Core tip: The $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (NKCC2), which was thought to be only expressed in the apical membrane of the epithelial cells in the thick ascending limb of Henle's loop, was recently found to be expressed in

the colon. However, the role and regulating mechanism of NKCC2 in the gut are still not completely understood. Our results provide direct evidence that vasopressin also plays an important role in the colonic epithelia by stimulating NKCC2 trafficking to the apical membrane and inducing NKCC2-mediated ion transport. The action of vasopressin on NKCC2 in the colon would be recognized to supplement the role of the kidney in modulating whole-body homeostasis and electrolyte balance in physiological or pathophysiological conditions.

Xue H, Zhang ZJ, Li XS, Sun HM, Kang Q, Wu B, Wang YX, Zou WJ, Zhou DS. Localization and vasopressin regulation of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter in the distal colonic epithelium. *World J Gastroenterol* 2014; 20(16): 4692-4701 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4692.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4692>

INTRODUCTION

The bumetanide-sensitive $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporters (NKCC) mediate the electroneutral movement of 1 Na^+ , 1 K^+ and 2 Cl^- ions across cell membranes^[1,2]. Two isoforms, NKCC1 and NKCC2, are currently known and are encoded by different genes. NKCC1 (SLC12A2) is widely distributed in the basolateral membrane of secretory epithelial cells^[3]. In contrast, NKCC2 (SLC12A1) is primarily localized to the apical membrane of the epithelial cells of the thick ascending limb of Henle's loop (TALH), where it mediates the apical entry of Na^+ , K^+ (or NH_4^+) and Cl^- ^[4]. NKCC2 has been thought to be a kidney-specific isoform. However, there is growing evidence for extra-renal expression, including expression in the pancreas and the gastrointestinal tract of rats and humans, where it might mediate Cl^- absorption^[5-8]. The NKCC2 protein expression and location as well as its mechanism of regulation in the mouse distal colon are not understood.

Vasopressin (also termed antidiuretic hormone) increases NaCl absorption in the TALH^[9,10] and Na and water absorption in cortical collecting duct cells^[11]. Several reports have verified that vasopressin has both long-term and short-term effects on NKCC2 expression and function in TALH cells. In the short term, the vasopressin-dependent phosphorylation of NKCC2 is associated with vesicular trafficking of the transporter to the luminal membrane^[12,13]. Long-term vasopressin exposure in the TALH or in collecting duct principal cells increases the expression of NKCC2^[14] and aquaporin 2^[15], respectively. The colon could also be a target for vasopressin^[16] because vasopressin stimulates NaCl and water absorption in *in vitro* preparations of mouse, rat, and human colons^[17-21]. However, little is known about the mechanism of ion transport that is induced by vasopressin in the colon. Specifically, the identity of the protein that mediates NaCl absorption in the colon and whether NKCC2 is involved in this process are currently unclear.

Previous studies have focused on the regulation of NKCC2 by vasopressin in the kidney. How vasopressin regulates colonic NKCC2 is unknown. The present study addresses this issue by showing the NKCC2 expression and spatial distribution in the mouse colonic epithelia. We investigated the NKCC2 redistribution and trafficking in the colonic epithelia following short-term exposure to vasopressin. We also explored whether NKCC2 is involved in the ion transport induced by vasopressin using the short circuit current method in isolated colonic mucosa. Our results show that the effects of vasopressin on colonic NKCC2 are similar to those described for the kidney. The significance of this finding for colonic epithelial physiology is discussed.

MATERIALS AND METHODS

Animals and tissue preparations

Male C57BL/6 mice weighing 20-25 g (Laboratory Animal Services Center, Capital Medical University, Beijing, China) were fed a normal diet with free access to water. The protocol was approved by the Animal Care and Use Committee of Chinese Capital Medical University. On the day of the experiments, 10 ng of dDAVP (a vasopressin analogue; Sigma) per animal^[12] or saline (as a control) was administered by intraperitoneal injection. To reduce the level of endogenous vasopressin, the animals were water-loaded by offering them a 5% dextrose/1% ethanol solution overnight. The water load was assessed by measuring the solution intake. The mice were killed by cervical dislocation. The distal colon was removed by opening the abdominal cavity 15 min, 30 min and 1 h after drug application. Intestinal segments were briefly rinsed with ice-cold PBS. Frozen sections (5 μm) were cut on a cryostat (Leica, CM3050S), mounted on glass slides and stored at -20°C . Tissues were cut into 2- μm -thick rings, fixed in 2% paraformaldehyde in PBS at pH = 7.4 for 1 h at room temperature and then rinsed with PBS and cryoprotected in 30% sucrose overnight. The approach of the tissue preparation and arrays was completely based on a previously described method^[22].

Immunofluorescence staining

Intestinal tissues were fixed in 2% (w/v) paraformaldehyde-PBS for 1 h at 25°C . Following fixation, the tissues were cryoprotected in 30% sucrose overnight in the cold, embedded in Tissue-Tek O.C.T medium and frozen in liquid nitrogen. The sections were rehydrated in PBS and incubated for 2 h in a blocking solution (BS) consisting of PBS, 10% goat serum or donkey serum and 0.1% Triton-X (pH = 7.4). Then, the sections were incubated with a primary antibody overnight at 4°C . After washing with PBS, the sections were incubated with the corresponding secondary antibody for 1 h at 25°C . The primary and secondary antibodies used in this study are summarized in Table 1. Immunostaining controls were performed by omitting the primary antibody or by using nonspecific IgG. The NKCC2 antibodies were purchased

Table 1 Primary and secondary antibodies used in this experiment

Antibody	Host species	Dilution	Source/Catalog No.
NKCC2	Rabbit	1:100	Santa cruz/sc-133823
NKCC2	Rabbit	1:150	Fitzgerald/70R-3806
NKCC1	Goat	1:100	Santa cruz/sc-21574
T4	Mouse	1:200	Developmental Studies Hybridoma Bank
Rabbit IgG	Donkey (Texas red)	1:400	Abcam
Goat IgG	Donkey (Alexa 488)	1:200	Invitrogen
Mouse IgG	Goat (cy3)	1:200	Invitrogen
Rabbit IgG	Goat (HRP)	1:2000	Santa cruz

NKCC2: Sodium-potassium-chloride cotransporter 2; NKCC1: Sodium-potassium-chloride cotransporter 1.

from two different commercial sources. NKCC1 and NKCC2 antibodies were preadsorbed with their corresponding control peptides (Santa Cruz sc-21547P 10 µg per 1 µg NKCC1 antibody; Fitzgerald 33R-6671, 5 µg per 1 µg NKCC2 antibody) to determine the specificity of the antibodies. The specimens were then examined using a fluorescence microscope (Nikon 80i, Japan) or a confocal laser scanning microscope (Leica TCS SP5 MP, Germany).

Fluorescence image analysis

Immunolabeled sections were examined using a confocal laser scanning microscope. Confocal images were converted to 2-channel (red and green) mode by subtracting DAPI (blue). The confocal images were analyzed using Image J software. Areas of NKCC2 labeling were highlighted in white using the Image J. Data from 4 to 12 selected areas were averaged in each image; 6 images were randomly selected for analysis from each measurement group of one animal ($n = 6$ images; $n' = 4-12$ selected areas), and data were collected from three animals ($n = 3$). The method described above was based on the recently published work from the laboratory of Prof. Ameen^[22,23]. Statistical analysis was performed using GraphPad Prism software. Differences among groups were determined using one-way ANOVA and the Tukey's post hoc method of multiple comparisons. The level of significance was set at $P < 0.05$.

Ussing chamber experiments

The distal colon was cut longitudinally along the mesenteric border. The serosa, muscularis and submucosa were stripped away with fine forceps to prepare the mucosa sample. The stripped mucosa was mounted in a modified Ussing chamber in a tissue holder (Easy Mount Chamber; Physiologic Instruments, San Diego, CA) with an aperture surface area of 0.3 cm², and the sample was bathed bilaterally in Krebs-Henseleit solution (KHS).

The short-circuit current was measured *in vitro* in the Ussing chambers. The transepithelial PD was then clamped at 0 mV, and the short-circuit current (I_{sc}) was recorded with a VCC MC6 voltage-current clamp am-

plifier (Physiologic Instruments, San Diego, CA). The transepithelial resistance (TR) (Ωcm^2) was measured by altering the membrane potential in a stepwise fashion (-0.1 mV) and applying the Ohmic relationship.

Solutions and chemicals

Krebs-Henseleit solution (KHS) (mmol/L): NaCl, 117; KCl, 4.7; MgCl₂, 1.2; KH₂PO₄, 1.2; NaHCO₃, 24.8; CaCl₂, 2.5; and glucose, 11.1. The solution was bubbled with 95% O₂ to 5% CO₂ to maintain a pH value of 7.4. The dDAVP, bumetanide, indomethacin, tetraethylammonium (TEA), tetrodotoxin (TTX), vasopressin and amiloride were purchased from Sigma (St Louis, MO, United States). Stock solutions of all the above chemicals were dissolved in DMSO. The final DMSO concentrations never exceeded 0.1% (v/v). Preliminary experiments indicated that the vehicle did not alter any baseline electrophysiological parameters.

Reverse transcription-polymerase chain reaction

RNA from the stripped mucosa and renal medulla was harvested using the Trizol RNA purification system (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The following primers were used for the PCR of NKCC2: forward 5'-TTGAGA-TTGGCGTGGTCATA-3' and reverse 5'-AAGCAT-GTCAGCCAGCTTTT-3'. The following primers were used for β -actin, an internal control: forward 5'-TGT TTG AGA CCT TCA ACA CC-3' and reverse 5'-CAG TAA TCT CCT TCT GCA TCC-3'. The amplification products and a DNA size marker (DNA maker C; SBS Genetech, Beijing, China) were separated by electrophoresis on a 1.5% agarose gel in 0.5 TRIS-borate-EDTA buffer containing ethidium bromide. The bands of the amplification products were viewed using ultraviolet light, and the images were taken using a GelDoc2000 (Bio-Rad Laboratories, Hercules, CA, United States).

Colonic epithelial protein isolation and membrane protein preparation

The serosa, muscularis and submucosa were stripped away with fine forceps to prepare the mucosa sample. The protocol was based on the manufacturer's instructions (Boogo Company, Shanghai W002). The preparation was homogenized in ice-cold tissue protein extraction reagent A, and then sonicated until the sample was completely dissolved. After freezing in liquid nitrogen and thawing at 25 °C three times, the samples were centrifuged at 5000 *g* at 4 °C for 10 min, and the supernatants were carefully collected. Then, the sample was centrifuged at 14000 *g* at 4 °C for 30 min to precipitate the membrane fragments. Protein extraction reagent B was added to the precipitates to centrifuge at 14000 *g* at 4 °C for 10 min. The supernatants were thoroughly removed, and then protein extraction reagent C was added.

Total protein preparation

Tissue was homogenized in 300 µL of cold lysis buffer,

and the total tissue homogenates were sonicated until they were completely dissolved. The sample was then centrifuged at 12000 rpm for 30 min at 4 °C. The protein concentration of the samples was measured using a Bradford assay kit.

Immunoblotting

Total NKCC2 proteins (20 µg) or membrane NKCC2 proteins (40 µg) were separated by 8% SDS/PAGE, and the separated proteins were electroblotted onto a PVDF membrane (Millipore), which was then washed for 10 min with TBST and immersed in blocking buffer containing 5% non-fat dry milk in TBST for 1 h at 25 °C. The blot was washed with TBST and then incubated with a polyclonal primary antibody against NKCC2 (Santa Cruz sc-133823) overnight at 4 °C. After washing in TBST, the blot was incubated with a secondary antibody against rabbit IgG (Santa Cruz) for 1 h at 25 °C. The blot was finally washed with TBST, and the protein bands were visualized with a chemiluminescence system (ECL Plus, Applygen Technologies Inc.). The resulting image was analyzed using Total Lab Quant software.

Statistical analysis

Data are expressed as mean \pm SD; statistical significance between two individual measurement groups was determined using an unpaired *t* test. Differences among groups were determined using one-way ANOVA and the Tukey's post hoc method of multiple comparisons. The level of significance was set at $P < 0.05$.

RESULTS

We first sought to investigate the expression of NKCC2 and determine the location of NKCC in the mouse colonic epithelia. Reverse transcription-polymerase chain reaction and immunostaining were performed. As shown in Figure 1A, a band of the expected size (1161 bp) was amplified in both the colonic mucosa and the kidney. As a negative control, samples lacking amplified products were obtained by using non-reverse-transcribed RNA from the mouse colonic mucosa or by omitting cDNA. Immunofluorescence staining revealed a high level of NKCC1 in the lower crypt of the colonic epithelia, which is the predominant site of secretion^[24]. In the crypt regions, NKCC1 was mainly localized to the basolateral membrane (Figure 1B), consistent with previous observations in mammalian intestines^[25]. In contrast, the NKCC2 immunoreactivity at the colonic surface epithelia was observed predominantly in the apical membrane (arrowhead, Figure 1C), consistent with observations in rat and human colonic tissues^[6,7]. The spatial distributions of NKCC1 and NKCC2 differed in the mouse colon. NKCC2 was located in the apical membrane of the mouse colonic surface epithelia, whereas NKCC1 was found in the lower crypt epithelia of the mouse distal colon. Preadsorption of NKCC1 or NKCC2 antibodies with the corresponding control peptides abolished the

immunoreactivity (Figure 1D and H), suggesting that both antibodies were specific. The NKCC2 antibody was also tested in the mouse kidney as a positive control. Greater NKCC2 abundance was detected in the apical regions of cells lining the thick ascending limb segment of the mouse nephron (Figure 1F). No staining was observed when the NKCC2 primary antibody was omitted (Figure 1G). T4 is a monoclonal antibody that is generated against a fusion protein encompassing the carboxy terminus (S760-S1212) of human NKCC^[26]. The antibody is known to recognize both the apical NKCC2 and basolateral NKCC1 isoforms of the Na-K-2Cl cotransporter^[26]. As expected, T4 immunoreactivity was observed in the apical membrane, further supporting the NKCC2 localization data (Figure 1E and I).

NKCC2 has been demonstrated to be modulated by vasopressin in the TAL^[12,13]; thus, we next investigated whether the apically expressed NKCC2 was regulated by short-term treatment with vasopressin. Short-term vasopressin treatment has been reported to induce NKCC2 apical vesicular trafficking in medullary TAL cells^[12]. Therefore, we investigated whether vasopressin could cause a redistribution of NKCC2 in the colonic epithelia. In this study, we used a V2-type receptor-specific vasopressin analogue, dDAVP, to examine its effect on NKCC2 trafficking. Higher magnification images of unstimulated colon sections indicated that NKCC2 immunoreactivity was also present in the intracellular compartments (Figure 2A). dDAVP (10 ng per animal, intraperitoneal injection) was administered to adult mice. At 30 min post-stimulation, more intense staining for NKCC2 was detected in the apical region of surface enterocytes, as shown in Figure 2C. Notably, at 1 h post-stimulation, the vasopressin-induced membrane recruitment of NKCC2 resulted in labeling intensities that were higher in the apical membrane than the 30 min post-stimulation intensities (Figure 2D, d). This observation was confirmed by densitometry: the NKCC2 fluorescence intensity on the apical membrane of enterocytes ranged from values that were approximately 1.5-fold to 3-fold higher than that in the untreated mouse (normalized to the apical membrane of the surface cells in untreated mice, $n = 3$ mice, $n = 6$ images, $n' = 4-12$ selected area $P < 0.001$, Figure 2I). The dramatic recruitment of NKCC2 to the apical membrane seemed to occur for at least 30 min because no significant differences could be detected between the 15 min post-stimulation and unstimulated states (Figure 2B). At the same time, we also determined whether NKCC1 was redistributed in response to vasopressin. In the control condition, the NKCC1 labeling was predominantly found at the basal and lateral membranes, and there was a partial punctate vesicular-like intracellular pattern in the colonic crypt base (Figure 2E arrow). After 15 min, 30 min and 1 h of stimulation, the NKCC1 localization and labeling intensity in the basolateral membrane did not change significantly, suggesting that vasopressin might mainly affect NKCC2 (Figure 2F, G, H and J).

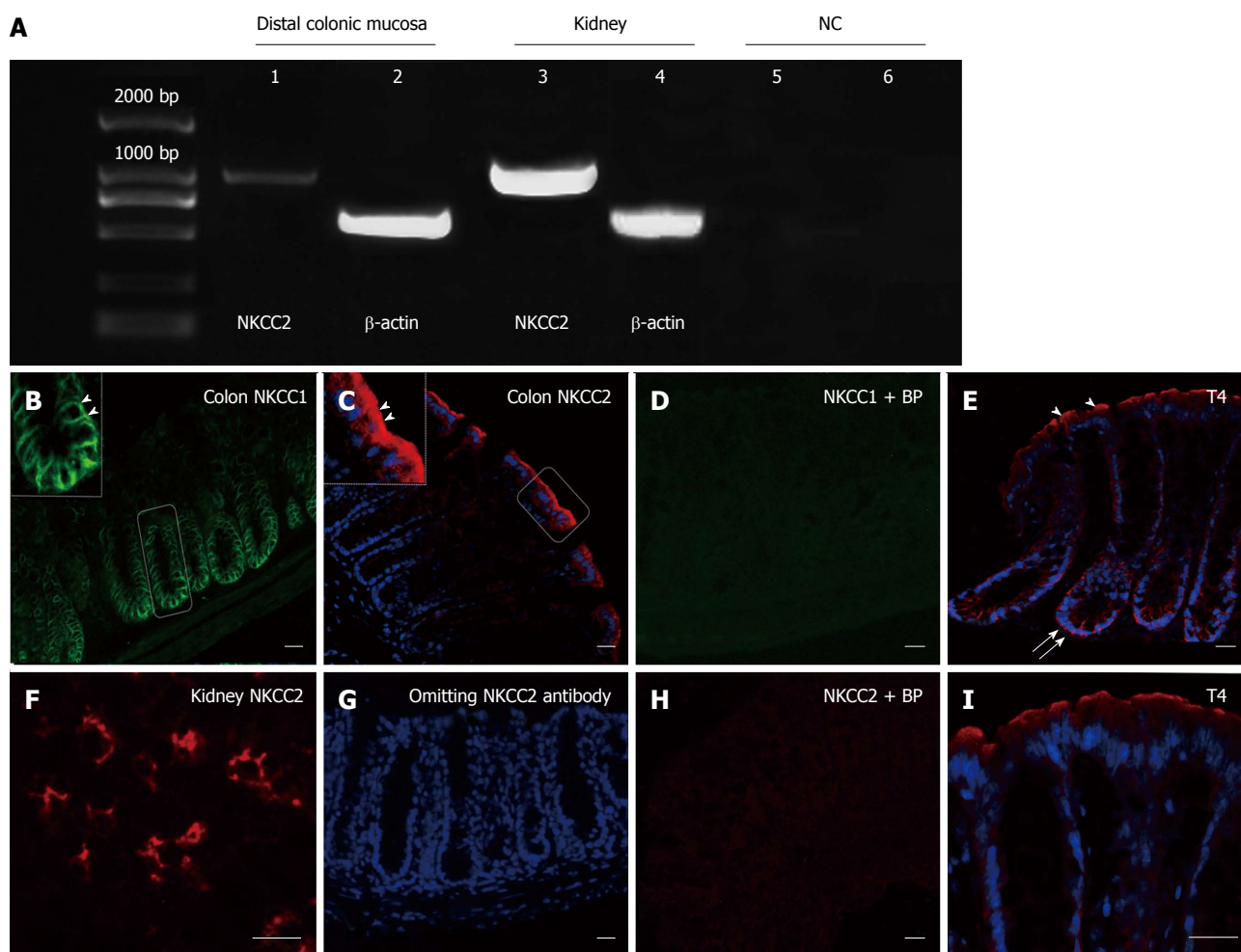


Figure 1 Expression and spatial distribution of Na⁺-K⁺-2Cl⁻ cotransporter in the mouse colon and kidney. RT-PCR identified mRNA transcripts for NKCC2 (1161 bp) in the mouse distal colon mucosa (lane 1) and kidney (lane 3) (A). In control experiments, no band was detected using non reverse transcribed RNA (lane 5) or by omitting cDNA (lane 6). The expression of β-actin is also shown as an internal control (lanes 2 and 4). Localization of the NKCC1 protein in the basolateral membrane along lower crypt epithelium (B) (arrowhead, the left inset is enlarged image of white rectangle) and NKCC2 in the apical membrane along the surface epithelium (C) (arrowhead, the left inset is enlarged image of white rectangle $n = 3$ mice) were clearly observed. Preadsorption of NKCC1 and NKCC2 antibodies with their corresponding blocking peptides (+ BP) resulted in no immunoreactivity (D, H). NKCC2 expression in the apical membrane of TAL cells in the kidney served as a positive control (F). Primary NKCC2 antibody was omitted also as a negative control (G). NKCC labeling with T4 antibody at the surface and crypt of the colonic epithelium (arrowhead: apical arrow: basolateral) (E) Higher magnification of surface epithelium (I). Scale bar = 20 μm; NC: Negative control; NKCC2: Na⁺-K⁺-2Cl⁻ cotransporter.

Immunoblot analysis of whole cell homogenates of the mouse colonic mucosa showed the strong signals for the NKCC2 at 140 KD, which were also found in the kidney; this finding also confirmed the NKCC2 expression in the colon. Western blotting showed no obvious change in the overall NKCC2 abundance, similar to its behavior in the kidney (Figure 3A); this finding indicated that vasopressin induced redistribution of NKCC2 from intracellular vesicles to the apical membrane without affecting the overall level of NKCC2 expression. To further substantiate this finding, protein samples that were enriched for plasma membranes were also analyzed. An equal amount of protein (40 μg) was loaded and we found that dDAVP caused a significant increase in the NKCC2 membrane abundance with time by comparison with unstimulated samples (Figure 3B). Densitometry analysis confirmed the significantly higher abundance in the dDAVP-treated mouse colonic mucosa relative to that in the untreated control, which is consistent with our

immunostaining data (Figure 3C, $n = 4$ mice, $P < 0.01$).

Because NKCC2, an absorptive isoform of NKCC, was expressed in the apical region of the colonic epithelia and regulated by short-term treatment with vasopressin, we investigated whether the apical NKCC2 was responsible for the observed vasopressin-induced electrolyte transport. To avoid complications due to the effects of vasopressin on the microcirculation and motility *in vivo*, isolated mucosa was used for these *in vitro* experiments. Based on the parameters reported in the previous study, we used a vasopressin concentration of 5×10^{-8} mol/l^[9]. Before the experiments were performed, the freshly isolated distal colonic segment was pretreated with indomethacin (10 μmol) and TTX (tetrodotoxin, 1 μmol), a neuronal Na⁺ channel blocker, on the serosal side to suppress endogenous prostaglandin production and the neurally mediated effects.

The serosal addition of vasopressin (5×10^{-8} mol) induced an immediate decrease of -11.41 ± 1.12 μA/

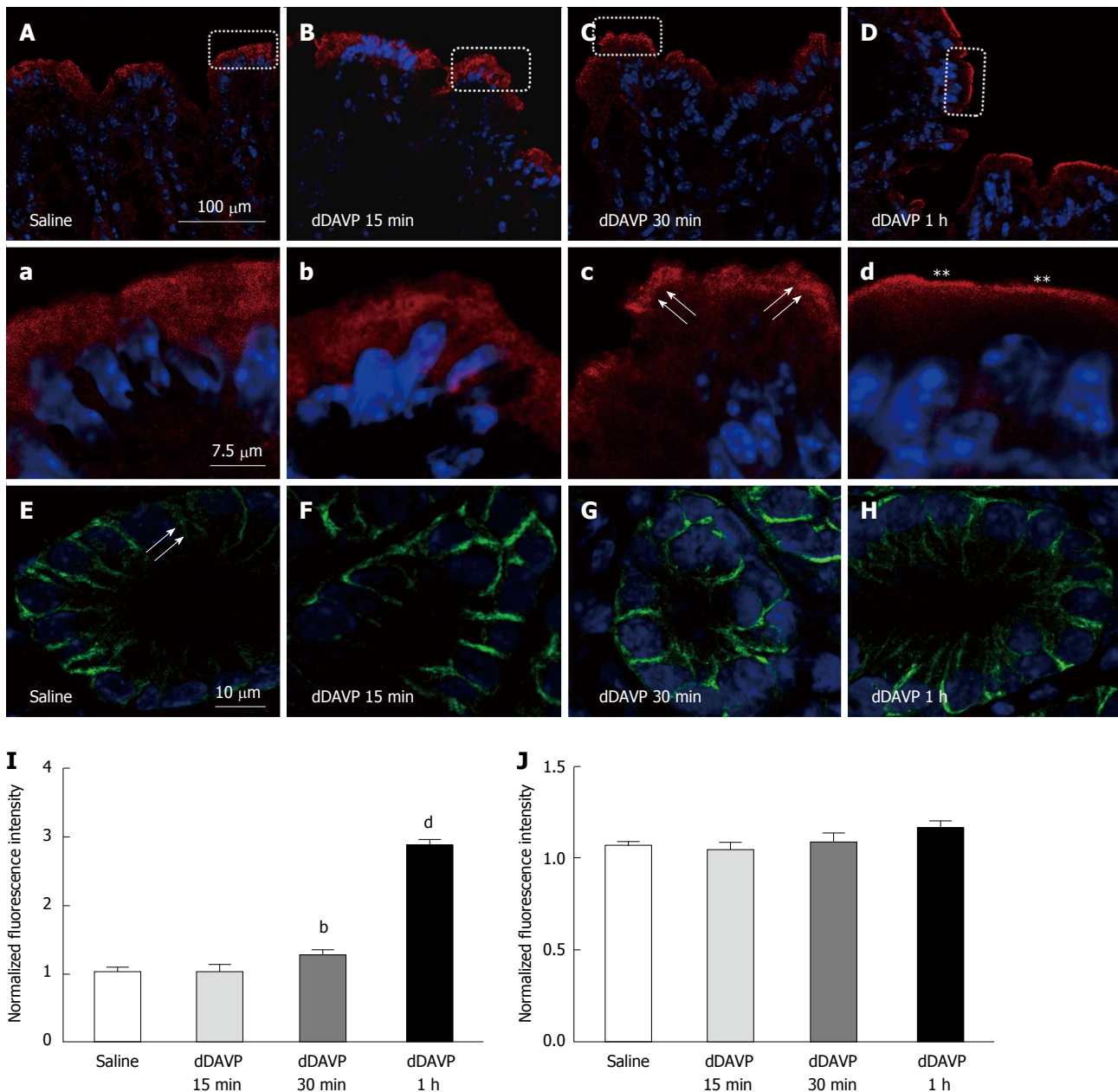


Figure 2 Effect of vasopressin on the cellular and subcellular location of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter and $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter 2. Vasopressin-induced redistribution of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (NKCC2) in the mouse distal colon (A-C). Mice were treated with saline (A) or dDAVP (10 ng) (B: 15 min; C: 30 min; D: 1 h), and NKCC2 and NKCC1 immunolabeling was performed. A-D: Lower magnification images. Higher magnification images of the white rectangles in the corresponding A-D images. The arrow and asterisk indicate increased apical NKCC2; arrowheads indicate NKCC2 localization. Most of the NKCC2 labeling appears to be intracellular in the control mice. NKCC2 recruitment to the apical membranes of enterocytes at 30 min and 1 h post-stimulation is shown (C and D arrow and asterisk). However, vasopressin seemed to have no obvious effects on the redistribution of NKCC1 (I, J). Apical membrane NKCC2 and basolateral NKCC1 fluorescence intensity in saline- or dDAVP-treated distal surface epithelia was normalized to the apical or basolateral membrane in the saline-treated mouse. A significant difference in NKCC2 and NKCC1 intensity was observed at 15 min, 30 min and 1 h compared with that in the unstimulated state (E-H). $N = 3$ mouse, $n = 6$ images, $n' = 4\text{-}12$ selected area;

cm^2 ($n = 13$) in I_{sc} , consistent with the results of the previous study^[20]. To determine whether NKCC2 was responsible for the vasopressin-induced decrease in I_{sc} , we pretreated the isolated distal colonic segment with bumetanide (10 μmol), a well-known inhibitor of the NKCC, on the apical side. As shown in Figure 4A and D, apical pretreatment with bumetanide significantly inhibited the vasopressin-induced I_{sc} response by 50% (from $-11.41 \pm 1.12 \mu\text{A}/\text{cm}^2$ to $-6.46 \pm 0.76 \mu\text{A}/\text{cm}^2$, $n = 9$, $P < 0.01$, Figure 4A), implying that apical NKCC2 was

involved in the vasopressin-induced response. Previous studies demonstrated an effect of vasopressin on Na^+ absorption in the guinea pig distal colon^[27]. Thus, we tested the effect of a blocker of the epithelial Na^+ channel, amiloride (10 $\mu\text{mol}/\text{L}$). The results indicated that the change in the vasopressin-induced I_{sc} decrease was not altered by amiloride (from $-11.41 \pm 1.12 \mu\text{A}/\text{cm}^2$ to $-11.18 \pm 1.46 \mu\text{A}/\text{cm}^2$, $n = 6$, $P > 0.05$, Figure 4B and D), indicating that the vasopressin-induced response involved an amiloride-insensitive mechanism. A similar inhibition

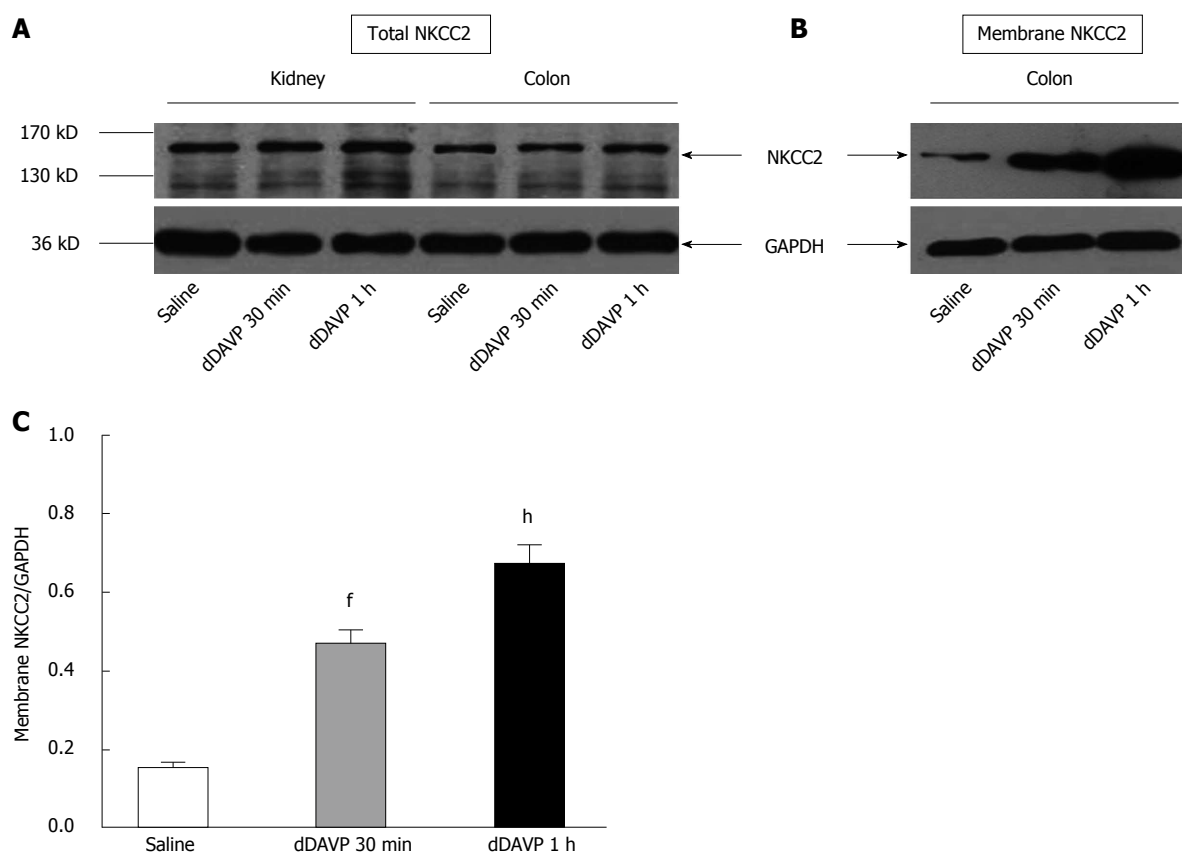


Figure 3 Western blotting analysis of total and plasma membrane $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter protein after saline or 1-D-Amino(8-D-arginine)-vasopressin injection. A: Each lane was loaded with 20 μg of protein from the mouse distal colon and kidney homogenates (control and dDAVP-treated $n = 3$ mice); B: Each lane was loaded with 40 μg of protein from the plasma membrane of the colonic mucosa ($n = 4$ mice); C: Summary of the densitometric analysis of the membrane NKCC2, normalized to GAPDH. Densitometric analysis revealed that the expression of NKCC2 was significantly higher in the treated mice than in the control mice ($P < 0.01$; $^hP < 0.01$, $n = 3$).

(33%), from $-11.18 \pm 1.46 \mu\text{A}/\text{cm}^2$ to $-7.61 \pm 0.96 \mu\text{A}/\text{cm}^2$, was observed for apical pretreatment with tetraethylammonium (TEA 5 mmol/L) (Figure 4C and D, $n = 6$, $P < 0.05$), a putative K^+ channel blocker, indicating that the vasopressin-induced downward deflection of I_{sc} was related to K^+ transport.

DISCUSSION

Previous studies have demonstrated that NKCC2 was expressed in the apical membrane of the colon and played an important role in Cl^- absorption^[6,7]. The present study not only extended these results regarding the cellular location and function of NKCC2 but also demonstrated that NKCC2 in the mouse colon is regulated by vasopressin. Our results confirmed the NKCC2 expression in the mouse distal colon and found a differential spatial distribution of NKCC1 and NKCC2. The absorptive isoform, NKCC2, was mainly located in the apical membrane of surface epithelia, whereas the secretory isoform, NKCC1, was located in the basolateral membrane of the lower crypt epithelia. The differential location pattern of NKCC1 and NKCC2 indicates their distinct roles, which are related to the functions of each cell population. The localization of NKCC2 corresponds to a possible func-

tional role in mediating apical $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ absorption in the colonic epithelia. The location of NKCC1 was consistent with previous observations showing the basolateral localization of NKCC1 in the secretory epithelia, supporting its role in chloride secretion^[28,29]. The immunofluorescence results showed that NKCC2 was only expressed in apical membrane of the epithelial cells in the colonic surface epithelium and was not expressed in the crypt epithelium. NKCC1 was only expressed in the basolateral membrane of the crypt epithelium and was not expressed in the surface epithelium. The results showed that the two isoforms were not located in the same cells or the same region.

Recently, the mechanism by which vasopressin regulates NKCC2 by inducing cellular redistribution in the kidney has been studied in more detail^[30,31]. Furthermore, the functional activity of NKCC2 depends on the transporter density at the apical region of the surface cells, which is regulated by endocytic and exocytic trafficking^[31,32]. Whether NKCC2 expression in the colon is regulated by vasopressin was unclear; our immunolocalization studies showed that a portion of NKCC2 was localized to intracellular vesicles in the unstimulated colonic epithelia. The increase in membrane-bound NKCC2 following short-term vasopressin treatment indicated a change

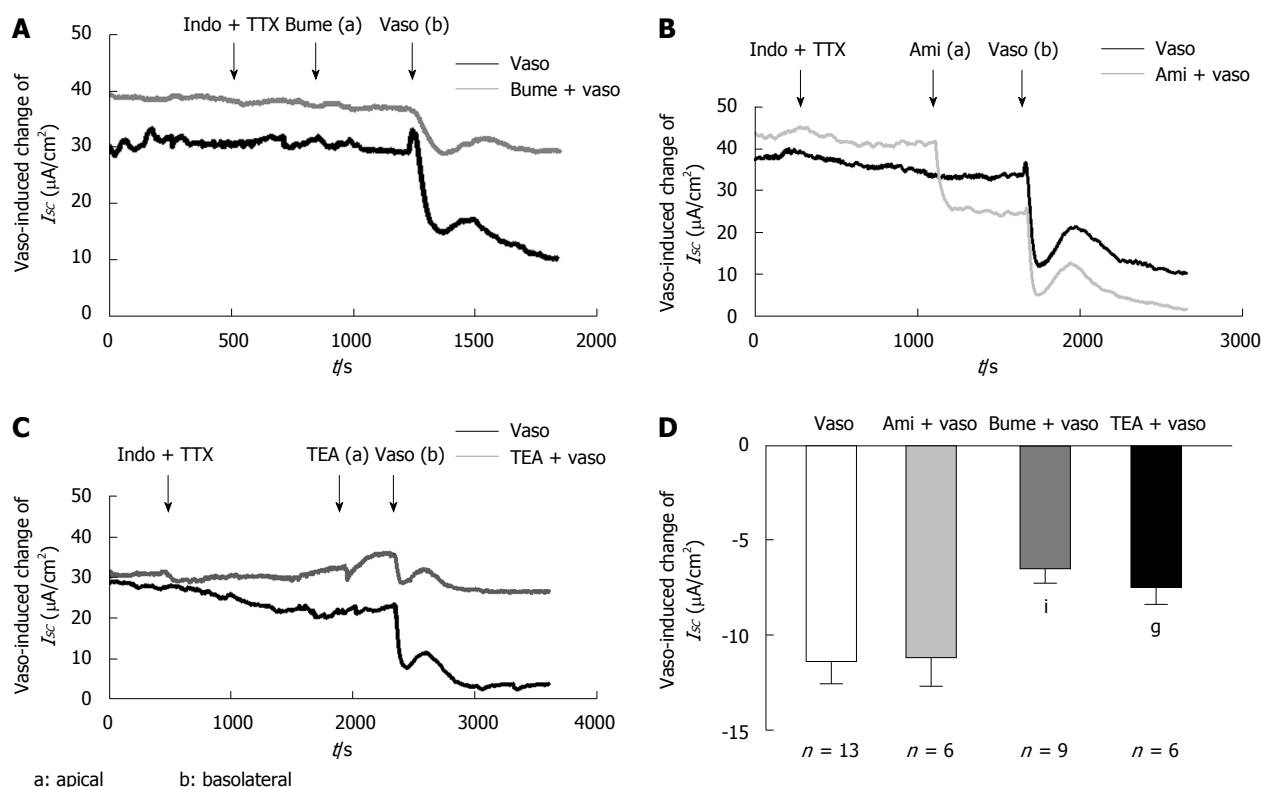


Figure 4 Apical $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter involvement in the serosal vasopressin-induced I_{sc} response. A: Representative I_{sc} recordings in response to indomethacin (10 μmol , basolateral), TTX (tetrodotoxin, 1 $\mu\text{mol/L}$, basolateral), bumetanide (10 $\mu\text{mol/L}$, apical), and vasopressin (5×10^{-8} mol/L, basolateral); B: Representative I_{sc} recordings in response to indomethacin (10 $\mu\text{mol/L}$, basolateral), TTX (tetrodotoxin, 1 $\mu\text{mol/L}$, basolateral), amiloride (10 $\mu\text{mol/L}$, apical), and vasopressin (5×10^{-8} mol/L, basolateral). Arrows indicate the time of drug addition; C: Representative I_{sc} recordings in response to indomethacin (10 $\mu\text{mol/L}$, basolateral), TTX (tetrodotoxin, 1 $\mu\text{mol/L}$, basolateral), TEA (5 mmol/L, apical), and vasopressin (5×10^{-8} mol/L, basolateral); D: Comparison of the effects of serosal vasopressin on the I_{sc} with or without apical pretreatment with bumetanide ($n = 9$), amiloride ($n = 6$) and TEA ($n = 6$). Values are mean \pm SE; ⁱ $P < 0.05$; ^g $P < 0.01$.

in intracellular trafficking because the total NKCC2 expression did not increase. Moreover, the 0.5 h and 1 h intervals that were examined were shorter than the time (4–6 h) required for new protein synthesis. The approximately 1.5- and 3-fold increase in the NKCC2 fluorescence intensity after the 30 min and 1 h stimulation, respectively, in the apical membrane may reflect the onset of NKCC2 membrane recruitment. NKCC2 trafficking was not observed after a 15 min stimulation, possibly because vasopressin required a longer time to become effective after the intraperitoneal injection. To further elucidate the process of NKCC2 trafficking, we compared the NKCC2 protein expression level in the plasma membrane in the control and stimulation conditions. Consistent with laser scanning confocal microscope (LSCM) observation, the abundance of NKCC2 was indeed greater after vasopressin stimulation. Previous studies have shown that in TAL cells, trafficking to the apical membrane is the mechanism of stimulating NKCC2 activity. This finding indicated that vasopressin could activate apical NKCC2 by stimulating transporter trafficking to the plasma membrane. In contrast to NKCC2, the distribution of NKCC1 did not change obviously after vasopressin stimulation. Whether vasopressin has an effect on NKCC1 in the colon remains to be investigated.

It was reported that vasopressin induced an im-

mediate decrease in the short-circuit current (I_{sc})^[17,18]. However, whether the apical NKCC2 is involved in the vasopressin-induced electrolyte transport in the distal colon remains unclear. Previous studies reported that vasopressin could enhance the amiloride-insensitive Cl^- -dependent Na^+ absorption in the normal rat distal colon^[17]. Another report indicated that vasopressin inhibited the electrogenic amiloride-sensitive Na^+ absorption in the guinea pig distal colon^[23]. In our study, apical pretreatment with amiloride, an epithelial sodium channel (ENaC) blocker, failed to affect the serosal vasopressin-induced I_{sc} decrease, indicating that amiloride-sensitive Na^+ absorption cannot be involved in the I_{sc} decrease. Apical pretreatment with bumetanide blunted the vasopressin-induced I_{sc} decrease by approximately 50%, suggesting that the effect depends at least partially on NKCC2. In other words, apical NKCC2 stimulation by vasopressin contributed to the electrolyte transport in the mouse distal colonic epithelia. Interestingly, the apical addition of TEA (5 mmol/L), a putative inhibitor of K^+ channel blocker, inhibited the vasopressin-induced I_{sc} decrease by 33%, indicating that an apical K^+ channel was involved in this response and that the current was partially mediated by K^+ secretion. Moreover, we also examined the effect of vasopressin in conjunction with basolateral pretreatment with bumetanide. The basolateral pretreatment with

bumetanide also inhibited the response by approximately 50% (Preliminary data). A similar blocking effect was observed for the pretreatment with basolateral bumetanide, also supporting that vasopressin mainly affects the NKCC2 cotransporter, which is predominantly located in the apical region of the distal colonic epithelia during this process. The possible explanations are that bumetanide is fat soluble and could cross the basolateral membrane to affect the apical NKCC2. Moreover, previous reports demonstrated that NKCC2 is more sensitive to inhibition by bumetanide than NKCC1^[33,34]. This finding, together with the finding that no NKCC1 redistribution was observed after stimulation by vasopressin, again suggests that vasopressin might mainly affect NKCC2. Taken together, our results suggest that NKCC2 is involved in the vasopressin-induced *I_{sc}* decrease in the mouse distal colonic epithelia.

Clearly, the kidney is not the only target organ through which vasopressin regulates the electrolyte balance of the whole body. The gastrointestinal tract is also a potent target of vasopressin because all vasopressin receptor subtypes are expressed throughout the gut in humans^[35], and these receptors could be activated by either circulating or local vasopressin. In the present study, we focused on the effects of vasopressin on apical NKCC2 in the mouse colon and found that vasopressin may induce NKCC2 trafficking and activate apical NKCC2 in the mouse distal colon *in vivo*. These effects could enhance the capacity of the distal colonic epithelia to reabsorb NaCl and water in response to short-term hormonal stimulation. These findings suggest a possible mechanism through which vasopressin regulates apical NKCC2 activity in the colon, and this mechanism resembles its mechanism of action in the kidney. Collectively, the colon and kidney are predicted to work synergistically in the electrolyte absorption regulated by vasopressin. Consequently, the action of vasopressin on NKCC2 in the colon would be recognized to supplement the role in modulating whole-body homeostasis and electrolyte balance under physiological or pathophysiological conditions.

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COMMENTS

Background

The Na⁺-K⁺-2Cl⁻ cotransporter (NKCC2), which was thought to be expressed only in the apical membrane of the epithelial cells in the thick ascending limb of Henle's loop, was recently found to be expressed in the rat and human distal colon also. However, the role and regulating mechanism of NKCC2 in the gut are still not completely understood.

Research frontiers

Previous studies have focused on the regulation of NKCC2 by vasopressin in the kidney. How vasopressin regulates colonic NKCC2 is unknown. The present study addresses this issue by showing the NKCC2 expression and spatial distribution in the mouse colonic epithelia. The results show that the effects of

vasopressin on colonic NKCC2 are similar to those described for the kidney.

Innovations and breakthroughs

Several reports have verified that vasopressin has both long-term and short-term effects on NKCC2 expression and function in thick ascending limb of Henle's loop cells. The colon could also be a target for vasopressin because vasopressin stimulates NaCl and water absorption in *in vitro* preparations of mouse, rat, and human colons. However, little is known about the mechanism of ion transport that is induced by vasopressin in the colon. Specifically, the identity of the protein that mediates NaCl absorption in the colon and whether NKCC2 is involved in this process are currently unclear. The present study provide direct evidence that vasopressin also plays an important role in the distal colonic epithelia by stimulating trafficking of NKCC2 to the apical membrane and inducing NKCC2-mediated ion transport at the apical region of the colonic epithelia.

Applications

The action of vasopressin on NKCC2 in the colon would be recognized to supplement the role of the kidney in modulating whole-body homeostasis and electrolyte balance under physiological or pathophysiological conditions.

Terminology

Endocytic trafficking involves the cellular internalization and sorting of extracellular molecules, plasma membrane proteins and lipids. Endocytosis is required for a vast number of functions, including nutrient uptake, cell adhesion and migration, receptor signaling, pathogen entry and cell polarity. Exocytic trafficking is the durable, energy-consuming process by which a cell directs the contents of secretory vesicles out of the cell membrane and into the extracellular space.

Peer review

The authors have conducted experiments using immunofluorescence, Ussing chamber, reverse transcriptase polymerase chain reaction, and Western blotting techniques to demonstrate, for the first time in mice, that vasopressin plays a role in the trafficking of NKCC2 to the apical membrane of distal colonic epithelia. The results are interesting and may suggest a mechanism through which vasopressin regulates apical NKCC2 activity in the colon.

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Controlled attenuation parameter for non-invasive assessment of hepatic steatosis in Chinese patients

Feng Shen, Rui-Dan Zheng, Yu-Qiang Mi, Xiao-Ying Wang, Qin Pan, Guang-Yu Chen, Hai-Xia Cao, Ming-Li Chen, Liang Xu, Jian-Neng Chen, Yi Cao, Rui-Nan Zhang, Lei-Ming Xu, Jian-Gao Fan

Feng Shen, Qin Pan, Guang-Yu Chen, Hai-Xia Cao, Yi Cao, Rui-Nan Zhang, Lei-Ming Xu, Jian-Gao Fan, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

Rui-Dan Zheng, Jian-Neng Chen, Research and Therapy Center for Liver Diseases, The Affiliated Dongnan Hospital of Xiamen University, Zhangzhou 363000, Fujian Province, China

Yu-Qiang Mi, Liang Xu, Tianjin Research Institute of Liver Diseases, Tianjin Hospital of Infectious Diseases, Tianjin 300192, China

Xiao-Ying Wang, Department of Pathology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

Ming-Li Chen, Department of Ultrasound, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

Author contributions: Shen F, Zheng RD, Mi YQ and Fan JG designed the study; Shen F, Wang XY, Chen GY, Cao HX, Chen ML, Xu L and Chen JN performed the research; Pan Q, Chen GY, Cao Y and Zhang RN carried out statistical analysis; Shen F, Xu LM and Fan JG wrote the manuscript; all the authors have read and approved the final revision to be published.

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Correspondence to: Jian-Gao Fan, MD, PhD, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, 1665 Kongjiang Road, Shanghai 200092, China. fanjiangao@gmail.com

Telephone: +86-21-25077340 Fax: +86-21-25077340

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sive controlled attenuation parameter (CAP) to assess liver steatosis.

METHODS: This was a multi-center prospective cohort study. Consecutive patients (aged ≥ 18 years) who had undergone percutaneous liver biopsy and CAP measurement were recruited from three Chinese liver centers. Steatosis was categorized as S0: $< 5\%$; S1: $5\%-33\%$; S2: $34\%-66\%$; or S3: $\geq 67\%$, according to the non-alcoholic fatty liver disease (NAFLD) activity score. The FibroScan® 502 equipped with the M probe (Echosens, Paris, France) was used to capture both CAP and liver stiffness measurement values simultaneously. Receiver operating characteristic curves were plotted, and the areas under the curves were calculated to determine the diagnostic efficacy. The accuracy of the CAP values at the optimal thresholds was defined by maximizing the sum of sensitivity and specificity (maximum Youden index).

RESULTS: A total of 152 patients were recruited, including 52 (34.2%) patients with NAFLD and 100 (65.8%) with chronic hepatitis B (CHB) virus infection. After adjustment, the steatosis grade (OR = 37.12; 95%CI: 21.63-52.60, $P < 0.001$) and body mass index (BMI, OR = 6.20; 95%CI: 2.92-9.48, $P < 0.001$) were found independently associated with CAP by multivariate linear regression analysis. CAP was not influenced by inflammation, fibrosis or aetiology. The median CAP values and interquartile ranges among patients with S0, S1, S2 and S3 steatosis were 211 (181-240) dB/m, 270 (253-305) dB/m, 330 (302-360) dB/m, and 346 (313-363) dB/m, respectively. The cut-offs for the CAP values in all patients with steatosis $\geq 5\%$, $\geq 34\%$ and $\geq 67\%$ were 253 dB/m, 285 dB/m and 310 dB/m, respectively. The areas under the curves were 0.92, 0.92 and 0.88 for steatosis $\geq 5\%$, $\geq 34\%$ and $\geq 67\%$, respectively. No significant differences were found in the CAP values between the NAFLD group and the CHB group in each steatosis grade.

Abstract

AIM: To evaluate the performance of a novel non-inva-

CONCLUSION: CAP appears to be a promising tool for the non-invasive detection and quantification of hepatic steatosis, but is limited by BMI.

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Key words: Fatty liver; Nonalcoholic; Controlled attenuation parameter; Transient elastography; Chronic hepatitis B

Core tip: We introduced a novel controlled attenuation parameter (CAP) which was acquired using the FibroScan® equipped with the M probe. This multi-center prospective cohort study was performed in Chinese nonalcoholic fatty liver disease or chronic hepatitis B patients. Following multivariate linear regression analysis, we found that CAP was significantly correlated with steatosis grade and was not influenced by inflammation, fibrosis or aetiology. Although it is less effective in identifying moderate to severe steatosis and limited by body mass index, we believe that CAP values are more useful than the measurement of 5% steatosis and may be used as a substitute for ultrasonography in epidemiological investigations of fatty liver.

Shen F, Zheng RD, Mi YQ, Wang XY, Pan Q, Chen GY, Cao HX, Chen ML, Xu L, Chen JN, Cao Y, Zhang RN, Xu LM, Fan JG. Controlled attenuation parameter for non-invasive assessment of hepatic steatosis in Chinese patients. *World J Gastroenterol* 2014; 20(16): 4702-4711 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v20/i16/4702.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4702>

INTRODUCTION

Hepatic steatosis is diagnosed when the accumulation of fatty droplets (mainly triglycerides) exceeds more than 5% of liver weight^[1]. The prevalence of hepatic steatosis is rising in association with the global increase in obesity and type 2 diabetes and is currently present in 2%-40% of the general population^[2], 50.9% of individuals with chronic hepatitis C (CHC)^[3], 46.4% of heavy drinkers^[4] and 50%-80% of obese individuals^[5]. In the past, simple steatosis was regarded as benign and reversible. However, the presence of other aetiologies may act in synergy with steatosis to aggravate liver injury, enhance oxidative stress, produce inflammation, increase susceptibility to apoptosis and even promote the progression of fibrosis^[6]. Therefore, it is necessary to accurately quantify the extent of hepatic steatosis and monitor its dynamic changes.

Although liver biopsy (LB) is regarded as the gold standard to assess hepatic steatosis, its use has several limitations, including sampling bias, intra- or inter-observer sampling variability, and the potential for severe complications^[7]. Therefore, patients opt to avoid such an invasive procedure and frequently refuse to repeat it.

As a result, there is a need for a simple and reliable non-invasive alternative that either complements or eliminates liver biopsy altogether.

Recently, a novel non-invasive tool based on ultrasound attenuation was developed to assess liver steatosis. The evaluation of ultrasound attenuation has been implemented with the FibroScan® (Echosens, Paris, France) using a novel proprietary algorithm called the controlled attenuation parameter (CAP)^[11]. In the existing literature, CAP displayed good diagnostic value for chronic liver diseases such as viral hepatitis^[8-9] and multi-aetiology cohorts^[10-12]. However, there is no research using CAP values to assess hepatic steatosis in the Chinese population. CHB is the most prevalent liver disease in China, and nonalcoholic fatty liver disease (NAFLD) is also highly prevalent, especially in the more affluent regions^[13]. Furthermore, the coexistence of hepatitis B virus (HBV) infection and NAFLD is a novel characteristic of liver disease in the Chinese population. Therefore, the aim of this study was to evaluate the performance of CAP measurements in assessing steatosis, in a cohort of consecutive NAFLD/CHB patients in China, using liver biopsy as the reference.

MATERIALS AND METHODS

Patients

Adults (aged ≥ 18 years) with NAFLD or HBV infection (with or without steatosis) were eligible for the study. Patients were prospectively recruited from three Chinese liver centers (Xinhua Hospital, Shanghai; Dongnan Hospital, Fujian; and Tianjin Hospital of Infectious Diseases, Tianjin) between March 2012 and March 2013. The ethics committees of the three hospitals approved the study, and all patients gave their written informed consent before participation.

Each patient had undergone percutaneous liver biopsy and transient elastography (TE) within 4 wk and met the diagnostic criteria for either NAFLD^[14] or CHB^[15]. Exclusion criteria were: (1) alcohol intake per week greater than 140 g in men and 70 g in women in the past 12 months; (2) other diseases that lead to fatty liver (*e.g.*, CHC, drug-induced liver disease, total parenteral nutrition, hepatolenticular degeneration, autoimmune liver disease, *etc.*); (3) previous liver transplantation; (4) other terminal disease or malignancy; (5) refusal to undergo LB or disqualified biopsy specimens; and (6) contraindications to FibroScan® examination (*e.g.*, ascites, implanted pacemakers, non-healing wounds in the upper-right quadrant of the abdomen, pregnancy, *etc.*) or unreliable CAP measurements [*e.g.*, success rate less than 60% or interquartile range (IQR) $> 30\%$].

Clinical evaluation and laboratory data

All patients received physical examinations at the time of TE measurement. Liver disease aetiology and anthropometric measurements, including body mass index [BMI, weight (kg)/height (m)²] and waist-to-hip ratio [WHR,

waist circumference (cm)/hip circumference (cm)] were obtained. Demographic information, such as age, sex and medical/drinking history were obtained from patient interviews during screening. Laboratory data, including liver biochemistry, fasting glucose, total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) within 4 wk of the liver biopsy and in fasting conditions were recorded. HBsAg, HBeAg and anti-HBe were determined with commercially available enzyme-linked immunosorbent assay kits. Serum HBV DNA was measured by the real-time PCR Cobas Taqman assay if HBV was positive.

Liver histology

Percutaneous LB was performed with an 18-gauge BARD Max-Core Disposable Biopsy Instrument (BARD Biopsy Systems, Tempe, AZ, United States) from the right lobe under real-time ultrasound guidance. Biopsy specimens were formalin-fixed, paraffin-embedded, sectioned, and stained with HE, Masson's trichrome stain and reticulin. Liver biopsy sections were interpreted by two experienced hepatopathologists who were blinded to the clinical data, and a consensus was required in the case of discordant results. The length of the sample was required to be ≥ 15 mm, and the sample was to contain at least 6 portal tracts (PTs). For both NAFLD and CHB samples, the liver sections were first evaluated for percentage of lipid deposition, and the presence of visible steatosis in $\geq 5\%$ of hepatocytes was considered to represent fatty liver^[16] which was evaluated by light microscopic examination of an HE liver section (4–5 μ m thick) under a 10 \times objective lens^[17]. Steatosis was categorised as S0: $< 5\%$; S1: 5%–33%; S2: 34%–66%; or S3: $\geq 67\%$, according to the NAFLD activity score (NAS)^[16].

The grades and stages of the liver samples were dependent on the liver disease aetiology. The METAVIR classification was used for CHB and fibrosis was staged from F0 to F4: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis^[18]. Fibrosis in NAFLD was staged as follows: F0, no fibrosis; F1, perisinusoidal or periportal fibrosis; F2, perisinusoidal and portal/periportal fibrosis; F3, bridging fibrosis; and F4, cirrhosis. Because of the different diagnostic criteria, METAVIR grades of A2–A3 in patients with CHB and NAS scores of ≥ 5 in patients with NAFLD were classified as moderate to severe inflammation, and F ≥ 2 was classified as significant fibrosis.

CAP and liver stiffness measurement

One certified operator with experience of more than 200 cases in each centre performed the TE examinations and was blinded to the liver histology. The FibroScan[®] 502 equipped with the M probe (Echosens, Paris, France) was used to capture both CAP and liver stiffness measurement (LSM) values simultaneously. CAP values and LSM values were expressed in units of decibels per metre (dB/

m) and kilopascal (kPa), ranging from 100 to 400 dB/m and 2.5 to 75 kPa, respectively. Details of the LSM and CAP measurement principle were provided in previous publications^[1,19]. Fasted patients were placed in the supine position with their right hand on their head in order to extend the intercostal space. The tip of the transducer probe was placed on the surface of the skin between the ribs and over the right lobe of the liver.

A reliable LSM was defined as more than 10 valid shots, a success rate of at least 60%, and an IQR $< 30\%$ of the median LSM value^[19]. Since there are no reliability criteria for CAP measurement, it was arbitrarily decided to use the reliability criteria for LSM. Therefore if the LSM was reliable according to those criteria, the corresponding median CAP value was also considered reliable.

Statistical analysis

Continuous variables and patient characteristics were expressed as either medians (IQR) or n (%), as appropriate. The χ^2 test and Fisher's exact test were used to compare categorical data. The Mann-Whitney test was used to compare two groups, and the Kruskal-Wallis test was used to compare more than three groups. Correlations between CAP values and continuous variables were assessed by Spearman correlation coefficients (ρ), and multivariate analysis was performed using linear regressions. The receiver operating characteristic (ROC) curves were plotted, and the areas under the curves (AUC) were calculated with 95% confidence intervals (CIs) to determine the diagnostic efficacy of CAP to differentiate between those with hepatic steatosis $\geq 5\%$, $\geq 34\%$ and $\geq 67\%$ versus controls. The accuracy of the CAP values at the optimal thresholds was defined by maximizing the sum of sensitivity and specificity (maximum Youden index). For each optimal cut-off value, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. All statistical analyses were performed using SPSS version 13 for Windows (SPSS, Chicago, IL, United States). Two-sided P values < 0.05 indicated statistical significance.

RESULTS

Patients' baseline characteristics

A total of 189 consecutive patients were screened within the study period. Of these patients, 15 were excluded due to withdrawal of consent, 6 for excessive alcohol consumption, 5 for disqualified biopsy specimens, and 11 due to the inability to acquire qualified CAP and/or LSM data. A total of 152 (80.4%) patients were finally included in the statistical analysis, including 52 (34.2%) with NAFLD and 100 (65.8%) with CHB. Demographic, anthropometric, laboratory, and FibroScan examination characteristics of the study population are outlined in Table 1. The median BMI (26.0 kg/m², IQR: 24.4–29.3), waist circumference (90.0 cm, IQR: 86.0–97.5), hip circumference (97.0 cm, IQR: 93.0–102.8) and WHR (0.94, IQR: 0.91–0.97) in the NAFLD group were significantly

Table 1 Baseline characteristics of patients with non-alcoholic fatty liver disease and chronic hepatitis B virus infection

Characteristics	All patients (<i>n</i> = 152)	NAFLD (<i>n</i> = 52)	CHB (<i>n</i> = 100)	<i>P</i> value
Demographics				
Male gender, <i>n</i> (%) ¹	106 (69.3)	36 (69.2)	70 (70.0)	0.992
Age (yr) ²	35 (28-49)	39 (29-50)	35 (27-49)	0.167
Anthropometrics				
BMI (kg/m ²) ²	24.9 (22.5-27.7)	26.0 (24.4-29.3)	23.9 (21.8-26.6)	< 0.001
< 18.5	7 (4.6)	0	7 (7.0)	
18.5-24.9	70 (46.1)	18 (34.6)	52 (52.0)	
25-29.9	61 (40.1)	24 (46.2)	37 (37.0)	
≥ 30	14 (9.2)	10 (19.2)	4 (4.0)	
WHR ²	0.92 (0.88-0.96)	0.94 (0.91-0.97)	0.90 (0.86-0.95)	0.001
Laboratory findings				
ALT (U/L)	64.8 (37.9-134.0)	55.0 (31.3-104.4)	69.0 (40.0-187.0)	0.022
AST (U/L)	44.0 (26.0-77.5)	33.4 (25.0-67.0)	46.3 (28.7-93.5)	0.042
ALP (U/L)	89.0 (68.0-109.0)	84.0 (63.1-109.0)	90.3 (73.3-109.0)	0.320
γ-GT (U/L)	54.3 (28.9-104.4)	60.0 (31.6-97.1)	52.0 (26.5-117.3)	0.770
Albumin (g/L)	42.7 (39.8-45.1)	43.5 (41.5-46.8)	42.1 (38.6-44.8)	0.017
Total bilirubin (μmol/L)	14.2 (11.6-20.2)	12.6 (10.5-15.5)	15.2 (12.1-22.3)	0.003
Direct bilirubin (μmol/L)	4.9 (3.7-7.8)	4.6 (3.8-5.6)	5.2 (3.7-9.0)	0.080
Total cholesterol (mmol/L)	4.6 (4.0-5.1)	4.7 (4.3-5.1)	4.3 (3.7-5.1)	0.018
Triglyceride (mmol/L)	1.4 (0.9-2.2)	2.0 (1.3-2.7)	1.3 (0.87-2.0)	0.001
HDL-C (mmol/L)	1.2 (1.0-1.3)	1.2 (1.1-1.4)	1.1 (1.0-1.3)	0.166
LDL-C (mmol/L)	2.5 (1.9-2.9)	2.8 (2.6-3.1)	2.3 (1.8-2.6)	< 0.001
Prothrombin time (s)	12.3 (11.1-13.0)	11.9 (10.4-12.4)	12.7 (12.0-13.4)	< 0.001
Fasting glucose (μmol/L)	5.3 (4.7-5.8)	5.2 (4.5-6.0)	5.3 (4.8-5.7)	0.819
Log10 (HBV-DNA, IU/mL)	-	-	3.3 (0.92-5.9)	
Liver histology				
Steatosis grade, <i>n</i> (%) ¹				< 0.001
S0 (< 5%)	63 (41.4)	0	63 (63.0)	
S1 (5%-33%)	44 (28.9)	19 (36.5)	25 (25.0)	
S2 (34%-66%)	32 (21.1)	23 (44.2)	9 (9.0)	
S3 (≥ 67%)	13 (8.6)	10 (19.2)	3 (3.0)	
Fibrosis stage, <i>n</i> (%) ¹				0.234
F0	-	28 (53.8)	36 (36.0)	
F1	-	13 (25.0)	30 (30.0)	
F2	-	6 (11.5)	20 (20.0)	
F3	-	4 (7.7)	8 (8.0)	
F4	-	1 (1.9)	6 (6.0)	
Significant fibrosis (F ≥ 2), <i>n</i> (%)	45 (29.6)	11 (21.2)	34 (34.0)	0.100
Moderate to severe inflammation, <i>n</i> (%) ^{1,3}	62 (40.8)	21 (40.4)	41 (41.0)	0.942
FibroScan® parameters				
Controlled attenuation parameter (dB/m)	262 (215-310)	310 (273-347)	236 (199-281)	< 0.001
Liver stiffness measurement (kPa)	7.5 (5.5-12.9)	6.1 (4.8-11.1)	8.0 (5.9-13.9)	0.025

All data are expressed as medians (IQR), or *n* (%), as appropriate; ¹Either χ^2 test or Fisher's exact test was used; ²Mann-Whitney test was used; ³METAVIR grades A2-A3 in patients with viral hepatitis and NAS score ≥ 5 in patients with NAFLD. BMI: Body mass index; WHR: Waist-to-hip ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GT: γ-glutamyl transpeptidase; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; NAFLD: Non-alcoholic fatty liver disease; CHB: Chronic hepatitis B.

higher than those in the CHB group.

The characteristics of liver histology are also shown in Table 1. The median length and the PTs of the liver biopsy samples were 18 mm (IQR: 17-19) and 8 (IQR: 7-10), respectively. The 63 patients with a steatosis score of 0%-5% (S0) all had CHB.

Association of CAP with different parameters

BMI, WHR, albumin, TC, TG, LDL-C and steatosis grade showed significant positive associations with CAP, while alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and direct bilirubin were negatively associated with CAP (Table 2). Parameters such as gender ($P = 0.49$), age ($P = 0.93$), alkaline

phosphatase ($P = 0.37$), γ-glutamyl transpeptidase ($P = 0.93$), HDL-C ($P = 0.24$), fasting glucose ($P = 0.20$), HBV DNA levels ($P = 0.42$, for CHB patients), prothrombin time ($P = 0.06$), moderate to severe inflammation ($P = 0.18$), significant fibrosis ($P = 0.55$) and LSM ($P = 0.43$) were not significantly correlated with CAP.

Multivariate linear regression analysis of CAP for steatosis and other parameters

Parameters which were significantly associated with CAP were entered into a multivariate linear regression model (stepwise methods). After adjustment, only steatosis grade (OR = 37.12; 95%CI: 21.63-52.60, $P < 0.001$) and BMI (OR = 6.2; 95%CI: 2.92-9.48, $P < 0.001$) were inde-

Table 2 Correlation and multivariate linear regression analyses for controlled attenuation parameter with other different parameters

Parameter	Spearman correlation		Multivariate linear regression		
	<i>r</i>	<i>P</i> value	OR	95%CI	<i>P</i> value
BMI	0.49	< 0.001	6.20	2.92-9.48	< 0.001
WHR	0.32	< 0.001	-	-	-
ALT	-0.25	0.002	-	-	-
AST	-0.25	0.002	-	-	-
Albumin	0.27	0.001	-	-	-
Total bilirubin	-0.24	0.003	-	-	-
Direct bilirubin	-0.17	0.039	-	-	-
Total cholesterol	0.18	0.034	-	-	-
Triglyceride	0.31	< 0.001	-	-	-
LDL-C	0.39	< 0.001	-	-	-
Steatosis grade	0.76	< 0.001	37.12	21.63-52.60	< 0.001

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; WHR: Waist-to-hip ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDL-C: Low density lipoprotein cholesterol.

pendent predictive factors of CAP (Table 2).

The median CAP (IQR) values in patients with S0, S1, S2 and S3 steatosis were 211 (181-240) dB/m, 270 (253-305) dB/m, 330 (302-360) dB/m, and 346 (313-363) dB/m, respectively ($P < 0.001$; Figure 1A). CAP values were significantly different between S0 *vs* S1 ($P < 0.001$) and S1 *vs* S2 ($P < 0.001$), but not between S2 *vs* S3 ($P = 0.224$). Moreover, the median CAP (IQR) values were 169 (148-207) dB/m, 235 (200-275) dB/m, 288 (254-340) dB/m and 331 (304-360) dB/m for patients with a BMI (kg/m^2) of < 18.5 , 18.5-24.9, 25-29.9 and ≥ 30 , respectively ($P < 0.001$; Figure 1B). To remove the confounding factors of liver steatosis and aetiology, we further analyzed the CAP values with BMI values in the 63 CHB patients with 0%-5% steatosis. The Kruskal-Wallis test was used, and significant differences ($P = 0.004$) were found between the CAP values and the different BMI levels (Figure 1C).

The CAP (IQR) values were 271 (258-291) dB/m for NAFLD patients and 270 (247-308) dB/m for CHB patients with S1 ($P = 0.670$), 328 (303-361) dB/m for NAFLD patients and 331 (289-350) dB/m for CHB patients with S2 ($P = 0.681$), and 341 (315-361) dB/m for NAFLD patients and 349 (310-378) dB/m for CHB patients with S3 ($P = 0.692$). Therefore, no significant differences were found between the CAP values of NAFLD and CHB patients in each steatosis grade (Figure 1D).

Diagnostic performance of CAP for different steatosis grades

The ROC curves of CAP to differentiate between steatosis grades are displayed in Figure 2A. CAP was found to be excellent for predicting fatty liver (steatosis $\geq 5\%$; AUC = 0.92, 95%CI: 0.88-0.97), for the detection of steatosis $\geq 34\%$ (AUC = 0.92, 95%CI: 0.87-0.97) and good for the detection of steatosis $\geq 67\%$ (AUC = 0.88, 95%CI: 0.82-0.94). Using the maximum Youden index, optimal cut-off values with the sensitivity, specificity,

PPV and NPV of CAP for hepatic steatosis $\geq 5\%$, $\geq 34\%$ and $\geq 67\%$ in patients were also calculated and are shown in Table 3.

ROC curves and AUCs of CAP values were also calculated between two steatosis grades to differentiate individual grades of steatosis: 1-grade difference, including S0 *vs* S1, S1 *vs* S2 and S2 *vs* S3; 2-grade difference, including S0 *vs* S2 and S1 *vs* S3; 3-grade difference, including S0 *vs* S3. All six potential pairs are shown in Figure 2B. CAP performance was excellent for differentiating between 2 or 3 grades, such as S0 *vs* S2 (AUC = 0.97), S1 *vs* S3 (AUC = 0.92) and S0 *vs* S3 (AUC = 0.99). However, it was poorer at differentiating between 1 grade than more than 2 grades, especially for S2 *vs* S3 (AUC = 0.62).

DISCUSSION

In China, it has been estimated that at least 10% of the general population is chronically infected with HBV, which is the most common cause of liver disease^[20]. In recent years, with the increasing pandemic of obesity, NAFLD has now become a major cause of liver-related morbidity and mortality, with a prevalence of 15% in China^[13]. Although clinical studies have found that HBV infection is associated with a lower prevalence of fatty liver, hypertriglyceridemia and metabolic syndrome^[21], hepatic steatosis has been associated with Entecavir treatment failure^[22]. Therefore, it is important to determine whether hepatic steatosis or steatohepatitis coexist in CHB patients.

The current "gold standard" diagnostic procedure is still LB. Due to the trauma, sampling error, complications and imperfect reproducibility of LB, its application is limited. Therefore, the development of a non-invasive quantitative measure of hepatic steatosis is necessary. The two existing methods mainly include serological and imaging methods. Serological methods such as the SteatoTest^[23], the Fatty Liver Index (FLI)^[24] and the Hepatic Steatosis Index (HSI)^[25] combine a number of biochemical markers and/or anthropometric characteristics that have been extensively developed in the last decade to diagnose fatty liver. The M65 enzyme-linked immunosorbent assay, which detects both caspase-cleaved and uncleaved cytokeratin-18, may also differentiate patients with simple steatosis from healthy individuals^[26]. However, the accuracy and diagnostic efficacy of these tests still need to be improved, thus, they are not yet recommended for use in clinical practice. The imaging methods include ultrasonography, computed tomography (CT) and magnetic resonance (MR)^[27]. Ultrasonography is the most common technique, and is accepted as an initial tool for fatty liver as it is non-invasive, non-ionizing, inexpensive and widely available. However, the major weaknesses of ultrasound include high operator- and machine-dependency and the ability to detect only patients with more than 30% steatosis. CT provides an accurate and a reliable visualisation of the whole liver, enabling the diagnosis not only of diffuse, but also of focal fatty deposits. However, CT is

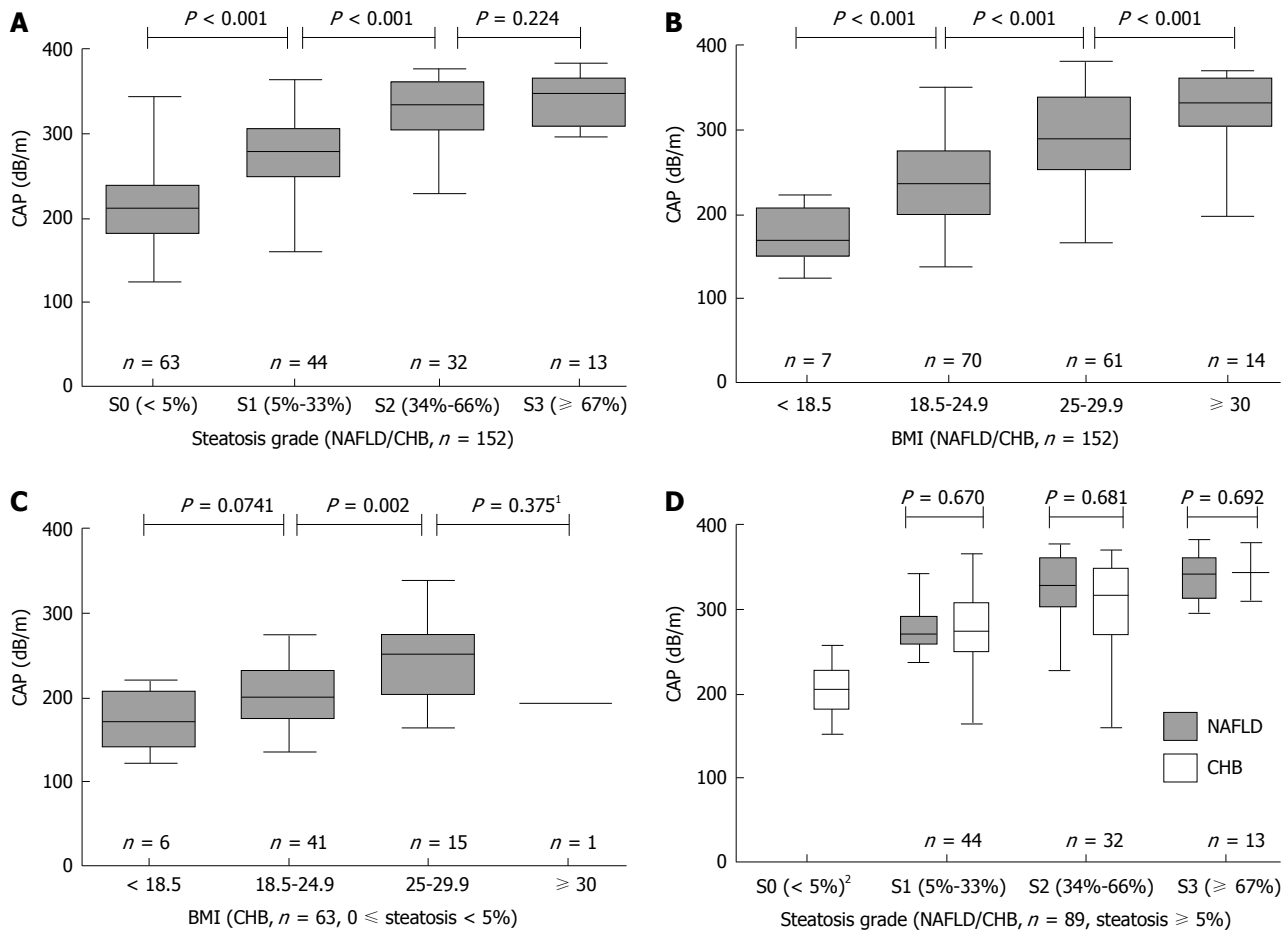


Figure 1 Controlled attenuation parameter distribution for different steatosis grades or body mass index levels. A: Controlled attenuation parameter distribution in patients with different steatosis grades ($n = 152$); B: CAP distribution in patients with different BMI levels ($n = 152$); C: CAP distribution in patients with BMI levels with either no steatosis or steatosis less than 5% ($n = 63$); D: CAP between NAFLD and CHB patients with the same degree of fatty deposition ($n = 89$). ¹Fisher's exact test was used; ²No NAFLD patients in S0. CAP: Controlled attenuation parameter; BMI: Body mass index; NAFLD: Non-alcoholic fatty liver disease; CHB: Chronic hepatitis B.

Table 3 Diagnostic accuracy of controlled attenuation parameter and its suggested optimal cut-off values

Steatosis	AUC (95%CI)	P value	Cut-off (dB/m)	Sensitivity (95%CI), %	Specificity (95%CI), %	PPV (95%CI), %	NPV (95%CI), %
S0 vs S1-S3 ($\geq 5\%$)	0.92 (0.88-0.97)	< 0.001	253	88.8 (79.9-94.2)	82.5 (70.5-90.6)	87.8 (78.8-93.4)	83.9 (71.9-91.6)
S0-S1 vs S2-S3 ($\geq 34\%$)	0.92 (0.87-0.97)	< 0.000	285	93.3 (80.7-98.3)	83.2 (74.4-89.5)	70.0 (56.6-80.8)	96.7 (90.1-99.2)
S0-S2 vs S3 ($\geq 67\%$)	0.88 (0.82-0.94)	< 0.001	310	92.3 (62.1-99.6)	79.1 (71.3-85.4)	29.3 (16.6-45.7)	99.1 (94.4-99.9)

AUC: Areas under the curve; PPV: Positive predictive value; NPV: Negative predictive value.

associated with radiation exposure, which limits its use in longitudinal studies and in children. Moreover, iron accumulation plays an important role in steatohepatitis during NAFLD^[28], and CT is strongly influenced by iron deposition in the liver, leading to misdiagnosis. MR, especially proton magnetic resonance spectroscopy (¹H MRS), has emerged as a fast, safe, non-invasive alternative for the quantification of hepatic fat content. ¹H MRS has been used in NAFLD patients with mild steatosis or advanced fibrosis, and can be performed easily without special devices^[29]. However, its use is limited due to high costs, low availability and a lack of standardisation.

FibroScan[®] is now widely used to obtain LSMs, which relate to liver fibrosis, and has shown good results for the

diagnosis of cirrhosis in chronic liver disease^[30], but was incapable of assessing steatosis. In 2010, Sasso *et al*^[10] first reported a novel attenuation parameter that was based on the ultrasonic properties of the radiofrequency back-propagated signals acquired by the FibroScan[®] guided by vibration-controlled transient elastography. This new parameter has the advantages of being a non-ionizing, relatively inexpensive, painless, and operator and machine independent method. The overall intraclass correlation coefficient for the determination of hepatic steatosis by means of CAP in HIV and/or hepatitis virus infection was 0.84 (95%CI: 0.77-0.88)^[31], suggesting that CAP measurement represents an observer-independent method.

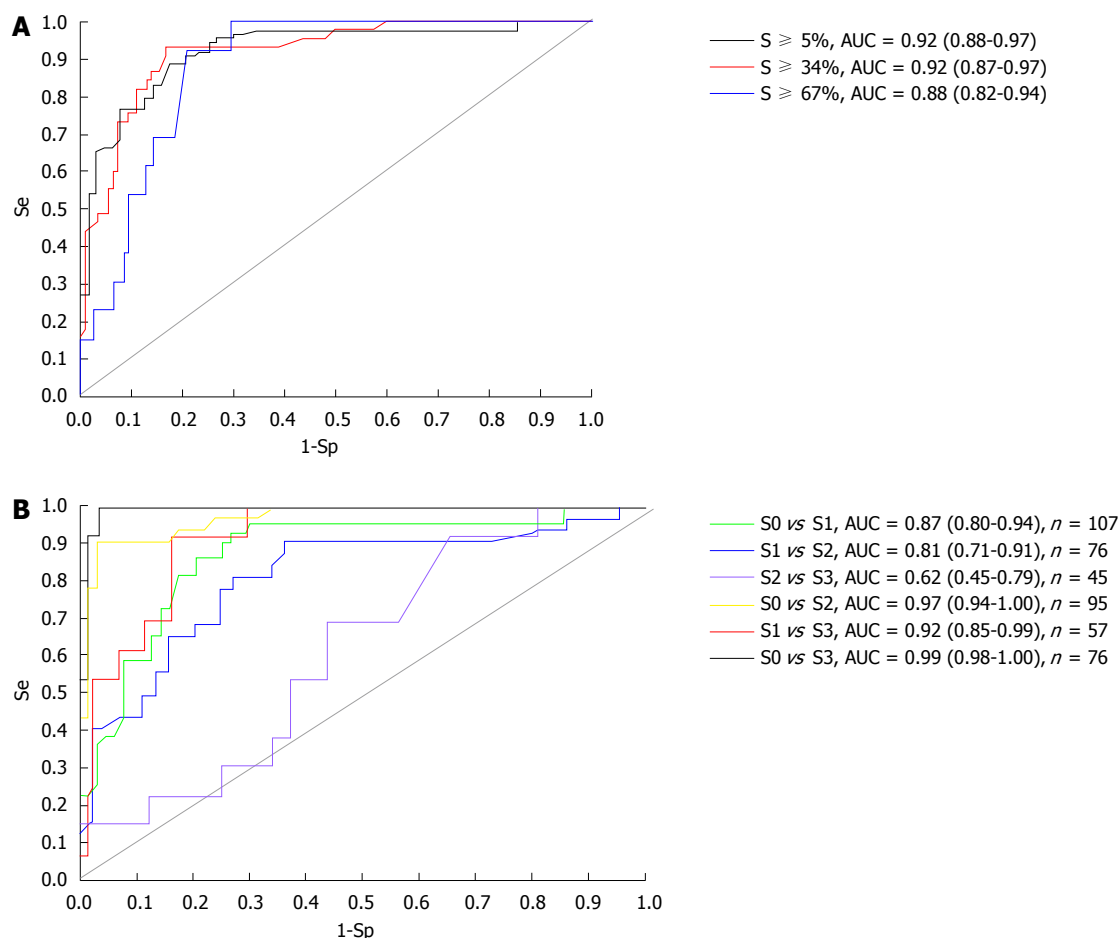


Figure 2 Receiver operating characteristic curves. A: Receiver operating characteristic curve for controlled attenuation parameter in the diagnosis of hepatic steatosis $\geq 5\%$, $\geq 34\%$ and $\geq 67\%$; B: Receiver operating characteristic curves and area under the curves (AUCs) between two steatosis grades. Se: sensitivity; Sp: specificity.

In the first publication^[10], CAP values were significantly correlated with steatosis grade (Spearman correlation $\rho = 0.81$, $P < 10^{-16}$), which were similar to our results ($P = 0.76$, $P < 0.001$). Our study identified median (IQR) CAP values for the S0, S1, S2 and S3 steatosis grades to be 211 (181-240) dB/m, 270 (253-305) dB/m, 330 (302-360) dB/m, and 346 (313-363) dB/m, respectively. CAP values were significantly different between S0 vs S1 and S1 vs S2 groups, but were not significantly different between the S2 vs S3 groups. Combined with other clinical trial results^[9,12,32], we believe that CAP values can identify more than 5% steatosis, but are less effective in identifying close steatosis grades, especially for moderate to severe steatosis. The reason for this may be that BMI was increasing together with the fat content in the liver, and the discriminability of the CAP measurement using the M probe was limited by BMI.

Inflammation and biochemical indicators such as ALT and bilirubin have been demonstrated to influence LSM measurement, and different liver disease aetiologies may lead to different cut-off values. Therefore, multivariate linear regression was used to evaluate which parameters are related to CAP values. After adjustment, only steatosis grade and BMI were independent predictive factors

of CAP values. In this work, CAP values did not appear to be influenced by inflammation, fibrosis or aetiology. These results were similar to those obtained in other studies^[9]. It was previously found that LSM values are significantly correlated with BMI, especially in NAFLD patients^[19]. In our study, BMI was also independently associated with CAP, and elevated BMI may influence the accuracy of CAP values in detecting liver steatosis. To control the confounding factors, we determined the impact of BMI in 63 CHB patients with steatosis less than 5%. Significant differences were still found, especially between the normal and overweight groups. In other studies, the optimal cut-off of CAP values for significant steatosis ($\geq 10\%$) in patients with BMIs ≥ 28 kg/m²^[12] was higher than in patients with low BMIs^[10] (283 dB/m vs 237.7 dB/m). The reason for this phenomenon might be that subcutaneous fat is involved in the measurement using the M probe in patients with a skin-liver capsule depth distance greater than 25 mm, strengthening the degree of attenuation^[33]. It is already known that subcutaneous adipose tissue may lead to overestimation of liver stiffness. Therefore, the choice of probe during an examination should depend on the distance between the probe and the liver. Compared with the M probe, the XL

probe reduces TE failure and facilitates reliable LSM in NAFLD patients^[34]. However, CAP measurements are currently available only using the M probe and under development for the XL probe.

In the present study which involved a Chinese population, we suggest CAP cut-off values of 253 dB/m, 285 dB/m, and 310 dB/m should be used for the diagnosis of $S \geq 5\%$ (S0 vs S1-S3), $S \geq 34\%$ (S0-S1 vs S2-S3), and $S \geq 67\%$ (S0-S2 vs S3), respectively. The ROC curves and corresponding AUCs were also calculated to assess the CAP performance. The results are consistent with several other reports^[8,10], suggesting that this non-invasive test has a high accuracy for the detection of steatosis. The performance of CAP was also excellent for the detection of significant steatosis ($\geq 10\%$) with AUCs of 0.84^[11], 0.81^[12] or 0.80^[8], higher than those for HSI (0.65), FLI (0.72) and M65 (0.68)^[12,26]. CAP provides a high ability to identify steatosis (more than 5%) compared with unenhanced CT or ultrasonography (more than 30%). CAP performance between two steatosis grades for differentiating individual grades of steatosis was also excellent; however, it showed poor accuracy in differentiating between adjacent grades of steatosis, especially for S2 vs S3 (AUC = 0.62).

Our study has some limitations. First, liver biopsies were used as the gold standard and interpreted by two experienced hepatopathologists, however, biopsies are not the best reference for liver steatosis measurement. Determination of the percentage of hepatocytes containing lipid vesicles is highly subjective, and steatosis grading corresponds only to a semiquantitative scale. Therefore, objective tools such as MRS together with liver biopsies may be better for the assessment of hepatic steatosis. Second, our sample size was limited due to the difficulty in obtaining liver biopsies from NAFLD patients and valid CAP measurements from obese patients. Thus, further studies including large cohorts of patients are needed to validate our preliminary data in patients with NAFLD or CHB. Third, this clinical trial was performed only in the Chinese Han population, and more tests should be carried out between different ethnicities.

In conclusion, our study shows that the novel CAP appears to be a promising tool for the non-invasive detection and quantification of hepatic steatosis in patients with either NAFLD or CHB. CAP values can be evaluated simultaneously with LSM to assess hepatic fibrosis. This new parameter has the advantages of being a simple, non-invasive, inexpensive, painless, and operator and machine independent method, and displays good application prospects. More than 5% steatosis is an acceptable pathological diagnosis of fatty liver and this clinical trial has shown that CAP values can be identified. Therefore, we believe that CAP may be an alternative method to ultrasonography for epidemiological investigations of fatty liver. However, this new method is limited by BMI, and CAP values in obese patients may be overestimated. The CAP method requires further evaluation in studies using adequate references, including studies using large samples

of patients with different aetiologies.

COMMENTS

Background

Although liver biopsy is regarded as the gold standard to assess hepatic steatosis, its use has several limitations. Recently, a novel non-invasive tool based on ultrasound attenuation was developed to assess liver steatosis. The aim of this study was to evaluate the performance of controlled attenuation parameter (CAP) measurements in assessing steatosis, in a cohort of consecutive nonalcoholic fatty liver disease (NAFLD)/chronic hepatitis B (CHB) patients in China, using liver biopsy as the reference.

Research frontiers

In 2010, a novel attenuation parameter was reported which was based on the ultrasonic properties of the radiofrequency back-propagated signals acquired by the FibroScan® guided by vibration-controlled transient elastography. In the first publication, CAP values were significantly correlated with steatosis grade. In their clinical trial, they believe that CAP can identify more than 5% steatosis, but is less effective in identifying close steatosis grades, and this new method is limited by BMI.

Innovations and breakthroughs

This study shows that the novel CAP appears to be a promising tool for the non-invasive detection and quantification of hepatic steatosis in patients with either NAFLD or CHB. They firstly reported in the Chinese population, and suggested that CAP cut-off values of 253 dB/m, 285 dB/m, and 310 dB/m should be used for the diagnosis of $S \geq 5\%$ (S0 vs S1-S3), $S \geq 34\%$ (S0-S1 vs S2-S3), and $S \geq 67\%$ (S0-S2 vs S3), respectively.

Applications

Ultrasonography is the most common technique, and is accepted as an initial tool for fatty liver. However, the major weaknesses of ultrasound include high operator- and machine-dependency and the ability to detect only patients with more than 30% steatosis. CAP can identify more than 5% steatosis and assess steatosis quantitatively and dynamically. Therefore, they believe that CAP may be an alternative method to ultrasonography for epidemiological investigations of fatty liver.

Terminology

FibroScan® is now widely used to obtain LSMs, which relate to liver fibrosis, and has shown good results for the diagnosis of cirrhosis in chronic liver disease. The evaluation of ultrasound attenuation has been implemented with the FibroScan® using a novel proprietary algorithm called the CAP. The new FibroScan® 502 equipped with the M probe was used to capture both CAP and LSM values simultaneously.

Peer review

A multi-center prospective cohort study involving patients from three Chinese liver centers who had undergone percutaneous liver biopsy shows that the novel CAP appears to be a promising tool for the non-invasive detection and quantification of hepatic steatosis in patients with either NAFLD or chronic hepatitis B. CAP values can be evaluated simultaneously with LSM. Though CAP was limited by BMI, it may be an alternative method to ultrasonography for epidemiological research of fatty liver. On the other hand, the CAP method requires further in-depth studies, also comparative analyses, involving similar observations from other research centers.

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Sonic hedgehog expression in a rat model of chronic pancreatitis

Luo-Wei Wang, Han Lin, Yi Lu, Wei Xia, Jun Gao, Zhao-Shen Li

Luo-Wei Wang, Han Lin, Wei Xia, Jun Gao, Zhao-Shen Li, Department of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
Yi Lu, Department of Respiratory Medicine, Shanghai Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China

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Correspondence to: Zhao-Shen Li, MD, PhD, Professor, Department of Gastroenterology, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, China. zhsl@1890.net

Telephone: +86-21-25070552 Fax: +86-21-55621735

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Abstract

AIM: To analyze the activation of sonic hedgehog (SHh) signaling pathways in a rat model of chronic pancreatitis.

METHODS: Forty Wistar rats were randomly divided into 2 groups: experimental group and control group (20 rats in each group). Dibutyltin dichloride was infused into the tail vein of the rats to induce chronic pancreatitis in the experimental group. The same volume of ethanol and glycerol mixture was infused in the control group. The expression of Ptch, Smo and Gli were analyzed using immunohistochemistry, and real-time reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: Compared with the control group, significant histological changes in terms of the areas of abnormal architecture, glandular atrophy, fibrosis, pseudo tubular complexes, and edema were observed at week 4 in the experimental group. The expression of Ptch1, Smo and Gli1 in the pancreatic tissue increased significantly in

the experimental group. Using RT-PCR, mRNA levels of Ptch, Smo and Gli in the experimental group increased significantly compared with the control group.

CONCLUSION: The SHh signaling pathway is aberrantly activated in rats with chronic pancreatitis. The SHh signaling pathway plays an important role in the development of chronic pancreatitis. These results may be helpful in studies focusing on the relationship between chronic pancreatitis and pancreatic cancer.

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Key words: Sonic hedgehog; Chronic pancreatitis; Pancreatic cancer; Ptch1; Smo; Gli1

Core tip: Chronic pancreatitis (CP) is a progressive inflammation of the pancreas in which pancreatic secretory parenchyma is destroyed and replaced by fibrous tissue, eventually leading to impairment of both exocrine and endocrine functions. Hedgehog (Hh) signaling is a developmental signaling pathway that is highly activated in the embryo and in the early postnatal phase. Studies on the Hh signaling pathway in human CP are restricted by limited availability of tissues. Therefore, the present study was carried out to analyze the activation of SHh signaling pathways in a rat model of CP.

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INTRODUCTION

Chronic pancreatitis (CP) is a progressive inflammation of the pancreas in which pancreatic secretory pa-

renchyma is destroyed and replaced by fibrous tissue, eventually leading to impairment of both exocrine and endocrine functions^[1]. On histology, the main findings in this disease are acinar loss, mononuclear cell infiltration, and fibrosis. It is also reported that each inflammatory attack in the pancreas can cause fat necrosis that seems to lead to both pseudocysts and fibrosis^[2]. Although CP has received increased attention over the past few years, the pathogenesis of CP has not yet been fully elucidated. Furthermore, patients with CP have a high risk of developing pancreatic cancer, and the molecular mechanism has also not been established^[1].

Hedgehog (Hh) signaling is a developmental signaling pathway that is highly activated in the embryo and in the early postnatal phase. Three vertebrate Hh homologs have been identified, including Sonic hedgehog (SHh), Indian hedgehog (IHh), and Desert hedgehog. Of these Hh homologs, SHh has been the most studied in terms of the Hh signaling pathway in vertebrates^[3]. Recently, aberrant activation of Hh signaling pathways has been described in various human neoplastic diseases^[4-6]. Overexpression of the Hh ligands has also been reported in pancreatic adenocarcinoma, and inhibition of the Hh pathway at the level of Smo can result in blockage of cell proliferation and induction of apoptosis in many pancreatic cell lines *in vitro*. Kaye *et al.*^[7,8] analyzed the expression, distribution, and function of human hedgehog-interacting protein in normal pancreas, CP and pancreatic adenocarcinoma, and found that there was enhanced activation of hedgehog signaling in CP and pancreatic adenocarcinoma. However, studies on the Hh signaling pathway in human CP are restricted by limited availability of tissues. Therefore, the present study was carried out to analyze the activation of SHh signaling pathways in a rat model of CP.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Use and Care Committee of the Second Military Medical University, Shanghai, China.

Animals and induction of CP

Forty 6-wk-old male Wistar rats (180-200 g) were obtained from the Animal Center of the Second Military Medical University. Rats were provided with food and water *ad libitum* and housed in a temperature- and humidity-controlled room with a 12-h dark-light cycle (lights on at 7 AM) for 1 wk before the experiment.

CP was induced as previously described^[9]. Briefly, dibutyltin dichloride (DBTC; Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) was dissolved in 100% ethanol (Changshu Yangyuan Chemical Co China, Changshu, China) and mixed with glycerol (Amresco, OH, United States), with a volume ratio of 2:3 (ethanol:glycerol). The final DBTC concentration was 8 mg/mL. DBTC was then infused slowly into the tail vein of rats at a dose of 8 mg/kg body weight. Another 20 rats which

acted as controls were infused with the same volume of ethanol and glycerol mixture. One day after the infusion, all rats received 0.6 mL soybean oil (Jinlongyu, China) by orogastric gavage once daily for 4 wk, and body weight was measured before and weekly after the gavage. The rats were killed by exsanguination under pentobarbital anesthesia (50 mg/kg, intraperitoneal) and the pancreas was removed for further analysis 4 wk after the gavage.

Samples and histological examination

The head of the pancreas taken from each rat was fixed in 10% formalin and embedded in paraffin, and then 4- μ m-thick sections were cut for histological examination, Sirius red staining, and immunohistochemistry. The caudal part of the pancreas was used for isolation of mRNA for real-time reverse transcription polymerase chain reaction (RT-PCR).

The pancreas sections were stained with hematoxylin and eosin for histological examination, and the histological changes were evaluated by an independent experienced pathologist, as previously described^[9]. Briefly, the areas of abnormal architecture were classified as 0, absent; 1, rare; 2, minimal (< 10%); 3, moderate (10%-50%); and 4, severe (> 50%). Glandular atrophy, fibrosis, and pseudo tubular complexes within these areas were each scored as 0, absent; 1, minimal (< 10%); 2, moderate (10%-50%); and 3, severe (> 50%). In addition, the content of inflammatory cells (mainly neutrophils) and edema were also scored on a scale of 0-4. The sections were stained using a Sirius red stain kit (Genmed Inc, Shanghai, China) according to the manufacturer's protocol for collagen deposition.

The expression of SHh proteins by immunohistochemistry

Immunostaining was performed to determine the expression of SHh proteins and to identify the activated SHh signaling pathway. The paraffin sections of pancreas were rehydrated and washed 3 times in phosphate buffered saline for 5 min. The sections were incubated with 0.3% H₂O₂ for 30 min to eliminate intrinsic peroxidase and then washed. The sections were probed with primary antibodies overnight at 4°C. The sections were incubated with goat polyclonal anti-Ptch1 (Patched1) antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, United States) diluted to 1:400, goat polyclonal anti-Gli1 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, United States) diluted to 1:500, and rabbit polyclonal anti-Smo (Smoothened) antibody (Abcam, Cambridge, United Kingdom) diluted to 1:400, at room temperature for 3 h.

RNA extraction and real-time RT-PCR

Total RNA was isolated from pancreatic tissues using TRIzol reagent (TaKaRa, Dalian, China), according to the manufacturer's instructions. cDNA synthesis was formed using the real-time RT-PCR kit (TaKaRa), according to the manufacturer's protocol. The specific primers for Ptch, Smo, Gli1 are listed in Table 1. The

Table 1 Nucleotide sequences of the primers used for real-time reverse transcription polymerase chain reaction

Genes	Forward (5'-3'), reverse (5'-3')
<i>Ptch1</i>	TGGTCACACGAACAATGG TGAAGTGGGCAGCTATGAAGTC
<i>Smo</i>	AGTTACATCGCAGCCTTC CACACTACTCCAGCCATC
<i>Gli1</i>	TGCTGACACTCTGGGATA CAGGGCCATAGTTGGTT

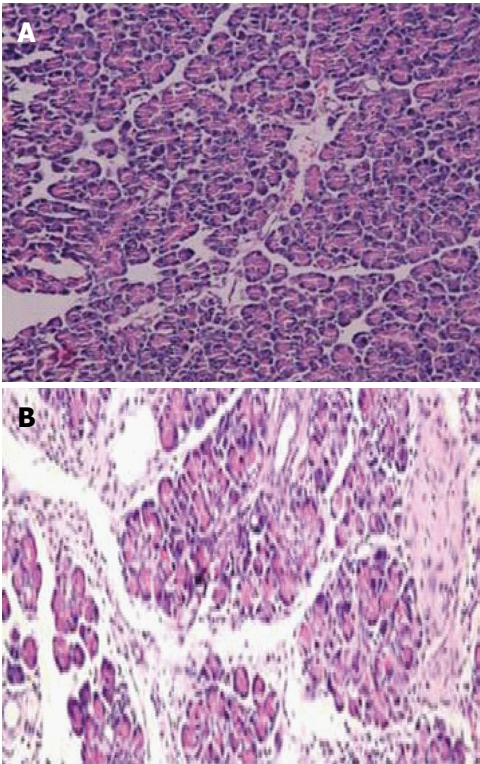


Figure 1 Histologic examination of pancreas ($\times 200$). A: Normal rats; B: Chronic pancreatitis rats. Dense inflammatory infiltrates, large regions of glandular atrophy, pseudo tubular complexes, edema, and fibrosis replaced the normal pancreas (B).

quantitation of target mRNA expression was performed on the ABI Model 7500 RT-PCR Sequence Detector (Applied Biosystems, Foster City, CA, United States) using a TaKaRa real-time PCR kit. The amplified PCR products were quantified by measuring the target and β -actin mRNA calculated cycle thresholds. The quality of specific mRNA in each sample was calculated from the standard curve and normalized with β -actin mRNA. Results were expressed as normalized values relative to the indicated cell line using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Statistics was performed using SPSS 16.0 (SPSS Inc, Chicago, IL, United states). All data are expressed as mean \pm SD. The Student's t test was used for statistical analysis. The relationships between the variables were assessed using Spearman rank correlation coefficient. A value of *P*

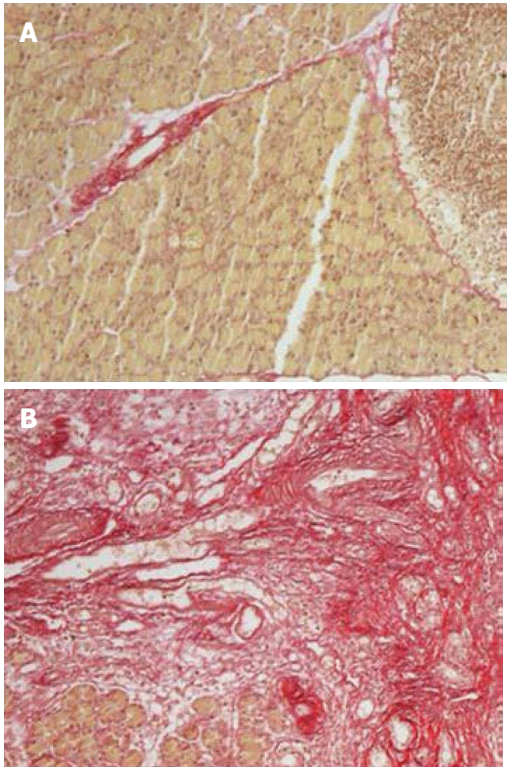


Figure 2 Sirius red staining of pancreas ($\times 200$). A: Normal rats; B: Chronic pancreatitis rats. Significantly higher expression of lobular and sublobular collagen deposition is seen in Figure B.

< 0.05 was considered statistically significant.

RESULTS

Evidence from the DBTC-induced CP model

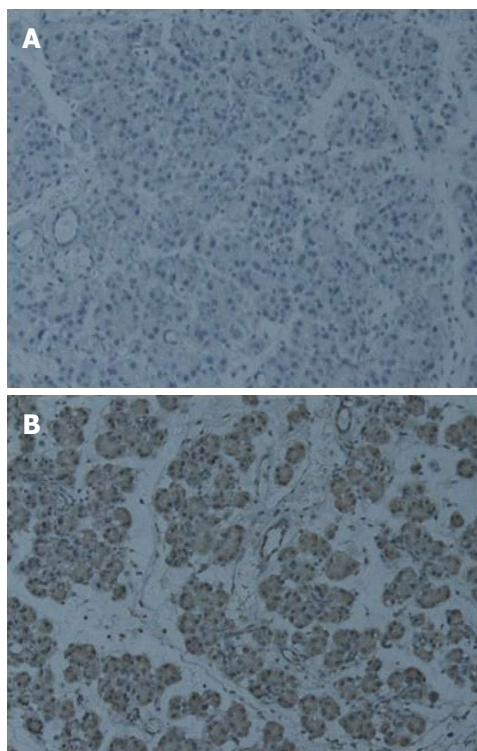
The survival rates of rats were 65% (13/20) and 95% (19/20) for the experimental group (DBTC treated group) and control group (ethanol and glycerol mixture treated group), respectively. There was no difference in body weight at baseline between the two groups, however, a significant reduction in body weight was observed in the experimental group at all time points (week 1, week 2, week 3 and week 4) compared with the controls (all $P < 0.05$). Histologic examination showed almost normal pancreas in the control rats (Figure 1A), however, dense inflammatory infiltrates, large regions of glandular atrophy, pseudo tubular complexes, edema, and fibrosis replaced the normal pancreas in DBTC treated rats (Figure 1B). Sirius red staining showed a significantly higher expression of lobular and sublobular collagen deposition in DBTC treated rats ($P < 0.05$) (Figure 2).

Expression of SHh proteins

Immunohistochemical staining revealed the expression of *Ptch1* in 10 of 13 (76.9%) CP rats (Table 2), while the expression of *Ptch1* was negative in control rats. *Ptch1* expression was mainly restricted to ductal, acinar, or islet compartments of the pancreas (Figure 3). *Smo* immunoreactivity was present in 9 (69.2%) rats in the

Table 2 Expression of *Ptch*, *Smo*, and *Gli1* mRNA (mean \pm SD)

Rats	<i>Ptch</i>	<i>Smo</i>	<i>Gli</i>
Ethanol and glycerol mixture treated	0.23 \pm 0.16	0.14 \pm 0.05	0.57 \pm 0.12
DBTC treated	2.38 \pm 0.42	3.85 \pm 1.03	4.63 \pm 1.49

**Figure 3** Immunohistochemical staining of *Ptch1* in pancreas (\times 200). A: Normal rats; B: Chronic pancreatitis rats. Significantly higher expression of *Ptch1* is seen in Figure B, and *Ptch1* expression was mainly restricted to ductal, acinar, or islet compartments of the pancreas.

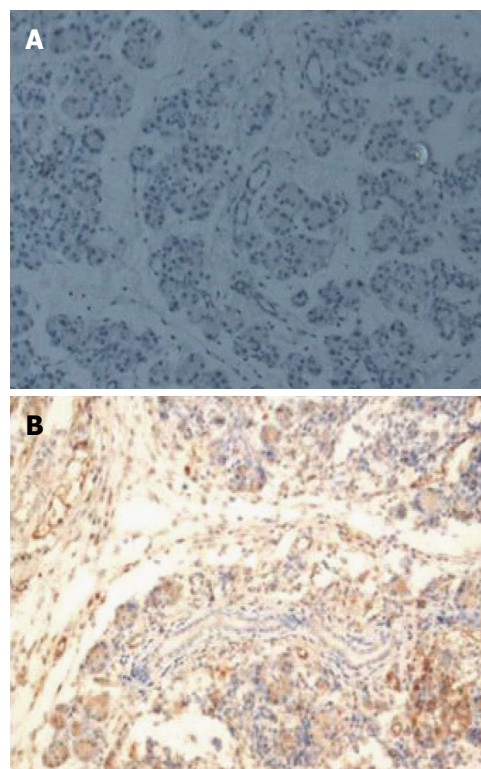
experimental group, and no *Smo* positive cells were present in the control group. The location of *Smo* expression was mainly restricted to ductal, acinar, or islet compartments of the pancreas (Figure 4). Eight (61.5%) CP rats displayed strong *Gli1* immunoreactivity, and no *Gli1* immunoreactivity was observed in the control group. *Gli1* expression was mainly restricted to ductal, acinar, or islet compartments of the pancreas (Figure 5).

Expression of *Ptch*, *Smo*, and *Gli1* mRNA

These results were confirmed at the mRNA level using quantitative real-time RT-PCR. Compared with the controls, the mRNA expression of *Ptch1*, *Smo*, and *Gli1* mRNA was significantly increased in rats treated with DBTC (Table 2).

DISCUSSION

Using immunohistochemistry, we found that SHh proteins are overexpressed in rats with DBTC-induced CP.

**Figure 4** Immunohistochemical staining of *Smo* in pancreas (\times 200). A: Normal rats; B: Chronic pancreatitis rats. Significantly higher expression of *Smo* is seen in Figure 4B, and the location of *Smo* expression was mainly restricted to ductal, acinar, or islet compartments of the pancreas.

This overexpression was confirmed by real-time RT-PCR. The SHh signaling pathway may play an important role in the development of CP.

Intercellular communication is essential for morphogenesis and patterning in vertebrates, and the Hh genes encode secreted proteins implicated in cell-cell interaction^[10]. Two transmembrane receptors, *Ptch1* and *Ptch2*, have been identified as receptors for processed Hh ligands, and *Ptch1* expression is more prominent than *Ptch2*. In the absence of ligand, *Ptch1* represses the activity of *Smo*. On ligand binding, the repression of *Smo* is alleviated, and *Smo* initiates a signaling cascade that results in the translocation of Gli transcription factors into the nucleus^[11]. There are three known Gli transcription factors, *Gli1*, *Gli2* and *Gli3*, in mammals, and *Gli1* seems to act as a transcriptional activator and the Hh transcriptional target genes. Should there be additional text here?^[12].

Although progress has been made toward understanding Hh signaling recently, further research is necessary to clarify the complex nature of this signaling pathway. SHh is the best studied Hh with the broadest expression pattern in organs such as the developing nervous system, limb buds, skin and gut^[11].

The expression of Hh signaling pathways is seen in the embryonic mouse pancreas, and is also observed in the adult mouse pancreas. *Ptch* and *Smo* are expressed in islet β cells. Activation of the Hh signaling pathway leads

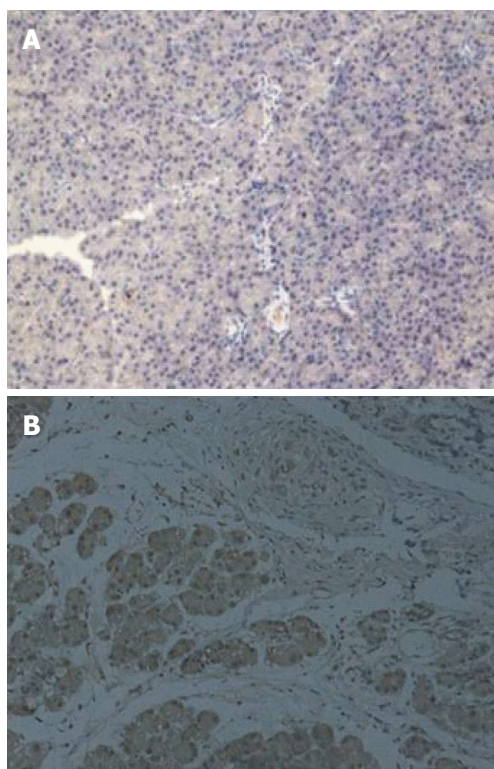


Figure 5 Immunohistochemical staining of Gli1 in pancreas (× 200). A: Normal rats; B: Chronic pancreatitis rats. Significantly higher expression of Gli1 is seen in Figure 5B, and Gli1 expression was mainly restricted to ductal, acinar, or islet compartments of the pancreas.

to increased insulin production, and this increase in insulin production can be prevented through inhibition of Hh signaling, indicating that Hh signaling in adult pancreatic islets acts mainly to regulate insulin production^[13]. In the human pancreas, Ptch and Smo are localized in the islet cells similar to that in mouse pancreas, and weakly expressed in normal ductal cells^[14].

Ptch, Smo, and Gli are reported to be overexpressed in human pancreatic cancer tissues and pancreatic cancer cell lines. SHh and its receptors are localized in malignant pancreatic ductal cells as well as in premalignant pancreatic lesions^[7,15]. Specific inhibition of Hh activity in Ptch expressing pancreatic cancer cells can reduce pancreatic cancer cell growth *in vitro*. The inhibition of Hh activity can not only reduce the development of pancreatic tumors, but also the growth of already established tumors. In addition, binding of the Hh ligands can reduce Hh signaling activity in pancreatic cancer cells expressing Ptch^[7,15-16].

It is reported that IHH and its receptors, Ptch and Smo, are overexpressed in human CP tissues and are localized in the ductal cells and tubular complexes. Compared to normal islets, the islet cells of CP tissues exhibit an abnormal localization pattern for Ihh^[17]. Walter *et al*^[18] established fibroblast cultures from human pancreatic adenocarcinomas and non-neoplastic pancreas tissues to identify differentially expressed genes in cancer-associated stromal fibroblasts. The results showed that Smo was upregulated in cancer-associated stromal fibroblasts, and the expressed Smo could transduce the SHh signal to

activate Gli1 expression. Furthermore, small interfering RNA knockdown of Smo blocked the induction of Gli1 in these cells^[18].

Due to the high risk of developing pancreatic cancer in CP patients, and the aberrant expression of Hh proteins, we examined the expression of SHh signaling pathways in a rat model of CP. The results showed that the receptors of SHh were overexpressed in CP. Given the role of Hh signaling in pancreatic cancer, these findings may provide a mechanism for the pathogenesis between CP and pancreatic cancer. The role of the microenvironment determining SHh expression and localization, and the role of SHh in CP require further investigation.

COMMENTS

Background

Chronic pancreatitis (CP) is a progressive inflammation of the pancreas, and patients with CP have a high risk of developing pancreatic cancer. Recently, aberrant activation of hedgehog (Hh) signaling pathways has been described in pancreatic cancer. Studies on the Hh signaling pathway in CP animal models are lacking.

Research frontiers

It has been reported that human hedgehog-interacting protein was activated in CP and pancreatic adenocarcinoma, but little is known about the role of Hh in CP development and its role in the transformation from CP to pancreatic cancer.

Innovations and breakthroughs

Previous studies have reported that SHh and its receptors are localized in malignant pancreatic ductal cells as well as in premalignant pancreatic lesions. Specific inhibition of Hh activity in Ptch-expressing pancreatic cancer cells can reduce pancreatic cancer cell growth *in vitro*. In this study, they found that SHh proteins are overexpressed in rats with DBTC-induced CP, and this overexpression was confirmed by real-time reverse transcription polymerase chain reaction. The SHh signaling pathway may play an important role in the development of CP.

Applications

The study results suggest that the SHh signaling pathway may play an important role in the development of chronic pancreatitis, and these findings may provide a mechanism for the pathogenesis between chronic pancreatitis and pancreatic cancer.

Peer review

The authors analyzed the expression of SHh and its related protein in a rat CP model, results showed SHh proteins are overexpressed. These findings can provide a new clue in the explanation of CP developing, even the mechanistic pathogenesis from chronic pancreatitis to pancreatic cancer.

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Improved biopsy accuracy in Barrett's esophagus with a transparent cap

Bai-Li Chen, Xiang-Bin Xing, Jin-Hui Wang, Ting Feng, Li-Shou Xiong, Jin-Ping Wang, Yi Cui

Bai-Li Chen, Xiang-Bin Xing, Jin-Hui Wang, Ting Feng, Li-Shou Xiong, Jin-Ping Wang, Yi Cui, Department of Gastroenterology, the First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Author contributions: Chen BL and Xing XB contributed equally to this work, and designed the research, analyzed the data and drafted the paper; Chen BL, Xing XB, Wang JH, Wang JP and Cui Y performed endoscopy and data acquisition; Cui Y offered suggestions to maintain the integrity of the work; Feng T was responsible for submission of the paper; all authors approved the final version to be published.

Correspondence to: Dr. Yi Cui, Department of Gastroenterology, the First Affiliated Hospital of Sun Yat-Sen University, No. 58, Zhongshan Road II, Guangzhou 510080, Guangdong Province, China. gzcuiyi@163.com

Telephone: +86-20-87332916 Fax: +86-20-87332916

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Abstract

AIM: To evaluate the efficacy of endoscopy with a transparent cap on biopsy positioning in Barrett's esophagus (BE).

METHODS: One hundred and sixty-eight patients with suspected BE at endoscopy were enrolled in our study from November 2007 to December 2009 and divided into two groups: transparent cap group ($n = 60$) and control group ($n = 108$). Endoscopy with or without a transparent cap and subsequent biopsy of suspected lesions were performed by five experienced endoscopists in our hospital. In both groups, two biopsy specimens were taken from each patient, and the columnar epithelium or goblet cells in histological assessment were used as the diagnostic standard for BE.

RESULTS: In the transparent cap group, 41 cases were tongue type, while 17 and two cases were identified as island type and circumferential type, respectively. In the

control group, 65 tongue-type cases were confirmed, with 38 island-type and five circumferential-type cases. Moreover, there was no significant difference with regard to the composition of endoscopic BE types in the two groups ($P > 0.05$). In the biopsy specimens, BE was detected in 50 cases in the transparent cap group (83.3%, 50/60), whereas the detection rate in the control group (69.4%, 75/108) was lower compared to that in the transparent cap group ($P < 0.05$). In addition, goblet cells were recognized in only eight cases (all with columnar epithelium) (8/60, 13.3%) in the transparent cap group, with 11 cases in the control group.

CONCLUSION: Transparent cap-fitted endoscopy can guide biopsy positioning in BE without other accompanying complications, thus increasing the detection rate of BE.

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Key words: Barrett's esophagus; Endoscopy; Transparent cap; Biopsy

Core tip: A transparent cap applied to endoscopy can guide biopsy positioning in Barrett's esophagus without other accompanying complications, thus increasing biopsy accuracy.

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INTRODUCTION

Barrett's esophagus (BE) is a premalignant lesion of



Figure 1 Improved transparent cap.

esophageal carcinoma, with a relative risk of developing adenocarcinoma ranging from 30- to 125-fold higher than in the healthy population^[1]. Endoscopy is considered to be an effective approach in the detection of BE. However, even when using current technologies, the detection rate of BE is low, and small lesions, such as tongue-type and island-type, are often missed^[2]. In addition, due to frequent cardiac peristalsis, the diagnostic rate of BE is decreased. We found that the use of a transparent cap on the tip of the endoscope can help exposure and retention of lesions in the small lumen in clinical practice. However, there is a lack of statistical data to show whether application of the transparent cap can help biopsy positioning and improve the diagnosis of BE.

Therefore, the goal of this study was to determine whether endoscopy with the transparent cap device was helpful in guiding accurate tissue sampling and increasing BE detection rate without accompanying complications.

MATERIALS AND METHODS

Diagnostic standards of BE

BE is the transformation of stratified squamous epithelium into a single layer of columnar epithelium in the lower esophagus, with or without intestinal metaplasia^[3]. The presence of specialized intestinal metaplasia is the precancerous state of esophageal adenocarcinoma. Visible extension of the columnar epithelium into the lower esophagus under endoscopy is defined as endoscopically suspected BE^[4]. In the present study, endoscopic BE was classified into three types: tongue, island and circumferential, according to the macroscopic appearance of the mucosa^[5]. In addition, columnar epithelium identified by histological assessment was a diagnostic criterion for BE^[6]. Columnar-lined esophagus was confirmed when the columnar mucosa of the cardiac (junctional), oxyntic or intestinal types was found on biopsy.

Patients

We recruited 168 patients (97 male; mean age: 48 years, range: 16-77 years) with endoscopically suspected BE from November 2007 to December 2009. Endoscopy and subsequent biopsy were performed by five experi-

enced endoscopists, two of whom were accustomed to operating endoscopes with a transparent cap. All patients were informed of the purpose of the study and gave signed informed consent, in accordance with the ethics committee guidelines of the hospital.

Device

An Olympus GIF-XQ240, GIF-H20 GASTROSCOPE (Olympus Optical Co, Ltd, Tokyo, Japan), standard biopsy forceps (FB-25k, Olympus, Tokyo, Japan) and an improved transparent cap with the rigid front-end part clipped with only 2-mm left outside^[7] (six-shooter, Saeed Multi-Band Ligator, Wilson-Cook Company, United States) were used in this study (Figure 1).

Methods

One hundred and sixty-eight patients with suspected BE were randomly allocated to an endoscopist and underwent endoscopy. Sixty patients underwent endoscopy with a transparent cap and 108 cases underwent endoscopy without a cap (control group). In both groups, routine endoscopy without magnification was performed to identify visible mucosal lesions of suspected BE. When lesions were found, the endoscope was retracted and the transparent cap was subsequently installed on the front end of the endoscope in the transparent cap group. When the lesions were large and obvious in some cases, especially the circumferential-type, methylene blue staining was performed to help decide on the biopsy site and improve the detection rate of goblet cells. Two specimens were taken from the suspected BE lesions, fixed in formaldehyde, and embedded in paraffin. Serial sections (4-5 μ m thick) were cut for hematoxylin and eosin staining. Histological assessment was undertaken by two experienced gastrointestinal histopathologists.

Statistical analysis

The data were presented as frequency and analyzed using the χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

In the transparent cap group, 41 cases were tongue-type, 17 were island-type, and two were circumferential-type. In the control group, 65 tongue-type cases were confirmed, along with 38 island-type and five circumferential-type cases. There was no significant difference with regard to the composition of endoscopic BE types in the two groups ($P > 0.05$) (Figures 2-5).

Comparison of detection rate

Histological assessment revealed that columnar epithelium was found in 50 cases in the transparent cap group (83.3%), with goblet cells present in only eight cases (all with columnar epithelium, 13.3%). In the control group, the detection rate of BE was 69.4% (75/108), which was significantly lower compared to the transparent cap group ($P < 0.05$). In addition, 11 cases in the control group ex-

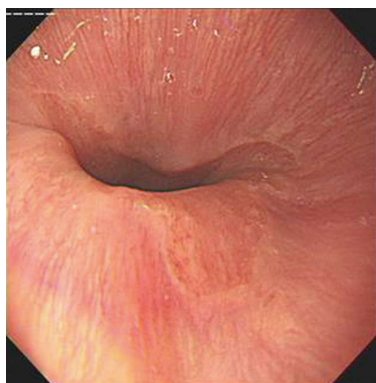


Figure 2 Tongue-type Barrett's esophagus (without transparent cap).

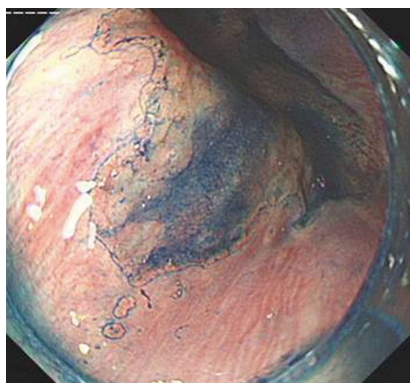


Figure 3 Same lesion as in Figure 1 (with transparent cap).

hibited goblet cells, accompanied by columnar epithelium (10.2%).

Complications

Severe complications, such as massive hemorrhage or perforation, were not observed in either group. However, in the control group, one case demonstrated active hemorrhage from a small blood vessel, and bleeding was subsequently stopped using a titanium clamp. In the transparent cap group, difficulty occurred when the endoscope with the transparent cap passed through the patient's throat.

DISCUSSION

Since the 1980s, the prevalence of cancer in the gastric cardia and lower esophagus has shown an increasing trend. Numerous studies have reported that nearly all lower esophageal adenocarcinoma originates from BE, and approximately 40% of cardiac cancer is associated with BE^[8]. Therefore, early diagnosis and long-term follow-up of BE are of great importance. Currently, diagnosis and surveillance of BE remain controversial in gastroenterology. A widely accepted and practical definition of BE, which is endorsed by the American College of Gastroenterology, is a change in normal esophageal squamous mucosa (of any length) that is visible endoscopically and demonstrates intestinal metaplasia on biopsy^[9].

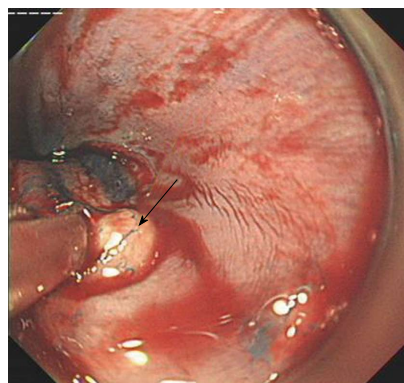


Figure 4 Targeted biopsy of the suspected Barrett's esophagus lesion (the same lesion as in Figure 1). The Z line is indicated by the arrow.



Figure 5 Intestinal metaplasia in circumferential-type Barrett's esophagus, indicated by methylene blue dye.

In the United Kingdom, the presence of intestinal metaplasia is not mandatory for the diagnosis of columnar-lined esophagus, which may be diagnosed when columnar mucosa of cardiac (junctional), oxyntic, or intestinal type is seen on biopsy^[10,11]. Recently, Kelty *et al*^[12] found that patients who had glandular mucosa on biopsy, without the presence of intestinal metaplasia, still carried a significant risk of cancer development over their lifetime, and more importantly, it added weight to the argument proposed by the British Society of Gastroenterology (BSG) that glandular mucosa should still be classified as BE, and such patients should be entered into a surveillance program. This is contrary to the current thinking in the United States, which demands the presence of specialized intestinal metaplasia to make the diagnosis^[9]. However, it should be acknowledged that biopsy protocols in the United States are more rigorous than elsewhere. A recent study also demonstrated that a minimum of eight biopsies are required from a columnar-lined esophagus to show intestinal metaplasia^[13]. In the present study, the biopsy was performed by experienced endoscopists and a similar number of biopsies were undertaken in each group. However, because the number of biopsies was fewer than that required by present guidelines, only 19 patients were found to have intestinal metaplasia in our study.

To date, the accurate biopsy of suspected lesions is the major approach in BE diagnosis. Prof. Raj Goyal from Harvard Medical School and Prof. Qin Huang from the Pathology Department of Brown Medical School have indicated that multiple repeated biopsies can reduce the misdiagnosis rate, and avoid sampling error. The current international four-quadrant biopsy approach recommends that, due to up-shift of the Z line in BE, endoscopic biopsy specimens should be taken between the Z line and gastroesophageal junction, and four specimens at intervals of 1-2 cm along the major axis of the lesion should be obtained for histological assessment^[14]. Therefore, the method and accuracy of biopsy are the basic guarantee of BE diagnosis.

Recently, transparent-cap fitted colonoscopy has been widely used in the screening of colorectal neoplasia based on the following benefits: improving and extending visualization^[15-17], shortening cecal intubation^[18-20] and reducing patient discomfort^[21]. However, the detection rate of adenoma remains controversial. Similarly, due to frequent peristaltic movement of the cardia, endoscopic vision is often unclear, making biopsy difficult and time consuming. We found that the transparent cap helped to expose the mucosa in the lumen with stenosis or frequent peristaltic movement, and guided biopsy positioning in our center^[22-24]. The question then arose as to whether the application of the transparent cap could enhance the detection rate of BE on biopsy. We thus used endoscopes with or without transparent caps to perform mucosal biopsy of suspected BE lesions and compared the detection rate of both methods. Using columnar epithelium as the diagnostic standard of BE, application of the transparent cap significantly raised the detection rate from 69.4% to 83.3% in our study. In contrast, the detection rate for goblet cells in the two groups did not differ significantly. The majority of the cases enrolled in this study were tongue- and island-type, increasing the difficulty of biopsy when compared with long-segment circumferential-type lesions. This resulted in difficulty in accurate positioning in the control group, and therefore, the detection rate of BE using a traditional endoscope without a transparent cap might miss several BE cases. In addition, application of the transparent cap did not lead to serious complications such as massive hemorrhage or perforation, indicating the safety of this approach.

High tension in the luminal wall and frequent peristaltic movement of the cardia often result in close contact of the mucosa with the front end of the endoscope, thus blurring vision. This increases the difficulty of observation and biopsy of the lesions, especially small lesions, such as tongue- or island-type, prolongs the operation time, and decreases the accuracy of the biopsy. Installation of a transparent cap protruding 2 cm from the front end of the endoscope leads to clear exposure of the lesion and stability of the front end of the endoscope, thus facilitating the targeted biopsy of suspected BE. Therefore, this approach makes early detection of intestinal epithelium, goblet cells and dysplasia possible, providing

a reliable basis for further diagnosis and treatment of BE. However, installation of the transparent cap causes increasing patient discomfort, therefore, patient tolerance should be taken into account and strengthened by improvement of techniques or use of sedatives.

In conclusion, transparent-cap-assisted biopsy improved the detection rate of BE without increasing complications. Although a number of new endoscope-assisted biopsy techniques, such as dye endoscopy, narrow-band endoscopic imaging^[25,26], and confocal endoscopy^[27], have facilitated biopsy and increased the detection rate of BE in recent years, the application of these techniques remains limited by the required experience of the endoscopist, high cost, and complex manipulation. The transparent-cap-assisted biopsy is more economic, simpler and safer, therefore, this technique is of more practical value and worthy of further promotion in clinical practice.

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COMMENTS

Background

Barrett's esophagus (BE) is a premalignant lesion of esophageal carcinoma, with a relative risk of developing adenocarcinoma ranging from 30- to 125-fold higher than in the healthy population. Endoscopy is considered to be an effective approach in the detection of BE. However, even when using current technologies, the detection rate of BE is low, and small lesions, such as tongue- and island-type are often missed. In addition, due to frequent peristalsis of the cardia, the diagnostic rate of BE is decreased.

Research frontiers

A transparent cap is widely used for endoscopic variceal ligation. The authors have found that the use of a transparent cap on the tip of an endoscope could help with exposure and retention of lesions in the small lumen in BE in clinical practice. However, there is a lack of statistical data.

Innovations and breakthroughs

To date, accurate biopsy of suspected lesions is the major approach in BE diagnosis. Due to frequent peristaltic movement of the cardia, endoscopic vision is often unclear. The authors found that a transparent cap helped to expose the mucosa in the lumen with stenosis or frequent peristaltic movement, and guided biopsy positioning in clinical practice.

Applications

Transparent-cap-fitted endoscopy can guide biopsy accurately in BE without increasing other complications, thus increasing the detection rate of BE.

Terminology

BE is the transformation of stratified squamous epithelium into a single layer of columnar epithelium in the lower esophagus, with or without intestinal metaplasia. The presence of specialized intestinal metaplasia is the precancerous state of esophageal adenocarcinoma.

Peer review

This was a good intervention study in which the authors tried to improve the macroscopic evaluation of the distal esophagus regarding BE detection.

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MT1M and MT1G promoter methylation as biomarkers for hepatocellular carcinoma

Xiang-Fen Ji, Yu-Chen Fan, Shuai Gao, Yang Yang, Jian-Jun Zhang, Kai Wang

Xiang-Fen Ji, Shuai Gao, Yang Yang, Jian-Jun Zhang, Department of Hepatology, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Yu-Chen Fan, Kai Wang, Department of Hepatology, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Yu-Chen Fan, Kai Wang, Institute of Hepatology, Shandong University, Jinan 250012, Shandong Province, China

Author contributions: Ji XF designed the study, carried out the experiments and drafted the manuscript; Fan YC revised the manuscript; Yang Y performed part of experiments; Gao S and Zhang JJ interpreted the data; Wang K revised the paper critically for important intellectual content.

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Correspondence to: Kai Wang, MD, PhD, Department of Hepatology, Qilu Hospital of Shandong University and Institute of Hepatology, Shandong University, No. 107, Wenhua Road, Jinan 250012, Shandong Province, China. wangdoc876@126.com
Telephone: +86-531-86630809 Fax: +86-531-8692754

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Abstract

AIM: To investigate the potential of promoter methylation of two tumor suppressor genes (TSGs) as biomarkers for hepatocellular carcinoma (HCC).

METHODS: A total of 189 subjects were included in this retrospective cohort, which contained 121 HCC patients without any history of curative treatment, 37 patients with chronic hepatitis B (CHB), and 31 normal controls (NCs). DNA samples were extracted from 400 μ L of serum of each subject and then modified using bisulfite treatment. Methylation of the promoters of the TSGs (metallothionein 1M, *MT1M*; and metallothionein 1G, *MT1G*) was determined using methylation-specific polymerase chain reaction. The diagnostic value of combined *MT1M* and *MT1G* promoter methylation was evaluated using the area under the receiver operating characteristic curves.

RESULTS: Our results indicated that the methylation status of serum *MT1M* (48.8%, 59/121) and *MT1G* (70.2%, 85/121) promoters in the HCC group was significantly higher than that in the CHB group (*MT1M* 5.4%, 2/37, $P < 0.001$; *MT1G* 16.2%, 6/37, $P < 0.001$) and NC group (*MT1M* 6.5%, 2/31, $P < 0.001$; *MT1G* 12.9%, 4/27, $P < 0.001$). Aberrant serum *MT1M* promoter methylation gave higher specificity to discriminate HCC from CHB (94.6%) and NCs (93.5%), whereas combined methylation of serum *MT1M* and *MT1G* promoters showed higher diagnostic sensitivity (90.9%), suggesting that they are potential markers for noninvasive detection of HCC. Furthermore, *MT1M* promoter methylation was positively correlated with tumor size ($r_s = 0.321$, $P < 0.001$), and HCC patients with both *MT1M* and *MT1G* promoter methylation tended to show a higher incidence of vascular invasion or metastasis ($P = 0.018$).

CONCLUSION: *MT1M* and *MT1G* promoter methylation may be used as serum biomarkers for noninvasive detection of HCC.

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Key words: *MT1M*; *MT1G*; Methylation; Serum biomarker; Hepatocellular carcinoma

Core tip: DNA methylation of tumor suppressor gene promoter regions appears to be a valuable biomarker in many tumors, including hepatocellular carcinoma (HCC). We found that aberrant serum metallothionein 1M (*MT1M*) promoter methylation gave higher specificity to discriminate HCC from chronic hepatitis B and normal controls. In contrast, combined methylation of serum *MT1M* and metallothionein 1G promoters showed higher diagnostic sensitivity. This indicates that they may be used as potential biomarkers for noninvasive detection of HCC.

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MT1G promoter methylation as biomarkers for hepatocellular carcinoma. *World J Gastroenterol* 2014; 20(16): 4723-4729 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4723.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4723>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common tumor and has the third highest mortality^[1]. The areas of highest incidence are Asia and Africa, which are linked to the wide prevalence of hepatitis B virus (HBV) infection^[2]. However, the incidence of HCC has been rapidly increasing in the United States and United Kingdom over the past 20 years, which is attributed to increased hepatitis C virus (HCV) infection^[3,4]. In addition, aflatoxin B1 exposure and alcohol addiction are also associated with hepatocellular carcinogenesis. Despite advanced treatment, patients with HCC have a dismal 5-year survival rate of about 5%, as a result of late diagnosis^[5]. Currently available screening tests to detect HCC mainly combine serum α -fetoprotein (AFP) and ultrasound (US). Regrettably, their effectiveness remains controversial, and the diagnostic rate of AFP meets with a low sensitivity and that of US depends on examiner expertise, patient data, presence of liver cirrhosis, and tumor size^[6,7]. Great efforts have been made to find new biomarkers for early detection of HCC. As a result, the potential value of tumor-associated DNA methylation as a biomarker has attracted much attention^[8,9].

It is well known that the silencing of tumor suppressor genes by promoter hypermethylation is responsible for carcinogenesis. Some studies have found that tumors shed methylated DNA sequences into the blood in the early stages^[10-13]. Moreover, Zhang *et al.*^[14] found that methylated DNA could be detected 1-9 years before the clinical diagnosis of HCC. Thus, methylated DNA has been suggested as an ideal biomarker because of its early appearance in the disease course, as well as its easy and noninvasive detection in biological samples. In addition, the DNA methylation pattern is more stable than protein and RNA expression, which changes markedly and unpredictably^[9].

The metallothioneins (MTs) are a superfamily of low-molecular-weight, cysteine-rich intracellular proteins, consisting of at least 10 functional members (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1X, MT2A, MT3, and MT4)^[15,16]. The role of MTs in metal homeostasis, protection against oxidative damage, cell proliferation and apoptosis, resistance to radiation and chemotherapy, as well as several aspects of the carcinogenic process, has been revealed^[17-21]. Some studies have shown that MT-1 and MT-2 are frequently downregulated in HCC^[22-25], and decreased MT expression might be an early event in HCC progression^[22]. MT downregulation may be concerned with hypermethylation of *MT* promoters, as shown in rat hepatoma^[26]. Moreover, others have reported that metallothionein 1M (MT1M)^[27] and metallothionein 1G (MT1G)^[28] are decreased in human HCC tissues by pro-

motor hypermethylation.

Therefore, in the present study, we hypothesized that methylation of *MT1M* and *MT1G* promoters could be detected in the serum of patients with HCC, and aimed to define optimal gene sets as noninvasive markers for early detection of HCC.

MATERIALS AND METHODS

Collection of serum specimens

After obtaining informed consent, we collected 189 serum samples from 121 patients with HCC, 37 patients with chronic hepatitis B (CHB), and 31 normal controls (NCs), based on clinical and laboratory examinations. Patients with HCC and CHB were recruited from those enrolled from July 2011 to March 2013 at Qilu Hospital, Shandong University in accordance with American Association for the Study of Liver Diseases Practice Guidelines for HCC and CHB, respectively^[29,30]. All cases of HCC included in our study were confirmed by pathological data. Serum samples were collected from HCC patients who did not receive curative treatments such as surgical resection, transcatheter arterial chemoembolization (TACE), or radiofrequency ablation before and during the study. Exclusion criteria included other tumors, co-infection with HCV or human immunodeficiency virus, and other causes of chronic liver diseases. The patient selection process is shown in Figure 1.

Tumor size was calibrated by computed tomography and presented as the longest diameter. AFP concentration > 20 ng/mL was regarded as abnormal^[31]. The study protocol was approved by the Ethics Committee of Qilu Hospital.

Serum DNA extraction and sodium bisulfite modification

DNA was extracted from 400 μ L of serum with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the DNA Purification from Blood or Body Fluids protocol. Bisulfite modification was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, United States) according to the manufacturer's instructions. After bisulfite treatment, all unmethylated cytosine residues were converted to uracil, whereas the methylated residues would have been resistant to this modification and remained as cytosine. The modified DNA was finally stored at -20 °C before methylation-specific polymerase chain reaction (MSP).

MSP

The primer pairs of *MT1M* and *MT1G* for MSP analysis were as described previously^[27,28] (Table 1). One microliter of bisulfite-treated DNA, 0.5 μ L each primer (10 μ mol/L), 10.5 μ L nuclease-free water, and 12.5 μ L Premix Taq (Zymo Research) were mixed together to form a 25- μ L MSP reaction mixture. The PCR protocol included an initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at the respective temperature (54 °C for *MT1M*, 59 °C for *MT1G*-U, and 50 °C for *MT1G*-M) for 40 s, primer ex-

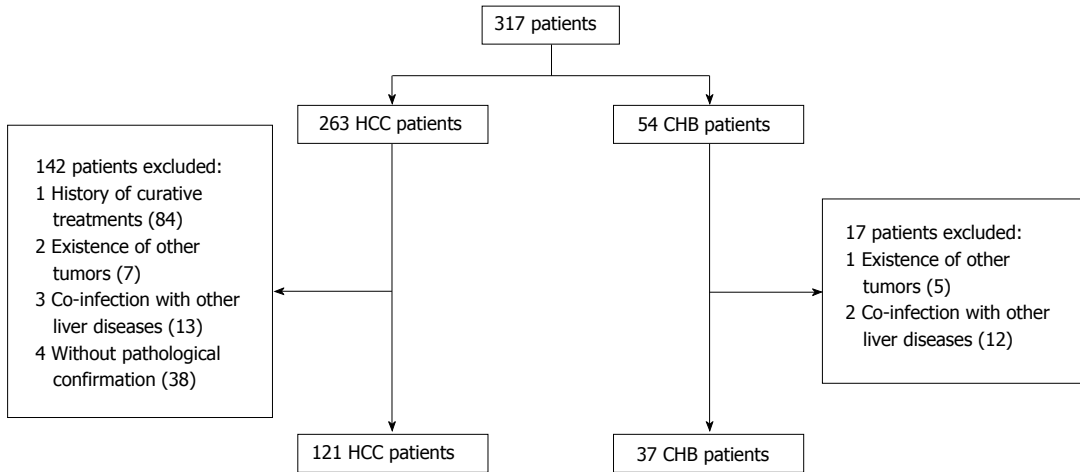


Figure 1 Patient selection process. HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis B.

Table 1 Primers for polymerase chain reaction

Primers	Sequences	Annealing temp (°C)	Size (bp)
<i>MT1M</i>			
U	5'-TTGAAAATGGTGGGGTGA-3'	54	163
	5'-AAACTATACACCAAATAATACACAATATCC-3'		
M	5'-GACGTTTCGCGACGTTAAG-3'	54	124
	5'-ACGCCGAATAATACGCAAT-3'		
<i>MT1G</i>			
U	5'-GGGGTGTGTTTGTGGTGTGTG-3'	59	135
	5'-AAACACCCACCCACCCCTT-3'		
M	5'-TTCGCGAGTCGGTGCAGAAAG-3'	50	96
	5'-CCGCGATCCCGACCTAACT-3'		

tension at 72 °C for 40 s, and a final extension at 72 °C for 10 min (Table 1). Water without DNA was used as a negative control. PCR products were electrophoresed on 2% agarose gels, stained with Gel Red, and visualized under UV illumination.

Statistical analysis

The differences in DNA methylation status of *MT1M* and *MT1G* promoters between different groups and the associations between gene methylation in HCC patients and clinical pathological variables were analyzed using the χ^2 test. Correlation between *MT1M* and *MT1G* promoter methylation and tumor size was calculated by Spearman rank correlation. Diagnostic value of combined methylation of *MT1M* and *MT1G* promoters and serum AFP level was evaluated by the area under the receiver operating characteristic curves (AUC). Differences were considered significant at $P < 0.05$. All statistical analyses were conducted with SPSS 16.0 software.

RESULTS

Methylation status in serum

The methylation status of *MT1M* or *MT1G* promoter in 121 patients with HCC, 37 patients with CHB and 31

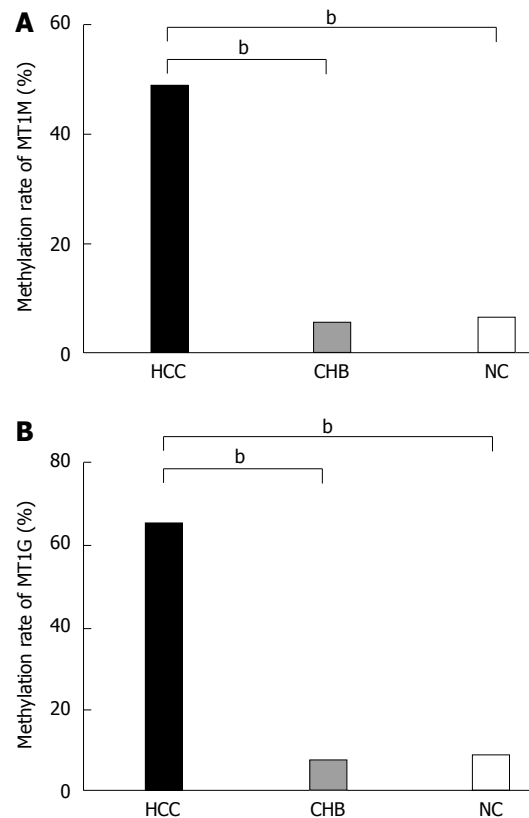


Figure 2 Percentage methylation of *MT1M* and *MT1G* in hepatocellular carcinoma, chronic hepatitis B and normal controls groups. A: Percentage methylation of *MT1M* was 48.8% (59/121) in the HCC, 5.4% (2/37) in the CHB, and 6.5% (2/31) in the NC groups; B: Percentage methylation of *MT1G* was 70.2% (85/121) in the HCC, 16.2% (6/37) in the CHB, and 12.9% (4/31) in the NC groups ($^bP < 0.001$).

NCs was compared (Figure 2). The methylation percentages were higher in HCC (48.8% for *MT1M* and 70.2% for *MT1G*) than in CHB (5.4% for *MT1M* and 16.2% for *MT1G*) or NCs (6.5% for *MT1M* and 12.9% for *MT1G*) ($P < 0.001$). However, no differences were found for either of them between the CHB and NC groups. Representative MSP results for methylated *MT1M* and *MT1G*

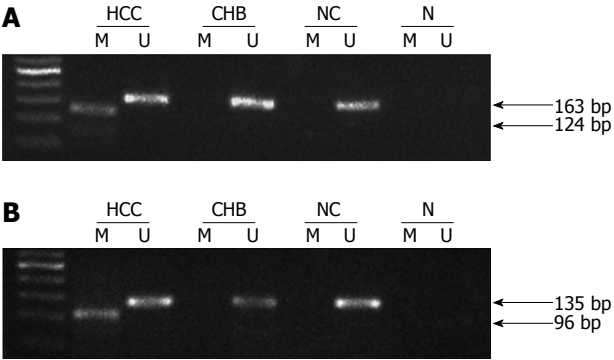


Figure 3 Representative methylation of metallothionein 1M and metallothionein 1G gene promoters by methylation-specific polymerase chain reaction. A: The methylated and unmethylated sequences of *MT1M* were 124 and 163 bp, respectively; B: The methylated and unmethylated sequences of *MT1G* were 96 and 135 bp, respectively. N: Negative control; M: Methylation-specific primers; U: Unmethylation-specific primers.

Table 2 Clinicopathological data and serum metallothionein 1M and metallothionein 1G promoter methylation in hepatocellular carcinoma patients

Characteristics	n	<i>MT1M</i>			<i>MT1G</i>		
		¹ M	² U	P value	¹ M	² U	P value
Total number	121	59	62		85	36	
Gender							
Male	100	50	50	0.552	70	30	0.896
Female	21	9	12		15	6	
Age (yr)							
≥ 55	67	32	35	0.807	46	21	0.670
< 55	54	27	27		39	15	
HBV infection							
Yes	100	50	50	0.552	69	31	0.512
No	21	9	12		16	5	
Vascular invasion or metastasis							
Yes	44	26	18	0.086	33	11	0.387
No	77	33	44		52	25	
Histological differentiation							
Poor	41	18	23	0.610	26	15	0.426
Moderate	50	27	23		38	12	
Well	30	14	16		21	9	
TNM stage							
I - II	64	26	38	0.058	44	13	0.115
III-IV	57	33	24		41	23	
Tumor multiplicity							
Single	72	26	36	0.741	52	20	0.565
Multiple	49	23	26		33	16	
Tumor size(cm)							
≥ 5	59	38	21	0.001	38	21	0.170
< 5	62	21	41		47	15	

¹Methylated; ²Unmethylated.

promoters are shown in Figure 3.

Correlation with clinicopathological parameters

For analysis of the correlation between methylation status of a single gene promoter in serum and clinicopathological features, there was a significant association between the methylation ratio of *MT1M* promoter and tumor size ($P = 0.001$) (Table 2). Further analysis revealed that the correlation was positive ($r_s = 0.321$, $P < 0.001$) (Table

Table 3 Correlation of metallothionein 1M and metallothionein 1G promoter methylation with tumor size

Gene	Tumor size (cm)		r_s	P value
	Methylated M (P ₂₅ -P ₇₅)	Unmethylated M (P ₂₅ -P ₇₅)		
<i>MT1M</i>	6.5 (4.0-9.0)	3.9 (2.2-6.4)	0.321	0.000
<i>MT1G</i>	4.4 (2.9-8.0)	5.0 (3.5-7.9)	-0.049	0.590

M: Median; P₂₅: First quartile; P₇₅: Third quartile; r_s : Spearman correlation coefficient; MT1M: Metallothionein 1M; MT1G: Metallothionein 1G.

Table 4 Vascular invasion or metastasis and a combination of metallothionein 1M and metallothionein 1G promoter methylation

Characteristic	n	<i>MT1M</i> and <i>MT1G</i>		
		¹ M	² U	P value
Vascular invasion or metastasis				
Yes	44	18	26	0.018
No	77	16	61	

¹Both *MT1M* and *MT1G* were methylated; ²One of *MT1M* and *MT1G* was methylated or neither of them methylated. MT1M: Metallothionein 1M; MT1G: Metallothionein 1G.

Table 5 Sensitivity and specificity of gene sets for hepatitis B virus (+) hepatocellular carcinoma detection in chronic hepatitis B group

No.	Marker	TP/FN	FP/TN	Sensitivity (%) TP/(TP + FN)	Specificity (%) TN/(TN + FP)
1	AFP	56/44	14/23	56.0	62.1
2	MT1M	50/50	2/35	50.0	94.6
3	MT1G	69/31	6/31	69.0	83.8
4	MT1M/MT1G	90/10	7/30	90.0	81.1

Sensitivity (%), TP/(TP + FN) and specificity (%), TN/(TN + FP) of each gene set were calculated and plotted. *MT1M/MT1G*, *MT1M* or *MT1G* promoter methylation. TP: True positive; FN: False negative; FP: False positive; TN: True negative.

3). Moreover, advanced TNM stage (III-IV) was associated with a more elevated percentage of serum *MT1M* promoter methylation than early TNM stage (I - II), although the difference was not significant ($P = 0.058$) (Table 2). In addition, HCC patients with both *MT1M* and *MT1G* promoters methylated (18/44) tended to show a higher incidence of vascular invasion or metastasis than those with only one or neither gene methylated (16/77) ($P = 0.018$) (Table 4). However, no significant relationships were observed between the methylation levels of *MT1M* and *MT1G* promoters and other parameters, such as sex, age, HBV infection, serum AFP levels, tumor multiplicity or TNM stage ($P > 0.05$).

Sensitivity and specificity for single or combination methylation

There were 100 HCC patients with HBV infection (Table 2). To discriminate HBV-associated HCC from CHB, *MT1M* and *MT1G* promoter methylation showed a

Table 6 Sensitivity and specificity of gene sets for hepatocellular carcinoma detection in normal controls group

No.	Gene	TP/FN	FP/TN	Sensitivity (%) TP/(TP+FN)	Specificity (%) TN/(TN+FP)
1	MT1M	59/62	2/29	48.8	93.5
2	MT1G	85/36	4/27	70.2	87.1
3	MT1M/MT1G	110/11	5/26	90.9	83.9

Sensitivity (%), TP/(TP + FN) and specificity (%), TN/(TN + FP) of each gene set were calculated and plotted. *MT1M/MT1G*, *MT1M* or *MT1G* promoter methylation. TP: True positive; FN: False negative; FP: False positive; TN: True negative; MT1M: Metallothionein 1M; MT1G: Metallothionein 1G.

moderate sensitivity (*MT1M*, 50%, 50/100; *MT1G*, 69%, 69/100) but a high specificity (*MT1M*, 94.6%, 2/37; *MT1G*, 83.8%, 6/37), whereas the sensitivity and specificity of AFP were 56% (56/100) and 62.1% (23/37), respectively (Table 5). To discriminate HCC from the NC group, the specificity was still high (*MT1M*, 93.5%, 2/31; *MT1G*, 87.1%, 4/31) (Table 6). Otherwise, combined methylation of *MT1M* and *MT1G* promoters gave a sensitivity up to 90.9% (110/121) but a lower specificity to discriminate HCC from the NC (83.9%, 5/31) or CHB (81.1%, 7/37) groups (Tables 5 and 6). Moreover, the AUC of combined methylation of *MT1M* and *MT1G* promoters was 0.855 (95%CI: 0.785-0.910), which was significantly higher than that of AFP (0.754; 95%CI: 0.673-0.824) ($P = 0.0446$) (Figure 4).

DISCUSSION

DNA methylation is suggested as a promising biomarker for cancer detection. However, most studies about DNA methylation have concentrated on the analysis of tumor tissue, which is invasive and not always available, as well as one single gene, which cannot provide enough diagnostic sensitivity. In the present study, we first demonstrated that aberrant methylation status of *MT1M* and *MT1G* promoters could be detected in the serum of patients with HCC, and the frequencies were 48.8% (59/121) and 70.2% (85/121) using MSP, which were significantly higher than those in the CHB and NC groups. This was consistent with previous studies in which *MT1M* and *MT1G* promoters were methylated in HCC tissues^[27,28]. From a diagnostic point of view, assaying a single gene, *MT1G* promoter methylation, showed a higher sensitivity of 70.2%, whereas *MT1M* promoter methylation gave a higher specificity to discriminate HCC from CHB (94.6%) and NCs (93.5%). However, combined methylation of *MT1M* and *MT1G* promoters significantly elevated the diagnostic sensitivity for HCC (90.9%). In addition, aberrant methylation status of *MT1M* and *MT1G* promoters was also observed in early HCC, including TNM stage I, well differentiated and small in size, as well as in patients with negative AFP. Thus, analysis of *MT1M* and *MT1G* promoter methylation showed potential value in early detection of HCC.

MT was first isolated in 1957. In addition to its func-

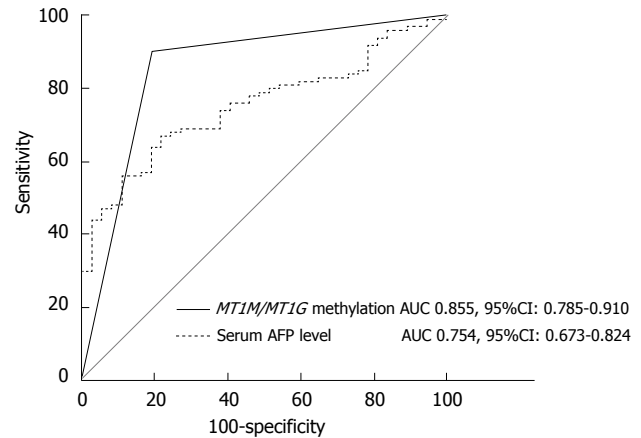


Figure 4 Receiver operating characteristic curves of α -fetoprotein and combined methylation of metallothionein 1M and metallothionein 1G promoters. *MT1M/MT1G*, *MT1M* or *MT1G* promoter methylation. AUC: Area under the ROC curve; MT1M: Metallothionein 1M; MT1G: Metallothionein 1G

tion in metal homeostasis and protection against oxidative damage, several studies have focused on its role in tumors. However, large discrepancies in MT exist between different tumor types. MT expression in tumors of the lung, nasopharynx, breast, kidney, ovary, testes, thyroid, salivary gland, and urinary bladder is increased^[20,21], but it is decreased in other tumors such as prostate cancer, colorectal cancer and HCC^[22-25,32-34]. Compared with overall MT expression in tumors, its isoforms appear more specific and play distinct roles in different tumor types, such as breast cancer, urological malignancies, and nasopharyngeal cancer^[35]. However, there are few reports on the expression of different isoforms of MT in HCC. MT1M and MT1G are two major isoforms that were recently reported to be downregulated in HCC tissues by promoter hypermethylation. Restored expression of MT1M in HCC cells impedes HCC cell growth, and low levels of MT1M are correlated with clinical TNM grade^[27]. *MT1G* acts as a TSG in HCC and patients with *MT1G* promoter methylation have a poorer prognosis, although the difference is not significant^[28].

In our present study, we also evaluated whether methylation status of serum *MT1M* and *MT1G* promoters in patients with HCC was associated with any clinicopathological parameter. *MT1M* promoter methylation was positively correlated with tumor size ($r_s = 0.321$, $P < 0.001$), suggesting that methylated *MT1M* promoter could reflect tumor load. In addition, patients with advanced TNM stage (III-IV) showed a higher elevated percentage of serum *MT1M* promoter methylation than those with early TNM stage (I-II), although the difference was not significant ($P = 0.058$). These differences from the previous study^[27] may have been due to the use of different biological samples of HCC in different regions. Surprisingly, HCC patients with combined methylation of *MT1M* and *MT1G* promoters tended to show a higher incidence of vascular invasion and lymph node or extrahepatic metastasis ($P = 0.018$). Tumor invasion in the portal vein is the main route for intrahepatic metas-

tasis, which is regarded as the most frequent metastatic site of HCC^[36]. Lymph node or extrahepatic metastasis is less common. Although curative resection remains a major effective method for HCC, the possibility of tumor recurrence, caused mainly by metastasis, leads to dismal prognosis. Therefore, combined methylation of serum *MT1M* and *MT1G* promoters may be a valuable prognostic marker for HCC. Also, our findings indicated that *MT1M* and *MT1G* may not only be tumor suppressors but also metastatic suppressors in HCC. However, the molecular mechanisms of this remain unclear. In previous studies, it was reported that *MT1G* methylation contributes to tumor invasion in prostate cancer and peripheral pulmonary adenocarcinoma^[37,38]. However, to the best of our knowledge, no studies have investigated *MT1M* and tumor invasion. Further study is necessary to elucidate the mechanism of how *MT1M* and *MT1G* promoter methylation synergistically acts on metastasis in HCC. However, no significant differences between serum *MT1M* and *MT1G* promoter methylation and sex, age and history of HBV infection were observed, thus the analysis of serum *MT1M* and *MT1G* promoter methylation enabled the detection of HCC independent of patient settings.

Our findings demonstrated that MT isoform gene expression may be specific and reciprocal in carcinogenesis and progression of HCC. They also support the concept that the clinical significance of MT expression in HCC might be further defined if specific MT isoforms were known for individual tumors^[26].

Our study had some limitations. First, the small number of HCC patients and NCs may have led to bias. Second, we do not have long-term follow-up data for HCC patients, which may reveal the predictive value of *MT1M* and *MT1G* promoter methylation in prognosis. Further study with a larger number of cases and longer follow-up is needed.

In conclusion, we demonstrated that *MT1M* and *MT1G* promoter methylation was frequently detected in serum of patients with HCC, and appeared to be a valuable diagnostic marker for noninvasive detection of HCC. Furthermore, we observed that *MT1M* promoter methylation was associated with tumor size and combined *MT1M* and *MT1G* promoter methylation in serum was easily detected in HCC patients with vascular invasion or metastasis, suggesting that it may be a useful prognostic marker as well.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common fatal tumors worldwide. Currently available screening tools for the diagnosis of HCC mainly depend on serum α -fetoprotein and ultrasound. However, the sensitivities and specificities of the two tools remain controversial.

Research frontiers

Great efforts have been devoted to searching for new biomarkers for early diagnosis of HCC. In the present study, we demonstrated that *MT1M* and *MT1G* promoter methylation might be noninvasive biomarkers for diagnosis of HCC.

Innovations and breakthroughs

The authors demonstrated aberrant methylation of serum *MT1M* and *MT1G* promoters in HCC and reported the potential value of the two gene promoters as biomarkers for noninvasive and early diagnosis of HCC.

Applications

Serum *MT1M* and *MT1G* gene promoter methylation might be applied in the early diagnosis of HCC as novel and noninvasive biomarkers.

Terminology

MT1M and *MT1G* are two major isoforms in the metallothionein superfamily, and are low-molecular-weight, cysteine-rich intracellular proteins. DNA methylation is an epigenetic event to alter gene expression and function, which refers to the covalent addition of a methyl group without changing the order of bases. A biomarker is a substance used as an indicator of a biological state.

Peer review

This was a diagnostic trial. *MT1M* and *MT1G* promoter methylation is reported as serum biomarkers for HCC, which might be interesting for clinical practice.

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Roles of sphincter of Oddi motility and serum vasoactive intestinal peptide, gastrin and cholecystokinin octapeptide

Zhen-Hai Zhang, Cheng-Kun Qin, Shuo-Dong Wu, Jian Xu, Xian-Ping Cui, Zhi-Yi Wang, Guo-Zhe Xian

Zhen-Hai Zhang, Cheng-Kun Qin, Jian Xu, Xian-Ping Cui, Zhi-Yi Wang, Guo-Zhe Xian, Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong Province, China

Shuo-Dong Wu, Departments of Minimally Invasive Surgery and the Second General Surgery, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China

Author contributions: Zhang ZH, Wu SD and Qin CK designed the study; Zhang ZH and Qin CK wrote and revised the manuscript; Xu J, Cui XP, Zhang ZH, Wang ZY and Xian GZ performed the research, and analyzed and interpreted the data; Zhang ZH acquired funding; Qin CK revised the manuscript critically for important intellectual content, and supervised the study; and all authors have read and approved the final version to be published.

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Correspondence to: Cheng-Kun Qin, PhD, Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jingwu Road, Jinan 250021, Shandong Province, China. zhangzhenhai410@126.com

Telephone: +86-531-68776363 Fax: +86-531-68776363

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(CCK-8) were detected at each stage in the process of pigment gallbladder stone formation by enzyme-linked immunosorbent assay.

RESULTS: The incidence of pigment gallstone formation was 0%, 0%, 16.7% and 66.7% in the 3-, 6-, 9- and 12-wk group, respectively. The frequency of myoelectric activity decreased in the 3-wk group. The amplitude of myoelectric activity had a tendency to decrease but not significantly. The frequency of the SO decreased significantly in the 9-wk group. The SO basal pressure and common bile duct pressure increased in the 12-wk group (25.19 ± 7.77 mmHg vs 40.56 ± 11.81 mmHg, 22.35 ± 7.60 mmHg vs 38.51 ± 11.57 mmHg, $P < 0.05$). Serum VIP was significantly elevated in the 6- and 12-wk groups and serum CCK-8 was decreased significantly in the 12-wk group.

CONCLUSION: Pigment gallstone-causing diet may induce SO dysfunction. The tension of the SO increased. The disturbance in SO motility may play a role in pigment gallstone formation, and changes in serum VIP and CCK-8 may be important causes of SO dysfunction.

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Abstract

AIM: To investigate roles of sphincter of Oddi (SO) motility played in pigment gallbladder stone formation in model of guinea pigs.

METHODS: Thirty-four adult male Hartley guinea pigs were divided randomly into two groups: the control group and pigment stone group. The pigment stone group was divided into 4 subgroups with 6 guinea pigs each according to time of sacrifice, and were fed a pigment lithogenic diet and sacrificed after 3, 6, 9 and 12 wk. SO manometry and recording of myoelectric activity of the guinea pigs were obtained by multifunctional physiograph at each stage. Serum vasoactive intestinal peptide (VIP), gastrin and cholecystokinin octapeptide

Key words: Pigment gallstone; Sphincter of Oddi; Manometry; Myoelectric activity; Guinea pig; Vasoactive intestinal peptide; Gastrin; Cholecystokinin octapeptide

Core tip: Biliary stasis is thought to be important in the development of pigment gallstones. Sphincter of Oddi (SO) motility may play an important role in the process of pigment gallstone formation. We used a guinea pig model of pigment gallstones to investigate whether SO dysfunction happens and what a role the sphincter plays in the process of pigment gallstone formation. The myoelectric activity and SO manometry were measured at different stages of stone formation. Pigment gallstone-causing diet may induce SO dysfunction. The

disturbance in SO motility may play a role in pigment gallstone formation, and changes in serum vasoactive intestinal peptide and cholecystokinin octapeptide may be important causes of SO dysfunction.

Zhang ZH, Qin CK, Wu SD, Xu J, Cui XP, Wang ZY, Xian GZ. Roles of sphincter of Oddi motility and serum vasoactive intestinal peptide, gastrin and cholecystokinin octapeptide. *World J Gastroenterol* 2014; 20(16): 4730-4736 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4730.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4730>

INTRODUCTION

In America, cholesterol gallstones or cholesterol mixed with calcium bilirubinate account for 80% of gallstones, whereas the remaining 20% are pigment or calcium bilirubinate stones^[1]. In Asia, pigment gallstones are common. Their treatment, especially for intrahepatic stones, is a challenge in biliary surgery^[2,3]. In Japan, cholesterol stones account for 58.3%, black gallstones for 23.7%, and pigment stones for 15.9% of gallstones^[4]. In China, pigment bile duct stones are found in about 10% of gallstone patients^[5].

However, the etiology and pathogenesis of pigment gallstones have not been fully clarified. Etiologically, many factors, including age, bacterial infection, malnutrition, biliary stasis, impaired intestinal barrier function, and congenital duodenal diverticulum, may be involved, but none of these has been proved unequivocally. Among these factors, biliary stasis is thought to be important. The sphincter of Oddi (SO) is the only gate through which bile is discharged into the duodenum. SO motility may play an important role in the process of pigment gallstone formation. To the best of our knowledge, no study has investigated the relationship between SO motility and pigment gallstone formation. Our study first investigated the myoelectric activity and SO manometry simultaneously in the process of pigment gallstone formation.

The aim of this study was to investigate the role of SO motility in pigment gallbladder stone formation in a guinea pig model. The myoelectric activity and SO manometry were measured at different stages of stone formation. As SO motility is controlled by neurological and hormonal factors, we detected the changes in serum vasoactive intestinal peptide (VIP), gastrin and cholecystokinin octapeptide (CCK-8) in the process of pigment gallstone formation.

MATERIALS AND METHODS

Laboratory animals and grouping

Thirty-four adult male Hartley guinea pigs weighing between 230 and 270 g were provided by the Huishan Jiangnan Laboratory Animal Company (license SCXK

SU:2009-0005). The guinea pigs were divided randomly into two groups (control group and pigment stone group) after 7 d with standard diet. The control group ($n = 10$) was fed with standard diet. The pigment stone group was divided into 4 subgroups with 6 guinea pigs each according to time of sacrifice, and was fed with a pigment lithogenic diet^[6] and was sacrificed after 3, 6, 9 and 12 wk.

Experimental method

Preparation of pigment gallstone-causing diet: The pigment gallstone diet comprised: corn flour 136.3 g/kg, soy bean flour 90.9 g/kg, flour 90.7 g/kg, fishmeal 63.6 g/kg, whole wheat 90.9 g/kg, salt 10 g/kg, yeast powder 10 g/kg, alfalfa meal 416.5 g/kg, lard oil 20 g/kg, sucrose 20 g/kg, cellulose 20 g/kg, cholesterol 1 g/kg, cholic acid 0.4 g/kg, vitamin C 0.05 g/kg, and casein 20 g/kg (purchased from Trophic Animal Feed High-Tech Co. Ltd., Nantong, China). A greenish yellow colored, granular calculus could be seen against the contrasting clear background in the gallbladders of the guinea pigs. The calculi were tested by infrared (IR) spectrometry to verify the sample as pigment gallstones. SO manometry and myoelectric activity of the guinea pigs were determined by multifunctional physiograph at 3, 6, 9 and 12 wk.

Measurement of myoelectric activity of SO

Preparation of two polar hook metal electrode: Two plexi-glasses were cut into strips (length, 5 cm; diameter, 1 cm; thickness, 0.5 cm). Two parallel cuts along the longitudinal axes were scored at 0.2-cm intervals. Then two acupuncture needles which were treated with insulating varnish were put into the gap and the plexi-glass was conglutinated by adhesives. The anterior extremity of the acupuncture needle (1 cm long) was exposed and the insulated paint at 0.5 cm of the apical part was scraped off to expose the metal of the needle. A microelectrode was formed by bending the needle point (0.2 cm long) about 120°. The handle of the needle in the posterior extremity was exposed by about 1 cm to connect with the leads. At the midpiece of the plexi-glasses, a cut along the transverse axes was scored to fix the needle by a silk thread.

Measurement of myoelectric activity

The guinea pig was anesthetized by injecting pentobarbital sodium (45 mg/kg) into the peritoneal cavity. The guinea pig was fixed in the supine position, and the skin of the superior abdomen was prepared and sterilized. A longitudinal incision was made and the papilla (entrance of the common bile duct into the duodenum) was determined. Two polar hook metal electrodes were inserted at 0.2 mm into the subserosa of the SO by megaloscope ($\times 10$ magnification). Care was taken not to insert too deep, to avoid penetrating the sphincter. The interelectrode distance was approximately 2 mm and the electrodes were hung by two silk threads to the experimental shelf to maintain the necessary tension. The experimental shelf was adjusted to maintain the necessary tension and correct angle. The output of the two signals were connected

with the two polar of the physiological recorder (BL-420 F; Chengdu Taimeng Software, China) and a syringe needle was inserted into the legs of the guinea pigs to connect with the earth pole of the recorder. The myoelectric signal was collected by the electrode and imported into the computer after handling by the physiological recorder, and it was stored after processing with the software system that was specialized in electromyographic signals. The setup parameters were as follows: scanning speed, 500 ms/div; sensitivity, 200 μ V; time parameter, 1 s; and frequency filtering, 10 Hz. The myoelectric figure was dealt with digital filtering of 10-30 Hz at last.

SO manometry

Preparation of manometry catheter: A pedo bi-lumen central venous catheter (4 F and 30 cm long) was chosen. The catheter was cut 0.2 cm apart from the distal end of the water out lateral aperture, and the head end was made blunt and round for inserting easily into the SO. Another lateral aperture (0.3 mm diameter) was made inferior to the water out lateral aperture. Manometry lumen (b) and water effusion lumen (a1) were marked and the SO manometry catheter was completed.

SO manometry: The a1 catheter lumen was infused with sterile water at a flow rate of 15 mL/h by a minimally compliant hydraulic capillary infusion system and connected to pressure transducers. A physiological recorder and relevant manometry program were used to record and analyze the tracings. Calibrating by air compression method and zero setting, 0 and 200 mmHg were taken for baseline calibration. The manometry catheter was inserted into the common bile duct from the duodenum and fixed by silk thread. The gallbladder duct also was ligated by silk thread. The catheter was withdrawn to the SO, and manometry was carried out. The frequency of SO phasic contraction, SO basal pressure, SO amplitude and common bile duct pressure were measured and recorded.

Detection of serum VIP, gastrin and CCK-8

Four milliliters of venous blood was obtained from the guinea pigs in the early morning before they were sacrificed and placed in a test tube. The blood was centrifuged at 1500 r/min for 15 min, and serum was isolated, placed in Eppendorf tubes, and stored at -70 °C. Serum VIP, gastrin and CCK-8 were measured by enzyme-linked immunosorbent assay (ELISA). The ELISA testing kit of guinea pigs was supplied by USCN Life Science Inc., Houston, TX, United States (VIP kit: E90380Gu, lot number: 20130123; GT kit E91224Gu, lot number: 20130221; CCK-8 kit: E91044Gu, lot number: 20130123).

Statistical analysis

Statistical analysis was carried out using Student's *t* test. Data were analyzed with SPSS version 11.5. The results were expressed as mean \pm SD. A two-tailed *P* value < 0.05

Table 1 Changes in sphincter of Oddi myoelectric activity in process of gallstone formation

Groups	Amplitude	Frequency
Control group	146.44 \pm 81.09	15.86 \pm 4.35
3-wk group	125.06 \pm 59.76	10.38 \pm 2.02 ^a
6-wk group	112.02 \pm 64.69	12.90 \pm 4.39
9-wk group	77.81 \pm 27.17	15.10 \pm 4.13
12-wk group	71.72 \pm 35.10	13.70 \pm 4.15

^a*P* < 0.05 vs the control group.

was considered statistically significant.

RESULTS

Condition of gallstone formation

In the guinea pigs in the control group, no pigment gallstones were found. The gallstone-formation rate in the pigment gallstone group was 0%, 0%, 16.7% and 66.7% in the 3-, 6-, 9- and 12-wk groups, respectively.

SO myoelectric activity

Compared with the control group, the frequency of myoelectric activity decreased significantly in the 3-wk group (*P* < 0.05). The amplitude of myoelectric activity had a tendency to decrease but not significantly (Table 1, Figure 1A and B).

SO manometry analysis

Compared with the control group, the frequency of the SO decreased significantly in the 9-wk group (*P* < 0.05). The SO basal pressure and common bile duct pressure increased significantly in the 12-wk group (*P* < 0.05) (Table 2, Figure 1C and D).

Changes in serum VIP, gastrin and CCK-8

Compared with the control group, serum VIP was significantly elevated in the 6- and 12-wk groups (*P* < 0.05) (Table 3). Serum gastrin was significantly decreased in the 3-wk group (*P* < 0.001) (Table 3). Serum CCK-8 was significantly decreased in the 12-wk group (*P* < 0.05) (Table 3).

DISCUSSION

Pigment gallstones are classified descriptively as black stones, which are hard, and brown stones, which are soft^[7]. Black stones often contain crystalline salts of calcium phosphate and/or calcium carbonate in one of its polymorphic forms, calcite, aragonite or valerite, and may also contain many metals found in bile^[8]. They form in sterile gallbladder bile, and the principal risk factor is hyperbilirubinemia. Other causes of black stones are gallbladder hypomotility secondary to diabetes mellitus^[9], total parenteral nutrition^[10], and truncal vagotomy^[11]. Parietal (gallbladder mucosa) factors may play a role in pigment stone formation^[10]. We investigated many aspects

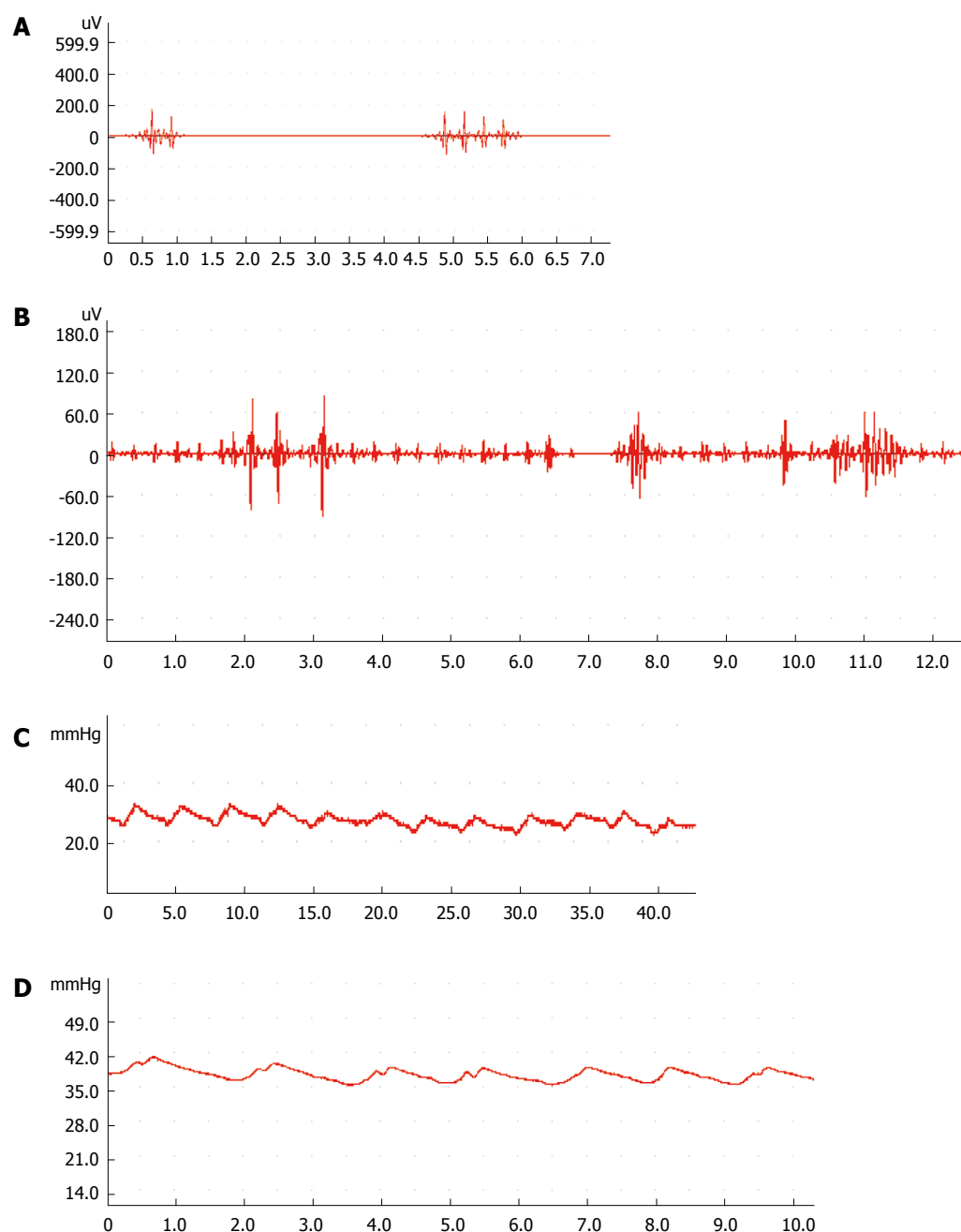


Figure 1 Myoelectric activity and sphincter of Oddi manometry. A: Myoelectric activity of the control group; B: Myoelectric activity of the 12-wk group; C: Sphincter of Oddi (SO) manometry of the control group; D: SO manometry of the 12-wk group.

relating to pigment gallstone formation and found that intestinal barrier function was correlated with pigment gallstone formation^[6,11,12]. Endotoxemia and increased biliary β -glucuronidase may play important roles^[13]. The structure and function of the SO are correlated significantly with bile duct pigment stones. Anatomical abnormalities and dysfunction of the SO play important roles in the formation of bile duct pigment stones^[14].

Gallbladder stasis resulting from mechanical obstruction is an important antecedent in the development of pigment gallstones and sludge. Patients with cholesterol gallstones have impaired gallbladder emptying^[15] and show dyspeptic symptoms with functional defects of

both upper and lower gastrointestinal tracts^[16,17]. Gallbladder emptying is also defective in patients with black pigment stones and such a defect is less severe than in patients with cholesterol stones^[15]. Pigment stones are common in patients with cirrhosis^[18], chronic hemolysis^[19], ileal Crohn's disease^[20], and conditions associated with impaired gallbladder kinetics^[19,21,22].

However, as the gate that regulates the flow of bile and pancreatic juice into the duodenum, and prevents the reflux of duodenal contents into the biliary and pancreatic duct, the role of SO motility in pigment gallstone formation has not been elucidated. Whether the SO dysfunction is present and what role it plays in pigment

Table 2 Sphincter of Oddi manometry in process of gallstone formation

Group	SO basal pressure (mmHg)	Common bile duct pressure (mmHg)	Amplitude of SO	Frequency of SO
Control group	25.19 ± 7.77	22.35 ± 7.60	8.52 ± 2.27	11.57 ± 2.94
3-wk group	29.72 ± 5.59	25.78 ± 5.30	11.83 ± 3.32	8.17 ± 3.54
6-wk group	27.07 ± 11.11	24.29 ± 10.79	8.35 ± 2.82	10.50 ± 5.20
9-wk group	33.09 ± 13.65	29.19 ± 13.84	11.71 ± 2.37	7.50 ± 1.73 ^a
12-wk group	40.56 ± 11.81 ^a	38.51 ± 11.57 ^a	6.15 ± 2.97	8.20 ± 2.59

^a*P* < 0.05 *vs* the control group. SO: Sphincter of Oddi.**Table 3** Changes in process of pigment gallstone formation

Group	3-wk	6-wk	9-wk	12-wk
VIP				
Control (pg/mL)	5.96 ± 2.97	4.37 ± 1.00	7.70 ± 2.08	8.68 ± 0.65
Pigment gallstone (pg/mL)	10.35 ± 2.59	14.70 ± 3.41 ^a	17.02 ± 6.26	25.30 ± 6.56 ^a
Gastrin				
Control (pg/mL)	17.83 ± 2.35	18.56 ± 5.77	17.42 ± 6.39	19.41 ± 4.58
Pigment gallstone (pg/mL)	2.44 ± 0.78 ^b	8.51 ± 0.33	36.51 ± 12.83	23.01 ± 4.76
CCK-8				
Control (pg/mL)	3482.63 ± 154.25	3650.73 ± 55.08	3606.74 ± 129.42	3599.28 ± 120.15
Pigment gallstone (pg/mL)	3524.50 ± 79.29	3597.64 ± 86.55	3498.01 ± 107.14	3436.09 ± 82.96 ^a

^a*P* < 0.05, ^b*P* < 0.01 *vs* the control group. VIP: Vasoactive intestinal peptide; CCK-8: Cholecystokinin octapeptide.

gallstone formation require further study. SO function may play an important role in cholesterol gallstone formation [23,24].

We used a guinea pig model of pigment gallstones to investigate whether SO dysfunction happens and what role the sphincter plays in the process of pigment gallstone formation. SO manometry and myoelectric activity were investigated at the same time. A MEDLINE search found that there were no studies of SO manometry and recording of myoelectric activity simultaneously in the process of gallstone formation.

We found that gallstones did not occur until 9 wk, when the incidence was 16.7%, and after 12 wk the incidence was 66.7%. The frequency of myoelectric activity decreased significantly in the 3-wk group. The amplitude of myoelectric activity tended to decrease but not significantly. As the most important indicators, SO basal pressure and common bile duct pressure increased gradually in the 12-wk group. We observed in the process of gallstone formation that SO tension increased and gallbladder stasis occurred. Disturbance of SO motor function impedes the flow of bile into the duodenum and may play an important role in gallstone formation.

The mechanism by which a cholesterol gallstone-causing diet induces SO dysfunction has not been fully elucidated. SO motility is controlled by numerous neurotransmitters and gastrointestinal hormones and their interactions [25].

VIP, an alkaline intestinal peptide composed of 28 amino acids, belongs to the secretin family. VIP relaxes gallbladder smooth muscle, decreases gallbladder pressure, and inhibits contractions induced by CCK [26,27]. VIP is thought to work as a neurotransmitter of the vagus nerve terminals [28,29]. Previously, we found that VIP2-R

mRNA expression level in controls was lower than in patients with gallbladder polyps or gallstones [30]. The study of the relationship between VIP and the SO showed that the myenteric plexuses of the sphincter and duodenum are in direct continuity with many interconnecting nerve trunks, some of which show nitric oxide synthase activity and VIP immunoreactivity [31]. VIP increases the phasic activity of the sphincter [32]. In the present study, serum VIP level in the pigment gallstone group was higher than that of the control group. We suggest that the role of VIP in the formation of pigment gallstones may be as follows. First, VIP decreases the resting pressure in the gallbladder, causing its dilation and bile stasis. Second, it inhibits the contraction induced by CCK. Third, it regulates secretion of mucoproteins of the gallbladder mucosa and alters the bile components. Fourth, increased plasma VIP results in SO contraction, thus preventing bile flow from entering the duodenum. We noted that the frequency of SO decreased significantly in the 9-wk group and the SO basal pressure and common bile duct pressure increased significantly in the 12-wk group. Elevation of VIP may play an important role in mechanism of SO dysfunction.

There have been few studies about the effect of gastrin on the SO [33,34]. A few reports show that gastrin can increase the sphincter of Oddi basal pressure amplitude. Chen suggested that patients with post-cholecystectomy pain had SO dysfunction with characteristics of high tension, which was related to elevation of serum gastrin [35]. In our study, we found that serum gastrin decreased significantly in the 3-wk group. This may have resulted from changes in diet and did not necessarily have any relationship with the formation of pigment gallstones.

As the most important neural or hormonal factors which control the motility of biliary tract, CCK may play

an important role in gallstone formation. Many studies have evaluated CCK-8 and its effect on the SO. Zhang *et al.*^[24] found that gallbladder cholestasis was observed during early stages of gallstone formation in Ch rabbits. Cholesterol gallstone model rabbits CCK-8 could not improve gallbladder cholestasis in the Ch group. Another study found that cyclic myoelectric activity of the SO at phases 2 and 3 of the migrating motor complex, and the excitatory response to CCK-8 were dramatically decreased in animals with chronic cholangitis^[36]. We found that the levels of serum CCK-8 were significantly decreased. As a hormone that stimulates SO motility, decreasing its level may play a role in sphincter dysfunction and gallstone formation.

In conclusion, our study found that a pigment gallstone-causing diet may induce SO dysfunction. The tension of the SO increased. Disturbance of SO motility may play a role in gallstone formation. The mechanism by which a cholesterol gallstone-causing diet induces SO dysfunction has not been fully elucidated. The disturbance of serum VIP and CCK-8 may be important causes of SO dysfunction. Control of diet and regulation of SO motility are important in the prevention of pigment gallstone formation.

COMMENTS

Background

Biliary stasis is thought to be important in the development of pigment gallstones. Sphincter of Oddi (SO) motility may play an important role in the process of pigment gallstone formation. This study investigated the role of SO motility in pigment gallbladder stone formation in a guinea pig model. The myoelectric activity and SO manometry were measured at different stages of stone formation. The changes in serum vasoactive intestinal peptide (VIP), gastrin and cholecystokinin octapeptide (CCK-8) were detected in the process of pigment gallstone formation. There were no prior studies of SO manometry and recording of myoelectric activity simultaneously in the process of gallstone formation.

Research frontiers

SO manometry and recording of myoelectric activity are the two most important methods in study of SO motility. SO manometry (SOM) is recognized as the standard diagnostic modality for sphincter of Oddi dysfunction (SOD). In this study, the authors clarified the impact of SO motility on the formation of pigment gallstone formation.

Innovations and breakthroughs

This study showed that SO motility played an important role in gallstone formation. This result is similar to those of previous reports. This is the first study to investigate SO manometry and recording of myoelectric activity simultaneously. SO motility is controlled by numerous neurotransmitters and gastrointestinal hormones and their interactions. Serum VIP, gastrin and CCK8 were detected at each stage in the process of pigment gallbladder stone formation by enzyme-linked immunosorbent assay. The disturbance of serum VIP and CCK8 may be important causes of SO dysfunction.

Applications

The study results suggest that disturbance of SO motility may play a role in gallstone formation. The disturbance of serum VIP and CCK8 may be important causes of SO dysfunction. Control of diet and regulation of SO motility are important in the prevention of pigment gallstone formation.

Terminology

SO dysfunction is a painful syndrome that presents as recurrent episodes of right upper quadrant biliary pain, or recurrent idiopathic pancreatitis. SOM is the only available method to measure SO motor activity directly. Additionally, it is the only modality for diagnosis of suspected SOD which has been demonstrated to be reproducible and predictive of positive therapeutic outcome results.

Peer review

The study investigated the roles of sphincter of Oddi motility played in pigment gallbladder stone formation in model of guinea pigs. The myoelectric activity and pressure of SO were measured at different stages of stone formation. The result shows that disturbance of SO motility may play a role in gallstone formation. The disturbance of serum VIP and CCK8 may be important causes of SO dysfunction. Control of diet and regulation of SO motility are important in the prevention of pigment gallstone formation.

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Association between *NOD2/CARD15* gene polymorphisms and Crohn's disease in Chinese Zhuang patients

Wei-Yan Long, Lan Chen, Cui-Liang Zhang, Rong-Mao Nong, Mei-Jiao Lin, Ling-Ling Zhan, Xiao-Ping Lv

Wei-Yan Long, Lan Chen, Cui-Liang Zhang, Rong-Mao Nong, Mei-Jiao Lin, Xiao-Ping Lv, Department of Gastroenterology, the First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Ling-Ling Zhan, Department of Clinical Experimental Medicine, the First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Author contributions: Long WY performed the experiments and wrote the manuscript; Zhang CL, Nong RM and Lin MJ collected samples; Chen L and Zhan LL analyzed the data and revised the manuscript; Lv XP designed the research and revised the paper.

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Correspondence to: Xiao-Ping Lv, Professor, Department of Gastroenterology, the First Affiliated Hospital of Guangxi Medical University, No. 6 Shuangyong Road, Nanning 530021, Guangxi Zhuang Autonomous Region, China. lxp58@hotmail.com

Telephone: +86-771-3277211 Fax: +86-771-3277285

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form method. The P268S, JW1 and N852S polymorphisms were amplified using polymerase chain reaction (PCR), detected by restriction fragment length polymorphism (RFLP), and verified by gene sequencing.

RESULTS: Heterozygous mutation of P268S in the *NOD2/CARD15* gene was detected in 10 CD cases (six Zhuang and four Han), two Han UC cases, and one Zhuang healthy control, and P268S was strongly associated with the Chinese Zhuang and Han CD populations ($P = 0.016$ and 0.022 , respectively). No homozygous mutant P268S was detected in any of the groups. No significant difference was found in P268S genotype and allele frequencies between UC and control groups ($P > 0.05$). Patients with CD who carried P268S were likely to be ≤ 40 years of age ($P = 0.040$), but were not significantly different with regard to race, lesion site, complications, and other clinical features ($P > 0.05$). Neither JW1 nor N852S polymorphisms of the *NOD2/CARD15* gene were found in any of the subjects ($P > 0.05$).

CONCLUSION: P268S polymorphism may be associated with CD susceptibility in the Zhuang population in the Guangxi Zhuang Autonomous Region, China. In contrast, JW1 and N852S polymorphisms may not be related to CD susceptibility in these patients.

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Key words: Crohn's disease; *NOD2/CARD15*; Single nucleotide polymorphisms

Core tip: In this study, P268S, JW1 and N852S polymorphisms of the *NOD2/CARD15* gene were genotyped using the PCR-RFLP method and gene sequencing, and the presence of P268S in Guangxi Zhuang Crohn's disease patients was identified. However, no JW1 or N852S mutants were found in this cohort.

Abstract

AIM: To assess the relationship between the P268S, JW1 and N852S polymorphisms and Crohn's disease (CD) susceptibility in Zhuang patients in Guangxi, China.

METHODS: Intestinal tissues from 102 Zhuang [48 CD and 54 ulcerative colitis (UC)] and 100 Han (50 CD and 50 UC) unrelated patients with inflammatory bowel disease and 72 Zhuang and 78 Han unrelated healthy individuals were collected in the Guangxi Zhuang Autonomous Region from January 2009 to March 2013. Genomic DNA was extracted using the phenol chloro-

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic recurrent inflammatory disease of the gastrointestinal tract and includes ulcerative colitis (UC) and Crohn's disease (CD). In recent years, the incidence of IBD has increased in Western populations with an East-West gradient existing in Europe and is progressively increasing in Asia^[1-3]. A recent systematic review revealed that the highest annual incidence of CD was 12.7 per 100000 person-years in Europe, 20.2 per 100000 person-years in North America, and 5.0 per 100000 person-years in Asia and the Middle East^[4]. The etiology and pathogenesis of IBD are not completely clear, which involve a complex interaction of factors such as genetics, immunology, environment, and infection^[5,6]. Several pathways may be crucial for intestinal homeostasis in IBD, for example barrier function, epithelial restitution, microbial defense, innate immune regulation, adaptive immunity regulation, reactive oxygen species (ROS) generation, and autophagy^[7].

Genetic susceptibility to CD shows significant ethnic differences. A recent meta-analysis of multiple genome-wide association studies confirmed that 71 CD susceptibility loci were detected in a European population^[8]. However, the majority of these genes could not be verified in the Asian region^[9-11]. *NOD2/CARD15* was the first verified predisposing gene for CD. Multiple single nucleotide polymorphisms (SNPs) of *NOD2/CARD15* were shown to be significantly associated with CD in Caucasian populations^[12-14]. Our previous studies confirmed that the R702W, G908R, and L1007fs SNPs of the *NOD2* gene were not associated with CD and UC in a Chinese Zhuang population from Guangxi Zhuang Autonomous Region, China^[15]. In recent years, some gene mutation sites of *NOD2/CARD15* such as P268S, JW1, N852S, D113N, D357A, I363F, and L550V were shown to confer CD susceptibility^[16-18]. The P268S SNP of the *NOD2/CARD15* gene was also associated with Chinese Han CD susceptibility and its clinical features^[19]. The JW1 SNP of the *NOD2/CARD15* gene was shown to be associated with CD in Chinese Han, Malay, and Indians in Malaysia^[17]. The N852S SNP of the *NOD2/CARD15* gene was found to be significantly associated with CD in Ashkenazi Jewish populations^[18]. However, there are no data on the correlation between the P268S, JW1, and N852S SNPs of the *NOD2/CARD15* gene and the Chinese Zhuang CD population in the Guangxi Zhuang Autonomous Region.

In view of the differences in data regarding the correlation between key regulatory genes and IBD susceptibility, the purpose of the present study was to investigate

whether the known gene SNPs (P268S, JW1 and N852S) of the *NOD2/CARD15* gene determine susceptibility to CD in the Guangxi Zhuang population from the Guangxi Zhuang Autonomous Region, China. Guangxi has a large Zhuang population in which genetic diseases and genetic SNPs are unique. Therefore, research on the correlation between the P268S, JW1, N852S SNPs of the *NOD2/CARD15* gene and CD in Chinese Zhuang patients from the Guangxi Zhuang Autonomous Region is needed.

MATERIALS AND METHODS

Specimen collection

Intestinal tissues from 102 Zhuang (48 CD and 54 UC) and 100 Han (50 CD and 50 UC) unrelated patients with IBD were collected at the Gastroenterology Department, First Affiliated Hospital of Guangxi Medical University, from January 2009 to March 2013. The control group included 72 Zhuang and 78 Han unrelated healthy individuals who did not have liver or gastrointestinal diseases. All patients had a well-established diagnosis of UC or CD based on the modified criteria framed by the World Gastroenterology Organization in 2010^[20]. This study was approved by the hospital ethics committee and all the patients or their families provided written informed consent.

DNA extraction

Intestinal mucosa samples were digested using 450 μ L of TES buffer (pH = 8.0) which consisted of Tris-HCl, ethylene diamine tetraacetic acid, and sodium chloride, 50 μ L sodium dodecyl sulfate (10%), and 5 μ L proteinase K (20 g/L) in a 56 °C water bath for 4-6 h. The supernatant was successively extracted by centrifugation at 12000 r/min for 10 min at 4 °C following the sequential addition of equal volumes of phenol, chloroform, and isoamyl alcohol (25:24:1), chloroform, and isoamyl alcohol (24:1). A white floc was precipitated from the final supernatant after the addition of 2.5 volumes of absolute ethanol and repeated aspiration. DNA was extracted from the white floc by centrifugation at 12000 r/min for 5 min at 4 °C after the addition of 75% ethanol. The DNA was dissolved by the addition of 50-120 μ L of TE and stored at -20 °C.

Genotyping of P268S, JW1 and N852S

The primer sequences were as published elsewhere^[18] and were synthesized by SHENGGONG Biotechnology Co., Ltd., Shanghai, China. PCR reaction mixture contained 2 μ L DNA template, 1 μ L each of forward and reverse primers (10 μ mol/L), 6 μ L H₂O, and 10 μ L of 2 \times PCR Master Mix (TIANGEN Biotechnology Co., Ltd., Beijing, China). Reaction conditions consisted of an initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at different temperatures (Table 1) for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. All of the PCR

Table 1 Polymerase chain reaction primers, annealing temperatures and polymerase chain reaction fragment sizes

Mutation	Primer	Annealing temperature (°C)	PCR fragment size (bp)
P268S	F-TGCCTCTTCTTCTGCCTTCC R-AGTAGAGTCCGCACAGAGAG	60	422
JW1	F-TGCAGTTTCTTGGGGAGAT R-TGTACCTGATCCAGCCCAAT	59	220
N852S	F-CTGTTTGCATGATGGGGG R-CAGCCGTCAGTCAATTTGTAG	55	151

PCR: Polymerase chain reaction.

Table 2 Enzymes and gene polymorphism analysis

Mutation	Base change	Enzyme	Restriction fragment size (bp)	
			Wild-type	Mutant
P268S	C→T	<i>Bam</i> HI	422	Heterozygote 422 + 247 + 175 Homozygote 247 + 175
JW1	C→T	<i>Xho</i> I	125 + 95	Heterozygote 220 + 125 + 95 Homozygote 220
N852S	A→G	<i>Alu</i> I	151	Heterozygote 151 + 129 + 22 Homozygote 129 + 22

products were electrophoresed on a 1.5% agarose gel with 1 × Tris-borate-EDTA buffer at 100 V for 30 min and then observed under ultraviolet illumination (Bio-Rad Gel Doc-2000, Hercules, CA, United States).

The PCR products of P268S, JW1, and N852S SNPs of the *NOD2/CARD15* gene were digested at 37 °C for 11 h with *Bam*HI, *Xho*I, and *Alu*I restriction enzymes, respectively (Fermentas, Pittsburgh PA, United States). The digestion reaction contained 5 µL of the PCR product, 2 µL of 10 × buffer, 1 µL of restriction enzyme, and 9 µL of H₂O in a total of 17 µL. Following enzymatic digestion, the fragments were separated and visualized using gel electrophoresis (Yito Bio-Instrument Company Ltd., Shanghai, China) (Table 2).

The DNA mutative samples which were found by PCR-RFLP were reamplified. The products of each SNP were purified using a PCR purification kit (QIAGEN, Hilden, Germany) and sequenced using ABI 3730XL sequencer (Applied Biosystems, Foster, United States).

Statistical analysis

SPSS version 16.0 software was used for the statistical analysis, while comparisons of genotype and allelic frequencies among the different groups were performed using Fisher's exact test. The Hardy-Weinberg equilibrium test was used to test the distributions of each mutation genotype frequency. Values of *P* < 0.05 were considered statistically significant.

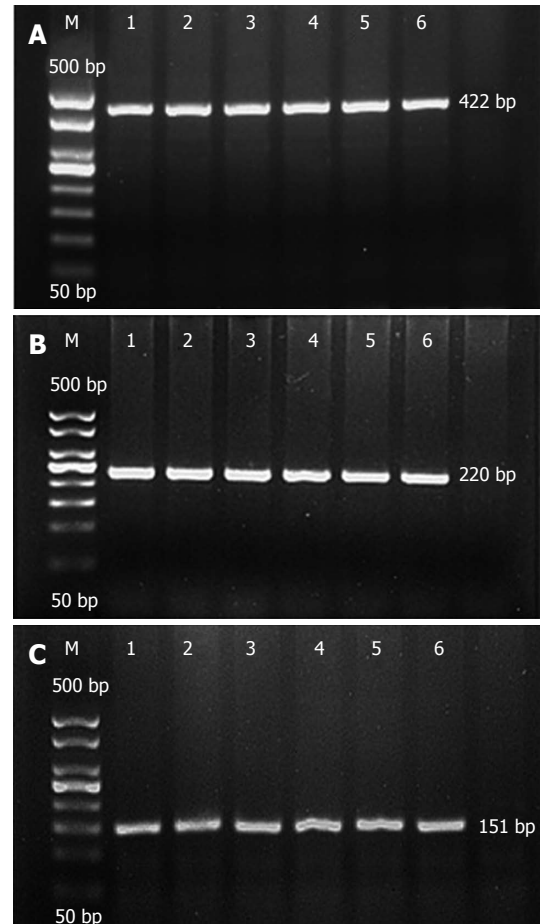


Figure 1 Electrophoresis of P268S, JW1, and N852S PCR products. A: P268S; B: JW1; C: N852S. M: Marker; 1, 2: Ulcerative colitis (UC), Crohn's disease (CD) of Han; 3, 4: UC, CD of Zhuang; 5, 6: healthy controls.

RESULTS

PCR

All three SNPs of the *NOD2/CARD15* gene were amplified by PCR, and the PCR products were then used for both RFLP analysis and gene sequencing. The target fragment sizes of the P268S, JW1, and N852S mutations were 422 bp (Figure 1A), 220 bp (Figure 1B), and 151 bp (Figure 1C), respectively.

PCR-RFLP

The PCR products of the P268S, JW1, and N852S mutations were digested using the *Bam*HI, *Xho*I, and *Alu*I enzymes, respectively. For P268S, a wild-type band of 422 bp was found in the majority of controls, CD patients, and UC patients, while heterozygous mutant bands of 422 bp, 247 bp, and 175 bp were found in six Zhuang CD cases, four Han CD cases, two Han UC cases, and one Zhuang healthy control, however, no homozygous mutants were detected (Figure 2A). For JW1, only wild-type bands of 125 bp and 95 bp were observed in all subjects (Figure 2B). Similarly, just one band of 151 bp was found in wild-type N852S in all subjects (Figure 2C), and no other mutant bands of JW1 or N852S were detected using PCR-RFLP fragment electrophoresis.

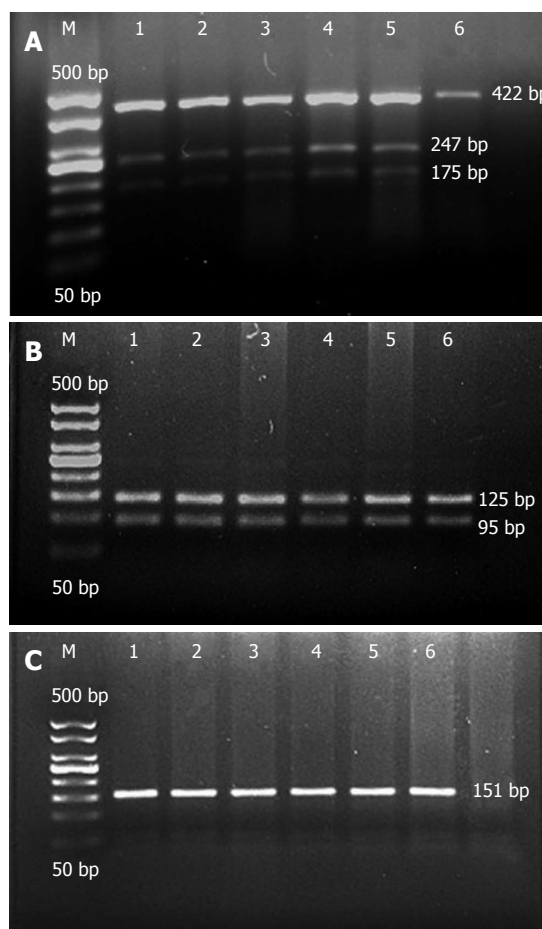


Figure 2 Electrophoresis of P268S, JW1, and N852S digestion products. A: M: Marker; 1-5: Heterozygote of P268S, 6: Wild-type of P268S. B: M: Marker; 1-6: Wild-type of JW1. C: M: Marker; 1-6: Wild-type of N852S.

DNA sequencing

The gene sequencing results of the P268S, JW1 and N852S variants were consistent with those found on PCR-RFLP. For both mutant P268S and JW1, it is a C to T substitution mutation, and for mutant N852S, it is an A to G substitution mutation. In our study, heterozygous (C/T) (Figure 3A) and wild-type (C/C) (Figure 3B) P268S were detected in controls, CD patients, and UC patients, but no homozygous P268S (T/T) was detected. However, only wild-type JW1 (C/C) (Figure 3C) and wild-type N852S (A/A) (Figure 3D) were observed, and no other types (C/T, T/T, A/G, G/G).

Distribution of genotype and allelic frequencies

The distributions of P268S, JW1, and N852S genotypes were in accordance with the Hardy-Weinberg equilibrium test results ($P > 0.05$). In our cohort, only the P268S heterozygous mutation was found in six (12.5%) of 48 Zhuang CD cases, four (8.0%) of 50 Han CD cases, 0 (0.0%) of 54 Zhuang UC cases, two (4.0%) of 50 Han UC cases, one (1.4%) of 72 Zhuang controls, and zero (0.0%) of 78 Han controls. No P268S homozygous mutations were found. The genotype and allelic frequencies of P268S in the Zhuang and Han populations with CD

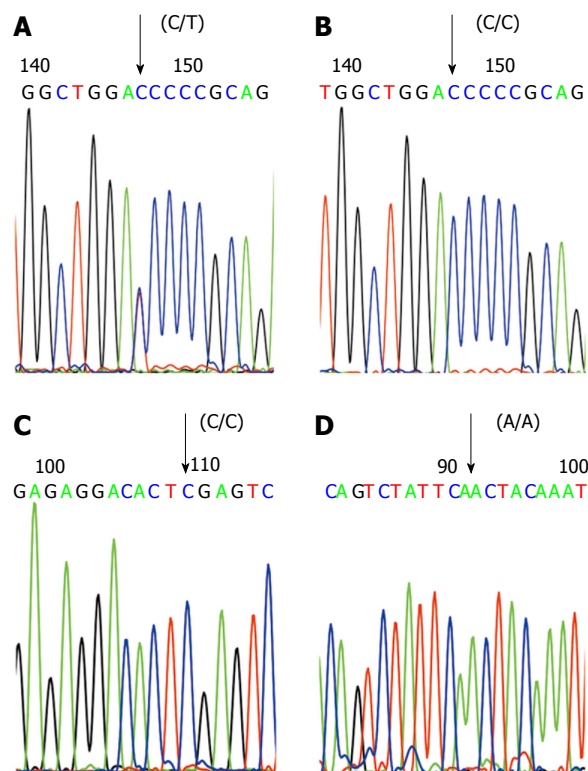


Figure 3 Gene sequencing analysis of P268S, JW1 and N852S polymerase chain reaction products. A: The forward sequencing map of the polymerase chain reaction (PCR) product of heterozygote P268S (C/T); B: The forward sequencing map of the PCR product of wild-type P268S (C/C); C: The forward sequencing map of the PCR product of wild-type JW1 (C/C); D: The forward sequencing map of the PCR product of wild-type N852S (A/A).

were significantly higher than those in the control group ($^aP = 0.016$, $^cP = 0.022$ under the genotypic model, and $^bP = 0.017$, $^dP = 0.022$ under the allelic model, respectively); however, the differences between the control group and the UC group were not statistically significant ($P > 0.05$). The JW1 and N852S genotypes were homozygous wild-type in all three groups of Zhuang and Han. No differences in genotype and allelic frequencies of JW1 and N852S were detected among the groups ($P > 0.05$) (Tables 3 and 4).

P268S genotype and clinical features of CD

A comparison between CD patients in Guangxi including Zhuang and Han with and without P268S mutations was performed. Eight of the ten patients with CD who carried the P268S mutation were ≤ 40 years of age ($^cP = 0.040$), which suggested that the P268S mutation may be correlated with younger onset of CD in Guangxi patients. However, this mutation was not associated with lesion location, gender, ethnic groups, complications, or lesion severity ($P > 0.05$) (Table 5).

DISCUSSION

The *NOD2/CARD15* gene is located on chromosome 16q12. The protein that is encoded by the *NOD2/CARD15* gene is highly expressed in intestinal mucosal

Table 3 Distribution of genotype and allele frequencies of mutations in Crohn's disease and ulcerative colitis patients compared with healthy controls in the Guangxi Zhuang population *n* (%)

Mutant	Genotype	Allele	Control	CD		UC	
				<i>P</i> value		<i>P</i> value	
P268S	CC	T	71 (98.6)	42 (87.5)	^a <i>P</i> ¹	54 (100.0)	NS ¹
	CT		1 (1.4)	6 (12.5)		0 (0.0)	
	TT		0 (0.0)	0 (0.0)	^c <i>P</i> ²	0 (0.0)	NS ²
			1 (0.7)	6 (6.2)		0 (0.0)	
JW1	CC	T	72 (100.0)	48 (100.0)	NS ¹	54 (100.0)	NS ¹
	CT		0 (0.0)	0 (0.0)		0 (0.0)	
	TT		0 (0.0)	0 (0.0)	NS ²	0 (0.0)	NS ²
			0 (0.0)	0 (0.0)		0 (0.0)	
N852S	AA	G	72 (100.0)	48 (100.0)	NS ¹	54 (100.0)	NS ¹
	AG		0 (0.0)	0 (0.0)		0 (0.0)	
	GG		0 (0.0)	0 (0.0)	NS ²	0 (0.0)	NS ²
			0 (0.0)	0 (0.0)		0 (0.0)	

¹Comparisons of genotype frequencies; ²Comparisons of allele frequencies. ^a*P* < 0.05 *vs* control using Fisher's exact test; ^c*P* < 0.05 *vs* control using Fisher's exact test. CD: Crohn's disease; UC: Ulcerative colitis; NS: No significance.

Table 4 Distribution of genotype and allele frequencies of mutations in Crohn's disease and ulcerative colitis patients compared with healthy controls in the Guangxi Han population *n* (%)

Mutant	Genotype	Allele	Control	CD		UC	
				<i>P</i> value		<i>P</i> value	
P268S	CC	T	78 (100.0)	46 (92.0)	^b <i>P</i> ¹	48 (96.0)	NS ¹
	CT		0 (0.0)	4 (8.0)		2 (4.0)	
	TT		0 (0.0)	0 (0.0)	^d <i>P</i> ²	0 (0.0)	NS ²
			0 (0.0)	4 (4.0)		2 (2.0)	
JW1	CC	T	78 (100.0)	50 (100.0)	NS ¹	50 (100.0)	NS ¹
	CT		0 (0.0)	0 (0.0)		0 (0.0)	
	TT		0 (0.0)	0 (0.0)	NS ²	0 (0.0)	NS ²
			0 (0.0)	0 (0.0)		0 (0.0)	
N852S	AA	G	78 (100.0)	50 (100.0)	NS ¹	50 (100.0)	NS ¹
	AG		0 (0.0)	0 (0.0)		0 (0.0)	
	GG		0 (0.0)	0 (0.0)	NS ²	0 (0.0)	NS ²
			0 (0.0)	0 (0.0)		0 (0.0)	

¹Comparisons of genotype frequencies; ²Comparisons of allele frequencies. ^b*P* < 0.05 *vs* control using Fisher's exact test; ^d*P* < 0.05 *vs* control using Fisher's exact test. CD: Crohn's disease; UC: Ulcerative colitis; NS: No significance.

Paneth cells^[21]. The *NOD2/CARD15* protein has two caspase recruitment domains and includes a nucleotide-binding domain and a leucine-rich repeat (LRR). The LRR may stimulate the secretion of defensin through the identification of bacterial muramyl dipeptide. The level of defensin decreased markedly in patients with CD and gene mutations^[22]. LRR may cause a defensive inflammatory reaction by combining bacterial lipopolysaccharide and activating NF- κ B^[23]. *NOD2/CARD15* is the first confirmed predisposing gene for CD, and the R702W, G908R, and L1007fs SNPs of the *NOD2/CARD15* gene were found to be significantly associated with CD in Caucasian populations^[12-14]. The mutant allele frequencies of these three mutations accounted for approximately 81% of the total CD mutations^[24]. Nevertheless, these SNPs

were not associated with CD in Japanese, Malaysian, Indian, or Hong Kong, Zhejiang, and Guangxi populations in China, and none of the patients with CD had heterozygous or homozygous variants of R702W, G908R, and L1007fs SNPs^[10,11,15,17,25,26]. In addition, the R702W, G908R, L1007fs, P268S, and JW1 SNPs were not correlated with IBD patients in Turkey, instead, the R702W mutation was significantly lower in the IBD group (1.5%) than in the control group (4.8%) (*P* < 0.05)^[27]. Thus, these findings indicate that the *NOD2/CARD15* genotype distribution has significant ethnic differences.

This is the first study to report the P268S, JW1, and N852S mutations of the *NOD2/CARD15* gene in patients with CD from the Guangxi Zhuang population of China, where the ethnic background is heteroge-

Table 5 Clinical characteristics of Crohn's disease patients in Guangxi with and without P268S mutations

Phenotype	n	P268S+	P268S-	P value
Age of onset				^a P
≤ 40 years	45	8 (17.8)	37 (82.2)	
> 40 years	53	2 (3.8)	51 (96.2)	
Location				NS
Ileum	58	6 (10.3)	52 (89.7)	
Colon/ileocolon	40	4 (10.0)	36 (90.0)	
Gender				NS
Male	51	7 (13.7)	44 (86.3)	
Female	47	3 (6.4)	44 (93.6)	
Ethnic groups				NS
Han	50	4 (8.0)	46 (92.0)	
Zhuang	48	6 (12.5)	42 (87.5)	
Comorbidities				NS
Luminal stenosis	31	4 (12.9)	27 (87.1)	
No luminal stenosis	67	6 (9.0)	61 (91.0)	
Severity				NS
Severe	42	3 (7.1)	39 (92.9)	
Mild-moderate	56	7 (12.5)	49 (87.5)	

NS: No significance. P268S+ Mutant P268S; P268S- Wild-type P268S. ^aP < 0.05 using Fisher's exact test.

neous with Han, Zhuang, and other ethnic groups. In this study, the P268S mutation genotype of *NOD2/CARD15* was found in some Zhuang and Han patients with CD and was detected only sporadically in healthy individuals and patients with UC in Zhuang and Han. The JW1 and N852S mutations of the *NOD2/CARD15* gene were not detected in Guangxi Zhuang or Han patients with IBD.

In recent years, several studies have reported that the P268S mutation of the *NOD2/CARD15* gene was found in Ashkenazi Jewish and Irish patients with CD^[16,28]. The population-attributable risk of the P268S-JW1 haplotype was 15.1% in Jewish patients with CD^[16]. Gasche *et al.*^[29] reported that the evolution of P268S occurred in the Middle East and that the mutant was associated with CD in Chinese Tu and Pakistani populations. The P268S SNP of the *NOD2/CARD15* gene was also reported to be closely related to CD in Indian patients^[17,30]. Similarly, the P268S mutant was confirmed to contribute to CD susceptibility and clinical features in a Han population in Guangdong, China^[19]. However, that finding was not in accordance with those of Juyal *et al.*^[31], in which the P268S mutant of the *NOD2/CARD15* gene was correlated with UC in North India. In our study, we confirmed that the P268S SNP may be involved in the susceptibility of Zhuang or Han patients to CD in Guangxi, China. Our results are in agreement with those from studies on Han and Tu patients with CD from other areas in China^[19,29]. However, the P268S homozygous variant was found in Han patients in Guangdong, China, and was not detected in our study population, which may be due to racial heterogeneity or our relatively small sample size. Compared to Europeans (31.2%)^[16], we found a lower frequency (12.5%) of mutant P268S in our Zhuang CD patients.

The N852S mutation of the *NOD2/CARD15* gene

was found to be significantly associated with CD in Ashkenazi Jewish populations^[18]; however, since it did not appear as a haplotype with R702W, G908R, and L1007fs of the *NOD2/CARD15* gene, it is thought to be an independent risk factor for CD^[32]. Our results indicated that N852S mutations of the *NOD2/CARD15* gene were not detected in Guangxi Zhuang patients with IBD. The JW1 mutant of the *NOD2/CARD15* gene was confirmed in Chinese Han in Malaysia^[17]. However, we did not find any heterozygous or homozygous mutations of JW1 in the Chinese Zhuang population from the Guangxi Zhuang Autonomous Region. These two novel loci have rarely been reported in China, and further studies are necessary to explore these loci in a larger cohort in China. In summary, the differences in these results may be attributed to the differences in race, geography, environment, and population.

Several studies have proved that the *NOD2/CARD15* gene is related to the clinical features of CD including onset location, age, complications, and disease severity^[33-35]. It was reported that P268S was related to ileal lesions ($P = 0.003$), lumen stenosis ($P = 0.007$), and age ≤ 20 years ($P = 0.028$) in a Chinese Han population with CD from Guangdong, China^[19]. In addition, Chua *et al.*^[17] reported that the JW1 mutant tended to correlate with luminal stenosis ($P = 0.055$) and age < 41 years ($P = 0.095$) in patients with CD in Malaysia. The results of the present study confirmed that P268S was only related to age ≤ 40 years ($P = 0.040$) in CD patients from the Chinese Zhuang population in the Guangxi Zhuang Autonomous Region. No important relationship was detected between mutant P268S and location, gender, ethnic group, lumen stenosis, and severity of CD. Our results were not in agreement with those studies on Chinese Han patients with CD from Guangdong or patients with CD from Malaysia. This difference may be due to racial heterogeneity, geographic environment, and a relatively small sample size.

In conclusion, this study is the first to demonstrate the relationship between the P268S SNP of the *NOD2/CARD15* gene and susceptibility to CD in a Zhuang population from the Guangxi Zhuang Autonomous Region, China. JW1 and N852S SNPs of the *NOD2/CARD15* gene were not found in the Zhuang population. Thus, we emphasize that genetic predisposition may be vital in the pathogenesis of IBD. However, the power of this conclusion may be limited by the relatively small sample size in this study. Further studies investigating risk factors and genetic susceptibility to IBD in a larger cohort of patients and in different ethnic groups are needed.

COMMENTS

Background

Inflammatory bowel disease (IBD) is a multifactorial disease with different susceptibility genes in various races. The P268S, JW1, and N852S polymorphisms of *NOD2/CARD15* have been confirmed in Crohn's disease (CD) susceptibility in Chinese Han and Ashkenazi Jewish populations, but there are no reports of a correlation between these three polymorphisms and the Chinese Zhuang CD population in the Guangxi Zhuang Autonomous Region.

Research frontiers

Nucleotide-binding oligomerization domain containing 2/caspase-activation and recruitment domain gene 15 (*NOD2/CARD15*) is the first confirmed predisposing gene for CD, and the P268S mutation of the *NOD2/CARD15* gene was found in Ashkenazi Jewish, Irish, Indian, Pakistani, and Chinese Han and Tu patients with CD, but not in CD in North India. The JW1 SNP of the *NOD2/CARD15* gene was shown to be associated with CD in Chinese Han, Malay, and Indians in Malaysia. The N852S mutation of the *NOD2/CARD15* gene was only found to be significantly associated with CD in Ashkenazi Jewish populations. The present study assessed whether these known SNPs were associated with IBD in Zhuang patients from Guangxi, China.

Innovations and breakthroughs

The Guangxi Zhuang Autonomous Region of China has the largest Zhuang population, thus genetic diseases and gene polymorphisms are unique. This study is the first to demonstrate the relationship between the P268S, JW1, and N852S polymorphisms of the *NOD2/CARD15* gene and susceptibility to CD in the Zhuang population from the Guangxi Zhuang Autonomous Region, China.

Applications

The P268S polymorphism may contribute to CD susceptibility in the Zhuang population in the Guangxi Zhuang Autonomous Region, China. However, JW1 and N852S SNPs may be absent or rare in this population.

Terminology

NOD2/CARD15 is located on chromosome 16q12, and encodes a protein with homology to plant disease resistance-related gene products. Mutant *NOD2/CARD15* responds to bacterial muramyl dipeptide and decreases NF- κ B activation, and these results implicate *NOD2/CARD15* in susceptibility to Crohn's disease. Polymerase chain reaction-restriction fragment length polymorphism is a popular technique used in genetic analysis. It has been used for the detection of intraspecies as well as interspecies variation.

Peer review

This brief paper demonstrates the relationship between the P268S polymorphism in *NOD2/CARD15* and Crohn's disease susceptibility in an ethnic Zhuang population. While the scientific findings in this paper are limited, documentation of genetic variation in CD susceptibility among various ethnic and regional groups is useful.

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Entecavir vs lamivudine therapy for naïve patients with spontaneous reactivation of hepatitis B presenting as acute-on-chronic liver failure

Yang Zhang, Xiao-Yu Hu, Sen Zhong, Fang Yang, Tao-You Zhou, Guo Chen, Yan-Yan Wang, Jian-Xing Luo

Yang Zhang, Xiao-Yu Hu, Sen Zhong, Guo Chen, Yan-Yan Wang, Department of Infectious Diseases, Affiliated Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu 610072, Sichuan Province, China

Yang Zhang, Xiao-Yu Hu, Fang Yang, Jian-Xing Luo, Department of Clinical Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu 610072, Sichuan Province, China

Tao-You Zhou, Center of Infectious Diseases, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Hu XY and Zhong S designed the study; Hu XY, Zhang Y, Chen G, Wang YY and Luo JX performed the majority of study; Zhang Y and Yang F analyzed the data; Zhang Y and Hu XY wrote the paper; and Zhou TY offered suggestions for revision of the paper.

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Correspondence to: Xiao-Yu Hu, Professor, Department of Infectious Diseases, Affiliated Hospital of Chengdu University of Traditional Chinese Medicine, 39 Shierqiao Road, Chengdu 610072, Sichuan Province, China. xiaoyuhu@aliyun.com

Telephone: +86-28-87766041 Fax: +86-28-87732407

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Abstract

AIM: To investigate the short-term and long-term efficacy of entecavir versus lamivudine in patients with spontaneous reactivation of hepatitis B presenting as acute-on-chronic liver failure (ACLF).

METHODS: This was a single center, prospective cohort study. Eligible, consecutive hospitalized patients received either entecavir 0.5 mg/d or lamivudine 100 mg/d. All patients were given standard comprehensive internal medicine. The primary endpoint was survival rate at day 60, and secondary endpoints were reduction in hepatitis B virus (HBV) DNA and alanine amino-

transferase (ALT) levels, and improvement in Child-Turcotte-Pugh (CTP) and model for end-stage liver disease (MELD) scores at day 60 and survival rate at week 52.

RESULTS: One hundred and nineteen eligible subjects were recruited from 176 patients with severe acute exacerbation of chronic hepatitis B: 65 were included in the entecavir group and 54 in the lamivudine group (full analysis set). No significant differences were found in patient baseline clinical parameters. At day 60, entecavir did not improve the probability of survival ($P = 0.066$), despite resulting in faster virological suppression ($P < 0.001$), higher rates of virological response ($P < 0.05$) and greater reductions in the CTP and MELD scores (all $P < 0.05$) than lamivudine. Intriguingly, at week 52, the probability of survival was higher in the entecavir group than in the lamivudine group [42/65 (64.6%) vs 26/54 (48.1%), respectively; $P = 0.038$]. The pretreatment MELD score (B, 1.357; 95%CI: 2.138-7.062; $P = 0.000$) and virological response at day 30 (B, 1.556; 95%CI: 1.811-12.411; $P = 0.002$), were found to be good predictors for 52-wk survival.

CONCLUSION: Entecavir significantly reduced HBV DNA levels, decreased the CTP and MELD scores, and thereby improved the long-term survival rate in patients with spontaneous reactivation of hepatitis B presenting as ACLF.

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Key words: Acute-on-chronic liver failure; Hepatitis B; Entecavir; Lamivudine; Survival

Core tip: This study compared the short-term and long-term efficacy of entecavir and lamivudine in patients with spontaneous reactivation of hepatitis B presenting as acute-on-chronic liver failure (ACLF). Entecavir significantly reduced hepatitis B virus DNA levels, decreased

the Child-Turcotte-Pugh and model for end-stage liver disease (MELD) scores, and thereby improved the long-term survival rate in patients with spontaneous reactivation of hepatitis B presenting as ACLF. Pretreatment MELD score and virological response at 30 d were good predictors of long-term survival.

Zhang Y, Hu XY, Zhong S, Yang F, Zhou TY, Chen G, Wang YY, Luo JX. Entecavir vs lamivudine therapy for naïve patients with spontaneous reactivation of hepatitis B presenting as acute-on-chronic liver failure. *World J Gastroenterol* 2014; 20(16): 4745-4752 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4745.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4745>

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a major type of liver failure in the Asian region. The incidence of ACLF was reported as 91.7% in an epidemiological investigation from China^[1]. ACLF has an extremely high short-term mortality rate, ranging from 50%-90%^[2,3], and 77% of patients died of multi-organ failure^[4]. Liver transplantation is the definitive therapy, but is limited by donor shortage and high costs^[5,6]. Thus, it is necessary to explore other effective therapies, especially in patients without liver transplantation.

In China, chronic HBV infection is the cause of approximately 90% of ACLF^[1]. In these patients, severe acute exacerbation (SAE) of chronic hepatitis B (CHB) often occurs spontaneously: it was seen in 15%-37% of patients with chronic HBV infection after 4 years of follow-up^[7]. Although there is no consensus definition of SAE, it usually refers to the abrupt reappearance or rise in serum hepatitis B virus (HBV) DNA in a patient with previously inactivated or resolved HBV infection^[8]. A high HBV DNA level ($> 10^5$ copies/mL) was suggested as useful to identify SAE of CHB in a previous study^[9]. Continuous high levels of viral replication is one of the key factors causing severe liver damage^[10]. Therefore, antiviral treatment should be promptly instituted.

Lamivudine is the first approved oral therapy for anti-HBV treatment, and has an excellent safety and tolerability profile. Early studies proved that the potential short-term benefits of lamivudine in patients with the disease are superior to historical controls^[11,12]. However, prolonged lamivudine monotherapy has been found to be associated with an increased risk of re-exacerbation after temporary relief of the initial SAE, because of treatment-induced HBV variants with YMDD mutations^[13,14]. Entecavir is another oral anti-HBV compound with potent activity. Its *in vitro* potency is 100- to 1000-fold greater than that of lamivudine^[15]. Furthermore, the cumulative rate of resistance to entecavir was only 1.2% in 5 years^[16]. Theoretically, entecavir may be more suitable for the long-term treatment of ACLF because of severe

reactivation of HBV.

However, the lack of large sample sizes, contemporary controls and long-term research, has lead to inconsistent clinical data with regard to the efficacy and safety of entecavir in these studies. This prospective cohort study was performed to compare the efficacy of entecavir and lamivudine in terms of the reduction in HBV DNA levels, improvement in biochemical and disease severity, likely improvement in survival and to identify prognostic factors in patients with severe reactivation of HBV presenting as ACLF.

MATERIALS AND METHODS

Patients

In this prospective cohort study, eligible consecutive hospitalized patients with ACLF were recruited from the Department of Infectious Diseases, Affiliated Hospital of Chengdu University of Traditional Chinese Medicine (TCM), from November 2007 to July 2011. All recruited patients were examined by clinicians and were enrolled into the study according to the criteria of ACLF^[17]. The inclusion criteria were: (1) age from 18 to 65 years; (2) the presence of hepatitis B surface antigen in the serum for at least 6 mo; (3) HBV DNA level $> 10^5$ copies/mL; (4) alanine aminotransferase (ALT) level > 5 times the upper limit of normal; and (5) acute hepatic insult manifesting as jaundice (serum total bilirubin ≥ 171 μ mol/L or a daily increase ≥ 17.1 μ mol/L) and coagulopathy [international normalized ratio (INR) ≥ 1.5 or prothrombin activity $< 40\%$], complicated within 4 wk by ascites and/or encephalopathy. The exclusion criteria were: (1) superinfection or co-infection with hepatitis A, C, D, E viruses, or human immunodeficiency virus; (2) coexistence of any other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, drug hepatitis or Wilson's disease; (3) hepatocellular carcinoma diagnosed by computed tomography; (4) coexistence of any other serious systemic or psychiatric diseases; (5) jaundice caused by obstructive or hemolytic diseases; (6) prolonged prothrombin time induced by blood system disease; and (7) a previous course of any antiviral, immunomodulator or cytotoxic/immunosuppressive therapy for chronic hepatitis or other illnesses within at least the preceding 12 mo.

The study protocol was in accordance with the Helsinki Declaration of 1975. The ethics committee of the Affiliated Hospital of Chengdu University of TCM approved the study. Written informed consent was obtained from each patient or their relatives before enrollment. Furthermore, the non-availability of artificial liver support therapy and liver transplantation facilities were also explained to the patients.

Study design

This was a prospective cohort study. All consecutive hospitalized patients spontaneously formed two cohorts (entecavir/lamivudine cohort), according to their preferences for antiviral therapy. Eligible subjects were given

comprehensive internal medicine for 60 d (study period), and were followed up until 52 wk after enrollment (follow-up period) or death.

The sample size was calculated based on the data from previous studies^[18,19], which suggested a survival rate in the lamivudine-treated group of 50% and a survival rate in the entecavir-treated group of approximately 65%. The match ratio was 1:1. The sample size in each group was 54, with a type I error (one-sided) of 5%, and a power of at least 80%^[20]. On the assumption of a rate of 10% loss to follow-up, a target sample size of 119 was required.

Treatment schedule

All eligible patients were requested to accept antiviral treatment both in the study period and in the follow-up period. The entecavir cohort was given entecavir 0.5 mg/d (Baraclude, Bristol-Myers Squibb, China), while the lamivudine cohort was given lamivudine 100 mg/d (QO, GlaxoSmithKline, China). In addition, each patient was given standard comprehensive internal medicine during the study period. This routinely included absolute bed rest, barrier nursing, high calorie diet (35-40 cal/kg per day), lactulose, and intensive care monitoring, intravenous plasma, maintenance water, electrolyte and acid-base equilibrium monitoring, and prevention and treatment of complications. Patients also received albumin, terlipressin, and proton pump inhibitors if required. Enteral or parenteral nutrition was provided for patients whose caloric requirement was not fulfilled by mouth.

Clinical and laboratory data

Patient clinical and laboratory data were collected prospectively. The latter included: (1) biochemical tests reflecting hepatocyte damage, for example serum ALT, total bilirubin (TBIL), albumin (ALB), and creatinine (CREA), all assayed using a colorimetric method (Automatic Analyzer 7170A, Hitachi, Japan); (2) INR for prothrombin time, performed following the manufacturer's instructions (STA-evolution, STAGO, France); (3) serum HBV DNA, determined by a fluorescent quantifying polymerase chain reaction (PCR) method with a low limit of detection of 1000 copies/mL (Lightcycler-480, Roche, Switzerland); and (4) HBV markers, for example HBV antigens and antibodies, detected using commercially available enzyme immunoassays (Alisei Quality System, RADIM, Italy).

The severity of liver disease was assessed by the Child-Turcotte Pugh (CTP) score^[21] and model for end-stage liver disease (MELD) score. The MELD score was calculated according to the following equation^[22]: $3.78 \times \ln[\text{total bilirubin (mg/dL)}] + 11.2 \times \ln \text{INR} + 9.57 \times \ln[\text{creatinine (mg/dL)}] + 6.43 \times (\text{constant for liver disease etiology: 0 if cholestatic or alcoholic, 1 otherwise})$.

Follow-up

Patients were examined every 15 d in the first 60 d, followed by every 3 mo up to 52 wk. Clinical and laboratory

data, adverse events, and compliance were monitored during the first 60 d of treatment, and both adverse events and compliance were monitored every 3 mo up to 52 wk.

Endpoints

The primary endpoint of this study was survival rate at day 60. Secondary endpoints were reduction in HBV DNA and ALT levels, and improvement in CTP and MELD scores at day 60, and survival rate at week 52. All patients were followed up and the outcome (recovery, bridging to artificial liver support therapy or liver transplantation, or death) of treatment was recorded. The date and cause of each death were documented.

Safety

The patients were questioned regarding adverse events. All adverse events, regardless of their possible association with antiviral drugs, were recorded.

Statistical analysis

Efficacy and safety analyses were performed according to intention-to-treat (ITT), and were conducted on the full analysis set (FAS). This set principally included the data from all patients receiving at least 1 dose of the study drugs. Partially missing data of the clinical evaluation were carried forward with the principle of the last visit carried forward. Deaths occurring in the study or follow-up period were not regarded as discontinued subjects.

Quantitative data were described by the mean \pm SD. Independent-samples *t* test or Mann-Whitney *U* tests were performed to compare differences between quantitative data. A χ^2 test or Fisher's exact test was performed to calculate differences between qualitative data. A survival curve was calculated by the Kaplan-Meier method and compared statistically using a log-rank test. Univariate and multivariate analyses were used to assess the associations between survival and independent variables, and multivariate Cox regression analysis with conditional stepwise forward was then used to calculate the relative risk ratios and 95%CI. All statistical tests were two-tailed, and a significance level (*P*) of 0.05 was used. The statistical tests were performed using the Statistical Package for the Social Sciences (SPSS version 17.0; SPSS Inc., Chicago, IL, United States).

RESULTS

Patients

Subject disposition during the study is shown in Figure 1. A total of 176 patients with SAE of CHB were assessed for eligibility, and 119 eligible patients were enrolled. One hundred and five patients completed the study, 57 in the entecavir group and 48 in the lamivudine group. Fourteen patients did not complete the study, but received treatment and had complete observations on at least one related record at a time point; the clinical data of these

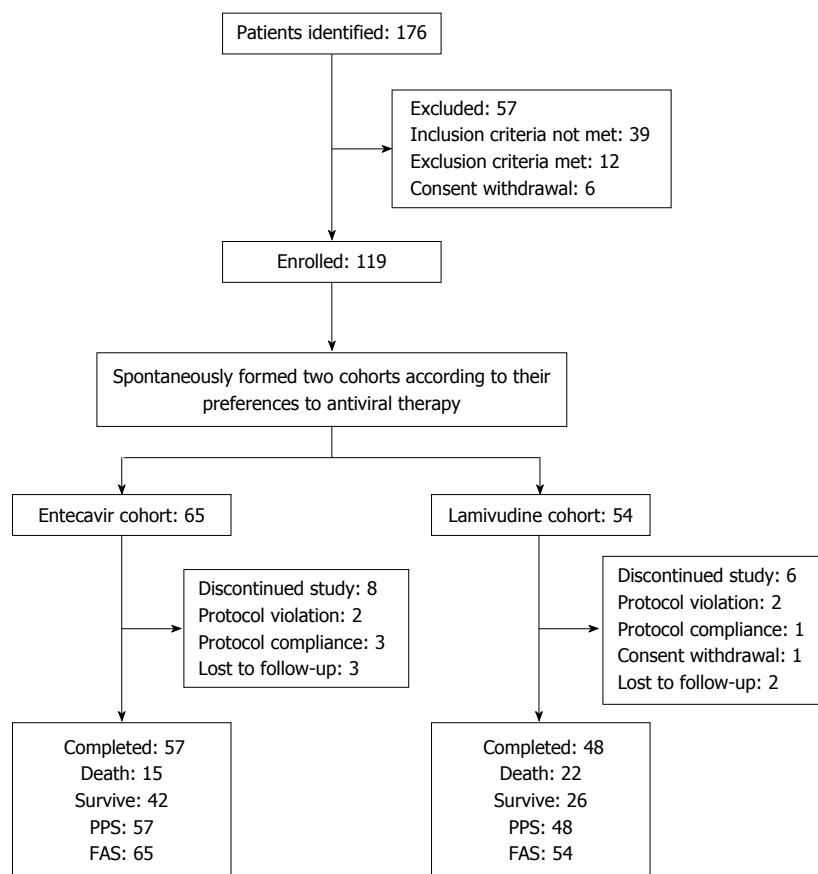


Figure 1 Patient disposition during the study. PPS: Per protocol set; FAS: Full analysis set.

patients were analyzed on the basis of ITT. The FAS population included 119, with 65 in the entecavir group and 54 in the lamivudine group.

Demographic data and baseline characteristics

Baseline characteristics of all patients are shown in Table 1. There was no significant difference in age, gender, serum ALT, ALB, TBIL, PTA, CREA, Na, CTP and MELD score, HBV DNA level, HBeAg (\pm), or complications between the two groups before treatment (all $P > 0.05$).

Virological and biochemical responses

Viral and host responses to nucleoside analog therapy in the two groups are presented in Table 2. Posttreatment HBV DNA levels in the entecavir and lamivudine group were significantly lower than pretreatment levels. Compared with patients in the lamivudine group, patients in the entecavir group had significantly lower HBV DNA levels at days 15, 30, 45, and 60 (all $P < 0.001$). Undetectable HBV DNA (lower than 1000 copies/mL) was observed in 33 of the 119 (27.7%) patients during treatment, and the entecavir group had a higher proportion of patients achieving undetectable viremia at days 45 and 60 (all $P < 0.05$). With regard to the ALT response between the two groups, there was a statistical difference at day 15 ($P < 0.01$), but no significant difference at days 30, 45, and 60 (all $P > 0.05$).

Outcomes with respect to severity scores

CTP and MELD scores in both groups of patients are presented in Table 3. Post-treatment CTP and MELD scores in the entecavir and lamivudine group were significantly lower than pretreatment scores. In addition, CTP scores at days 45 and 60 in the entecavir group were superior to those in the lamivudine group, with statistical significance ($P < 0.05$). Similarly, post-treatment MELD scores in the entecavir group were significantly lower than those in the lamivudine group ($P < 0.05$).

Short-term and long-term survival rate

Of the 119 patients in FAS, 51 (78.5%) survived in the entecavir group and 35 (64.8%) survived in the lamivudine group at day 60 ($P = 0.066$ by log-rank test, Figure 2A). However, at the end of the follow-up period of 52 wk, 42 (64.6%) in the entecavir group and 26 (48.1%) in the lamivudine group survived ($P = 0.038$ by log-rank test, Figure 2B).

The causes of death were analyzed for 37 patients who had a complete and reliable death record. The major causes were hepatorenal syndrome (8/57, 14.0%), multiple organ failure (3/57, 5.3%), encephalopathy (2/57, 3.5%) and upper gastrointestinal bleeding (2/57, 3.5%) in the entecavir group; hepatorenal syndrome (10/48, 20.8%), multiple organ failure (5/48, 10.4%), encephalopathy (4/48, 8.3%), liver failure (2/48, 4.2%) and upper gastrointestinal bleeding (1/48, 2.1%) in the lamivudine

Table 1 Baseline data comparison between entecavir and lamivudine groups *n* (%)

Group	Entecavir (<i>n</i> = 65)	Lamivudine (<i>n</i> = 54)	<i>t</i> / <i>χ</i> ²	<i>P</i> value
Age (yr)	42.8 ± 13.1	45.6 ± 11.4	1.230	0.221
Males	41 (63.1)	36 (66.7)	0.166	0.683
Total bilirubin (μmol/L)	331.6 ± 74.8	320.1 ± 82.4	0.797	0.427
ALT level (U/L)	352.5 ± 77.2	345.2 ± 89.5	0.478	0.634
HBeAg-positive	21 (32.3)	23 (42.6)	1.339	0.247
anti-HBc IgM	40 (61.5)	29 (53.7)	0.743	0.389
HBV DNA (log10 copies/mL)	7.0 ± 1.4	7.2 ± 1.6	0.727	0.469
Prothrombin activity (%)	24.7 ± 6.0	25.1 ± 5.7	0.370	0.712
Albumin (g/L)	28.7 ± 6.9	29.4 ± 5.3	0.611	0.543
Creatinine (μmol/L)	106.3 ± 42.1	109.7 ± 38.6	0.455	0.650
Sodium (mmol/L)	130.6 ± 11.4	127.2 ± 12.6	1.544	0.125
Ascites	52 (80.0)	45 (75.9)	0.217	0.641
Hepatic encephalopathy I - II	14 (21.5)	13 (24.1)	0	1
III-IV	3 (4.6)	2 (3.7)	0	1
Spontaneous bacterial peritonitis	15 (23.1)	11 (20.4)	0.127	0.722
CTP points	11.2 ± 2.4	10.7 ± 2.1	1.196	0.234
MELD points	27.2 ± 6.5	26.8 ± 6.3	0.339	0.735

All values are expressed as mean ± SD or number (%). ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; CTP: Child-Turcotte Pugh; MELD: Model for end-stage liver disease.

group. There were no significant differences between the two groups (all *P* > 0.05).

Predictive factors for long-term survival

On univariate analysis, gender (*P* = 0.020), HBeAg (±) (*P* = 0.048), undetectable HBV DNA at day 30 (*P* = 0.035), pretreatment CTP score (*P* = 0.026), and MELD score (*P* = 0.020) were found to be significantly associated with long-term survival. However, age, platelet count, serum ALT, HBV DNA level, and treatment with entecavir were not associated with fatal outcomes. In the forward Cox regression analysis, pretreatment MELD (B, 1.357; 95%CI: 2.138-7.062; *P* = 0.000), and undetectable HBV DNA at day 30 (B, 1.556; 95%CI: 1.811-12.411; *P* = 0.002) were found to be unfavorable predictors of long-term survival.

Safety

None of the patients developed drug-induced severe lactic acidosis or other serious adverse events, and all patients tolerated the therapy without dose modification or early discontinuation.

DISCUSSION

In this prospective cohort, compared with lamivudine treatment, entecavir treatment did not improve short-term prognosis, despite resulting in faster virological suppression, higher rates of virological response, and a greater reduction in CTP and MELD scores at day 60 in patients with ACLF. Intriguingly, continuation of en-

Table 2 Virological, biochemical responses and severity scores

Characteristics	Entecavir (<i>n</i> = 65)	Lamivudine (<i>n</i> = 54)	<i>t</i> / <i>χ</i> ² / <i>z</i>	<i>P</i> value
Virological				
Serum HBV DNA level (log10 copies/mL)				
Day 15	4.6 ± 1.1	5.4 ± 1.2	3.790	< 0.001
Day 30	3.9 ± 1.0	4.8 ± 1.3	4.266	< 0.001
Day 45	3.6 ± 0.8	4.5 ± 1.0	5.454	< 0.001
Day 60	3.4 ± 0.5	4.4 ± 0.9	7.653	< 0.001
Undetectable HBV DNA <i>n</i> (%)				
Day 15	3 (4.6)	0 (0.0)	1.024	0.312
Day 30	15 (23.1)	6 (11.1)	2.906	0.088
Day 45	21 (32.3)	8 (14.8)	4.897	0.027
Day 60	24 (36.9)	9 (16.7)	6.039	0.014
Biochemical				
Serum ALT level (U/L)				
Day 15	187.4 ± 67.5	231.6 ± 81.1	3.231	0.002
Day 30	86.2 ± 22.4	94.7 ± 37.3	1.410	0.163
Day 45	54.4 ± 19.6	60.8 ± 28.7	1.222	0.226
Day 60	45.1 ± 20.8	51.6 ± 22.3	1.455	0.149
Severity				
CTP score				
Day 15	10.4 ± 2.7	10.2 ± 3.4	0.348	0.348
Day 30	8.7 ± 3.6	9.9 ± 4.2	1.678	0.096
Day 45	7.4 ± 2.1	8.5 ± 3.7	2.035	0.044
Day 60	6.6 ± 2.4	8.2 ± 3.5	2.478	0.016
MELD score				
Day 15	25.2 ± 7.1	25.7 ± 8.4	0.350	0.727
Day 30	22.6 ± 6.7	24.5 ± 7.2	1.489	0.139
Day 45	17.8 ± 7.4	20.6 ± 8.2	1.956	0.052
Day 60	13.7 ± 4.6	16.1 ± 6.5	2.352	0.020

All values are expressed as mean ± SD. ALT: Alanine aminotransferase; CTP: Child-Turcotte Pugh; MELD: Model for end-stage liver disease.

tecavir treatment significantly benefited 52-wk survival. In addition, pretreatment MELD score and virological response at day 30 were significantly related to long-term prognosis.

Chronic HBV infection is a rapid, dynamic process with vast amounts of virus and infected cells produced and killed each day. When patients with chronic HBV infection receive nucleoside analogs, HBV DNA levels are generally attenuated. Frequent sampling of viral load showed a bi-phasic decline. During the initial phase, the antiviral drug, *via* almost complete blocking of virus replication in hepatocytes, reduces the release of free virus into the peripheral blood, resulting in a fast decline in serum HBV DNA level. In the subsequent slow viral decline phase, virus-infected liver cells are degraded because of the basic immunity response of the host^[23,24]. Thus, early rapid viral decline is dependent on the efficient inhibition of viral production, as determined by the dose and potency of antiviral therapy. As seen in previous studies^[25], entecavir, with a stronger and more potent activity, achieved faster viral decline and higher rates of virological response.

In previous studies, early rapid viral decline was reported to be associated with the prognosis of patients with ACLF because of severe reactivation of HBV^[26-28]. However, another cohort trial suggested that a fast viral decline conferred no survival benefit and did not prevent

Table 3 Univariate analysis of baseline predictors of 52-wk survival *n* (%)

Group	Survivors (<i>n</i> = 68)	Non-survivors (<i>n</i> = 51)	<i>t</i> / χ^2 / <i>z</i>	<i>P</i> value
Age (yr)	39.4 ± 11.7	43.2 ± 9.9	1.871	0.064
Males	38 (55.9)	39 (76.5)	5.409	0.020
Platelet count (10 ³ /μL)	93.5 ± 38.2	87.3 ± 34.1	0.917	0.361
ALT level (U/L)	367.3 ± 80.4	345.0 ± 72.6	1.560	0.121
HBeAg-positive	20 (29.4)	24 (47.1)	3.895	0.048
HBV DNA (log10 copies/mL)	7.2 ± 1.4	6.9 ± 1.5	1.122	0.264
HBV DNA undetectable (< 10 ³ copies/mL)				
Day 15	3 (4.4)	0 (0.0)	2.309	0.129
Day 30	17 (25.0)	5 (9.8)	4.466	0.035
Day 45	20 (29.4)	9 (17.6)	2.189	0.139
Day 60	21 (30.9)	12 (23.5)	1.020	0.313
Treatment with entecavir	42 (61.8)	23 (45.1)	3.266	0.071
CTP points (> 10/< 10)	30/38	33/18	4.958	0.026
MELD points (> 25/< 25)	28/40	32/19	5.423	0.020

All values are expressed as mean ± SD or number (%). ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; CTP: Child-Turcotte Pugh; MELD: Model for end-stage liver disease.

rapid progression of the disease to multi-organ failure^[18]. In a recent study in Hong Kong, fast viral decline was found to be related to increased 48-wk mortality, which may have led to an exaggerated immune response and exacerbated the liver injury^[29]. In the present study, entecavir treatment in patients with ACLF resulting from SAE of CHB rapidly reduced serum HBV DNA levels ($P < 0.001$), improved the CTP and MELD scores ($P = 0.016$ and 0.020 , respectively) at day 60, and thus reduced 52-wk mortality ($P = 0.038$).

Host immunity in the pathogenesis of liver failure has been widely recognized. Cytotoxic T-lymphocytes (CTLs), the core of cellular immunity, play a key role in the clearance of intracellular virus, which is most closely associated with cell apoptosis or necrosis^[30]. High levels of HBV replication is the major pathogenesis of ACLF because of reactivation of hepatitis B. Persistent viral replication causes a vigorous cellular immune response (especially in CTLs), resulting in severe hepatic damage. In this study, antiviral therapy with entecavir, compared with lamivudine, was found to be more effective in improving survival in patients with ACLF. This may be attributed to the weak activity of lamivudine, leading to the delayed commencement (median time 7–30 d) and viral suppression during the initial 4–8 wk of high viral replication^[31]. Conversely, entecavir, which is more potent and has lower resistance, was more likely to achieve rapid and persistent action. By rapidly suppressing HBV replication and reducing serum viral load, entecavir lowers the expression of HBV antigens on the liver cell membrane, and consequently partially blocks CTLs from damaging hepatic cells^[32]. Furthermore, a continuous decline in serum viral load caused a reduction in the number of infected hepatic cells^[24]. This might prevent vigorous im-

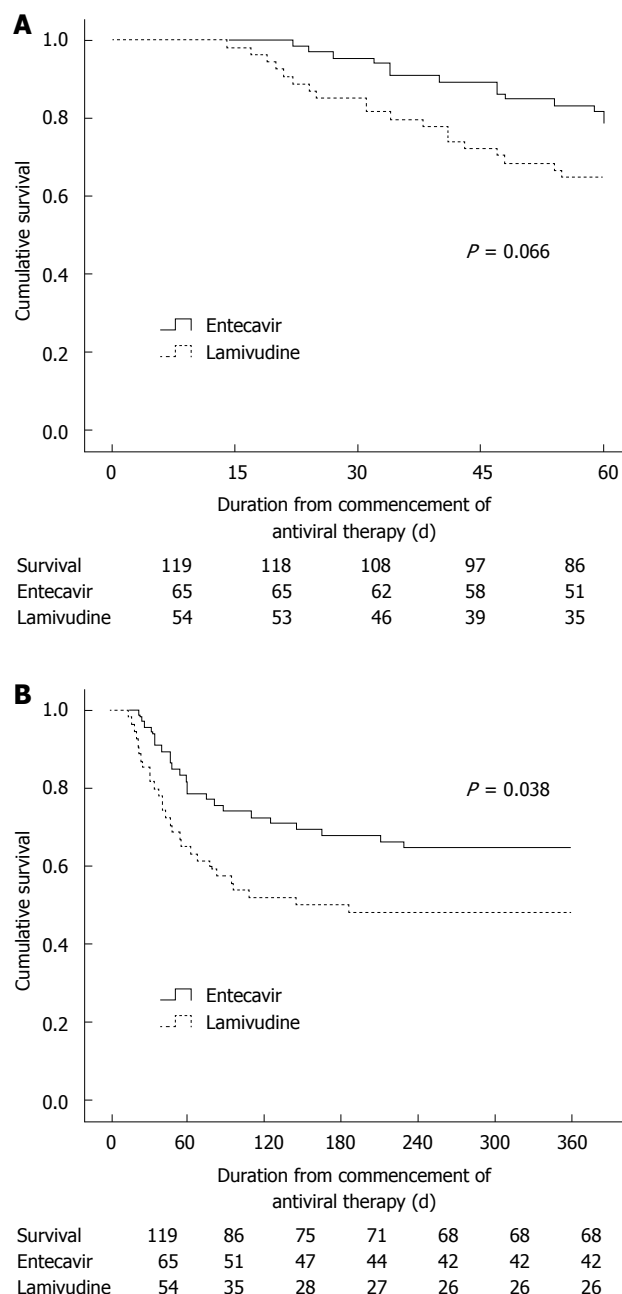


Figure 2 Survival curve for the entecavir and lamivudine groups as determined by the Kaplan-Meier method. A: 60-d; B: 52-wk.

une damage in normal hepatic cells.

Univariate analysis showed that gender, HBeAg status, HBV DNA response status at day 30, CTP and MELD scores were significantly associated with mortality. On multivariate analysis, only pretreatment MELD score and virological response at day 30 were found to be independent predictors of 52-wk survival. A previous study suggested that pretreatment HBV DNA load was related to the prognosis of patients with chronic severe hepatitis B^[26]. However, our results showed that there was no significant difference ($P = 0.264$), despite the higher mean pretreatment HBV DNA load [(7.2 ± 1.4) log10 copies/mL] in survivors than [(6.9 ± 1.5) log10 copies/mL] in non-survivors. This may be correlated with the differ-

ent baseline information in the subjects, sample size and study design. To obtain a more precise conclusion, more studies with a larger sample size, and a similar subject and trial design are needed. Moreover, it seems that virological response, which occurred late, did not always improve long-term patient survival. This might be associated with severe damage to residual hepatocytes, which limits the regenerative capacity of the liver^[53]. Therefore, potent nucleoside drugs, such as entecavir or tenofovir, should be given as quickly as possible.

This study also had some limitations. One limitation of the trial was that grouping was not in accordance with the randomization principle. Effective randomization can eliminate bias in grouping and improve the comparability of research data. The results of randomized, controlled trials (RCT) are considered to be evidence of the highest grade. However, it was not ethical to conduct an ideal RCT for such serious diseases. Therefore, we conducted a prospective cohort study. To accurately estimate the effectiveness of the therapeutic agents and improve the validity of the cohort study, our study was designed using rigorous methods that mimicked those of an RCT, such as inclusion and exclusion criteria, and statistical methods including ITT analysis. Another limitation was the lack of a placebo. Previous studies have indicated that entecavir or lamivudine could effectively improve the prognosis of ACLF patients compared with placebo. In our study, there was no need to repeat this conclusion. Moreover, it was medically unethical that patients with liver failure should undergo placebo therapy. Thus, the trial was designed to directly compare entecavir and lamivudine.

In conclusion, antiviral treatment with entecavir significantly reduced HBV DNA levels, decreased the CTP and MELD scores, and thereby improved the long-term survival rate in patients with spontaneous reactivation of hepatitis B presenting as ACLF. Entecavir was well tolerated throughout the study. Pretreatment MELD score and virological response at day 30 were related to 52-wk survival. To obtain a more objective and accurate conclusion, larger and longer-term multi-center studies are needed. In addition, it is critical to investigate whether combined nucleoside analogs can achieve faster viral decline, and whether relationships exist between the prognosis of patients and host immunity, especially cellular immunity, such as T helper 17 cells, regulatory T cells, and CTLs.

COMMENTS

Background

Acute-on-chronic liver failure (ACLF) is a severe clinical syndrome. Spontaneous acute exacerbation of chronic hepatitis B is a leading cause of ACLF. Liver transplantation is the definitive therapy, but is limited by donor shortage and high costs. Thus, it is necessary to explore other effective therapies.

Research frontiers

Lamivudine improved short-term prognosis in ACLF patients. However, continuous lamivudine therapy increased the risk of re-exacerbation after temporary relief, because of hepatitis B virus (HBV) variants with YMDD mutations. Entecavir is another oral anti-HBV compound with potent activity and low resistance. Theoretically, entecavir may be more suitable for the long-term treatment of

ACLF due to severe reactivation of HBV.

Innovations and breakthroughs

The present study demonstrates that entecavir significantly reduced HBV DNA levels, decreased the Child-Turcotte-Pugh and model for end-stage liver disease (MELD) scores, and thereby improved the long-term survival rate in patients with spontaneous reactivation of hepatitis B presenting as ACLF. The pretreatment MELD score and virological response at 30 d affected patient survival.

Applications

The data from this study provide a rational basis for nucleoside analog treatment in clinical practice for patients with severe reactivation of HBV presenting as ACLF.

Terminology

ACLF is a clinical syndrome where acute hepatic insult, manifesting as jaundice and coagulopathy, is complicated within 4 wk by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease.

Peer review

The results in the study are interesting and may help clinicians to select anti-HBV therapy.

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Hepatoprotective effect of *Cichorium intybus* L., a traditional Uighur medicine, against carbon tetrachloride-induced hepatic fibrosis in rats

Guo-Yu Li, Hong-Ying Gao, Jian Huang, Jin Lu, Jing-Kai Gu, Jin-Hui Wang

Guo-Yu Li, Jing-Kai Gu, Research Center for Drug Metabolism, College of Life Science, Jilin University, Changchun 130012, Jilin Province, China

Guo-Yu Li, Jin-Hui Wang, School of Pharmacy, Shihezi University, Shihezi 832002, Xinjiang Uygur Autonomous Region, China
Hong-Ying Gao, Jian Huang, Jin Lu, Jin-Hui Wang, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang 110016, Liaoning Province, China

Author contributions: Li GY, Gao HY and Lu J carried out the experiments; Li GY, Gao HY, Gu JK and Wang JH participated in the design of the study, performed the statistical analysis and drafted the manuscript; all authors read and approved the final manuscript.

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Correspondence to: Jing-Kai Gu, PhD, Research Center for Drug Metabolism, College of Life Science, Jilin University, Qianjin Street, 9 Changchun 130012, Jilin Province, China. gujk@mail.jlu.edu.cn

Telephone: +86-431-85155381 Fax: +86-431-85155380

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Abstract

AIM: To investigate the hepatoprotective effect of a *Cichorium intybus* L. extract (CIE) on CCl₄-induced hepatic fibrosis in rats.

METHODS: Seventy-two male Wistar albino rats were randomly divided into six groups of twelve rats each. The normal control group was allowed free access to food and water. Liver injury was performed in the remaining five groups with an *i.p.* injection of a 1.0 mL/kg CCl₄ and olive oil (2:3 v/v) mixture, twice weekly for 8 weeks. All rats, with the exception of the injury model group, were intragastrically (*i.g.*) administered quantum satis (*q.s.*) dosages [CIE group: 6, 18, and

54 mg/kg, respectively; Fu Fang Bie Jia Ruan Gan Pian (FFBJRGP) group: 780 mg/kg]. The oral administration of different drugs was performed on the day before CCl₄ administration and subsequently once per day for 8 wk. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), hexadecenoic acid (HA), laminin (LN), hydroxyproline (Hyp), and glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD) in the rat livers were measured. Histopathological changes in the liver were assessed for each group using HE staining and a Masson Trichrome examination. The expression of transforming growth factor- β_1 (TGF- β_1) and α -smooth muscle actin (α -SMA) was examined by immunohistochemical analysis.

RESULTS: CIE at oral doses of 6, 18, and 54 g/kg per day showed a significant hepatoprotective effect, especially at a dose of 54 g/kg per day. CIE doses reduced the levels of AST (149.04 ± 34.44 , $P < 0.01$), ALT (100.72 ± 27.19 , $P < 0.01$), HA (548.50 ± 65.09 , $P < 0.01$), LN (28.69 ± 3.32 , $P < 0.01$) and Hyp (263.33 ± 75.82 , $P < 0.01$). With regards to hepatoprotective activity, the CIE dose of 54 g/kg per day produced the largest significant effect by increasing GSH (3.11 ± 0.81), SOD (269.98 ± 33.77 , $P < 0.01$) and reducing MDA (2.76 ± 0.51 , $P < 0.01$) levels in the liver. The expressions of TGF- β_1 and α -SMA were measured by immunohistology and found to be significantly reduced by CIE in a dose-dependent manner.

CONCLUSION: CIE may effectively protect against CCl₄-induced hepatic fibrosis in rats; thus, it is a promising anti-fibrotic therapeutic agent.

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Key words: *Cichorium intybus* L. extract; Traditional Uighur medicine; Hepatic fibrosis; Carbon tetrachloride

Core tip: *Cichorium intybus* L. extract (CIE) is a traditional Uighur medicine that is commonly used in China and other Asian countries to nourish and improve the liver. The present study demonstrated that CIE has a hepatoprotective effect against CCl₄-induced hepatotoxicity in rats. We propose that the increased levels of antioxidant enzymes and reduced levels of malondialdehyde are the major mechanism of CIE for preventing the development of liver fibrosis induced by CCl₄.

Li GY, Gao HY, Huang J, Lu J, Gu JK, Wang JH. Hepatoprotective effect of *Cichorium intybus* L., a traditional Uighur medicine, against carbon tetrachloride-induced hepatic fibrosis in rats. *World J Gastroenterol* 2014; 20(16): 4753-4760 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4753.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4753>

INTRODUCTION

Cichorium intybus L. (Compositae), a traditional Uighur medicine, is a perennial herb from the asteraceae family with many commercial uses. Historically, *Cichorium intybus* L. (also known as chicory) was grown by ancient Egyptians as a medicinal plant, vegetable crop and for animal forage^[1]. In addition to its alimentary use, chicory also has a history of medicinal use. Chicory roots have antimicrobial^[2-4], anti-bacterial^[5-8], anti-diabetic^[9], immunoenhancement^[10], anti-hepatotoxic^[11-13], anti-hyperuricemia and anti-hypertriglyceridemia activities^[14,15]. Chicory has been used as a digestive aid, diuretic, laxative, and mild sedative^[16,17]. The water extract of *Cichorium intybus* L. showed a remarkable antioxidative effect on low density lipoprotein (LDL), and inhibitory effects on the production of thiobarbituric acid reactive substances and degradation of the fatty acids of LDL^[18]. The potential anti-inflammatory activities of chicory have been investigated. An ethyl acetate chicory root extract produced a marked inhibition of prostaglandin E2 (PGE2) production in human colon carcinoma HT29 cells treated with the pro-inflammatory agent TNF- α ^[19]. Additionally, hepatoprotective agents have been discovered in the seeds of the plant^[20].

Hepatic fibrosis is characterized by the excessive deposition of extracellular matrix (ECM) proteins, such as hyaluronic acid (HA), laminin (LN) and hydroxyproline (Hyp), which leads to severe pathophysiological disturbances, including remodeling of the liver architecture, development of intrahepatic shunts, liver insufficiency, portal hypertension, esophageal varices, ascites and encephalopathic coma. Despite the high incidence of hepatic fibrosis worldwide, no generally accepted anti-fibrogenic therapy is available^[21-23]. Presently, many studies are assessing potential anti-fibrogenic drugs that have been used in traditional Chinese medicine for thousands of years^[24].

The aim of the present study was to investigate the

effects of a *Cichorium intybus* L. extract (CIE) on markers of liver function in the serum, antioxidant enzyme levels and hepatic histopathology of the liver in rats with CCl₄-induced hepatotoxicity.

MATERIALS AND METHODS

Preparation of plant material

The herbs of *Cichorium intybus* L. were purchased from Shenyang and authenticated by pharmacognosist Dr. Jimin Xu. Ten kilograms of *Cichorium intybus* L. was powdered, decocted, refluxed three times with 100 L of ethanol, and then filtered. The filtrates were concentrated by rotary vacuum evaporation. The ethanolic extract was suspended in distilled water and extracted three times with the same volume of ethyl acetate. The extracts were concentrated once again by rotary vacuum evaporation and then lyophilized using a freeze dryer. The lyophilized powder was dissolved in sterilized distilled water before oral administration to experimental animals.

Experimental animals and design

Seventy-two male Wistar albino rats (180-250 g) were obtained from Shenyang Pharmaceutical University. The institutional ethics committee of Shenyang Pharmaceutical University approved all the experiments. Rats were housed in specific standard laboratory conditions for one week, including a temperature-controlled environment (25 °C \pm 2 °C), a relative humidity of 50% \pm 5%, and with a regular 12 h light/12 h dark cycle. All animals were fed with a standard rodent chow diet and water *ad libitum*. Rats weighing 180-250 g were used for the analysis of CCl₄-induced hepatotoxicity. Rats were randomly divided into six groups of twelve rats each. The normal control group was allowed free access to food and water. Liver injury was created in the remaining five groups with the *i.p.* injection of a 1.0 mL/kg CCl₄ and olive oil (2:3 v/v) mixture, twice weekly for a total of 8 weeks. All rats, with the exception of the model group, were given intragastric (*g.s.*) administration of quantum satis (*q.s.*) dosages [CIE groups: 6, 18, and 54 mg/kg; Fu Fang Bie Jia Ruan Gan Pian (FFBJRGP) group: 780 mg/kg]. The oral administration of drugs was performed the day before CCl₄ administration and then once per day for 8 wk.

Rats were euthanized by cervical dislocation the day after the last intragastric administration. Blood and liver tissue samples were harvested for further examination. The serum was obtained by centrifugation at 835 *g* for 10 min at 4 °C and then stored at -20 °C until subsequent use. Liver tissue was collected for the measurement of GSH, SOD and MDA levels, and determination of histopathological changes.

Measurement of markers of liver function

The blood was centrifuged at 835 *g* at 4 °C for 10 min to separate the serum. Markers of liver function, including aspartate transaminase (AST) and alanine transaminase (ALT), were measured with a UV spectrophotometer

Table 1 Effect of a *Cichorium intybus* L. extract on organ coefficients in rats with hepatic fibrosis

Treatment	Liver coefficient %	Spleen coefficient %	Kidney coefficient %
Control	2.18 ± 0.17	0.16 ± 0.01	0.65 ± 0.02
CCl ₄	3.59 ± 0.62 ^b	0.26 ± 0.05 ^b	0.68 ± 0.08
6 g/kg CIE + CCl ₄	3.45 ± 0.27	0.29 ± 0.07	0.67 ± 0.09
18 g/kg CIE + CCl ₄	3.20 ± 0.21 ^d	0.27 ± 0.11	0.66 ± 0.07
54 g/kg CIE + CCl ₄	3.10 ± 0.13 ^d	0.22 ± 0.04	0.65 ± 0.04
FFBJRGP (780 mg/kg)	3.38 ± 0.37	0.24 ± 0.06	0.64 ± 0.08

Values are expressed as the mean ± SD, *n* = 12. Compared with control group, ^b*P* < 0.01; Compared with CCl₄-exposed group, ^d*P* < 0.01. CIE: *Cichorium intybus* L. extract; FFBJRGP: Fu Fang Bie Jia Ruan Gan Pian.

(Shimadzu UV-2401).

Serum HA, LN and Hyp levels were measured using an enzyme-linked immunosorbent assay kit obtained from Sigma-Aldrich Chemicals Co., United States.

Measurement of lipid peroxidation by malondialdehyde formation

The detection of measurement of lipid peroxidation by malondialdehyde (MDA) using a kit estimated liver peroxidation. The liver tissue was prepared as a 10% homogenate (w/v). The tubes were kept at 95 °C for 40 min. After cooling, tubes were centrifuged at 1484 *g* for 10 min, and the supernatant was subsequently measured with a UV spectrophotometer (Shimadzu UV-2401) at 532 nm. The protein content was determined using a Coomassie brilliant blue protein kit, and the data are expressed as nmol MDA per milligram of protein of liver tissue (nmol/mg protein).

Determination of antioxidant enzyme activity

A commercially available kit from the Jiancheng Biological Engineering Institute (Nanjing, China) determined the SOD activity following the protocol provided by the manufacturer. Data were expressed as SOD U/mg protein. A GSH kit from Jiancheng Biological Engineering Institute (Nanjing, China) measured the GSH activity using a modified protocol. The reaction was measured at 420 nm, and the enzyme activity was calculated as mg/g protein.

Histopathological observations in the liver

Rat liver tissue was fixed with 10% formalin for 24 h, dehydrated with a sequence of ethanol solutions, and embedded in paraffin. Serial sections were cut at a 5-μm thickness and stained with hematoxylin-eosin (HE) and masson trichrome. Stained tissue sections were assessed for the detection of changes in the magnitude of liver injury using a photomicroscope.

Immunohistological analysis

The activation of HSCs was identified by the immunohistochemical analysis using monoclonal α-SMA and

Table 2 Effect of a *Cichorium intybus* L. extract on serum alanine aminotransferase and aminotransferase levels in rats

Treatment	AST (U/L)	ALT (U/L)
Control	60.84 ± 13.36	48.74 ± 8.98
CCl ₄	711.14 ± 165.78 ^b	738.81 ± 231.66 ^b
6 g/kg CIE + CCl ₄	194.86 ± 44.25 ^d	123.65 ± 29.93 ^d
18 g/kg CIE + CCl ₄	154.29 ± 41.97 ^d	120.18 ± 28.26 ^d
54 g/kg CIE + CCl ₄	149.04 ± 34.44 ^d	100.72 ± 27.19 ^d
FFBJRGP (780 mg/kg)	132.75 ± 34.78 ^d	71.57 ± 15.65 ^d

Values are expressed as the mean ± SD, *n* = 12. Compared with control group, ^b*P* < 0.01; Compared with CCl₄-exposed group, ^d*P* < 0.01. AST: Aminotransferase; ALT: Alanine aminotransferase; CIE: *Cichorium intybus* L. extract; FFBJRGP: Fu Fang Bie Jia Ruan Gan Pian.

TGF-β₁ antibodies (Abcam, United Kingdom) in deparaffinized tissue sections. The primary antibody was diluted to 1:50 and the biotinylated goat anti-rabbit secondary antibody was diluted to 1:100. In this experiment, we used sections that were not incubated with the primary antibody as the negative control.

Statistical analysis

All data were expressed as the mean ± SD. Data were analyzed with a one-way analysis of variance. Fisher's LSD test was used to calculate statistical significance, using SPSS software. Values of *P* < 0.05 and *P* < 0.01 were considered statistically significant.

RESULTS

The ability of CIE to attenuate CCl₄-induced increases in organ coefficients

Organ coefficients of the liver, spleen and kidney were evaluated in rats. Similar to previous studies^[25,26], liver and spleen coefficients were significantly increased in rats that were exposed to CCl₄ (*P* < 0.01); however, there was no difference in the kidney coefficient among all treated groups. As shown in Table 1, the CCl₄-induced increase in the liver coefficient was reduced by 18 and 54 mg/kg CIE (*P* < 0.01, *P* < 0.05 respectively). A non-statistically significant protective effect against an increase in the spleen coefficient was observed in rats treated with CIE (6, 18 and 54 mg/kg) and FFBJRGP treatment (780 mg/kg). Groups treated with CIE showed a dose-dependent attenuation of CCl₄-induced changes in the liver and spleen coefficients.

Effect of CIE on serum AST and ALT levels in rats

The leakage of AST and ALT in the blood indirectly reflects liver failure caused by CCl₄-induced hepatotoxicity. Table 2 shows that AST and ALT levels were significantly increased after the administration of CCl₄ compared with the control group (*P* < 0.01). Pretreatment with CIE (6, 18, and 54 g/kg) significantly reduced the elevation of AST and ALT (*P* < 0.01) compared with the CCl₄ group. Similarly, pretreatment with 780 mg/kg FFBJRGP signifi-

Table 3 Effect of a *Cichorium intybus* L. extract on serum hexadecenoic acid, laminin and hydroxyproline levels in rats

Treatment	HA (pg/mL)	LN (ng/mL)	HyP (μg/g)
Control	315.46 ± 49.54	18.27 ± 3.50	165.57 ± 21.34
CCl ₄	756.85 ± 57.82 ^b	40.47 ± 2.14 ^b	395.17 ± 94.31 ^b
6 g/kg CIE + CCl ₄	595.62 ± 76.19 ^d	27.92 ± 3.37 ^d	320.42 ± 63.35 ^d
18 g/kg CIE + CCl ₄	573.13 ± 65.42 ^d	28.17 ± 2.87 ^d	289.00 ± 89.99 ^d
54 g/kg CIE + CCl ₄	548.50 ± 65.09 ^d	28.69 ± 3.32 ^d	263.33 ± 75.82 ^d
FFBJRGP (780 mg/kg)	442.72 ± 18.84 ^d	30.70 ± 3.30 ^d	306.90 ± 51.85 ^d

Values are expressed as the mean ± SD, *n* = 12. Compared with control group, ^b*P* < 0.01; Compared with CCl₄-exposed group, ^d*P* < 0.01. HA: Hexadecenoic acid; LN: Laminin; HyP: Hydroxyproline; CIE: *Cichorium intybus* L. extract; FFBJRGP: Fu Fang Bie Jia Ruan Gan Pian.

cantly reduced the elevation of AST and ALT (*P* < 0.01).

Effect of CIE on serum HA, LN and Hyp levels in rats

The serum levels of HA, LN and Hyp were significantly increased after CCl₄ administration compared with the control group (*P* < 0.01, Table 3). Treatment with FFBJRGP (780 mg/kg) significantly decreased the serum levels of HA, LN and Hyp compared with the CCl₄ group (*P* < 0.05, *P* < 0.01). In addition, the serum levels of HA, LN and Hyp showed a dose-dependent decrease in the CIE groups (6, 18, and 54 mg/kg) compared with the CCl₄ group (*P* < 0.05, *P* < 0.01).

Effect of CIE on GSH, MDA and SOD levels in the rat liver

GSH and SOD are antioxidants that can scavenge lipid peroxide radicals. MDA is an end product of the breakdown of polyunsaturated fatty acids and related esters, and its formation is an index of lipid peroxidation in many organ homogenates. Administration of CCl₄ caused a significant decrease in the level of GSH and SOD and an increase in the MDA concentration compared with the normal control group (*P* < 0.01, Table 4). Pretreatment with 6, 18, and 54 g/kg of CIE significantly raised the level of SOD compared with the CCl₄ group (*P* < 0.01, *P* < 0.05). The concentration of GSH showed an increase after CIE (6, 18, 54 g/kg) treatment, but the effect did not reach statistical significance. In addition, rats treated with 18 and 54 g/kg of CIE showed a significant reduction in MDA levels in the liver homogenate compared with the CCl₄-exposed group (*P* < 0.01).

Effect of CIE as determined by a histopathological evaluation

Without magnification, the liver tissue from the control group was observed to be deep red, moist and glossy. In the CCl₄ group, the livers appeared swollen and had lost the luster on their surface. Pretreatment with CIE showed a dramatic and dose-dependent attenuation of the markers for liver injury (Figure 1).

Figure 2 shows a magnified view of the changes in liver histopathology from the control, CCl₄, CIE (6, 18, 54 g/kg) and FFBJRGP groups, as determined by an HE

Table 4 Effect of a *Cichorium intybus* L. extract on liver glutathione, superoxide dismutase and malondialdehyde levels in rats

Treatment	GSH (mg/gprot)	MDA (nmol/mgprot)	SOD (U/mgprot)
Control	6.58 ± 0.62	1.93 ± 0.18	334.12 ± 13.75
CCl ₄	2.52 ± 0.69 ^b	4.01 ± 1.00 ^b	193.58 ± 20.76 ^b
6 g/kg CIE + CCl ₄	2.93 ± 0.99	3.73 ± 0.61	223.23 ± 38.20 ^d
18 g/kg CIE + CCl ₄	3.03 ± 0.76	3.25 ± 0.73 ^d	241.29 ± 40.21 ^d
54 g/kg CIE + CCl ₄	3.11 ± 0.81	2.76 ± 0.51 ^d	269.98 ± 33.77 ^d
FFBJRGP (780 mg/kg)	4.44 ± 1.28 ^d	2.04 ± 0.70 ^d	274.43 ± 32.62 ^d

Values are expressed as the mean ± SD, *n* = 12. Compared with control group, ^b*P* < 0.01; Compared with CCl₄-exposed group, ^d*P* < 0.01. GSH: Glutathione; MDA: Malondialdehyde; SOD: Superoxide dismutase; CIE: *Cichorium intybus* L. extract; FFBJRGP: Fu Fang Bie Jia Ruan Gan Pian.

stain. Microscopic analysis showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein in the control group (Figure 2A). However, CCl₄-exposed tissue exhibited severe histopathological changes, such as centrilobular hepatic necrosis, Kupffer cells, ballooning degeneration and infiltrating lymphocytes (Figure 2B). Pretreatment with 780 mg/kg of FFBJRGP or CIE (6, 18, 54 g/kg) prevented the histopathological changes associated with CCl₄-induced hepatotoxicity (Figure 2C-F) to varying degrees.

The histopathological changes in fibrosis that occurred in the CCl₄ and CIE groups are shown in Figure 3. The livers of rats exposed to CCl₄ showed an extensive accumulation of connective tissue that resulted in the formation of continuous fibrotic septa, nodules of regeneration and noticeable alterations in the central vein compared with the normal control (Figure 3A and B). The groups treated with CIE and FFBJRGP showed a less pronounced destruction of the liver architecture, without fibrosis (Figure 3C-F). Based on a microscopic examination, the severe hepatic fibrosis induced by CCl₄ was markedly reduced by treatment with CIE. These data correlate with the results of the serum aminotransferase and hepatic antioxidant enzyme levels.

Immunohistochemical effects of CIE

A histological examination of the liver of rats exposed to CCl₄ revealed an increase and expansion of fibrous septa compared with normal control rats. α-SMA and TGF-β₁ expressions increased in the liver of CCl₄ rats compared with normal control rats (Figure 4A, B and Figure 5A, B). Treatment with CIE decreased α-SMA and TGF-β₁ staining (Figure 4D-F and Figure 5D-F). Similarly, α-SMA and TGF-β₁ expressions were significantly attenuated in the FFBJRGP group (Figure 4C and Figure 5C).

DISCUSSION

In the present study, CIE exhibited a hepatoprotective effect, as demonstrated by a significant decrease in AST and ALT concentrations and the prevention of histopathological changes in the liver of rats with CCl₄-

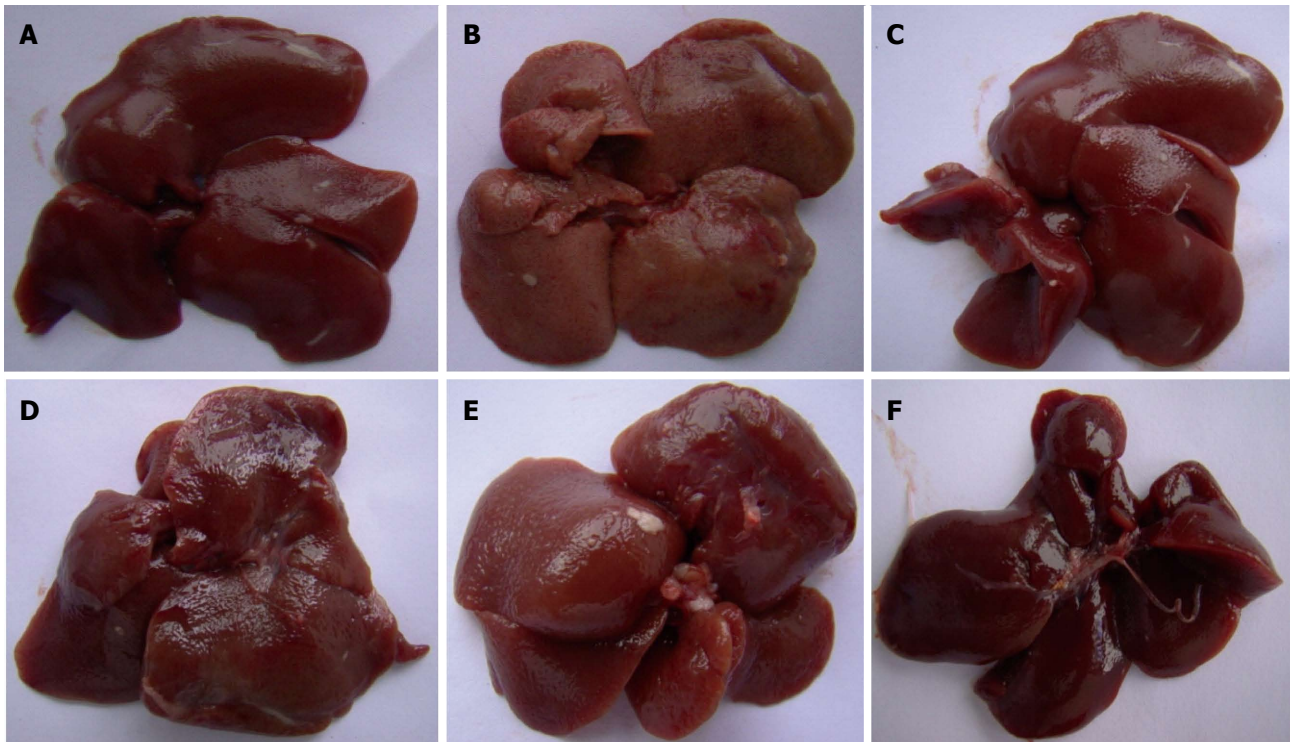


Figure 1 Liver morphology. A: Normal group; B: CCl₄-treated group; C: Positive-drug + CCl₄; D: 6 g/kg CIE + CCl₄; E: 18 g/kg CIE + CCl₄; F: 54 g/kg CIE + CCl₄. CIE: *Cichorium intybus* L. extract.

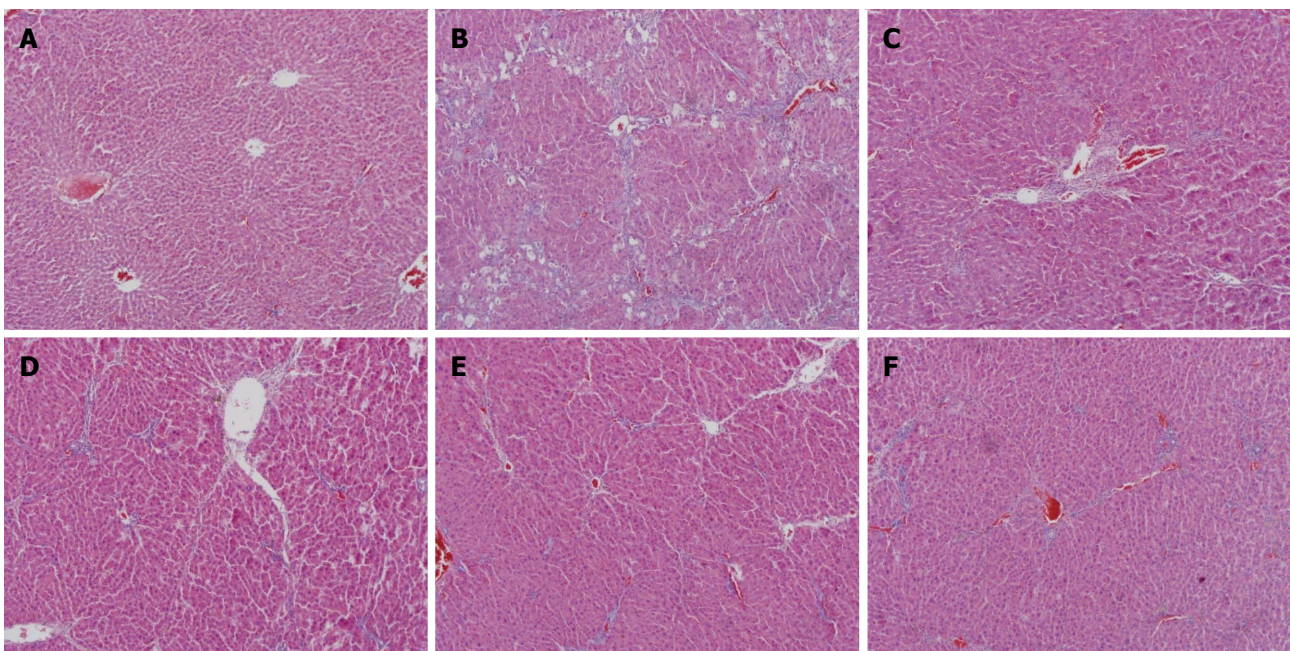


Figure 2 Hematoxylin-eosin (original magnification $\times 100$) of liver sections in rats. A: Normal group; B: CCl₄-treated group; C: Positive-drug + CCl₄; D: 6 g/kg CIE + CCl₄; E: 18 g/kg CIE + CCl₄; F: 54 g/kg CIE + CCl₄. CIE: *Cichorium intybus* L. extract.

induced hepatotoxicity. Moreover, CIE attenuated the reduction of GSH and SOD, and decreased MDA levels in rats with CCl₄-induced hepatotoxicity.

Transaminases are typically located in the cytoplasm; therefore, a decrease in the structural integrity of the liver can be reflected by an increase in the serum levels

of these enzymes. It is generally accepted that the toxicity of carbon tetrachloride depends on the cleavage of the carbon-chlorine bond to generate a trichloromethyl free radical. This free radical reacts rapidly with oxygen to form a trichloromethyl peroxy radical that may contribute to hepatotoxicity and the subsequent increase in hepatic

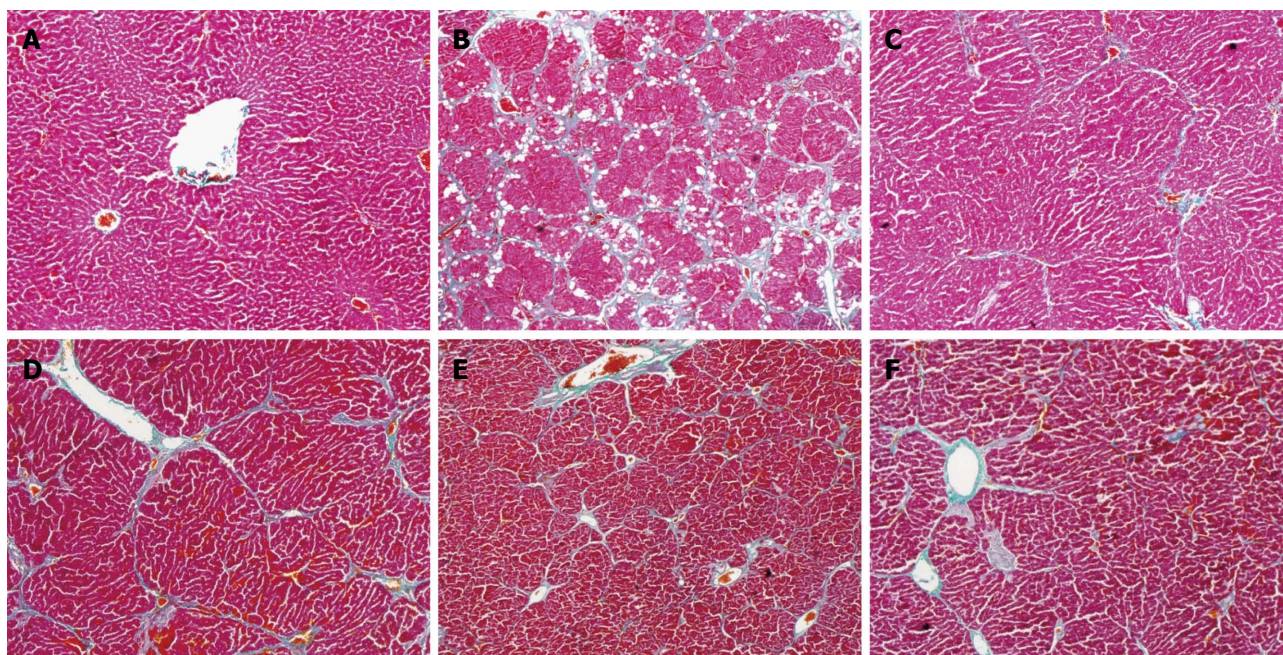


Figure 3 Masson Trichrome examinations (original magnification $\times 40$) of liver sections in rats. A: Normal group; B: CCl₄-treated group; C: Positive-drug + CCl₄; D: 6 g/kg CIE + CCl₄; E: 18 g/kg CIE + CCl₄; F: 54 g/kg CIE + CCl₄. CIE: *Cichorium intybus* L. extract.

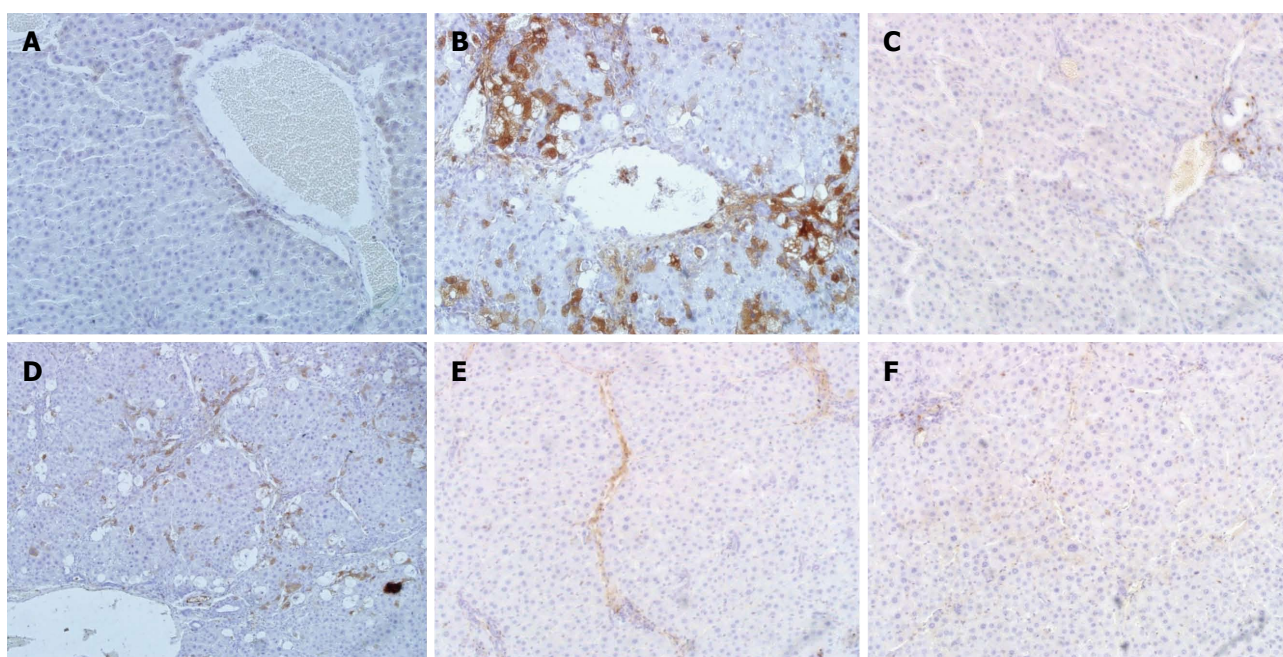


Figure 4 Immunohistochemical examination of transforming growth factor- β 1 positive cells (original magnification $\times 200$) of liver sections in rats. A: Normal group; B: CCl₄-treated group; C: Positive-drug + CCl₄; D: 6 g/kg CIE + CCl₄; E: 18 g/kg CIE + CCl₄; F: 54 g/kg CIE + CCl₄. CIE: *Cichorium intybus* L. extract.

enzymes^[27]. In the present study, the serum levels of the hepatic enzymes AST and ALT were increased, reflecting the hepatocellular damage in the CCl₄-induced injury model. However, treatment with CIE lowered the AST and ALT levels of CCl₄-exposed animals. Moreover, the liver index and serum levels of HA, LN, Hyp were increased in rats exposed to CCl₄. CIE markedly decreased the serum levels of HA, LN, Hyp and the liver index, suggesting that the extract has hepatoprotective effects.

In addition, these effects on liver function correlate with the histopathological changes observed from the microscopic examination of CIE-treated animals. Centrilobular hepatic necrosis, fatty changes, Kupffer cells, ballooning degeneration and infiltrating lymphocytes were found in rats exposed to CCl₄. Treatment with CIE prevented these histopathological changes. Thus, our results suggest that attenuating the elevation of certain markers of liver failure may be the mechanism of protective action of

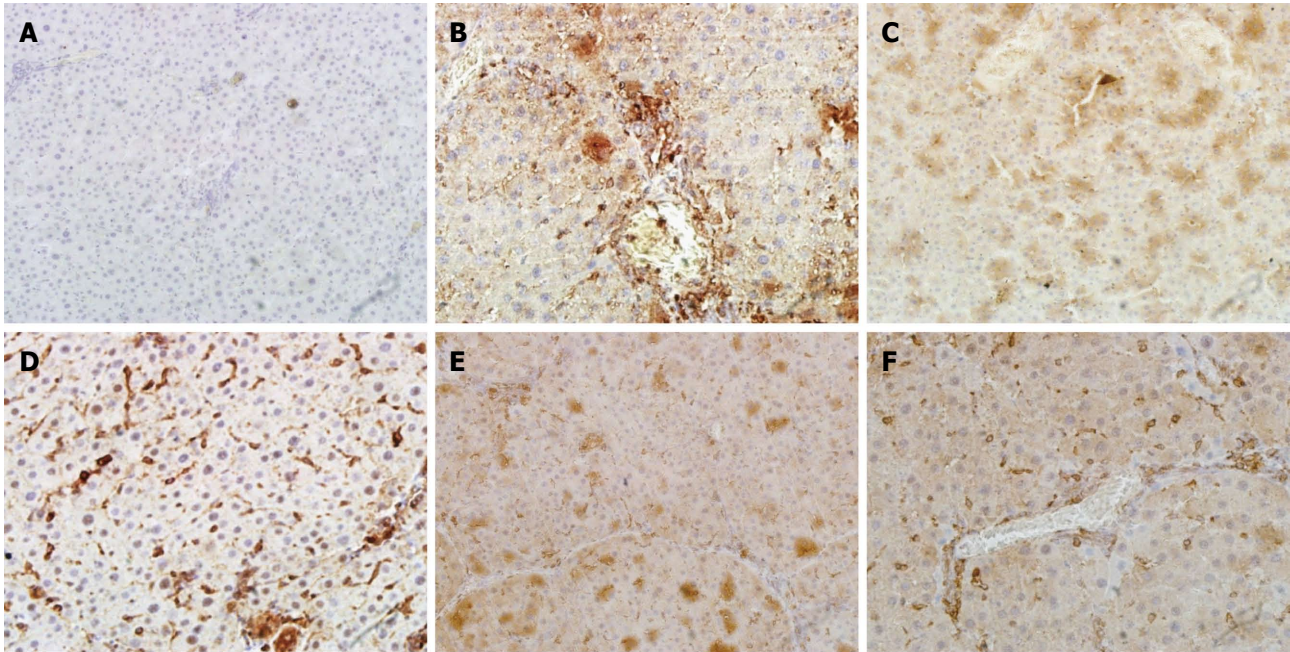


Figure 5 Immunohistochemical examination of α -smooth muscle actin positive cells (original magnification $\times 200$) of liver sections in rats. A: Normal group; B: CCl₄-treated group; C: Positive-drug + CCl₄; D: 6 g/kg CIE + CCl₄; E: 18 g/kg CIE + CCl₄; F: 54 g/kg CIE + CCl₄. CIE: *Cichorium intybus* L. extract.

CIE against CCl₄-induced hepatotoxicity.

In conclusion, we demonstrated that CIE has hepatoprotective effects against CCl₄-induced hepatotoxicity in rats. We propose that the observed enhancement of antioxidant enzymes and reduction in malondialdehyde are the major mechanisms of action of CIE in the prevention of CCl₄-induced liver fibrosis. However, additional work is required to establish the efficacy of CIE as a potent anti-hepatic fibrosis drug.

ACKNOWLEDGMENTS

We are grateful to Mr. Jimin Xu for identification of the plant materials.

COMMENTS

Background

Cichorium intybus L. (Compositae) is a traditional Uighur medicine with many commercial uses. Historically, *Cichorium intybus* L. was grown by the ancient Egyptians as a medicinal plant, vegetable crop and for animal forage. Previous studies have reported that *Cichorium intybus* L. exhibits various pharmacological effects, including anti-microbial, anti-hyperuricemia and anti-hypertriglyceridemia activities. Here, the authors investigated the effects of *Cichorium intybus* L. on markers of liver function, antioxidant enzyme levels and hepatic histopathology in the livers of rats with CCl₄-induced hepatotoxicity.

Research frontiers

Although *Cichorium intybus* L. has a long history of medicinal and alimentary use, there is no experimental research into its potential anti-hepatic fibrosis effects. This study demonstrates that this line of research is useful for the discovery of new anti-hepatic fibrosis drugs.

Innovations and breakthroughs

The present study is the first to report the anti-hepatic fibrosis effects of a *Cichorium intybus* L. extract on CCl₄-induced hepatotoxicity.

Applications

This study establishes the efficacy of the *Cichorium intybus* L. extract as a potent anti-hepatic fibrosis drug.

Terminology

Hepatic fibrosis is a dynamic process characterized by excessive deposition of extracellular matrix components, which can ultimately cause liver cirrhosis. In spite of the high incidence of hepatic fibrosis worldwide, no generally accepted anti-fibrogenic therapy is available. Current research is focused on the study of the potential anti-hepatic fibrosis effects of traditional Chinese medicines that have been used in China for thousands of years.

Peer review

This is an interesting study in which the authors use a CCl₄-induced model of hepatotoxicity to evaluate the protective effects of a *Cichorium intybus* L. extract. Interestingly, the results suggest that a *Cichorium intybus* L. extract enhances the levels of antioxidant enzymes and reduces malondialdehyde levels. These effects may be the major mechanism of action by which the *Cichorium intybus* L. extract prevents CCl₄-induced liver fibrosis.

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***Helicobacter pylori* isolates from ethnic minority patients in Guangxi: Resistance rates, mechanisms, and genotype**

Li-Juan Zhao, Yan-Qiang Huang, Bing-Pu Chen, Xiao-Qiang Mo, Zan-Song Huang, Xiao-Feng Huang, Lian-Deng Wei, Hong-Yu Wei, Yuan-Hong Chen, Hua-Ying Tang, Gan-Rong Huang, Yan-Chun Qin, Xiao-Hua Li, Lu-Yao Wang

Li-Juan Zhao, Yan-Qiang Huang, Bing-Pu Chen, Xiao-Qiang Mo, Zan-Song Huang, Xiao-Feng Huang, Lian-Deng Wei, Hong-Yu Wei, Yuan-Hong Chen, Hua-Ying Tang, Gan-Rong Huang, Yan-Chun Qin, Xiao-Hua Li, Lu-Yao Wang, Department of Medical Microbiology and Immunology, You Jiang Medical College for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

Author contributions: Huang XF, Wei LD and Wei HY performed the majority of experiments; Chen YH, Tang HY, Huang GR, Qin YC, Li XH and Wang LY provided vital reagents and analytical tools and were also involved in editing the manuscript; Chen BP, Mo XQ and Huang ZS co-ordinated and provided the collection of all the human material in addition to providing financial support for this work; Zhao LJ and Huang YQ designed the study and wrote the manuscript; Zhao LJ and Huang YQ contributed equally to this work.

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Correspondence to: Yan-Qiang Huang, Associate Professor, Department of Medical Microbiology and Immunology, You Jiang Medical College for Nationalities, 98 Countryside Road, Baise 533000, Guangxi Zhuang Autonomous Region, China. hyq77615@163.com

Telephone: +86-776-2853272 Fax: +86-776-2853272

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and subjected to drug sensitivity testing. The 23S rRNA gene of *H. pylori* isolates was amplified by PCR and analyzed by PCR-RFLP and direct sequencing to detect point mutations. REP-PCR was used for genotyping of *H. pylori* isolates, and NTsys_2 software was used for clustering analysis based on REP-PCR DNA fingerprints. Factors potentially influencing genotype distribution of *H. pylori* isolates were analyzed.

RESULTS: The rate of clarithromycin resistance was 31.3%. A2143G and A2144G mutations were detected in the 23S rRNA gene of all clarithromycin-resistant *H. pylori* isolates. At a genetic distance of 78%, clarithromycin-resistant *H. pylori* isolates could be divided into six groups. Significant clustering was noted among *H. pylori* isolates from patients with peptic ulcer or gastritis.

CONCLUSION: The rate of clarithromycin resistance is relatively high in ethnic minority patients in Guangxi. Main mechanisms of clarithromycin resistance are A2143G and A2144G mutations in the 23S rRNA gene. Clarithromycin-resistant *H. pylori* isolates can be divided into six groups based on REP-PCR DNA fingerprints. Several factors such as disease type may influence the genotype distribution of *H. pylori* isolates.

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Key words: *Helicobacter pylori*; Antibiotic resistance; Mechanism; Clarithromycin; Genotype

Abstract

AIM: To investigate the rate of *Helicobacter pylori* (*H. pylori*) resistance to clarithromycin among ethnic minority patients in Guangxi, explore the underlying mechanisms, and analyze factors influencing genotype distribution of *H. pylori* isolates.

METHODS: *H. pylori* strains were isolated, cultured

Core tip: The present study showed that the rate of *Helicobacter pylori* (*H. pylori*) resistance to clarithromycin was 31.3% in ethnic minority patients in Guangxi, slightly higher than that in Dongguan in 2009 but significantly higher than the reported resistance rate in 2008 in the same region (Guangxi). The significant increase in the rate of *H. pylori* resistance to clarithro-

mycin in this region may be caused by the long-term and/or wide use of clarithromycin, which can decrease the populations of sensitive bacteria and promote the propagation of drug-resistant bacteria. Of note, this study also found that there existed multidrug resistant *H. pylori* strains (resistant to amoxicillin, metronidazole, tetracycline, and levofloxacin).

Zhao LJ, Huang YQ, Chen BP, Mo XQ, Huang ZS, Huang XF, Wei LD, Wei HY, Chen YH, Tang HY, Huang GR, Qin YC, Li XH, Wang LY. *Helicobacter pylori* isolates from ethnic minority patients in Guangxi: Resistance rates, mechanisms, and genotype. *World J Gastroenterol* 2014; 20(16): 4761-4770 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4761.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4761>

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a frequent cause of human chronic gastritis and peptic ulcer, and is also involved in the pathogenesis of gastric cancer and mucosa-associated lymphoid tissue lymphoma^[1-4]. The wide application of antibiotics in *H. pylori* eradication therapy has led to the increasing prevalence of *H. pylori* resistance to antibiotics. Antibiotic resistance is a major cause of treatment failure. Due to the differences in doctors' prescribing habits, patients' medical history, and diets, the rates of *H. pylori* infection and antibiotic resistance vary among different countries or regions^[5-10]. Therefore, monitoring and research of antibiotic-resistant *H. pylori* strains from different regions can help not only understand the status of antibiotic resistance and guide clinical medication, but also overcome antibiotic resistance, increase the rate of eradication and avoid the emergence of drug-resistant strains. Guangxi is home to many ethnic minorities besides the Han Chinese, including Zhuang, Yao and Miao. Particularly, Zhuang has the largest population. These ethnic minorities have distinct living and eating habits. Additionally, the level of economic development and the standard of living are relatively low in this region, and there are fewer types of antibiotics available. Therefore, it is possible that the rates of *H. pylori* infection and antibiotic resistance in this region are significantly different from those in other regions of China. In the present study, we investigated the rate of *H. pylori* resistance to clarithromycin among ethnic minority patients in this region, explored the mechanism of clarithromycin resistance, and analyzed factors potentially influencing clarithromycin resistance, with an aim to reduce the rate of *H. pylori* resistance to antibiotics and improve the effect of treatment.

MATERIALS AND METHODS

H. pylori strains

Between May 2011 and May 2012, 164 gastric mucosal biopsies were collected from patients with gastritis or

peptic ulcer at the Department of Gastroscopy, the Affiliated Hospital of Youjiang Medical University for Nationalities. The samples were inoculated into Columbia medium and cultured for 5-7 d in a microaerobic bag at 37 °C. The suspected strains were confirmed as *H. pylori* by Gram-staining and urease, oxidase and catalase tests.

Drug sensitivity testing

The bacterial suspension was adjusted to a density of 1.0×10^8 CFU/mL and plated on the Columbia sheep blood agar. After the clarithromycin discs were plated, the plates were incubated at 37 °C for 5 d. The diameter of inhibition zone was measured. According to the criteria recommended by the 2012 Clinical and Laboratory Standards Institute (CLSI), strains were considered sensitive to clarithromycin when the diameter of inhibition zone was ≥ 17 mm and resistant to clarithromycin when the diameter was ≤ 13 mm.

H. pylori DNA extraction and PCR amplification

Genomic DNA was isolated from *H. pylori* cells using a commercial kit (Generay Biotech, Shanghai, China) according to the manufacturer's instructions. The A2144G and A2143G loci were amplified by PCR in a 50- μ L reaction system consisting of 29.5 μ L ddH₂O, 6.3 μ L $10 \times$ PCR buffer, 5.0 μ L dNTPs (25 mmol/L), 0.5 μ L Taq polymerase (5 kU/L), 3.7 μ L of each forward and reverse primer (10 μ mol/L), and 1.3 μ L DNA template. The primers were designed as previously described^[5] and their sequences were 5'-CCA CAG CGA TGT GGT CTCAG-3' (forward) and 5'-CTC CAT AAG AGC CAA AGCCC-3' (reverse), which yields a fragment of 425 bp. PCR cycling parameters were pre-denaturation at 94.0 °C for 4 min, 32 cycles of denaturation at 94.0 °C for 40 s, annealing at 61.5 °C for 1 min, and extension at 72.0 °C for 1 min, and a final extension at 72.0 °C for 7 min. PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet light.

PCR-RFLP

The two most common mutations associated with clarithromycin resistance (A2143G and A2144G) result in the generation of two new restriction sites for *Bbs* I and *Bsa* I. To examine whether these two mutations were present, the above PCR products (8 μ L) were incubated with *Bbs* I at 37 °C for 24 h or with *Bsa* I at 50 °C for 24 h. After enzyme digestion, the reaction products (10 μ L) were resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet light.

DNA sequencing

PCR products for 1 sensitive strain and 5 clarithromycin-resistant strains were randomly selected for DNA sequencing. DNA sequencing was performed by Generay Biotech (Shanghai, China). DNATool 6.0 program was used to analyze the 23S rRNA gene sequences of

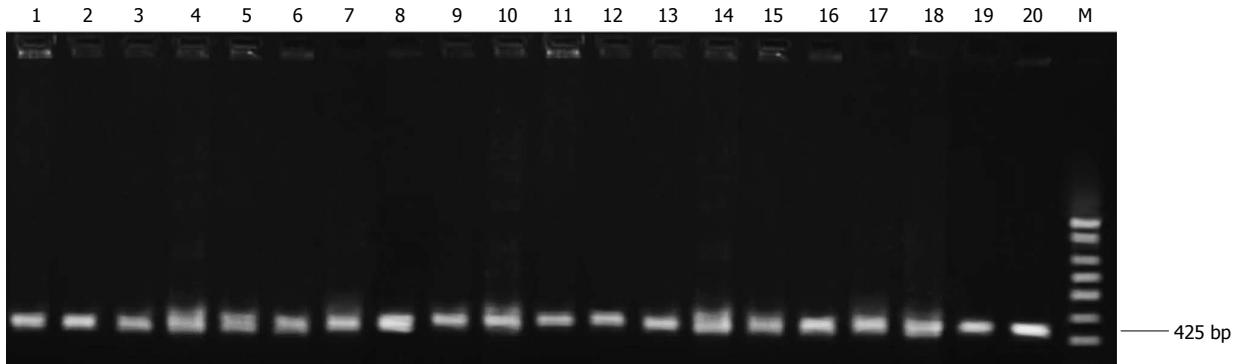


Figure 1 PCR amplification of the 23S rRNA gene of *Helicobacter pylori* strains. 1-10: Randomly selected clarithromycin-sensitive strains; 11-20: Randomly selected clarithromycin-resistant strains; M: 100 bp DNA ladder.

WT 5'-GCA AGA CGG AAA GAC CCC GTG GAC-3'

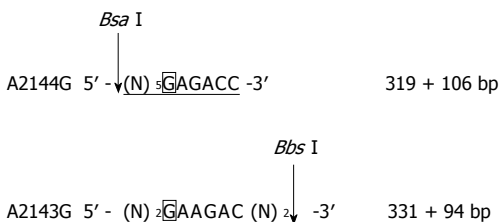


Figure 2 Two new restriction sites for *Bbs* I and *Bsa* I caused by clarithromycin resistance-associated mutations A2143G and A2144G in the 23S rRNA gene of *Helicobacter pylori* strains.

clarithromycin-sensitive and -resistant strains, and the sequences were compared with that of HPJ99 strain (NC-000921) deposited in the genome database (National Center for Biotechnology Information, NCBI).

REP-PCR

REP-PCR was performed in a 25- μ L reaction system consisting of 14.25 μ L ddH₂O, 2.5 μ L 10 \times PCR buffer, 0.5 μ L dNTPs, 0.5 μ L Taq polymerase (2 U), 0.5 μ L of each forward and reverse primer, 0.25 μ L MgCl₂ and 5 μ L DNA template. The primers were designed as previously described^[6] and their sequences were 5'-CGGIC-TAcIGCIGcIII-3' (forward) and 5'-ICGICITFATCIG-GCCTAC-3' (reverse), where I represents inosine. PCR cycling parameters were pre-denaturation at 95.0 $^{\circ}$ C for 30 s, 80 $^{\circ}$ C for 2 min, 65 cycles of denaturation at 95.0 $^{\circ}$ C for 30 s, annealing at 40 $^{\circ}$ C for 1 min, and extension at 65.0 $^{\circ}$ C for 8 min, and a final extension at 65.0 $^{\circ}$ C for 8 min. PCR products were resolved by 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet light.

Clustering analysis

Based on REP-PCR DNA fingerprints, the band was scored 1 if there was a mobility shift and 0 if there was not. NTsys_2 software was used for clustering analysis.

Statistical analysis

The rate of clarithromycin resistance is expressed as a percentage (%).

RESULTS

Bacterial isolation

A total of 115 clinical isolates were identified as *H. pylori*, of which 82 were isolated from patients with peptic ulcer and 33 from patients with gastritis.

Rate of clarithromycin resistance

Drug sensitivity testing revealed that there were 36 *H. pylori* strains that were resistant to clarithromycin, and the rate of clarithromycin resistance was 31.3% (36/115).

PCR-RFLP analysis of the 23S rRNA gene

Ten each of clarithromycin-sensitive and -resistant strains were randomly selected for PCR-RFLP analysis of the 23S rRNA gene. A 425-bp fragment of interest was amplified in all clarithromycin-sensitive and -resistant strains (Figure 1).

Clarithromycin resistance-associated mutations A2143G and A2144G result in the generation of two new restriction sites for *Bbs* I and *Bsa* I (Figure 2). Although the 425-bp fragment could be amplified from all *H. pylori* strains, only the fragment from the 10 clarithromycin-resistant strains could be digested by *Bbs* I and *Bsa* I, and the digestion resulted in the generation of two bands (319 bp and 106 bp for *Bsa* I digestion, and 331 bp and 94 bp for *Bbs* I digestion, Figure 3). This finding suggests the presence of A2143G and A2144G mutations in clarithromycin-resistant strains. In contrast, the fragment from the 10 clarithromycin-sensitive strains could not be digested by *Bbs* I and *Bsa* I, indicating the absence of A2143G and A2144G mutations in clarithromycin-sensitive strains (Figure 3). Of note, the 425-bp fragment could not be completely digested by both enzymes in all clarithromycin-resistant strains.

DNA sequencing

PCR products for 1 sensitive strain and 5 clarithromycin-resistant strains were randomly selected for DNA sequencing. The obtained DNA sequences were compared with that of HPJ99 strain deposited in the NCBI genome database. The results confirmed the presence of A2143G and A2144G mutations in clarithromycin-resistant strains,

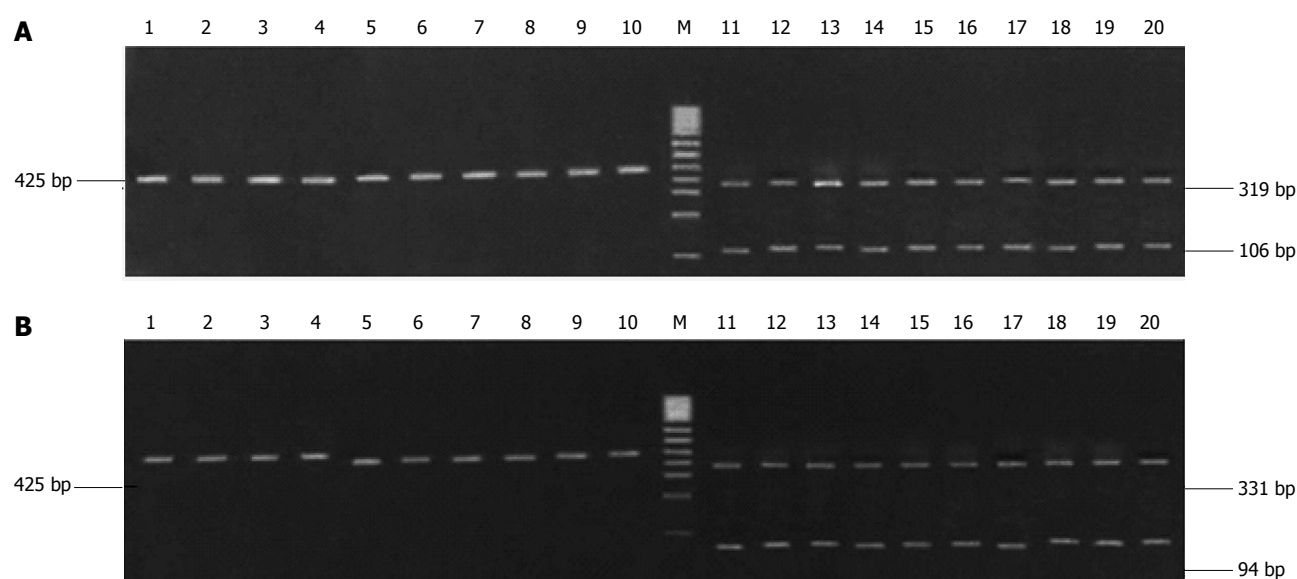


Figure 3 Digestion of the amplified fragment of the 23S rRNA gene from *Helicobacter pylori* isolates with restriction endonuclease *Bbs* I (A) and *Bsa* I (B). 1-10: Randomly selected clarithromycin-sensitive strains; 11-20: Randomly selected clarithromycin-resistant strains; M: 100 bp DNA ladder.

Table 1 Clinical characteristics of 26 patients from whom clarithromycin-resistant *Helicobacter pylori* strains were isolated

No.	Strain	Gender	Age	Ethnicity	Region	Disease type	History of medication	Family history of gastric disease	Antibiotic resistance
1	GXHP039	F	46	H	LY	PU	+	-	CA
2	GXHP051	F	46	H	BS	PU	+	+	CA
3	GXHP071	F	39	Ch	TY	CG	-	-	C
4	GXHP080	F	54	Ch	BS	CG	+	-	CAS
5	GXHP043	F	47	H	BS	PU	+	-	CA
6	GXHP093	M	46	Ch	TL	CG	-	-	CASG
7	GXHP004	F	49	Ch	TY	PU	-	-	C
8	GXHP014	F	46	Ch	BS	PU	+	-	CAS
9	GXHP016	F	57	Ch	BS	PU	-	+	CASG
10	GXHP019	M	52	Ch	LY	PU	-	+	C
11	GXHP021	M	51	Ch	TL	PU	+	-	CASG
12	GXHP023	M	48	Ch	LY	PU	+	-	CAG
13	GXHP026	M	41	Ch	TY	PU	-	-	C
14	GXHP121	M	46	H	TY	CG	+	-	C
15	GXHP085	M	53	Ch	TY	CG	-	+	C
16	GXHP034	M	51	Ch	BS	PU	-	-	CAG
17	GXHP090	M	41	Ch	BS	CG	-	-	C
18	GXHP067	M	41	H	BS	PU	+	-	C
19	GXHP056	M	39	H	TY	PU	-	+	CA
20	GXHP100	F	47	H	LY	CG	-	-	CA
21	GXHP104	F	46	H	TY	CG	+	-	CG
22	GXHP107	F	56	H	BS	CG	-	+	CAS
23	GXHP110	F	59	H	BS	CG	+	+	CAG
24	GXHP113	M	46	H	TL	CG	-	+	C
25	GXHP115	M	53	H	LY	CG	-	+	CAG
26	GXHP120	M	46	H	BS	CG	+	+	C

PU: Peptic ulcer; CG: Gastritis; Ch: Chuang; H: Han; M: Male; F: Female; BS: Baise; TY: Tianyang; LY: Lingyu; TL: Tianlin; +: A clear history; -: An unclear history; C: Clarithromycin; G: Gentamycin; S: Streptomycin; A: Amoxicillin.

but not in clarithromycin-sensitive strains (Figure 4).

REP-PCR analysis

Twenty-six each of randomly selected clarithromycin-resistant and -sensitive *H. pylori* isolates and the standard strain ATCC43504 were subjected to REP-PCR analysis, and the results are shown in Tables 1 and 2 and Figure 5.

Clustering analysis

The NTsys-2 software was used to analyze the similarity among the randomly selected clarithromycin-resistant and -sensitive *H. pylori* isolates. At a genetic distance of 78%, 26 clarithromycin-resistant *H. pylori* isolates could be classified into 6 genotypes (Figure 6), and 26 clarithromycin-sensitive *H. pylori* isolates could be classified into 4

J99 1428504	CCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGTGACGCCTGCCCGG
S 1	CCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGTGACGCCTGCCCGG
R1 1	CCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGTGACGCCTGCCCGG
R2 1	CCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGTGACGCCTGCCCGG
R3 1	CCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGTGACGCCTGCCCGG
R4 1	CCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGTGACGCCTGCCCGG
R5 1	CCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGTGACGCCTGCCCGG
J99 1428324	GACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCAGTGAAATTG
S 81	GACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCAGTGAAATTG
R1 81	GACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCAGTGAAATTG
R2 81	GACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCAGTGAAATTG
R3 81	GACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCAGTGAAATTG
R4 81	GACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCAGTGAAATTG
R5 81	GACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCAGTGAAATTG
J99 1428264	TAGTGGAGGTGAAAAATTCCTCCTACCCGCGGCAAGACGGAAAGACCCCGTGACCTTTAC
S 241	TAGTGGAGGTGAAAAATTCCTCCTACCCGCGGCAAGACGGAAAGACCCCGTGACCTTTAC
	A2143G, A2144G
	↓
R1 241	TAGTGGAGGTGAAAAATTCCTCCTACCCGCGGCAAGACGGAGGGACCCCGTGACCTTTAC
R2 241	TAGTGGAGGTGAAAAATTCCTCCTACCCGCGGCAAGACGGAGGGACCCCGTGACCTTTAC
R3 241	TAGTGGAGGTGAAAAATTCCTCCTACCCGCGGCAAGACGGAGGGACCCCGTGACCTTTAC
R4 241	TAGTGGAGGTGAAAAATTCCTCCTACCCGCGGCAAGACGGAGGGACCCCGTGACCTTTAC
R5 241	TAGTGGAGGTGAAAAATTCCTCCTACCCGCGGCAAGACGGAGGGACCCCGTGACCTTTAC
J99 1428204	TACAACCTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTA
S 301	TACAACCTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTA
R1 301	TACAACCTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTA
R2 301	TACAACCTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTA
R3 301	TACAACCTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTA
R4 301	TACAACCTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTA
R5 301	TACAACCTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTA
J99 1428144	AGGGCTTTGGCTCTTATGGAG
S 361	AGGGCTTTGGCTCTTATGGAG
R1 361	AGGGCTTTGGCTCTTATGGAG
R2 361	AGGGCTTTGGCTCTTATGGAG
R3 361	AGGGCTTTGGCTCTTATGGAG
R4 361	AGGGCTTTGGCTCTTATGGAG
R5 361	AGGGCTTTGGCTCTTATGGAG

Figure 4 Comparison of the 23S rRNA gene sequence of *Helicobacter pylori* isolates with that of HPJ99 strain. S: A clarithromycin-sensitive isolate; R1-5: clarithromycin-resistant strains.

genotypes (Figure 6).

Factors influencing genotype distribution of *H. pylori* isolates

Factors influencing genotype distribution of *H. pylori* isolates may include disease type, ethnicity, gender, age, region, history of antibiotic medication, history of gastric diseases, and multidrug resistance. The frequencies of the presence of these factors in the patients are presented in Tables 3 and 4.

DISCUSSION

Rate of *H. pylori* resistance to clarithromycin in ethnic minority patients in Guangxi

Antibiotic resistance is a main factor affecting therapeutic effects in patients with *H. pylori* infection. Particularly, the rates of *H. pylori* resistance to clarithromycin and metronidazole have been increasing year by year, and accordingly, the rate of *H. pylori* eradication achieved

with regimens containing either of the two antibiotics becomes lower and lower. The rate of *H. pylori* resistance to antibiotics varies among different countries or regions, although it shows an upward trend^[11-13]. From 1996 to 2004 in Japan, the prevalence of *H. pylori* resistance to clarithromycin has increased to 30%^[16]. In Vietnam in 2008^[17], the rate of clarithromycin resistance was 33%, and the rate in Ho Chi Minh City (49%) was obviously higher than that in Hanoi (18.5%). In China, the rate of *H. pylori* resistance to antibiotics varies among different regions. For example, a study on *H. pylori* resistance to antibiotics in Beijing, Shanghai and Wenzhou^[18] showed that the rate of resistance to metronidazole was highest, followed by clarithromycin. In 2009 in Dongguan, the rate of *H. pylori* resistance to clarithromycin was 27.6%^[19].

The present study showed that the rate of *H. pylori* resistance to clarithromycin was 31.3% in ethnic minority patients in Guangxi, slightly higher than that in Dongguan in 2009 but significantly higher than the reported resistance rate in 2008 in the same region (Guangxi). The

Table 2 Clinical characteristics of 26 patients from whom clarithromycin-sensitive *Helicobacter pylori* strains were isolated

No.	Strain	Gender	Age	Ethnicity	Region	Disease type	History of medication	Family history of gastric disease
1	GXHP069	M	46	Ch	TL	CG	+	+
2	GXHP070	M	43	Ch	LY	CG	+	+
3	GXHP096	F	36	Ch	BS	CG	-	+
4	GXHP119	F	48	H	TY	CG	-	-
5	GXHP094	F	46	Ch	BS	CG	+	+
6	GXHP074	M	47	Ch	TL	CG	+	-
7	GXHP116	F	46	H	BS	CG	-	+
8	GXHP075	M	45	Ch	LY	CG	-	-
9	GXHP076	M	48	Ch	TY	CG	-	+
10	GXHP079	M	53	Ch	TL	CG	+	+
11	GXHP078	M	52	Ch	BS	CG	+	+
12	GXHP101	M	42	H	BS	CG	-	+
13	GXHP102	M	56	H	BS	CG	+	-
14	GXHP005	M	35	Ch	BS	PU	-	+
15	GXHP006	M	41	Ch	TL	PU	-	+
16	GXHP007	M	60	Ch	TL	PU	+	+
17	GXHP008	M	38	Ch	BS	PU	-	-
18	GXHP024	F	50	Ch	TL	PU	+	+
19	GXHP025	F	39	Ch	BS	PU	-	-
20	GXHP027	F	59	Ch	BS	PU	-	-
21	GXHP040	M	51	H	BS	PU	+	+
22	GXHP041	M	52	H	BS	PU	+	+
23	GXHP052	F	53	H	BS	PU	-	-
24	GXHP053	F	47	H	BS	PU	+	+
25	GXHP054	F	49	H	BS	PU	-	-
26	GXHP050	M	72	H	LY	PU	-	+

PU: Peptic ulcer; CG: Gastritis; Ch: Chuang; H: Han; M: Male; F: Female; BS: Baise; TY: Tianyang; LY: Lingyu; TL: Tianlin; +: A clear history; -: An unclear history.

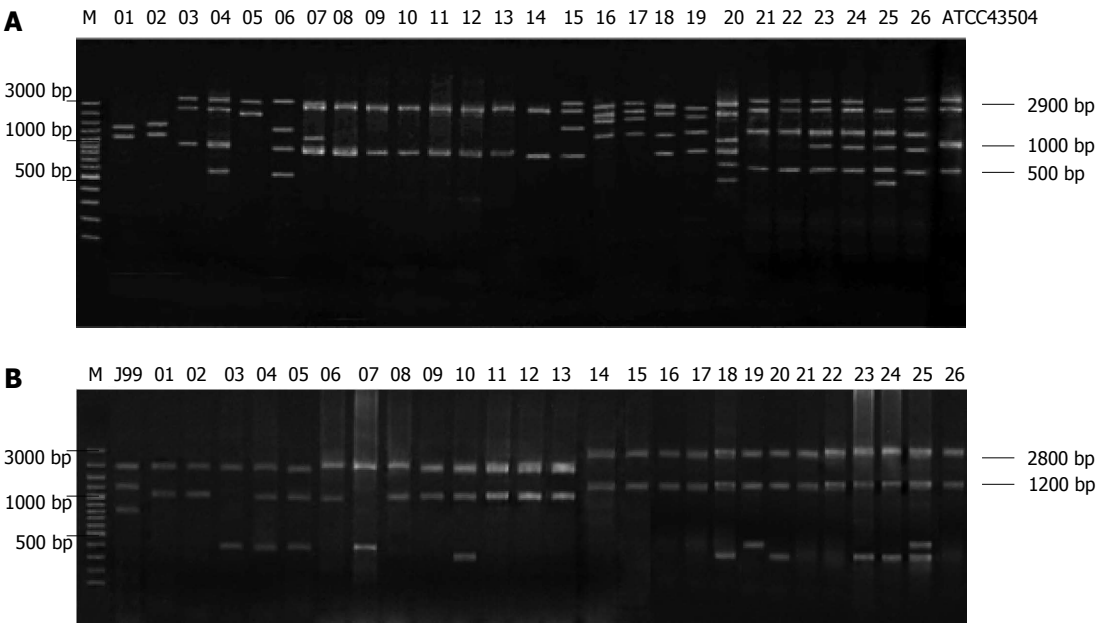


Figure 5 REP-PCR analysis of 26 clarithromycin-resistant (A) and clarithromycin-sensitive (B) *Helicobacter pylori* isolates. M: λ DNA/EcoR I-Hind III marker; 1-26: Clarithromycin-resistant *Helicobacter pylori* isolates; ATCC43504: A standard *Helicobacter pylori* strain.

significant increase in the rate of *H. pylori* resistance to clarithromycin in this region may be caused by the long-term and/or wide use of clarithromycin, which can decrease the populations of sensitive bacteria and promote the propagation of drug-resistant bacteria. Of note, this

study also found that there existed multidrug resistant *H. pylori* strains (resistant to amoxicillin, metronidazole, tetracycline, and levofloxacin)^[20]. Therefore, we recommend that, in order to improve the rate of *H. pylori* eradication, tests for *H. pylori* antibiotic resistance should be

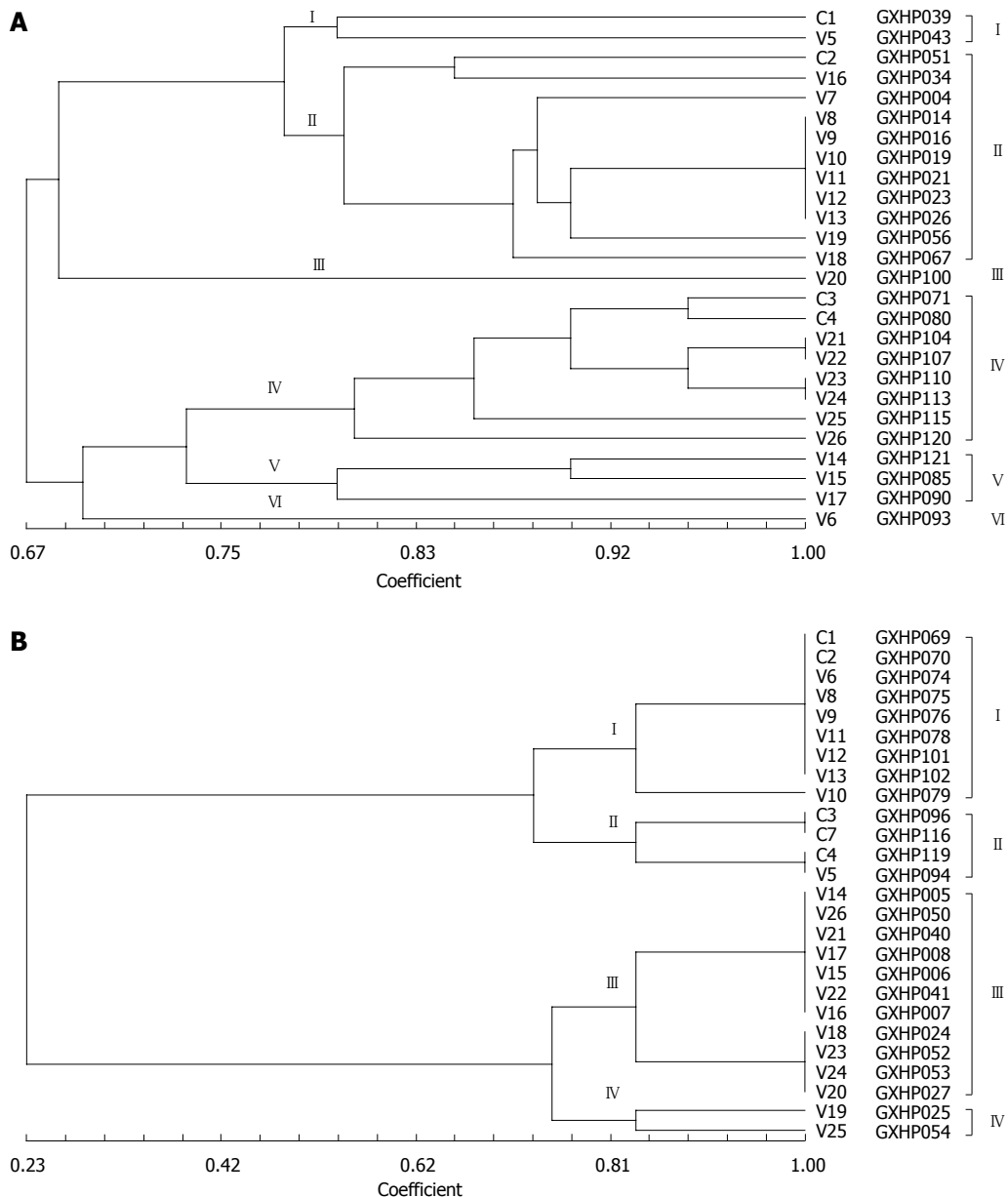


Figure 6 Dendrogram of REP-PCR DNA fingerprints for clarithromycin-resistant *Helicobacter pylori* strains. A: 6 genotypes; B: 4 genotypes.

Table 3 Frequencies of the presence of factors influencing genotype distribution of clarithromycin-resistant strains

	CG	PU	Ch	H	M	F	TY	TL	LY	BS	HMR	HM	FHGD	30-40 yr	41-50 yr	51-60 yr
Group I	0	2	0	2	2	0	0	0	1	1	2	2	0	0	2	0
Group II	0	11	8	3	4	7	3	1	2	5	4	5	4	1	6	4
Group III	1	0	0	1	1	0	0	0	1	0	1	1	1	0	1	0
Group IV	8	0	2	6	5	3	2	1	1	4	4	4	5	1	3	4
Group V	3	0	2	1	0	3	2	0	0	1	1	1	1	0	2	1
Group VI	1	0	1	0	0	1	0	1	0	0	1	0	0	0	1	0

CG: Gastritis; PU: Peptic ulcer; Ch: Chuang; H: Han; M: Male; F: Female; TY: Tianyang; TL: Tianlin; LY: Lingyu; BS: Baise; HMR: History of multidrug resistance; HM: History of medication; FHGD: Family history of gastric disease.

established in this region, sensitive antibiotics should be selected based on antibiotic sensitivity of *H. pylori* strains, and the research on the mechanisms of antibiotic resistance should be enhanced.

Mechanisms of *H. pylori* resistance to clarithromycin in ethnic minority patients in Guangxi

Clarithromycin, as a new generation of macrolide antibiotic, is acid-stable and can be dissolved in gastric

Table 4 Frequencies of the presence of factors influencing genotype distribution of clarithromycin-sensitive strains

	CG	PU	Ch	H	M	F	TY	TL	LY	BS	HM	FHGD	30-40 yr	41-50 yr	51-60 yr
Group I	9	0	7	2	0	9	1	2	2	4	2	6	0	6	3
Group II	4	0	2	2	4	0	1	0	0	3	1	3	1	3	0
Group III	0	11	5	6	4	7	1	2	1	7	3	7	3	3	5
Group IV	0	2	1	1	2	0	1	0	0	1	0	0	1	1	0

CG: Gastritis; PU: Peptic ulcer; Ch: Chuang; H: Han; M: Male; F: Female; TY: Tianyang; TL: Tianlin; LY: Lingyu; BS: Baise; HM: History of medication; FHGD: Family history of gastric disease.

juice that has a low pH level. Therefore, it has good oral bioavailability and its concentration is high in the gastric mucosa. Additionally, clarithromycin has few adverse reactions. Since clarithromycin monotherapy can achieve an eradication rate between 42%-54%, it is one of the currently known antibiotics that have the strongest effect on *H. pylori*. Compared with triple therapy without clarithromycin, clarithromycin-containing triple therapy can increase the rate of *H. pylori* eradication by 10%-20%. Therefore, clarithromycin is the main antibiotic used in regimens for *H. pylori*. However, significant resistance of *H. pylori* to clarithromycin has been observed, and the rate of clarithromycin resistance has increased, especially after an initial failure^[21-23]. Clarithromycin exerts antibacterial effects by penetrating the bacteria cell wall, binding to the domain V of the 23S ribosomal RNA of the 50S subunit of the bacterial ribosome, inhibiting peptidyl transferase activity, interfering with amino acid translocation and thereby suppressing bacterial protein synthesis. With regard to the mechanisms of *H. pylori* resistance to clarithromycin, the consensus view is point mutations in the domain V of the 23S ribosomal RNA, which reduce the binding force between clarithromycin and the ribosome. Most of the discovered mutations are A to G transition mutations, and currently known mutations include A2144G, A2143G, A2142G, A2142C, G2115A, G2141A, A2142T and A2143C. Some mutations can occur even in other chromosomal segments. Since the minimum inhibitory concentrations of clarithromycin are higher in *H. pylori* strains bearing A2144G and A2143G mutations, these strains have more stable resistance and higher growth rate^[24-26]. Therefore, A2143G and A2144G mutations are most common in clinically isolated clarithromycin-resistant *H. pylori* strains. However, point mutations in clarithromycin-resistant *H. pylori* strains can vary among different regions and different ethnicity groups.

This study detected the A2143G and A2144G mutations in the 23S rRNA gene in *H. pylori* isolates using PCR-RFLP, which is a simple and high-sensitivity method. Both mutations were detected in all 10 randomly selected clarithromycin-resistant isolates, but not in clarithromycin-sensitive isolates. Direct DNA sequencing of the 23S rRNA gene in 5 randomly selected clarithromycin-resistant isolates confirmed the above finding. Therefore, A2143G and A2144G mutations are closely related to *H. pylori* resistance to clarithromycin in ethnic minority patients in Guangxi. Future studies should develop methods to repair or avoid these mutations to reduce the rate

of clarithromycin resistance and improve the effectiveness of prevention and control.

Genotype variation in *H. pylori* isolates in Guangxi and factors influencing genotype distribution

To investigate the association of genotypes of *H. pylori* isolates from ethnic minority patients in Guangxi with disease type, ethnicity and multidrug resistance, REP-PCR was used to genotype clarithromycin-resistant *H. pylori* isolates from patients of different ethnicity or with different disease types, and the results showed that strains isolated from different patients had different DNA fingerprints (Figure 5A).

The NTsys_2 software was used to perform clustering analysis. The dendrogram of REP-PCR DNA fingerprints for clarithromycin-resistant *H. pylori* strains (Figure 6A) showed a similarity of 100% among GXHP014, GXHP016, GXHP019, GXHP021, GXHP023 and GXHP026; between GXHP104 and GXHP107; and between GXHP110 and GXHP113. These three groups shared a similarity of 90%. In addition, GXHP104 and GXHP107 had a similarity of 95.2% to GXHP110 and GXHP113. At a genetic distance of 78%, the 26 strains of clarithromycin-resistant *H. pylori* were divided into 6 groups, which are as follows: (1) group I: This group includes GXHP039 and GXHP043, which were isolated from a woman with peptic ulcer in Baise and a woman with the same disease in Lingyun, respectively. The two women, ranging in age between 41 and 50 years old, had a history of clarithromycin use, but their family history of gastric disease was unknown. The two strains were also resistant to amoxicillin, streptomycin, and gentamicin; (2) group II: This group includes GXHP051, GXHP034, GXHP004, GXHP014, GXHP016, GXHP019, GXHP021, GXHP023, GXHP026, GXHP056 and GXHP067. All the 11 strains were isolated from patients with peptic ulcer. GXHP034, GXHP004, GXHP014, GXHP016, GXHP019, GXHP021, GXHP023, GXHP026 and GXHP056 came from 9 Zhuang patients (6 females and 3 males) in Baise, Tianlin, Lingyun, or Tianyang. They had an unknown history of medication or a family history of gastric diseases. GXHP014, GXHP019, GXHP021 and GXHP026 were also resistant to amoxicillin and streptomycin. GXHP051, GXHP056 and GXHP067 came from 3 Han patients in Baise or Tianlin, all of whom had a history of clarithromycin use and a family history of gastric disease. These three strains were also resistant to amoxicillin, streptomycin and gen-

tamicin. In this group, one patient was in the 30-40 age group, 6 in the 41-50 age group, and 4 in the 51-60 age group; (3) group III: This group includes only GXHP100. This strain came from a Han male with chronic gastritis in Lingyun. He had a history of clarithromycin use and family history of gastric disease. The strain was also resistant to amoxicillin. The patient was in the 41-50 age group; (4) group IV: This group includes GXHP071, GXHP080, GXHP104, GXHP107, GXHP110, GXHP113, GXHP115 and GXHP120. The 8 strains came from 8 patients with chronic gastritis (5 males and 3 females) in Baise, Tianlin, Lingyun, or Tianyang. There were 6 Han patients and 2 Zhuang patients. Four patients had a history of clarithromycin use and five patients had a family history of gastric diseases. Four strains were also resistant to amoxicillin and gentamicin. In this group, one patient was in the 30-40 age group, 3 in the 41-50 age group, and 4 in the 51-60 age group; (5) group V: This group includes GXHP085, GXHP090 and GXHP121. All 3 strains came from 3 females with chronic gastritis in Baise or Tianlin. There were 1 Han patient and 2 Zhuang patients. One patient had both a history of clarithromycin use and a family history of gastric diseases. One strain was also resistant to amoxicillin and gentamicin. In this group, 2 patients were in the 41-50 age group, and 1 in the 51-60 age group; and (6) group VI: This group includes only GXHP093. This strain came from a Zhuang female with chronic gastritis in Tianlin. She had an unknown history of clarithromycin use or family history of gastric diseases. The strain was also resistant to amoxicillin and streptomycin. The patient was in the 41-50 age group.

Based on the above data, it can be found that factors influencing genotype distribution include disease type, ethnicity, gender, age, region, history of medication, family history of gastric disease, and multidrug resistance. The frequencies of the presence of these factors influencing genotype distribution of clarithromycin-resistant strains are shown in Table 3. Main factors influencing genotype distribution of clarithromycin-resistant strains may be: (1) disease type: Gastritis and peptic ulcer were main influencing factors, because group II and group IV strains were isolated from patients with gastritis and those with peptic ulcer, respectively; (2) ethnicity: Zhuang and Han were main influencing factors, because GXHP014, GXHP016, GXHP019, GXHP021, GXHP023 and GXHP026, which shared a similarity of 100%, were all isolated from Zhuang patients; GXHP104 and GXHP107, which shared a similarity of 100%, as well as GXHP110 and GXHP113, which also shared a similarity of 100%, were all isolated from Han patients; (3) family history of gastric disease: Since GXHP104 and GXHP107, which shared a similarity of 100%, were isolated from Han patients with a family history of gastric diseases, while GXHP110 and GXHP113, which also shared a similarity of 100%, were isolated from Han patients without a family history of gastric diseases, we speculate that family history of gastric disease may be

associated with the genotype distribution; (4) multidrug resistance: Since the frequency of the presence of multidrug resistance is high (50%-100%) in *H. pylori* isolates of various genotypes, there may exist an association between multidrug resistance and the genotype distribution.

We also analyzed the genotypes of clarithromycin-sensitive *H. pylori* strains using the same method and found that the main factors influencing the genotype distribution of clarithromycin-sensitive strains were disease type and patient gender (Figures 5B and 6B, Table 2). Compared with clarithromycin-resistant strains, the genotype distribution of clarithromycin-sensitive strains was influenced by fewer factors. This discrepancy may be partly explained by the genetic mutations in clarithromycin-resistant strains. In addition, we found that region, age and history of medication had relatively small impact on genotype distribution. However, since the number of strains analyzed in the present study is relatively small, we could not use statistical methods to analyze the influence of various factors on genotype distribution. Future studies should carefully address this issue.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a frequent cause of human chronic gastritis and peptic ulcer, and is also involved in the pathogenesis of gastric cancer and mucosa-associated lymphoid tissue lymphoma. The wide application of antibiotics in *H. pylori* eradication therapy has led to the increasing prevalence of *H. pylori* resistance to antibiotics. Antibiotic resistance is a major cause of treatment failure.

Research frontiers

Antibiotic resistance is a main factor affecting therapeutic effects in patients with *H. pylori* infection. Particularly, the rates of *H. pylori* resistance to clarithromycin and metronidazole have been increasing year by year, and accordingly, the rate of *H. pylori* eradication achieved with regimens containing either of the two antibiotics becomes lower and lower.

Applications

The rate of clarithromycin resistance is relatively high in ethnic minority patients in Guangxi. Main mechanisms of clarithromycin resistance are A2143G and A2144G mutations in the 23S rRNA gene. Clarithromycin-resistant *H. pylori* isolates can be divided into six groups based on REP-PCR DNA fingerprints. Several factors such as disease type may influence the genotype distribution of *H. pylori* isolates.

Peer review

The manuscript is very interesting. The authors try to investigate the rate of *H. pylori* resistance to clarithromycin among ethnic minority patients in Guangxi, the home to many ethnic minorities besides the Han Chinese. The research is well designed. The data are very interesting.

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Intravenous infusion of mesenteric lymph from severe intraperitoneal infection rats causes lung injury in healthy rats

Yan-Min Zhang, Shu-Kun Zhang, Nai-Qiang Cui

Yan-Min Zhang, Tianjin Medical University, Intensive Care Unit, Tianjin Nankai Hospital, Tianjin 300100, China
Shu-Kun Zhang, Tianjin Nankai Hospital, Tianjin 300100, China
Nai-Qiang Cui, Department of Surgery, Tianjin Nankai Hospital, Tianjin 300100, China

Author contributions: Zhang YM performed the majority of experiments and wrote the manuscript; Zhang SK provided vital reagents and analytical tools; Cui NQ designed the study and revised the manuscript.

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Correspondence to: Nai-Qiang Cui, Chief, Department of Surgery, Tianjin Nankai Hospital, 6 Changjiang Road, Nankai District, Tianjin 300100, China. summer9531@163.com
Telephone: +86-22-23746095 Fax: +86-22-27435365

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Abstract

AIM: To investigate whether mesenteric lymph from rats with severe intraperitoneal infection (SII) induces lung injury in healthy rats.

METHODS: Twenty adult male specific pathogen-free Wistar rats were divided into two groups. Animals in the SII group received intraperitoneal injection of *Escherichia coli* (*E. coli*) at a dose of 0.3 mL/100 g. Control rats underwent the same procedure, but were injected with normal saline rather than *E. coli*. We ligated and drained the mesenteric lymphatic vessels and collected the mesenteric lymph. Mesenteric lymph collected from SII or control rats was infused intravenously into male healthy rats at a rate of 1 mL/h for 4 h. At the end of the infusion, all rats were sacrificed. Lungs were removed and examined histologically, and wet-to-dry weight (W/D) ratio and myeloperoxidase (MPO) activity were determined. Enzyme-linked immunosorbent assay (ELISA) was performed to determine the levels

of the proinflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-6. We performed Western blot to investigate the activation of Toll-like receptor (TLR)-4, and nuclear factor (NF)- κ B p65.

RESULTS: Compared with the control infusion group, there were obvious pathological changes in the SII group. The W/D ratio was significantly increased in the SII compared to control infusion group (5.86 ± 0.06 vs 5.37 ± 0.06 , $P < 0.01$). MPO activity significantly increased in the SII infusion rats with a mean level of 0.86 ± 0.02 U/g compared to 0.18 ± 0.05 U/g in the control group ($P < 0.01$). The concentrations of TNF- α and IL-6 were significantly increased in the SII infusion group. The concentration of TNF- α was significantly increased in the SII infusion rats compared to control infusion rats (2104.46 ± 245.91 vs 1475.13 ± 137.82 pg/mL, $P < 0.01$). The concentration of IL-6 was significantly increased in the SII infusion rats with a mean level of 50.56 ± 2.85 pg/mL compared to 43.29 ± 2.02 pg/mL ($P < 0.01$). The expression levels of TLR-4 (7496.68 ± 376.43 vs 4589.02 ± 233.16 , $P < 0.01$) and NF- κ B (8722.19 ± 323.96 vs 6498.91 ± 338.76 , $P < 0.01$) were significantly increased in the SII infusion group compared to the control infusion group. The infusion of SII lymph, but not control lymph, caused lung injury.

CONCLUSION: The results indicate that SII lymph is sufficient to induce acute lung injury.

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Key words: Severe intraperitoneal infection; Mesenteric lymph; Acute lung injury; Toll-like receptor 4; Nuclear factor κ B

Core tip: We previously speculated that the lymphatic pathway plays a leading role in the early lung injury

caused by severe intraperitoneal infection (SII), and that the mesenteric lymph may be the original source of organ damage. Here, we infused mesenteric lymph from rats with SII into healthy rats to investigate the effect on lung tissues. We confirmed that the damage to the remote organ was caused *via* the mesenteric lymphatic pathway.

Zhang YM, Zhang SK, Cui NQ. Intravenous infusion of mesenteric lymph from severe intraperitoneal infection rats causes lung injury in healthy rats. *World J Gastroenterol* 2014; 20(16): 4771-4777 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4771.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4771>

INTRODUCTION

For intensive care unit (ICU) patients, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are the most common and life-threatening diseases^[1]. In the field of abdominal surgery, severe intraperitoneal infection (SII) caused by some primary diseases, such as perforation peritonitis, severe acute pancreatitis, biliary tract infection, or celiac abscess, is known as the main cause of sepsis or multiple organ dysfunction syndrome (MODS). According to the intestinal lymphatic hypothesis of SIRS/MODS proposed by Deitch *et al*^[2], early in intraperitoneal infection, endotoxin and endogenous inflammatory mediators can enter mesenteric lymphatic vessels and then the lacteal and systemic circulation *via* the thoracic duct.

Septic peritonitis induced by SII is a clinically relevant polymicrobial sepsis model in rodents^[3-5]. Multiple Toll-like receptor (TLR)-dependent pathways are activated during sepsis^[6]. Within the TLR family, TLR-4 appears to have a prominent role in the pathogenesis of microbial as well as sterile inflammatory states^[7]. Endotoxin signaling is mainly *via* TLR-4. Endotoxin binds to TLR-4 and leads to activation of nuclear factor (NF)- κ B to induce the production of proinflammatory cytokines^[8].

We have previously studied endotoxin distribution in the viscera and body fluids in rats with intraperitoneal infection after translocation of endogenous endotoxin. The level of endotoxin in the thoracic duct lymph was significantly higher than that in the portal vein blood^[9], and blocking the backflow of abdominal lymph can attenuate ALI in SII rats^[10]. Thus, the lymphatic but not portal vein pathway is speculated to play the leading role in the early lung injury caused by SII, and at the same time, the lymph in the thoracic duct may be the original source of organ damage.

In the present study, we infused mesenteric lymph from rats with SII into healthy rats, and examined its effect on lung tissues. We aimed to confirm whether damage to the remote organ was caused *via* the mesenteric lymphatic pathway, and whether the lymph from SII rats was sufficient to cause lung injury.

MATERIALS AND METHODS

Animals

Twenty adult male specific pathogen-free Wistar rats were purchased from the Chinese Academy of Military Medical Sciences [Animal license for SCXK (Army), 2009-003]. The animals (250-300 g) were maintained in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the Tianjin Nankai Hospital Animal Care and Use Committee.

Experimental design

This study aimed to test whether SII mesenteric lymph was sufficient to induce lung injury. Mesenteric lymph samples collected from both control and SII rats was infused into different healthy rats. In the initial experiment, lymph was collected from the SII or control group for 4 h after the end of the infection period. The collected lymph specimens were centrifuged at 2000 rpm at 4 °C for 10 min and stored at -80 °C. The collected SII and control lymph specimens were infused intravenously into rats at a rate of 1 mL/h for 4 h. The 20 rats were divided equally into the SII infusion and control infusion groups. The volume of lymph infusion was 0.35 mL/100 g, which was based on the fact that the total lymph was produced by the rats during the entire lymph collection period. At the end of the 4-h infusion, the rats were killed and lung tissues were harvested to assess injury.

SII and lymph cannulation models

After a 7-d acclimatization period, rats underwent mesenteric lymph duct cannulation, followed by SII or control infusion, as previously described. The SII group received intraperitoneal injection of *Escherichia coli* (*E. coli*) at a dose of 0.3 mL/100 g. Two hours after injection of *E. coli*, rats were anesthetized with 10% chloral hydrate (3 mL/kg). We surgically exposed the superior mesenteric artery and mesenteric lymphatic vessels, and ligated and drained the latter and collected the mesenteric lymph. Control group rats underwent the same procedure, but were injected with normal saline rather than *E. coli*. The body temperature was maintained above 36.3 °C with the use of heating pads or lamps as necessary. Mesenteric lymph was collected continuously into heparin-wetted sterile tubes that were placed on ice. The lymph samples were centrifuged for 10 min at 2000 rpm to remove all cellular components and stored at -80 °C until use. A total of 20 rats were used for lymph collection.

Lymph infusion protocol

Male Wistar rats underwent laparotomy as well as internal jugular vein cannulation. The pooled SII or control group mesenteric lymph specimens were infused intravenously *via* the jugular vein catheter and microinjection pump at a rate of 1 mL/h for 4 h. At the end, the rats were killed and lung injury was assessed by the wet and dry weight (W/D) ratio of the lung, the levels of inflammatory cytokines, and lung myeloperoxidase (MPO; marker of

pulmonary leuko sequestration).

Lung W/D ratio

The inferior lobe of the left lung was removed and weighed after gently wiping away the moisture on the surface. The specimen was placed in an 80 °C oven for 48 h and weighed to measure the dry weight. The W/D ratio was calculated for each group.

MPO assay

Frozen lung tissue was homogenized and processed for measurement of MPO activity. The MPO Activity Assay kit (Nanjing Jiancheng Bioengineering Institute, China) was used for MPO determination, according to the instructions provided with the kit.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the levels of the proinflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-6, using commercially available kits from RayBiotech Inc., Norcross, GA, United States. The optical density was measured on an ELISA plate scanner (KHB ST-360; Shanghai Danding Company, China) at 490 nm. The results were expressed as picograms of TNF- α or IL-6 per milliliter of lung tissue.

Lung histology

The lungs harvested from rats were immediately fixed in 10% buffered formalin. After fixation, the tissue samples were dehydrated and embedded in paraffin blocks. The sections were cut (4 μ m thick) and stained with hematoxylin and eosin (HE). The slides were viewed under a standard light microscope.

Western blot

Lung tissues immediately frozen in liquid nitrogen were disrupted using a homogenizer with RIPA cell lysis buffer (150 mmol NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mmol Tris-HCl, pH = 7.5, and 2 mmol EDTA) containing a mixture of protease inhibitors (Roche, Switzerland). Protein samples were separated by 8%-15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skimmed milk for 2 h, the membrane was incubated overnight with a primary antibody at 4 °C. The membrane was then triple-washed with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated with a secondary antibody for 2 h at room temperature. Horseradish-peroxidase-conjugated IgG in 5% skimmed milk (1:1000) was used as the secondary antibody. The target protein was detected using the ECL Western Blotting Analysis System (Santa Cruz Biotechnology, Santa Cruz, CA, United States) and X-ray film. The expression levels of TLR-4 (Cell Signaling Technology, Inc, United States), NF- κ B (Cell Signaling Technology, Inc, United States), and GAPDH (Cell Signaling Technology, Inc, United States) were measured.

Statistical analysis

Mean \pm SD values were calculated to summarize all outcome measurements. The *t* test was used to compare the means of two groups and *P* < 0.05 was considered significant. All statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL, United States).

RESULTS

Lung W/D ratio

To investigate the effect of mesenteric lymph of SII rats on lung edema, we measured lung W/D ratios. Lung W/D ratios were obviously increased in SII lymph-treated rats, compared with the control group (Figure 1A).

MPO activity in lung tissues

Neutrophil infiltration in lung tissue plays an important role in lung injury induced by SII lymph. The MPO assay is widely used to quantify the number of neutrophils in tissues and serves as an index of inflammation, because MPO is an enzyme that is released mainly from neutrophils. Compared with the control infusion group, the lung MPO activity was increased in the SII infusion group (Figure 1B). There is a growing body of evidence that involves polymorphonuclear leukocytes (PMNs) in the pathophysiological progress of SII. The increased MPO activity in the lung tissue after SII suggested activation of an inflammatory response.

TNF- α and IL-6 levels in lung tissues

To investigate the mechanism of the lung damage induced by mesenteric lymph from SII, we studied the levels of TNF- α and IL-6 in lung tissues. Compared with the control infusion group, lung tissue TNF- α and IL-6 levels increased significantly in the SII infusion group (Figure 2).

Lung histology

Lung injury is characterized by edema, hemorrhage and PMN infiltration. The control infusion group showed normal appearance of lung tissues. In the SII infusion group, the majority of the interstitial capillaries were congested with erythrocytes. The lung tissue also showed hemorrhage and edema between alveoli. Inflammatory cell infiltration increased septal thickness (Figure 3).

Expression of TLR-4 and NF- κ B

Activation of TLR-4 and NF- κ B signaling pathways plays a key role in the regulation of inflammatory mediator production. Therefore, we performed Western blot to investigate the activation of TLR-4 and NF- κ B p65. The expression of TLR-4 and NF- κ B was significantly increased after injection of SII lymph, compared with the control group (Figure 4).

DISCUSSION

The role of gut and gut-derived factors in the pathogen-

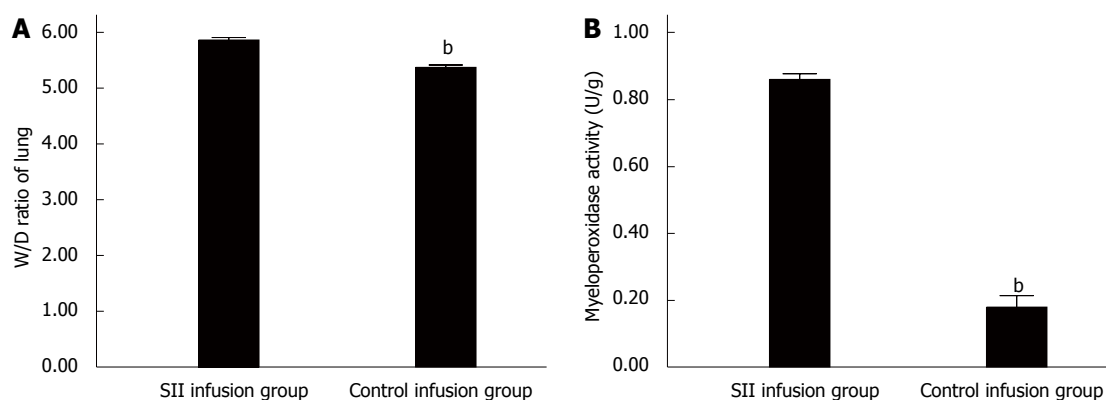


Figure 1 Change in lung wet-to-dry weight ratio and myeloperoxidase level. Severe intraperitoneal infection (SII) lymph infusion increased inflammatory injury. Myeloperoxidase (MPO) activity (A) and wet-to-dry weight (W/D) ratio (B) were measured in lung collected from SII and control lymph-infused rats 4 h after infusion. The results (mean \pm SD) are the summary of two separate experiments ($n = 10$ rats in each group). ^b $P < 0.01$ vs SII infusion group.

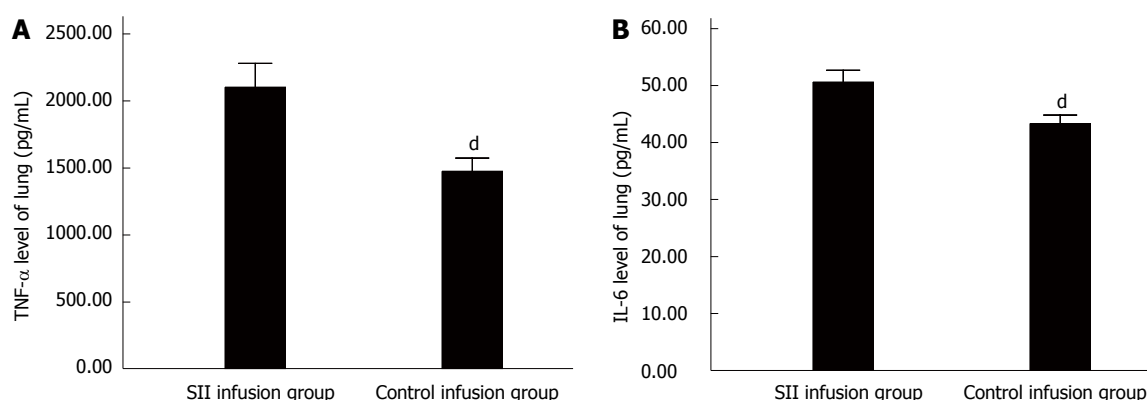


Figure 2 Tumor necrosis factor- α and interleukin-6 levels in lung tissues. Severe intraperitoneal infection (SII) lymph infusion enhances sepsis-induced inflammatory cytokine responses in the lung. Concentrations of tumor necrosis factor- α (A) and interleukin (IL)-6 (B) were measured from lung extracts of SII and control lymph-infused rats. Data are mean \pm SD of 10 rats in each group. Results are representative of two separate experiments. ^d $P < 0.01$ vs SII infusion group.

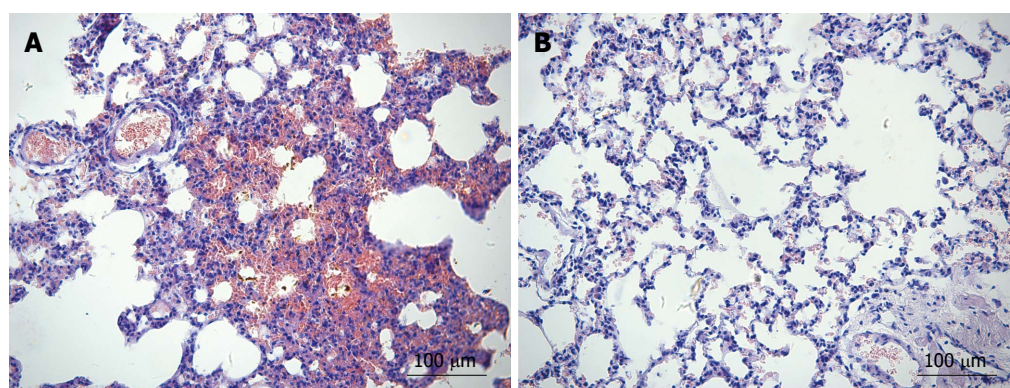


Figure 3 Tissue sections of lungs of severe intraperitoneal infection and control infusion rats stained with hematoxylin and eosin. Evidence of increased interstitial edema and inflammatory cell infiltration was found in rats injected with SII lymph (A). In contrast, control lymph-infused rats showed no evidence of lung injury (B). Magnification, $\times 200$.

esis of ARDS and MODS has been studied extensively and its understanding has evolved over the past two decades. Initially, it was believed that gut barrier failure during shock led to systemic inflammation and MODS by the translocation of bacteria and their products from the gut into the systemic circulation^[11]. However, several studies, including that by Moore *et al*^[12], in which neither

bacteria nor endotoxin was found in the portal blood of severely injured trauma patients, raised doubts about the clinical relevance of bacterial translocation. Recently, new insights into the pathogenesis of gut-induced ALI and inflammation after systemic insults, such as traumatic hemorrhage or major thermal injury have emerged, leading to the gut-lymph hypothesis of MODS^[2,13,14]. This

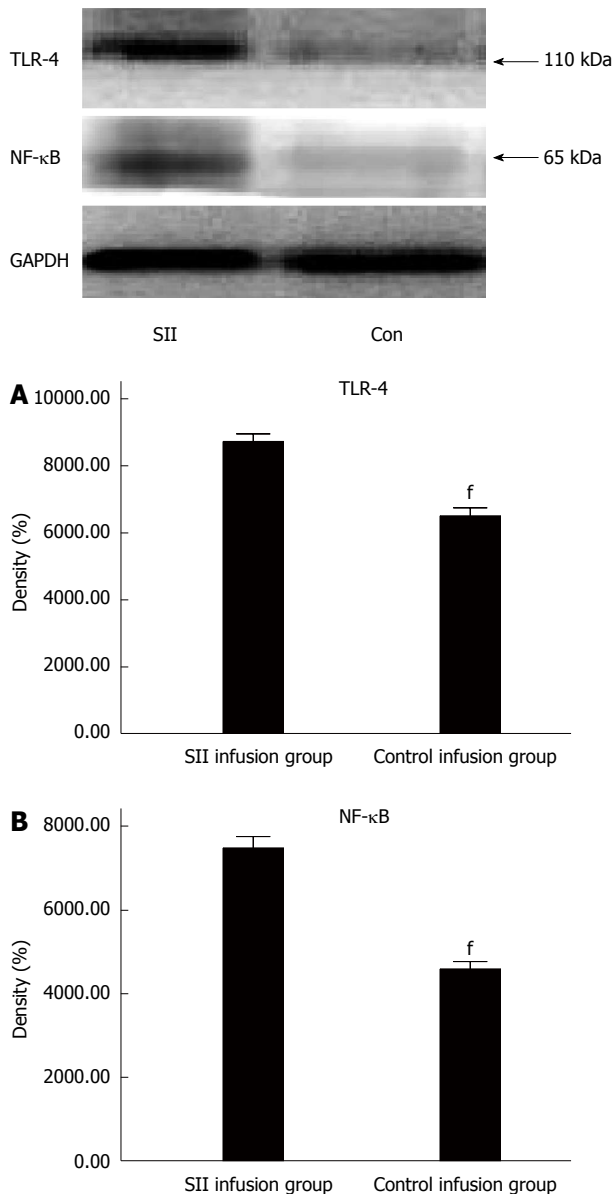


Figure 4 Compared with the control infusion group, toll-like receptor-4 and nuclear factor-κB expression levels were significantly increased in the severe intraperitoneal infusion group. Density of Toll-like receptor (TLR)-4 (A) and nuclear factor (NF)-κB (B) in lung protein extracts of SII and control rats was examined using Western blotting. All results (mean ± SD) are representative of two experiments ($n = 10$ rats in each group). ^f $P < 0.01$ vs SII infusion group.

hypothesis states that toxic products produced by the gut during shock are carried *via* the mesenteric lymph into the systemic circulation, resulting in multiple organ failure^[15].

MPO exists in azurophilic granules of neutrophils and accounts for about 5% of the dry weight of cells. In ALI, neutrophils first retain in pulmonary capillaries, adhere to endothelial cells, leave the pulmonary vascular bed, and release a series of inflammatory factors, causing diffuse lung damage. MPO is a characteristic neutrophil aggregation enzyme, is a sign of neutrophil degranulation, and can be used to assess the degree of neutrophil infiltration. Bradley *et al*^[16] showed a significant correlation between the activation of MPO and neutro-

phil count. Its increased activity in the tissue shows the number of PMNs and tissue damage. We used MPO as an index of evaluating lung inflammation. The experimental results show that the activity of lung tissue MPO increased after infusing mesenteric lymph of SII rats to healthy rats, which correlates with neutrophil chemotaxis and lung inflammatory response. Increased lung W/D ratio suggests lung tissue edema and increased pulmonary capillary permeability. Consistent with the pathological observations, lung tissue was damaged.

It has been found that, the process of sepsis in stress states, such as trauma and infection, causes enterogenic infection and multiple organ failure. However, ALI that occurs early and severely is often the direct cause of death in patients. ALI is a clinical syndrome characterized by pulmonary edema, and its severe phase is ARDS. Numerous studies have shown that Gram-negative bacillus endotoxin induces inflammatory cells such as neutrophils, mononuclear macrophages and endothelial cells to release a large number of cytokines and inflammatory mediators during the course of ARDS. TNF-α plays an important role in lung tissue damage and dysfunction in SII rats. TNF-α is a strong cytokine, and the amount of TNF-α is related to the degree of tissue damage. TNF-α can lead to injury of lung tissue and vascular endothelial cells, and significantly increase lung permeability, protein content of bronchial lavage fluid, and white blood cell count. TNF-α also inhibits the release of the alveolar surface-active substances, increases edema, and causes ARDS^[17]. The level of IL-6 is often used as a sign of cytokine cascade activation, which reflects the correlation between host inflammatory response and disease severity. If the level of IL-6 continues to increase, it is often accompanied by complications and mortality^[18]. It has been suggested that the level of IL-6 can serve as an indicator of the prognosis^[19,20].

TNF-α and IL-6 are both proinflammatory cytokines, and high expression levels can promote the generation of vascular active substances, activate complement and neutrophils, promote neutrophils to cross the vascular endothelium and gather in tissue, and form a cascade effect of inflammation to increase tissue injury. We showed that the levels of TNF-α and IL-6 in lung tissue increased significantly, which indicated an inflammatory reaction in the lungs. Reducing macrophages and NF-κB nuclear translocation can partly mediate these inflammatory effects^[21]. ALI or ARDS is characterized by neutrophil- and macrophage-mediated injury and release of inflammatory cytokines and proteases^[22]. Tiesi *et al*^[23] tested the hypothesis that gut-derived factors in mesenteric lymph are capable of simultaneously leading to an immune-mediated state as well as inducing systemic inflammation through a TLR-4-dependent pathway. TLR-4 stimulation can mediate ALI induced by SII, because TLR-4 induces the release of the inflammatory cytokines that are critical for the activation of powerful immune responses^[24]. TLR-4 stimulation is related to TLR-4-TRIF-TRAF6-NF-κB signaling, and the severity of ALI is controlled by this key pathway^[25]. NF-κB activation regulates expression of

cytokines, such as TNF- α and IL-6, at the transcriptional level^[26]. We found that infusion of mesenteric lymph of SII rats increased lung injury, for example, vascular leakage, neutrophil activation, inflammatory cytokine levels, and NF- κ B expression enhancement.

The potential clinical significance of the gut lymph hypothesis is that systemic organ and cellular injury/dysfunction can be abrogated by limiting gut injury and/or by preventing or neutralizing the production of biologically active SII lymph. In recent years, several preclinical studies have tested the strategy and found that gut-directed or lymph neutralizing-directed resuscitation approaches could limit SII-induced lung injury^[27,28] and systemic inflammation. Successful strategies have included: (1) ligation of mesenteric lymph ducts can attenuate lung injury caused by SII and reduce systemic inflammatory response and damage of other organs; and (2) *in vitro* experiments have shown that mesenteric lymph of sepsis can activate neutrophils and cause dysfunction of endothelial cells^[29,31].

In the present study, we infused the mesenteric lymph of SII rats into healthy rats *via* the internal jugular vein, which could lead to ALI in healthy rats. This supports the intestinal lymphatic hypothesis of MODS. In addition, in the experiments, the amount of mesenteric lymph infused was equal to the drainage amount of mesenteric lymphatic vessels from SII rats. This dose can fully induce lung injury in healthy rats. This indicates that the mesenteric lymph of SII rats itself is sufficient to cause lung damage.

The mesenteric lymph of SII rats can cause lung injury, and it may be associated with some toxic agents contained in lymph. On the basis of previous trials, if we drain mesenteric lymph from SII patients, it will have a profound effect on ICU patients who have sepsis-induced ARDS or MODS.

COMMENTS

Background

The role of gut and gut-derived factors in the pathogenesis of acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) has been studied extensively and its understanding has evolved over the past two decades. The authors investigated the pathway and biological foundation of lung injury caused by severe intraperitoneal infection (SII) to better understand the relationship between lung and gut disease.

Research frontiers

To reveal the pathway and biological foundation of lung injury induced by intestinal barrier damage and SII.

Innovations and breakthroughs

The authors infused mesenteric lymph of SII rats into healthy rats and examined its effect on lung tissue. The authors confirmed damage to the remote organ caused *via* the mesenteric lymphatic pathway, and the hypothesis that lymph from SII is sufficient to cause lung injury.

Applications

Mesenteric lymph from SII patients could have a profound effect on intensive care unit patients who have sepsis-induced ARDS or MODS.

Terminology

The lymphatic system is an important bypass of tissue fluid reflex to blood. The blind side of capillary lymphatic vessels starts from the tissue space, and merges into large lymphatic vessels gradually. Lymphatic vessels collect systemic

lymph and flow into veins *via* the right lymphatic duct and thoracic duct.

Peer review

A simple but important rat study of the role of mesenteric lymph in lung infection. The findings of the study are significant, and may have favorable clinical outcomes.

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Proteomic analysis of liver mitochondria from rats with nonalcoholic steatohepatitis

Lin Li, De-Zhao Lu, You-Ming Li, Xue-Qun Zhang, Xin-Xin Zhou, Xi Jin

Lin Li, You-Ming Li, Xue-Qun Zhang, Xin-Xin Zhou, Xi Jin, Department of Gastroenterology, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

De-Zhao Lu, College of Life Sciences, Zhejiang Chinese Medical University, Hangzhou 310003, Zhejiang Province, China

Author contributions: Li L and Lu DZ performed the majority of experiments; Li YM, Zhang XQ and Zhou XX provided vital reagents and analytical tools and were also involved in editing the manuscript; Jin X designed the study and wrote the manuscript.

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Correspondence to: Dr. Xi Jin, the First Affiliated Hospital, College of Medicine, Zhejiang University, No. 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. jxfl007@hotmail.com

Telephone: +86-571-87236603 Fax: +86-571-87236611

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and further analyzed using two-dimensional electrophoresis combined with matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry. Bioinformatic analyses of assigned gene ontology and biological pathway was used to study functional enrichments in the abundant proteomic data.

RESULTS: Eight up-regulated and sixteen down-regulated proteins were identified that showed greater than 1.5-fold differences between the controls and the NASH group. These dysregulated proteins were predicted to be involved in different metabolic processes including fatty acid β -oxidation processes, lipid metabolic processes, cell-cycle arrest, cell polarity maintenance, and adenosine triphosphate/sex hormone metabolic processes. Novel proteins that may be involved in NASH pathogenesis including the trifunctional enzyme Hadha, thyroxine, prohibitin, aldehyde dehydrogenase ALDH1L2, UDP-glucuronosyltransferase 2B31, and carbamoyl-phosphate synthase were identified using bioinformatics tools. The decreased expression of Hadha in NASH liver was verified by Western blotting, which was used as a complementary technique to confirm the proteomic results.

CONCLUSION: This novel report on the liver mitochondrial proteome of a NASH model may provide a reservoir of information on the pathogenesis and treatment of NASH.

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Key words: Nonalcoholic steatohepatitis; Hadha; Proteomics; Rat model

Core tip: Nonalcoholic fatty liver disease (NAFLD) is a major worldwide cause of chronic liver diseases, and nonalcoholic steatohepatitis (NASH) plays a critical role as a "turning point" in the development of NAFLD. Nevertheless, the pathogenesis of NASH remains unclear,

Abstract

AIM: To explore mitochondrial dysfunction in nonalcoholic steatohepatitis (NASH) by analyzing the proteome of liver mitochondria from a NASH model.

METHODS: The NASH rat model was established by feeding rats a fat-rich diet for 24 wk and was confirmed using hematoxylin and eosin staining of liver tissue and by changes in the levels of serum alanine transaminase, aspartate aminotransferase, triglyceride, total cholesterol and other markers. Liver mitochondria from each group were isolated using differential centrifugation. The mitochondrial samples were lyzed, purified

and mitochondrial dysfunction is known to be actively involved. To date, no study has reported on specific protein expression patterns in NASH mitochondria. We have, for the first time, performed a proteomic analysis of mitochondria from NASH rats, aiming to provide a protein reservoir for in-depth analyses of NASH mechanisms and for the exploration of potential therapeutics.

Li L, Lu DZ, Li YM, Zhang XQ, Zhou XX, Jin X. Proteomic analysis of liver mitochondria from rats with nonalcoholic steatohepatitis. *World J Gastroenterol* 2014; 20(16): 4778-4786 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4778.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4778>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease in North America, and its prevalence has been estimated to be as high as 35% in some populations^[1]. Currently, NAFLD is considered to be a hepatic manifestation of the metabolic syndrome that develops progressively from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis^[2]. Of these stages, NASH is a vital stage due to its role as the “turning point” in NAFLD development. Hepatic steatosis is known to be reversible; however, NASH is typically irreversible and the rate of progression to liver cirrhosis accelerates when NASH occurs. A recent study showed that 41% and 5.4% of NASH patients progress to liver fibrosis and end-stage liver disease, respectively^[3]. NASH is therefore a major risk factor for cryptogenic liver cirrhosis^[4] and requires more in-depth investigation.

Although Day and James^[5] previously outlined a “two-hit hypothesis” for NAFLD, the general mechanisms underlying the pathogenesis of NASH remain unclear. Currently, oxidative and endoplasmic reticulum stress, inflammatory factor release, and in particular, hepatic mitochondrial dysfunction are considered to be actively involved in NASH^[6]. Mitochondria play a major role in cell energy-generating processes and integrate several signaling pathways to control cellular life and death. Accumulating evidence has shown that NAFLD is characterized by mitochondrial alterations that depend on the activation of intracellular stress cascades or receptor-mediated pathways^[7]. Furthermore, NASH is considered a mitochondrial disease^[8], which reinforces the importance of mitochondrial dysfunction in its pathogenesis.

How mitochondrial dysfunction affects the initiation and progression of NASH remains unclear. Previous studies have shown that impaired adenosine triphosphate (ATP) formation and augmented generation of reactive oxygen species by the damaged respiratory chain may underlie the deterioration in mitochondrial dysfunction in NASH^[9]. Technological advancements have gradually uncovered the complex mechanism of mitochondrial dysfunction in NASH. For example, proteomic techniques

have provided a novel and high-throughput method for elucidating the complex pathogenesis of NAFLD^[10]. Using two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF-TOF) mass spectrometry (MS), our group reported unique protein expression patterns in a rat model of different stages of NAFLD^[11] and in a mouse model of ischemia/reperfusion injury and ischemic preconditioning^[12]. Recently, we also identified serum biomarkers enabling NAFLD diagnosis using a combination of surface-enhanced laser desorption/ionization (SELDI)-TOF-MS and bioinformatics^[13].

However, the specific protein expression pattern in NASH liver mitochondria has rarely been reported. Considering the importance of NASH in the clinical progression of NAFLD and the complexity of mitochondrial dysfunction in disease initiation and progression, we report the first proteomic analysis of liver mitochondria from the NASH rat model. We aimed to provide a protein reservoir for future in-depth analyses of NASH pathogenesis and progression.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Review Board of the First Affiliated Hospital, School of Medicine, Zhejiang University, China. All animal studies were conducted according to the regulations and guidelines for the use and care of experimental animals of the Department of Gastroenterology, the First Affiliated Hospital, School of Medicine, Zhejiang University, China.

Establishment of the NASH rat model

Twenty four Sprague-Dawley rats (*Rattus norvegicus*) weighing 160-170 g were purchased from the Medical Science Institution of Zhejiang Province (Hangzhou, China) and were randomly divided into NASH ($n = 12$) and control ($n = 12$) groups, as reported previously^[14]. All rats received food and water ad libitum and were maintained on a 12/12-h light/dark cycle. The control group was provided with a basic diet, whereas the NASH group was fed a fat-rich diet, as described previously^[15]. After 24 wk, the rats were euthanized by femoral exsanguination, and alanine transaminase (ALT), aspartate aminotransferase (AST), triglyceride (TG), total cholesterol (TCh), and hepatic TG levels were measured. The hepatic index, which was used to describe the lipid overload in the liver, was calculated as the ratio between liver wet weight and body weight. Liver sections were stained using hematoxylin and eosin (HE) and observed for hepatic steatosis and inflammation using an Olympus microscope. The severity of hepatic injury was estimated according to the histological activation index (HAI) as described previously^[16].

Isolation of mitochondria and sample preparation

Liver mitochondria from each group were isolated using differential centrifugation. First, liver sections were ex-

cised from the euthanized rats, washed with 0.25 mol/L sucrose and homogenized (1/10, w/v, 600 μ g) in a JA-17 rotor using MSHE (0.22 mol/L mannitol, 0.07 mol/L sucrose, 0.5 mmol/L EGTA, 0.1% bovine serum albumin, and 2 mmol/L Hepes/KOH, pH 7.4) at 4 °C for 5 min. The supernatant was then centrifuged at 10300 *g* for 10 min. Next, intact purified mitochondria were isolated using Percoll (Sigma, CA, United States) to remove contaminating organelles and broken mitochondria. The pellet (mainly the mitochondrial fraction) was then re-suspended in 5 mL of MSHE (225 mmol/L mannitol, 1 mmol/L EGTA, 25 mmol/L Hepes, and 0.1% bovine serum albumin) supplemented with 20 mL of 30% Percoll. This solution was spun at 95000 *g* in a Hitachi RP50T rotor for 30 min. The fraction with a density of 1.052-1.075 g/mL was then collected and washed twice with MSHE at 6300 *g* for 10 min to remove the Percoll. Finally, the purified mitochondria were washed twice using 150 mmol/L KCl and MSHE sequentially.

The mitochondrial samples were then processed for the experiment. First, lysis buffer containing 30 mmol Tris, 8 mol Urea, and 4% CHAPS 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfate) was added to the microfuge tubes to re-suspend the purified mitochondria and the suspension was stored at 4 °C for at least 2 h. The mitochondrial suspension, while on ice, was sonicated intermittently for 10 s bursts followed by 10-s cooling periods. This process was repeated 15 times. After sonication, the samples were centrifuged at 12000 *g* at 4 °C for 30 min. The supernatant was transferred to a new tube and any pellet was discarded. Contaminants were further removed using a 2-D Clean Up Kit (GE Healthcare, United States), and the protein concentrations were calculated using a 2-D Quant Kit (GE Healthcare, CT, United States).

2-DE and image analysis

Three-hundred micrograms of protein was diluted with rehydration solution comprising 8 mol urea, 2% CHAPS, 13 mmol/L DTT, 0.5% IPG buffer, and 0.002% bromophenol blue (pH = 3-10) to a total volume of 450 μ L. The protein solution was loaded onto six 24-cm Immobiline dry strips pH = 3-10 (GE Healthcare), which were used for isoelectric focusing on an Ettan IPGphor 3 IEF system (GE Healthcare) for a total of 66000 volt-hours. 2-D SDS-PAGE was performed on all six polyacrylamide gels simultaneously using an Ettan DALT Six electrophoresis unit at 5 W/strip for 45 min and then at 15 W/strip until the bromophenol blue reached the bottom of the gels. Protein spots on the gels were visualized using routine methods, and the 2-D images were scanned using a high-resolution image scanner at 300 pixels per inch. Image Master 2D Platinum 6.0 (GE Healthcare) software was used to match and analyze the protein spots. Triplicate gels for each sample were used to reduce experimental errors. An average increase or decrease greater than 1.5-fold between the NASH and control groups was used to identify the differentially expressed proteins.

In-gel digestion and MALDI-TOF-TOF/MS identification

The differentially expressed proteins were excised from silver stained gels. Each spot was destained by washing with a 1:1 solution of 30 mmol potassium ferricyanide and 100 mmol sodium thiosulfate followed by equilibration in 200 mmol ammonium bicarbonate for 20 min. After washing twice with Milli-Q water, the gel spots were dehydrated by adding acetonitrile and dried in a SpeedVac (Thermo Savant, United States) for 15 min. Subsequently, the gel spots were rehydrated in 5 μ L trypsin solution (20 ng/ μ L in 200 mmol NH_4HCO_3) and incubated at 37 °C overnight. After eluting twice with 30 μ L 50% acetonitrile and 5% trifluoroacetic acid, 1 μ L peptide mixture was mixed with 1 μ L cyano-4-hydroxycinnamic acid (10 mg/mL, Sigma, United States) and then saturated with 50% acetonitrile in 0.05% trifluoroacetic acid.

The mixture was analyzed using a MALDI-TOF-TOF mass spectrometer (4800 Proteomics Analyzer; Applied Biosystems, United States). Data were acquired in a positive MS reflector mode at a scan range of 800-4000 Da. Five monoisotopic precursors (S/N > 200) were selected for tandem mass analysis. To interpret the mass spectra, a combination of peptide mass fingerprints and peptide fragmentation patterns were used for protein identification against the National Center for Biotechnology Information (NCBI) non-redundant protein database using the Mascot search engine (www.matrixscience.com). All mass values were considered monoisotopic, and the mass tolerance was set to 150 ppm.

Western blotting and bioinformatics analyses

Hadha protein levels in liver from the NASH and control groups were examined using Western blotting with a primary mouse polyclonal antibody raised against Hadha (1:500; Abcam, United Kingdom) and an ECL chemiluminescence kit (Santa Cruz, Texas, United States). Bands were normalized by comparing to a western blot of the same samples, but probed with a mouse anti-GAPDH antibody. The sequences of Hadha and all uncoupling proteins (UCPs) were obtained from the NCBI gene and protein databases and aligned using CLUSTAL X version 2.0.8 using the SLIM substitution matrix and other default parameters, as described previously^[17]. For more information, the gene ontology (GO) database was used to complete functional enrichment analyses of the proteomic data we obtained.

Statistical analysis

Each experiment was performed in triplicate, and the data were expressed as the mean \pm SE. Student's *t*-tests were used to compare two unpaired groups and were executed by SPSS 17.0. Differences were considered statistically significant at *P* < 0.05.

RESULTS

Establishment of the NASH rat model

The NASH rat model was established successfully as

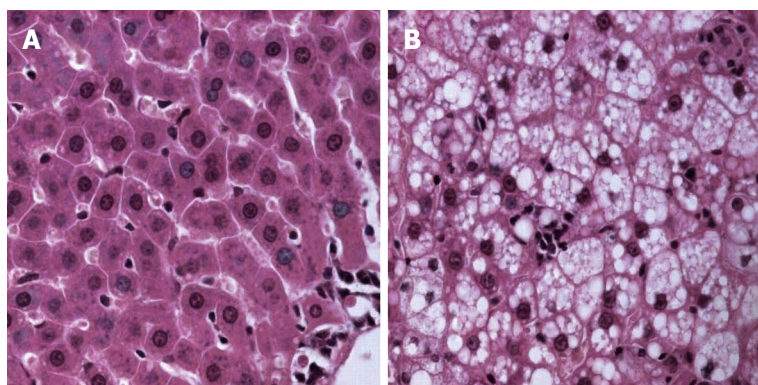


Figure 1 Pathology of nonalcoholic steatohepatitis and control group liver after hematoxylin and eosin staining. A: Control group; B: Nonalcoholic steatohepatitis group.

Table 1 Comparison of hepatic and serological markers in nonalcoholic steatohepatitis liver

Marker	Group	
	NASH	Control
Hepatic index (%)	3.79 ± 0.25 ^a	2.51 ± 0.19
ALT (IU/L)	153.51 ± 16.39 ^a	47.44 ± 14.06
AST (IU/L)	221.63 ± 37.28 ^a	104.31 ± 16.07
TG (mmol/L)	0.93 ± 0.12 ^a	0.55 ± 0.11
Tch (mmol/L)	2.95 ± 0.41 ^a	1.41 ± 0.23
Hepatic TG (mmol/L)	5.66 ± 0.71 ^a	1.43 ± 0.14
HAI	3.69 ± 0.51 ^a	1.31 ± 0.16

Values are expressed as the mean ± SE, ^a*P* < 0.05. NASH: Nonalcoholic steatohepatitis; ALT: Alanine transaminase; AST: Aspartate aminotransferase; TG: Triglyceride; Tch: Total cholesterol; HAI: Histological activation index.

confirmed by both serological and pathological changes. After feeding the rats a 24-wk high-fat diet, HE staining of NASH liver revealed varying degrees of fat deposition and mild to moderate chronic portal and intra-acinar inflammation (Figure 1). When compared with the control group, serum ALT, AST, TG and Tch levels, hepatic TG, hepatic index, and the HAI were all significantly increased in the NASH group (Table 1).

Quantitative proteomic analysis

The liver mitochondrial protein profiles of NASH and control rats were determined using a 2-DE approach. Briefly, 800-1000 protein spots per gel were detected using the Image Master 2D Platinum 6.0 software for image analysis. Differentially expressed spots were detected using a 2-D pattern comparison between the NASH and control groups. Using Student's *t*-tests, 34 protein spots showed changes of more than 1.5-fold between the NASH and control groups at a statistical significance of *P* < 0.05 (Figure 2). These differentially expressed protein spots were picked from the preparative gels and subjected to in-gel digestion and MALDI-TOF-TOF mass analysis. A total of eight up-regulated and sixteen down-regulated proteins were matched and identified, as displayed in Table 2. These dysregulated proteins were predicted to be involved in different metabolic processes including fatty

acid β -oxidation processes, lipid metabolic processes, cell-cycle arrest, cell polarity maintenance, ATP metabolic processes, and sex hormone metabolic processes.

The 24 identified proteins were selected using a peptide matching method. Therefore, it is not surprising that over half were similar to proteins from other Rodentia species, such as *Mus musculus*, *Cavia porcellus*, *Heterocephalus glaber*, and *Cricetulus griseus*. Because we used *R. norvegicus* as the NASH rat model in this study and to increase the reliability of our results, we selected seven proteins that matched proteins from *R. norvegicus* from the UniProt database (Table 3). Interestingly, only protein Ndufb10 (UniProt ID: D4A0T0) was up-regulated, whereas the other six proteins were down-regulated in the NASH group compared with the control group. Moreover, the GO cellular component analysis showed that only Agmatinase (UniProt ID: Q0D2L3) was located exclusively in the mitochondrion, whereas the other proteins were expressed in both the cytoplasm and mitochondria. In addition, with the exception of estradiol 17- β -dehydrogenase 8 (UniProt ID: Q6MGB5), which is hydrophobic, the other six proteins are hydrophilic. The proteomics data has provided basic information that may serve as a protein reservoir, although further functional studies on specific proteins are urgently required.

Validation and bioinformatics analyses of Hadha

Among the dysregulated proteins from *R. norvegicus*, Hadha (UniProt ID: Q64428) was annotated as a mitochondrial inner membrane protein. Bioinformatics analyses revealed that the sequence of rat Hadha was similar to known UCPs. Because UCP2 is known to be actively involved in NAFLD due to its uncoupling activity^[18], we tested Hadha expression levels in our samples using Western blotting. As shown in Figure 3, the steady-state level of hepatic Hadha was almost 50% less in the NASH group compared with the control group. This expression pattern is consistent with the 2-DE results and the peptide mass fingerprinting for Hadha, as shown in Figure 4.

DISCUSSION

NASH is considered a vital stage in NAFLD develop-



Figure 2 Representative two-dimensional electrophoresis image of proteins from the control and nonalcoholic steatohepatitis rat livers. The numbered spots denote proteins that exhibited modified expression levels in nonalcoholic steatohepatitis (NASH) liver.

ment because of its propensity to progress to advanced disease stages. Although NASH has been investigated intensively and mitochondrial dysfunction is known to be actively involved, the underlying mechanism remains unclear. Mitochondria play a major role in cellular life and death by controlling cell energy-generating processes. More importantly, the mitochondrion has its own genome, which produces organ-specific proteins and exerts vital biological functions^[19]. A previous study by our group revealed the protein profiles during different stages of NAFLD^[11]; however, the global mitochondrial protein expression in NASH remains unknown. In the current study, we systematically analyzed the liver mitochondrial proteome in a high-fat diet-induced NASH rat model and produced a novel protein reservoir that may help to elucidate the mechanism underlying NASH progression.

Of the 24 significantly dysregulated proteins, some have already been reported to participate in NASH progression. For example, of the up-regulated proteins, SOGA was identified as the target of adiponectin in reducing glucose production^[20] and thus, may be involved in the development of NASH. Intriguingly, iodothyro-

nine 5' monodeiodinase, which functions in the deiodination of thyroxine to T₃, was significantly increased in liver mitochondria from NASH rats. A previous study also showed its potential association with lipid peroxidation, which is vital to NASH progression^[21]. Taken together, these results reveal the potential involvement of thyroxine in NASH, although further investigations are needed. Furthermore, Nomura *et al.*^[22] showed enhanced ADP-ribosylation of phosphoglucomutase in patients with excess alcohol intake. Considering the similarity in pathology between alcoholic liver disease and NAFLD, its effect in NASH is also worth investigating. Finally, the increased UDP-glucuronosyltransferase 2B31 level in NASH liver was partially in line with a previous finding that UDP-glucuronosyltransferase levels in mouse liver increased in obesity and in fasting-induced steatosis^[23].

Similarly, among down-regulated proteins, ALDH1L2 has been identified as the mitochondrial homolog of 10-formyltetrahydrofolate dehydrogenase, an abundant enzyme involved in folate metabolism^[24]. A previous study showed that ALDH1L2 was a likely source of CO production from 10-formyltetrahydrofolate in mito-

Table 2 Dysregulated proteins in liver mitochondria of nonalcoholic steatohepatitis rats

	Spot no.	Protein GI	Protein name	Fold change	GO molecular function
Up-regulated	611	157822175	dehydrogenase 1 β subcomplex 10 (Ndufb10)	3.05	metabolic process/electron transport/ATP synthesis
	605	257467641	Suppressor of glucose by autophagy (SOGA)	2.97	suppressing glucogenesis
	510	29436756	Nuclear mitotic apparatus protein 1	2.68	microtubule binding/tubulin binding
	220	76779273	Hspd1 protein	2.54	ATP binding/insulin binding
	349	149029483	Mitochondrial F1 complex, alpha subunit, isoform 1	2.49	ATP synthesis/ADP binding
	51	202549	Iodothyronine 5' monodeiodinase	2.37	deiodination of thyroxine to T3
	135	148676986	Phosphoglucosmutase 5	1.81	carbohydrate metabolism/ Ca^{2+} homeostasis
	35	344244087	UDP-glucuronosyl-transferase 2B31	1.65	transferase activity/transferring hexosyl groups
Down-regulated	310	158631196	Peptidyl-prolyl cis-trans isomerase H isoform 2	-106	isomerase activity/ribonucleoprotein complex binding
	208	225690572	Leucine-rich repeat- containing protein 49 isoform 3	-106	protein-protein interaction/component of immune system
	183	300796253	Mitochondrial 10-formyltetrahydrofolate dehydrogenase (ALDH1L2)	-106	folate metabolism/CO production
	221	123244269	Microtubule-actin crosslinking factor 1	-106	ATPase activity/calcium ion binding/microtubule binding
	132	148687612	DEAH (Asp-Glu-Ala-His) box polypeptide 37, isoform CRA_b	-3.88	ATP binding/helicase and hydrolase activity
	248	60688189	Agmat protein	-2.85	agmatinase activity/hydrolase activity/metal ion binding
	244	351700869	Zinc finger protein 613	-2.52	nucleic acid binding/zinc ion binding
	258	47087119	Estradiol 17- β -dehydrogenase 8	-2.24	3-hydroxyacyl-CoA dehydrogenase activity/estradiol 17- β -dehydrogenase activity/oxidoreductase activity/testosterone 17- β -dehydrogenase (NAD+) activity
	122	6679299	Prohibitin	-2.14	histone deacetylase binding/transcription regulatory region DNA binding
	237	8393186	carbamoyl-phosphate synthase	-1.88	ATP binding/calcium ion binding/carbamoyl-phosphate synthase (ammonia) activity
	234	20304123	3-mercaptopyruvate sulfurtransferase	-1.87	3-mercaptopyruvate sulfurtransferase activity/thiosulfate sulfurtransferase activity/transferase activity
	179	149027156	Mitochondrial 3-oxoacyl-coenzyme A thiolase, isoform CRA_f	-1.85	acetyl-CoA C-acyltransferase activity/transferase activity
	260	148747393	Trifunctional enzyme subunit, alpha, mitochondrial precursor (Hadha)	-1.76	3-hydroxyacyl-CoA dehydrogenase activity
	156	347800699	T-complex protein 1 subunit theta	-1.74	NAD binding/enoyl-CoA hydratase activity/fatty-acyl-CoA binding/long-chain-3-hydroxyacyl-CoA dehydrogenase activity
	257	11968102	Ornithine aminotransferase, mitochondrial precursor	-1.60	ATP binding/nucleotide binding/unfolded protein binding
					2-oxoglutarate 5-5-aminotransferase activity/ornithine-oxo-acid transaminase activity/pyridoxal phosphate binding
	206	351715881	GRIP and coiled-coil domain	-1.55	protein binding-containing protein 2

The -10^6 -fold change denotes very low expression in the NASH group compared with the control group. GIs in bold italics are proteins that are known to be located exclusively in mitochondria. Underlined proteins are known to be located in the inner mitochondrial membrane. ATP: Adenosine triphosphate (ATP); GO: Gene ontology.

chondria^[25]. Therefore, by influencing folate metabolism, ALDH1L2 may also be involved in NASH progression. Moreover, increased and decreased expression of prohibitin was found in cells expressing hepatitis C virus core protein^[26] and in regenerating the liver^[27], respectively. We observed decreased prohibitin levels in NASH liver, which may act by blocking cell proliferation and inducing apoptosis, as reported previously in human hepatoma cells^[28]. In addition, depletion of carbamoyl-phosphate synthase was reported in liver during sepsis and was found to act as a marker of mitochondrial damage^[29]. We also observed decreased carbamoyl-phosphate synthase

in NASH rat liver, which indicated the involvement of mitochondrial damage in NASH progression. Nevertheless, many other proteins that may play a potential role in NASH are reported here for the first time, providing a novel protein reservoir for disease development that requires further confirmation.

As potentially redundant information may be produced from the study of a single protein and the potential interaction between different proteins, it is vital to enrich protein function using pathway analyses. Because we used a NASH rat model, we focused our analyses on protein information from *R. norvegicus*. Based on these

Table 3 Dysregulated proteins that match uniprot proteins from *Rattus norvegicus*

	UniProt ID	Gravity ¹	Fold change	GO biological process
Up-regulated	D4A0T0	-0.937	3.05	Biological process
Down-regulated	Q0D2L3	-0.033	-2.85	Putrescine biosynthetic process/spermidine biosynthetic process
	Q6MGB5	0.137	-2.24	Androgen metabolic process/estrogen and fatty acid biosynthetic process/oxidation-reduction process
	P07756	-0.177	-1.88	Anion homeostasis/carbamoyl phosphate biosynthetic process/cellular response to cAMP and fibroblast growth factor stimulus/hepatocyte differentiation and liver development/metabolic process
	P97532	-0.292	-1.87	3-Mercaptopyruvate sulfurtransferase activity
	Q64428	-0.081	-1.76	Fatty acid β -oxidation/fatty acid and lipid metabolic process/oxidation-reduction process/response to insulin stimulus
	P04182	-0.113	-1.60	Ornithine metabolic process

¹Gravity values < 0 indicate hydrophilicity, whereas values > 0 indicate hydrophobicity.

two criteria, we selected seven of the dysregulated proteins and examined common biological pathways that they shared. Several NASH-related pathways have been reported previously including insulin binding, fatty acid and lipid metabolic processes, and oxidation reduction processes, as shown in Table 3. We also discovered several pathways that are novel including androgen/estrogen metabolic processes, cellular response to cAMP and fibroblast growth factor stimulus, and 3-mercaptopyruvate sulfurtransferase activity. These pathways may indicate that novel mechanisms are involved in the progression of NASH and require further investigation.

Hadha was among the significantly dysregulated proteins and has been previously reported to be a candidate gene for NAFLD identification^[30]. UCPs are known for their role in oxidative phosphorylation and their participation in NAFLD pathogenesis^[18,31]. In this study, we confirmed the significant decrease in Hadha using Western blotting, but the detailed mechanism is, as yet, unknown. Several explanations are possible: Firstly, Hadha may influence the metabolism of fatty acid *via* its role in dehydrating 3-hydroxyacyl-CoA. Secondly, each protein in the mitochondrial inner membrane may possess uncoupling activity, as was previously proposed by several researchers^[32]. Because Hadha is located in the mitochondria

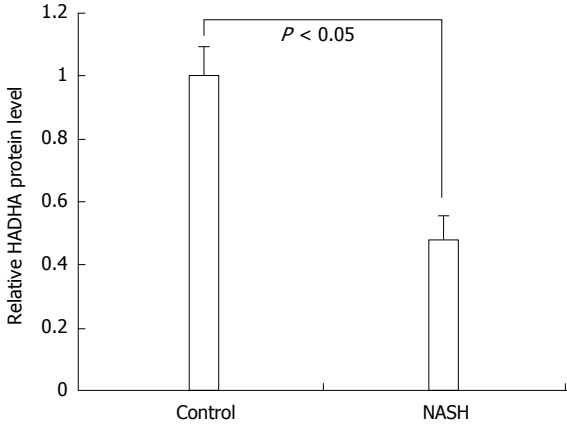
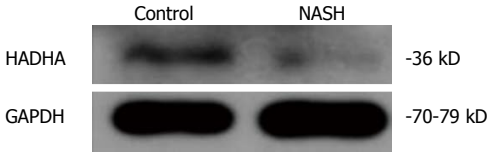


Figure 3 Significantly decreased hadha levels in nonalcoholic steatohepatitis rat mitochondria using Western blotting. NASH: Nonalcoholic steatohepatitis.

drial inner membrane and shares sequence similarity with UCPs, it may participate in NASH progression by influencing ATP synthesis and levels of oxidative stress.

This study has several limitations that should be acknowledged. Firstly, only 12 of the 24 selected proteins were found to be located exclusively in mitochondria (Table 2). The other 12 proteins are also found in the cytoplasm. A possible explanation may be the multi-function characteristics of these proteins. For instance, microtubule-actin crosslinking factor 1 may exert its ATPase activity in mitochondria, but its calcium ion binding function in the cytoplasm. Secondly, although the high-fat diet-induced rat model developed steatohepatitis that was morphologically similar to human NASH, it would be more convincing if the dysregulation of these proteins was verified in human patients. Thirdly, of the 24 identified proteins, only Hadha was verified, and future studies will confirm the other 23 proteins and their functions. Fourthly, the data on Hadha level is preliminary. Our result contrasted with the increase in Hadha mRNA in NAFLD human subjects as described by Kohjima *et al.*^[33]. Nevertheless, our result is at the protein level, whereas their result is at the mRNA level, which suggests the potential for post transcriptional regulation, such as miRNA regulation and methylation. Our next step will be to investigate both gene and protein expression of Hadha in larger animals and in patient samples. Finally, the 2-DE method itself has shortcomings as it does not generally detect proteins with high (> 150 kD) or low (< 10 kD) molecular masses, or very basic or hydrophobic proteins, thus limiting the proteomic coverage of most biological samples.

In summary, the differential proteomic approach that we employed enabled the identification of important

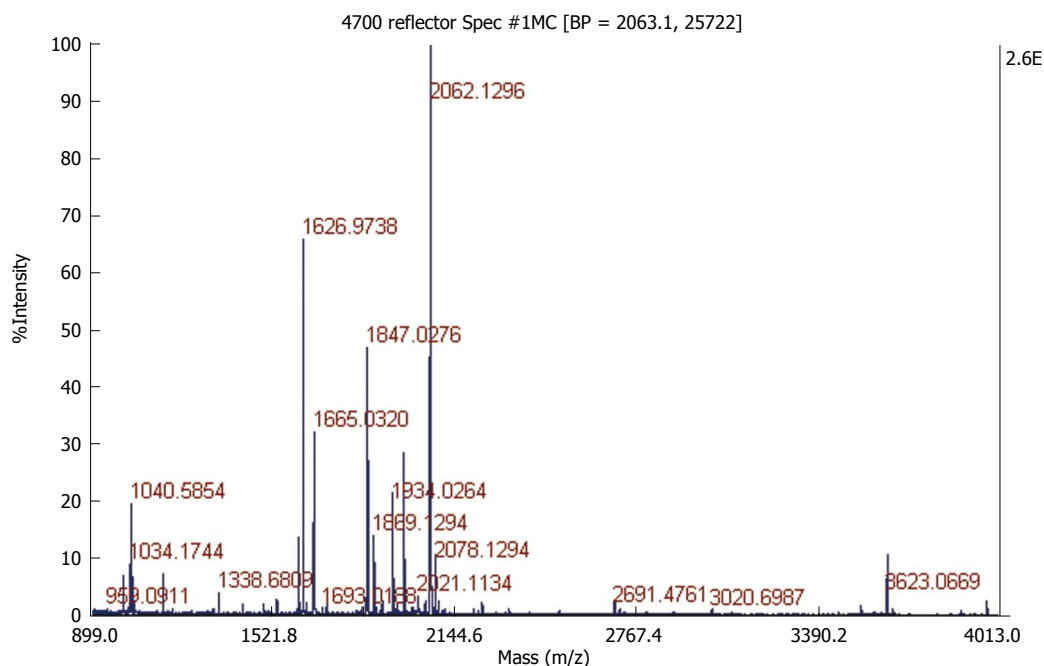


Figure 4 Peptide mass fingerprinting of an in-gel tryptic digest of the Hadha protein.

changes in mitochondrial protein expression in a NASH rat model, which provides a protein reservoir for future investigation of the mechanism of NASH development. In addition, the down-regulation of Hadha in NASH liver and its sequence similarity to known UCPs implies its potential role in NASH *via* uncoupling activity. Nevertheless, the underlying molecular mechanism and the regulatory network involved require further investigation.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease in North America, and its prevalence has been estimated to be as high as 35% in some populations. The spectrum of NAFLD includes simple steatosis, nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis. Of these stages, NASH is critical due to its role as the “turning point” in NAFLD development, with a greater percentage of patients with NASH progressing to end-stage liver diseases of unknown etiology.

Research frontiers

Currently, oxidative and endoplasmic reticulum stress, inflammatory factor release, and in particular, hepatic mitochondrial dysfunction are considered to be actively involved in NASH. Nevertheless, how mitochondrial dysfunction affects the initiation and progression of NASH remains unclear. Previous studies have shown that impaired adenosine triphosphate formation and increased generation of reactive oxygen species by a damaged respiratory chain may underlie the deterioration of mitochondrial dysfunction in NASH. With the development of proteomics, novel protein candidates have been revealed to participate in the pathogenesis and progression of NASH. Our group has reported unique protein expression patterns in a rat model of different stages of NAFLD and in a mouse model of ischemia/reperfusion injury and ischemic preconditioning. Recently, we also identified serum biomarkers enabling NAFLD diagnosis using a combination of surface-enhanced laser desorption/ionization-TOF-MS and bioinformatics.

Innovations and breakthroughs

The authors provide, for the first time, the liver mitochondrial proteome of the NASH rat model. Furthermore, Western blotting verified a decrease in Hadha and indicates its potential involvement in the progression of NASH.

Applications

This model proteome provides a protein reservoir for further investigation of the pathogenesis and progression of NASH.

Terminology

Hadha is the official acronym for “hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit”. This gene provides instructions for making part of an enzyme complex called mitochondrial trifunctional protein, which is essential for fatty acid oxidation.

Peer review

This study provided a protein reservoir for in-depth investigation of the mechanism of NASH. Moreover, the down-regulation of Hadha in NASH liver and its sequence similarity to known uncoupling proteins implies its potential role in NASH *via* uncoupling activity. All of these results were encouraging but putative; more verification of the other proteins is required and the application of this study to humans is anticipated.

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Point shear wave elastography method for assessing liver stiffness

Giovanna Ferraioli, Carmine Tinelli, Raffaella Lissandrin, Mabel Zicchetti, Barbara Dal Bello, Gaetano Filice, Carlo Filice

Giovanna Ferraioli, Raffaella Lissandrin, Mabel Zicchetti, Gaetano Filice, Carlo Filice, Department of Infectious Diseases, Fondazione IRCCS Policlinico San Matteo, Medical School University of Pavia, 27100 Pavia, Italy

Carmine Tinelli, Clinical Epidemiology and Biometric Unit, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy

Barbara Dal Bello, Department of Pathology, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy

Author contributions: Ferraioli G and Tinelli C contributed equally to this work; Ferraioli G, Tinelli C and Filice C designed the research; Ferraioli G, Lissandrin R, Zicchetti M and Dal Bello B performed the research; Tinelli C analyzed the data; Ferraioli G, Zicchetti M and Lissandrin R wrote the manuscript; Tinelli C, Dal Bello B, Filice G and Filice C reviewed the manuscript; and Ferraioli G, Tinelli C, Dal Bello B, Zicchetti M, Lissandrin R, Filice G and Filice C approved the final version of the manuscript; all the authors of the Liver Fibrosis Study Group performed the research, reviewed the manuscript and approved the final version. **Supported by** The FibroScan device was made available for this study by Echosens (Paris, France), and the iU22 ultrasound equipment was provided by Philips Medical Systems (Bothell, WA, United States)

Correspondence to: Giovanna Ferraioli, MD, Researcher, Ultrasound Unit, Department of Infectious Diseases, Fondazione IRCCS Policlinico San Matteo, Medical School University of Pavia, Via Taramelli 5, 27100 Pavia, Italy. giovanna.ferraioli@unipv.it

Telephone: +39-382-502887 Fax: +39-382-501505

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Abstract

AIM: To estimate the validity of the point shear-wave elastography method by evaluating its reproducibility and accuracy for assessing liver stiffness.

METHODS: This was a single-center, cross-sectional study. Consecutive patients with chronic viral hepatitis scheduled for liver biopsy (LB) (Group 1) and healthy volunteers (Group 2) were studied. In each subject 10

consecutive point shear-wave elastography (PSWE) measurements were performed using the iU22 ultrasound system (Philips Medical Systems, Bothell, WA, United States). Patients in Group 1 underwent PSWE, transient elastography (TE) using FibroScan (Echosens, Paris, France) and ultrasound-assisted LB. For the assessment of PSWE reproducibility two expert raters (rater 1 and rater 2) independently performed the examinations. The performance of PSWE was compared to that of TE using LB as a reference standard. Fibrosis was staged according to the METAVIR scoring system. Receiver operating characteristic curve analyses were performed to calculate the area under the receiver operating characteristic curve (AUC) for $F \geq 2$, $F \geq 3$ and $F = 4$. The intraobserver and interobserver reproducibility of PSWE were assessed by calculating Lin's concordance correlation coefficient.

RESULTS: To assess the performance of PSWE, 134 consecutive patients in Group 1 were studied. The median values of PSWE and TE (in kilopascals) were 4.7 (IQR = 3.8-5.4) and 5.5 (IQR = 4.7-6.5), respectively, in patients at the F0-F1 stage and 3.5 (IQR = 3.2-4.0) and 4.4 (IQR = 3.5-4.9), respectively, in the healthy volunteers in Group 2 ($P < 10^{-5}$). In the univariate analysis, the PSWE and TE values showed a high correlation with the fibrosis stage; low correlations with the degree of necroinflammation, aspartate aminotransferase and gamma-glutamyl transferase (GGT); and a moderate negative correlation with the platelet count. A multiple regression analysis confirmed the correlations of both PSWE and TE with fibrosis stage and GGT but not with any other variables. The following AUC values were found: 0.80 (0.71-0.87) for PSWE and 0.82 (0.73-0.89) for TE ($P = 0.42$); 0.88 (0.80-0.94) for PSWE and 0.95 (0.88-0.98) for TE ($P = 0.06$); and 0.95 (0.89-0.99) for PSWE and 0.92 (0.85-0.97) for TE ($P = 0.30$) for $F \geq 2$, $F \geq 3$ and $F = 4$, respectively. To assess PSWE reproducibility, 116 subjects were studied, including 47 consecutive patients scheduled for LB (Group 1) and 69

consecutive healthy volunteers (Group 2). The intraobserver agreement ranged from 0.83 (95%CI: 0.79-0.88) to 0.96 (95%CI: 0.95-0.97) for rater 1 and from 0.84 (95%CI: 0.79-0.88) to 0.96 (95%CI: 0.95-0.97) for rater 2. The interobserver agreement yielded values from 0.83 (95%CI: 0.78-0.88) to 0.93 (95%CI: 0.91-0.95).

CONCLUSION: PSWE is a reproducible method for assessing liver stiffness, and it compares with TE. Compared with patients with nonsignificant fibrosis, healthy volunteers showed significantly lower values.

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Key words: Chronic viral hepatitis; Liver biopsy; Liver fibrosis; Ultrasound; Elastography; Sensitivity and specificity

Core tip: The results of this study show that point shear-wave elastography (PSWE) is a highly reproducible method for assessing liver stiffness that is characterized by high levels of intraobserver and interobserver agreement, both overall and for single measurements. The PSWE performance compares with that of transient elastography (TE), the most widely accepted method for the noninvasive assessment of liver fibrosis. Compared with TE, routine ultrasound with elastography is advantageous because it allows the evaluation of other parameters that are complementary to stiffness, is highly accurate for the diagnosis of cirrhosis and can be used to screen for focal liver lesions.

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INTRODUCTION

The prognosis and management of chronic viral hepatitis depend on the extent and progression of liver fibrosis, which constitute the most important predictor of disease outcome and influence the indication for antiviral treatment^[1].

In the last decade, methods to noninvasively quantify liver fibrosis have been developed. The first available method was transient elastography (TE)^[2-6]. Several studies have demonstrated a high accuracy of TE in identifying significant fibrosis ($F > 2$) and cirrhosis ($F = 4$) in patients with chronic hepatitis C^[7-11].

The recent guidelines for the management of hepatitis C infection from the European Association for the Study of the Liver allow the use of TE, instead of liver biopsy (LB), in patients with chronic hepatitis C for as-

sessing liver disease severity prior to therapy at a safe level of predictability^[12]. TE has been approved by the French National Health Authority for the evaluation of fibrosis in treatment-naïve patients with chronic hepatitis C and no comorbidities^[13].

Shear wave elastography techniques have been implemented in conventional real-time ultrasound systems, and several studies have shown their accuracy in the assessment of liver fibrosis^[14-22]. Compared with TE, these techniques have the advantage of B-mode image guidance; thus, they can allow the user to choose the best acoustic window for correctly performing an examination in real time.

The aim of this study was to estimate the validity of a new point shear wave elastography (PSWE) technique by evaluating the reproducibility of measurements and the accuracy of this method in the assessment of liver fibrosis. The performance of PSWE was compared to that of TE using liver histology as a reference standard.

MATERIALS AND METHODS

Subjects and study design

This was a single-center, cross-sectional study. All consecutive patients with chronic viral hepatitis who were scheduled for liver biopsy at the Infectious Diseases Department of Policlinico San Matteo were enrolled in the study (Group 1). Consecutive healthy volunteers were also enrolled (Group 2).

The accuracy of PSWE in the assessment of liver fibrosis was prospectively estimated in consecutive patients in Group 1. LB was performed on the same day as the PSWE and TE measurements, as day-case procedures. Examinations were performed in the morning after an overnight fast. The patients' characteristics, epidemiological data and biochemical test results were recorded.

The reproducibility of PSWE measurements was prospectively assessed in consecutive subjects in Group 1 and Group 2. Two expert raters (rater 1 and rater 2) independently performed 10 consecutive measurements in each subject. All the subjects were asked to fast for at least six hours prior to the examination. The intraobserver agreement was assessed by comparing the median values of all the measurements and by comparing several combinations of measurements or single measurements. The interobserver agreement was assessed by comparing the median value of the 10 measurements performed in the same subject by each rater and by comparing combinations of measurements or single measurements.

Moreover, the results of liver stiffness measurements performed in patients with nonsignificant fibrosis (F0-F1) were compared to the values obtained in the healthy volunteers in Group 2.

Three physicians, each of whom was blinded to the other's results, independently performed the measurements. The PSWE measurements were performed by

G.F. and M.Z., and the TE measurements were performed by M.Z. and R.L.

The study protocol was approved by the institutional Ethics Committee. The participants provided written informed consent.

Liver biopsy

LB was performed by three experienced physicians (C.F., G.M. and E.B.) using a 17-gauge modified Menghini needle (Hepafix; Braun, Melsungen, Germany). The same intercostal space used for the TE and PSWE measurements was chosen for LB. The specimens were assessed on site by a single expert liver pathologist (B.D.B.) who was blind to both the TE and PSWE results. Liver fibrosis and necroinflammatory activity were evaluated semiquantitatively according to the METAVIR system^[23]. Steatosis was graded according to the method of Kleiner *et al.*^[24] as S0, steatosis in fewer than 5% of hepatocytes; S1, 5%-33%; S2, 34%-66%; and S3, more than 66%.

Transient elastography

TE measurements were performed using the M probe of the FibroScan[®] device by two physicians (M.Z. and R.L.) with experience performing at least 50 TE procedures. During the acquisition, the patients lay in the dorsal decubitus position with the right arm in maximum abduction. The results were expressed in kilopascals (kPa). Only examinations with 10 valid measurements and an interquartile range/mean (IQR/M) < 30% for values greater than 7.1 kPa were considered reliable^[2,4,25].

Point shear wave elastography

The examinations were performed using the iU22 ultrasound system (Philips Healthcare, Bothell, WA, United States) with a convex broadband probe and the ElastPQ[®] technique. As with other shear wave elastography methods, this technique generates shear waves inside the liver using radiation force from a focused ultrasound beam. The ultrasound machine monitors the shear wave propagation using a Doppler-like ultrasound technique and measures the velocity of the shear wave. The shear wave velocity is displayed in meters per second (m/s) or in kPa through Young's modulus $E = 3 (\nu S^2 \rho)$, where E is Young's modulus, νS is the shear wave velocity and ρ is the density of the tissue. If the amount of non-shear wave motion exceeds a threshold, the system does not display a calculation.

The two raters performing the PSWE measurements (G.F. and M.Z.) had seven years and two years, respectively, of experience in real-time elastography studies. They received training in PSWE measurements for two days before the study began. The examinations were performed in the right lobe of the liver through intercostal spaces, with the subject lying supine with the right arm in maximal abduction. Using a real-time B-mode image, the rater selected a vessel-free area, at least 1.5 cm below Glisson's capsule, where a fixed region of interest of 0.5 cm × 1.5 cm was placed by moving a trackball. The pa-

tients were instructed to hold their breath while the rater pressed a button that launched the data acquisition. Each rater performed 10 valid measurements, which were expressed in kPa. Measurements < 1 kPa were rejected by the raters.

Statistical analysis

Sample size considerations for the accuracy of PSWE:

A total sample size of 130 subjects, which included 65 subjects with the disease, *i.e.*, a prevalence of approximately 50%, was estimated to achieve 88% power to detect changes in sensitivity and in specificity from 0.75 to 0.90 using a two-sided binomial test. The target significance level was 0.05.

Sample size considerations for reproducibility of PSWE:

A sample size of 100 subjects, with two observations per subject, was estimated to achieve 97% power to detect a concordance correlation of 0.95 under the alternative hypothesis when the concordance correlation under the null hypothesis was 0.90 using an F-test with a significance level of 0.05.

Descriptive statistics were produced for the demographic, clinical and laboratory characteristics of this study sample of patients. The Shapiro-Wilk test was used to test the normal distribution of quantitative variables. For quantitative variables that were normally distributed, the results were expressed as mean ± SD; otherwise, medians and interquartile ranges (IQR; 25th-75th percentile) were reported. Qualitative variables were summarized as counts and percentages. A one-way ANOVA or the Kruskal-Wallis analysis of variance by ranks, with a Bonferroni correction, was used to analyze differences among patients undergoing liver biopsy. Pearson's or Spearman's rank coefficient was used to identify correlations between two study variables.

Linear regression was used for the multivariate model. A frequency distribution was obtained to choose optimal cut-off values of PSWE and to maximize the sum of the sensitivity and specificity for different fibrosis thresholds: F0-F1 *vs* F2-F4 ($F \geq 2$), F0-F2 *vs* F3-F4 ($F \geq 3$) and F0-F3 *vs* F4 ($F = 4$). For TE, we used cut-off values determined in a previous study^[26]. The diagnostic performance of PSWE, TE and their combinations was assessed using receiver operating characteristic (ROC) curves and an area under the ROC (AUC) curve analysis. Comparisons of AUCs were performed using the method described by DeLong *et al.*^[27] for correlated data. The Obuchowski measure was used to take into account all the pairwise comparisons between stages to minimize the spectrum effect and the risk of multiple testing^[28].

Interobserver reproducibility was assessed by calculating Lin's concordance correlation coefficient (CCC)^[29]. The CCC combines measures of both precision and accuracy to determine the degree of deviation of the observed data from the line of perfect concordance (*i.e.*, the line at 45 degrees on a square scatterplot). The CCC increases in value as a function of the proximity of the

data's reduced major axis to the line of perfect concordance (the accuracy of the data) and as a function of the tightness of the data about its reduced major axis (the precision of the data). CCC values range from 0 to +1. As CCC values approach 1, the measurement differences between the different raters become negligible and more consistent. The interobserver agreement was classified as poor (CCC = 0.00-0.20), fair to good (CCC = 0.40-0.75) or excellent (CCC > 0.75)^[30]. The CCCs were reported with 95% confidence intervals (CIs).

The data analysis was performed with the STATA statistical package (release 11.1, 2010, Stata Corporation, College Station, Texas, United States) and MedCalc (version 11.2, 2011 MedCalc Software bvba, Ostend, Belgium).

RESULTS

From August 2011 through April 2013, one hundred and thirty-four consecutive subjects in Group 1 and sixty-nine subjects in Group 2 were prospectively studied. Data from some patients in Group 1 have been reported in previous studies^[18,26].

Performance of PSWE

One hundred forty patients were eligible during the recruitment period. Six patients were excluded because they were undergoing antiviral therapy. Due to patient recruitment from our referring physicians, there were no patients with overt cirrhosis or ascites in this series of patients. One hundred thirty-four patients met the inclusion criteria. LB was performed in all the patients on the same day as the PSWE and TE measurements, and no complications were observed. The specimen length was adequate for liver histology in all but one patient. TE was feasible in all but one patient, whereas the PSWE measurements failed in five patients. The PSWE measurement failures were due to narrow intercostal spaces in four cases and obesity in one.

The characteristics of the 134 patients are summarized in Table 1. The mean length of the LB specimens was 2.5 (0.78) cm. The results of the statistical analysis performed on the data from the 102 patients with chronic hepatitis C are provided hereafter.

In the univariate analysis, the PSWE and TE values showed a high correlation with the fibrosis stage and low correlations with the degree of necroinflammation, aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT). The following values were obtained for PSWE and TE: (1) for liver fibrosis: $r = 0.61$ ($P < 10^{-5}$) and $r = 0.68$ ($P < 10^{-5}$); (2) for the degree of necroinflammation: $r = 0.39$ ($P < 10^{-5}$) and $r = 0.40$ ($P < 10^{-5}$); (3) for AST: $r = 0.37$ ($P = 0.0002$) and $r = 0.32$ ($P = 0.001$); and (4) for GGT: $r = 0.48$ ($P < 10^{-5}$) and $r = 0.43$ ($P < 10^{-5}$), respectively. The PSWE and TE values showed a moderate negative correlation with the platelet count ($r = -0.34$, $P < 0.0002$; $r = -0.36$, $P < 10^{-5}$). No correlations with other variables, including steatosis,

Table 1 Main clinical and demographic characteristics of patients with chronic viral hepatitis *n* (%)

Characteristics	All patients (<i>n</i> = 134)	Patients with chronic hepatitis C (<i>n</i> = 102)
Sex, females	29 (23.4)	20 (21.3)
Age, yr (SD)	43.70 (11.4)	45.2 (11.0)
BMI, kg/m ² (SD)	25.1 (4.5)	25.2 (4.9)
AST, IU/L (IQR)	46 (26-78)	46 (30-83)
ALT, IU/L (IQR)	69 (41-122)	70 (43-127)
Alkaline phosphatase, IU/L (IQR)	69 (59-87)	70 (59-95)
GGT, IU/L (IQR)	49 (26-80)	50 (36-88)
Total bilirubin, M/L (IQR)	0.62 (0.46-0.94)	0.60 (0.44-0.89)
Platelet count, 10 ³ /mm ³ (SD)	227.8 (76.0)	227.7 (70.1)
Prothrombin time, % (SD)	94.0 (15.5)	94.4 (17.0)
HCV infection	102 (76.1)	-
HBV infection	28 (20.9)	-
Other ¹	4 (3.0)	-
Fibrosis score (METAVIR) ²		
F0	14 (10.5)	6 (5.9)
F1	56 (42.1)	44 (43.6)
F2	29 (21.8)	24 (23.8)
F3	20 (15.0)	17 (16.8)
F4	14 (10.5)	10 (9.9)
Activity grade (METAVIR)		
A0	12 (9.0)	5 (4.9)
A1	77 (58.0)	57 (56.4)
A2	28 (21.0)	27 (26.7)
A3	16 (12.0)	12 (11.9)
Steatosis grade		
S0	83 (62.4)	62 (61.3)
S1	30 (22.6)	23 (22.8)
S2	16 (12.0)	12 (11.9)
S3	4 (3.0)	4 (4.0)
LSM, kPa (IQR)	6.6 (5.0-8.9)	6.5 (5.0-8.9)
PSWE, kPa (IQR)	5.3 (3.9-6.6)	5.2 (3.9-6.6)

¹HCV/HBV coinfection: *n* = 2; HCV/HIV coinfection: *n* = 2; ²Data for one patient missing due to inadequate biopsy specimen length. SD values represent means, and IQR values represent medians. BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; LSM: Liver stiffness measurement; PSWE: Point shear wave elastography.

were found.

A multiple regression analysis that included METAVIR stage, METAVIR grade, AST, GGT and platelet count confirmed the correlations, for both PSWE and TE, with fibrosis stage and GGT, but not with any other variables. The corresponding coefficients for METAVIR stage and GGT were 1.66 (95%CI: 0.85-2.46; $P < 10^{-5}$) and 0.007 (95%CI: 0.003-0.011; $P = 0.002$), respectively, for PSWE; and 3.05 (95%CI: 1.96-4.14; $P < 10^{-5}$) and 0.007 (95%CI: 0.001-0.060; $P = 0.002$), respectively, for TE.

After corrections for gender and age, both the PSWE and TE values differed significantly between the patients with chronic hepatitis C at the F0-F1 stage (*n*=50) and the healthy volunteers (*n* = 69) (Figure 1).

Liver stiffness assessment: Comparison of PSWE and TE: The median values, interquartile ranges, ranges, numbers of outliers and *P* values of the measurements obtained for each fibrosis stage using PSWE and TE are shown in Figure 2.

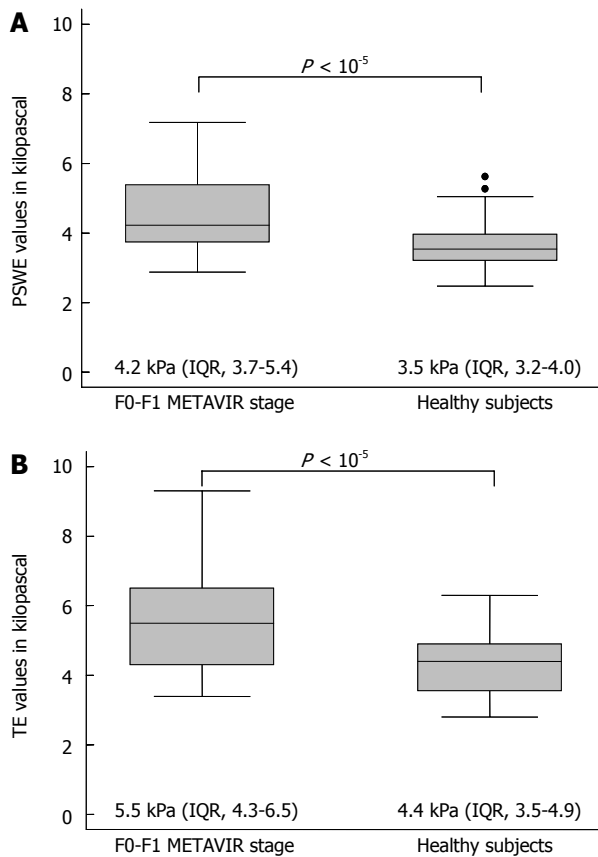


Figure 1 Box and whisker plots of stiffness values in patients at METAVIR stage F0-F1 and in healthy subjects. Box plots show interquartile range (box), median (line within box), range (whisker) and outliers (circle). Median values (IQR) and *P* values of differences are given. A: Point shear wave elastography (PSWE) values; B: Transient elastography (TE) values.

The optimal cut-off values for different levels of fibrosis were determined by analyzing the ROCs for PSWE. For TE, we used cut-off values obtained in a previous study^[26]. The cut-off values of PSWE and TE for each METAVIR stage, along with the AUCs, sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio, are presented in Table 2. Figure 3 shows the ROC curves for significant ($F \geq 2$) and severe fibrosis ($F \geq 3$), as well as for cirrhosis ($F = 4$). For staging advanced fibrosis ($F \geq 3$), TE had higher accuracy than PSWE, but this difference did not reach statistical significance. The Obuchowski measures were good for both PSWE [0.80 (95%CI: 0.73-0.86)] and TE [0.83 (95%CI: 0.77-0.90)].

Intraobserver and interobserver agreement of PSWE measurements

In total, 116 subjects were studied, including 47 consecutive patients scheduled for liver biopsy (Group 1) and 69 consecutive healthy volunteers (Group 2). The characteristics of the 116 subjects are summarized in Table 3. TE and PSWE were feasible in all the subjects.

Intraobserver agreement: For the 116 subjects in Group 1 and Group 2, the intraobserver agreement ranged from

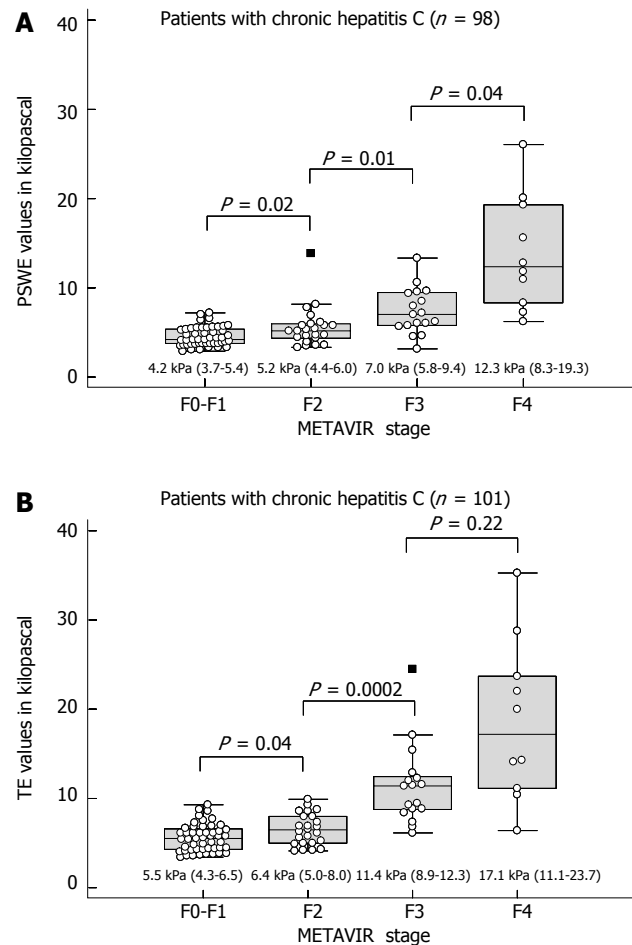


Figure 2 Distribution of stiffness values according to METAVIR fibrosis stage. Median values, interquartile ranges, ranges, numbers of outliers and *P* values are given for each fibrosis stage. A: Point shear wave elastography (PSWE); B: Transient elastography (TE).

0.83 (95%CI: 0.79-0.88) to 0.96 (95%CI: 0.95-0.97) for rater 1 and from 0.84 (95%CI: 0.79-0.88) to 0.96 (95%CI: 0.95-0.97) for rater 2 (Table 4).

When each group was considered separately, the CCC ranged from 0.77 (95%CI: 0.68-0.87) to 0.96 (95%CI: 0.94-0.97) for both raters in Group 1, from 0.80 (95%CI: 0.66-0.94) to 0.81 (95%CI: 0.67-0.94) for rater 1 and from 0.82 (95%CI: 0.69-0.94) to 0.83 (95%CI: 0.72-0.95) for rater 2 in Group 2.

Interobserver agreement: For the 116 subjects in Group 1 and Group 2, the interobserver agreement yielded CCC values ranging from 0.83 (95%CI: 0.78-0.88) to 0.93 (95%CI: 0.91-0.95) (Table 4).

When each group was considered separately, the interobserver agreement ranged from 0.76 (95%CI: 0.66-0.86) to 0.93 (95%CI: 0.88-0.96) in Group 1 and from 0.75 (95%CI: 0.58-0.95) to 0.83 (95%CI: 0.70-0.95) in Group 2.

DISCUSSION

This study was undertaken to assess the validity of

Table 2 Performance of point shear wave elastography ($n = 98$) and transient elastography ($n = 101$) in patients with chronic hepatitis C

Parameter	Method	$F \geq 2$	$F \geq 3$	$F = 4$
Cut-off in kPa	PSWE	5.7	5.8	7.2
	TE	6.9	7.3	9.3
AUC	PSWE	0.80 (0.71-0.87)	0.88 (0.80-0.94)	0.95 (0.89-0.99)
	TE	0.82 (0.73-0.89)	0.95 (0.88-0.98)	0.92 (0.85-0.97)
Sensitivity %	PSWE	62.0 (47.2-75.3)	85.2 (66.3-95.8)	90.0 (55.5-99.7)
	TE	62.7 (48.1-75.9)	89.9 (70.8-97.6)	90.0 (55.5-99.7)
Specificity %	PSWE	91.7 (80.0-97.7)	84.5 (74.0-92.0)	88.6 (80.1-94.4)
	TE	83.7 (70.3-92.7)	80.8 (69.9-89.1)	87.8 (79.2-93.7)
PPV %	PSWE	88.6 (73.3-96.8)	67.6 (49.5-82.6)	47.4 (24.4-71.1)
	TE	80.0 (64.1-91.1)	63.2 (45.7-78.4)	45.0 (23.1-78.5)
NPV %	PSWE	69.8 (57.0-80.8)	93.7 (84.7-98.3)	98.7 (93.1-100)
	TE	68.3 (55.0-79.7)	95.2 (86.5-99.0)	98.7 (93.2-100)
+LR	PSWE	7.4 (2.8-19.5)	5.5 (3.1-9.7)	7.9 (4.3-14.7)
	TE	3.8 (2.0-7.5)	4.6 (2.8-7.6)	7.4 (4.1-13.3)
-LR	PSWE	0.4 (0.3-0.6)	0.2 (0.07-0.4)	0.1 (0.02-0.7)
	TE	0.4 (0.3-0.6)	0.1 (0.05-0.4)	0.1 (0.02-0.7)

In parentheses: 95%CI. PSWE: Point shear wave elastography; TE: Transient elastography; kPa: Kilopascals; AUC: Area under the curve; PPV: Positive predictive value; NPV: Negative predictive value; +LR: Positive likelihood ratio; -LR: Negative likelihood ratio.

PSWE, *i.e.*, the repeatability of measurements and the performance of this method. The results show that PSWE is a highly reproducible method for assessing liver stiffness because it was characterized by very high levels of intraobserver and interobserver agreement, both overall and for single measurements. Moreover, the reproducibility of the method was similar in healthy subjects and in patients with chronic viral hepatitis. Ultrasound imaging techniques are subject to user dependency; nonetheless, we observed a high interobserver agreement rate that was similar to that reported for TE^[9]. Nevertheless, good interobserver agreement rates have been reported for other shear wave elastography ultrasound-based techniques, suggesting that the method itself has low variability and requires only a short period of training to be performed reliably^[31-33]. Indeed, the benefits of image guidance will likely reduce the learning curve and the variations between measurements^[33].

The results of this study show that TE and PSWE results are directly and linearly correlated with the stages of fibrosis determined using histology. Furthermore, the performance of PSWE compares with that of TE, the first available technique and the most widely accepted method for noninvasive assessment of liver fibrosis. In our series, liver stiffness did not correlate with liver steatosis in either the univariate or multivariate analysis; thus, steatosis was not a confounding variable. This result is similar to those observed in other studies using shear wave elastography techniques integrated into ultrasound systems, and this result appears to indicate that the value obtained was a true estimate of the stiffness of the liver^[14,16,18,19,34]. The influence of necroinflammation on liver stiffness is controversial; some studies have found an influence^[9,11,14,19,34], and others have not^[2-4,16,18,22].

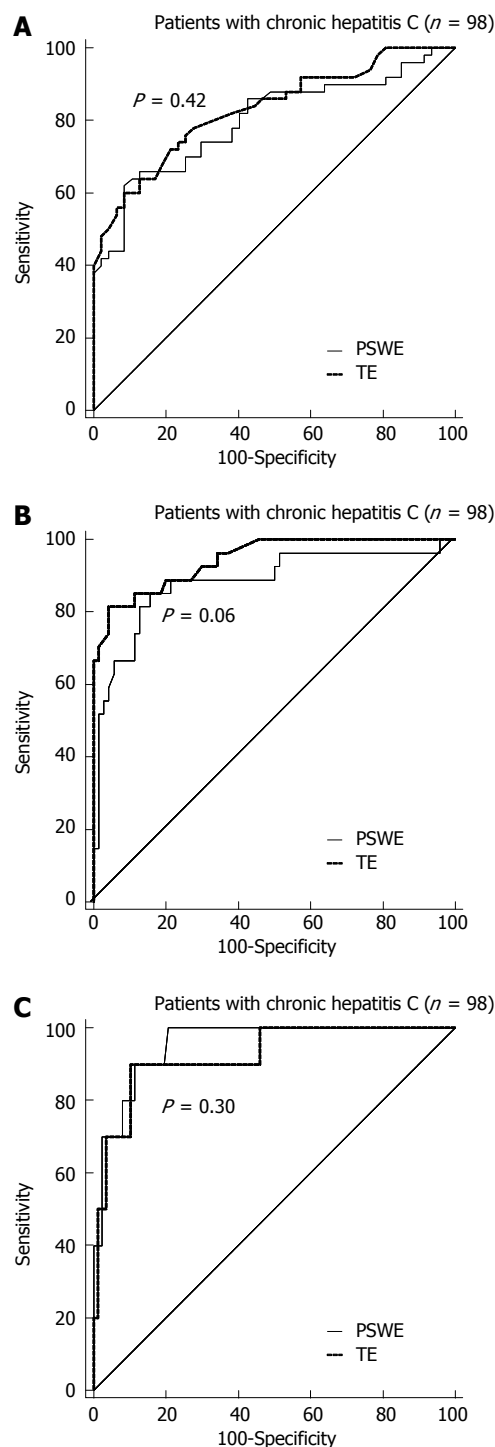


Figure 3 Receiver-operating characteristic curves for different fibrosis thresholds. The performance of point shear wave elastography (PSWE) was compared to that of transient elastography (TE). P values for differences between areas under the curve are given. A: F0-F1 vs F2-F4 ($F \geq 2$); B: F0-F2 vs F3-F4 ($F \geq 3$); C: F0-F3 vs F4 ($F = 4$).

In our series, we found no correlation between liver stiffness and necroinflammation. A positive correlation with GGT was found, which is in agreement with the results of a study by Forns *et al.*^[35], which identified this variable as an independent predictor of liver fibrosis.

The diagnostic accuracy of PSWE was similar to that reported by some other studies, which used a dif-

Table 3 Characteristics of the subjects assessed to determine the reproducibility of point shear wave elastography measurements

Characteristic	Total <i>n</i> = 116	Group 1 <i>n</i> = 47	Group 2 <i>n</i> = 69
Sex, females (%)	44 (37.9)	10 (21.3) ^b	34 (49.3)
Age, yr (SD)	41.9 (13.5)	44.1 (12.5) ^a	38.9 (13.8)
BMI, kg/m ² (SD)	23.2 (4.1)	23.7 (2.7)	23.0 (4.9)
PSWE measurement, rater 1 (IQR)	3.95 (3.3-5.2)	5.34 (4.1-7.3) ^b	3.53 (3.2-4.1)
PSWE measurement, rater 2 (IQR)	3.92 (3.3-5.2)	5.34 (4.1-7.1) ^b	3.52 (3.1-4.0)

SD values represent means, and IQR values represent medians. ^a*P* < 0.05; ^b*P* < 0.01. PSWE: point shear wave elastography; BMI: body mass index. Group 1: Patients with chronic viral hepatitis; Group 2: Healthy volunteers.

ferent point shear wave elastography method that also included acoustic radiation force impulse (ARFI); those studies reported no improvement in accuracy relative to TE for staging liver fibrosis^[14,20,21,36]. Rizzo *et al*^[16] found that ARFI was more accurate than TE for the staging of both significant and severe liver fibrosis. However, those results were not confirmed by a recent meta-analysis that compared ARFI with TE and found comparable diagnostic accuracies of both methods for the diagnosis of severe fibrosis and a slightly but significantly higher diagnostic accuracy of TE for the diagnosis of significant fibrosis and cirrhosis^[17].

PSWE is a recently developed method that is part of the second generation of ultrasound elastography methods. These methods differ from the first-generation TE in several aspects, including the generation of shear waves within the organ by a focused ultrasound beam and the capability of focusing the beam at different locations within the organ under ultrasound image guidance. These properties should improve the feasibility of stiffness measurements in obese patients and patients with ascites; they may also improve the accuracy of PSWE relative to TE. However, the current study demonstrated that neither the feasibility nor the accuracy of PSWE was higher than that of TE. This finding could be attributable to the fact that the patients in our series had a body mass index within the normal range and the absence of patients with ascites. On the other hand, compared with TE, routine ultrasound systems with an elastography technique are advantageous in that they also allow the evaluation of other parameters that are complementary to stiffness, they are highly accurate for the diagnosis of cirrhosis and they could be used to screen for focal liver lesions^[20,37].

The optimal cut-offs identified for each fibrosis stage, which were based on the maximal sensitivity and specificity, were close to each other. However, the diagnostic accuracy, assessed with AUCs, was high, suggesting that the PSWE method is acceptable for staging

Table 4 Intraobserver and interobserver agreement of point shear wave elastography measurements performed by two raters

Measurements	Rater 1 CCC (95%CI)	Rater 2 CCC (95%CI)	Rater 1 vs Rater 2 CCC (95%CI)
1, 2, 3, 4, 5 vs 6, 7, 8, 9, 10	0.96 (0.95-0.97)	0.96 (0.95-0.97)	-
1, 3, 5, 7, 9 vs 2, 4, 6, 8, 10	0.95 (0.93-0.96)	0.96 (0.94-0.97)	-
1, 2, 3, 4, 5	-	-	0.91 (0.88-0.94)
6, 7, 8, 9, 10	-	-	0.93 (0.91-0.95)
1, 3, 5, 7, 9	-	-	0.93 (0.91-0.95)
2, 4, 6, 8, 10	-	-	0.92 (0.90-0.94)
All	-	-	0.93 (0.90-0.95)
1 vs 5	0.93 (0.91-0.95)	0.85 (0.80-0.90)	-
5 vs 10	0.83 (0.79-0.88)	0.88 (0.84-0.92)	-
2 vs 5	0.87 (0.84-0.91)	0.84 (0.79-0.89)	-
3 vs 8	0.93 (0.91-0.95)	0.84 (0.79-0.88)	-
1	-	-	0.89 (0.85-0.93)
2	-	-	0.83 (0.78-0.88)
5	-	-	0.85 (0.80-0.90)
8	-	-	0.84 (0.79-0.90)

CCC: Concordance correlation coefficient; CI: Confidence interval.

liver fibrosis but needs to be refined. On the other hand, liver histology could be an imperfect gold standard because it is affected by intraobserver and interobserver variabilities in fibrosis assessment and represents only 1/50000 of the entire liver mass^[38]. Moreover, a recent study showed that liver biopsy exhibited a relative lower level of performance compared with FibroTest and TE when evaluated similarly for the diagnosis of advanced fibrosis^[39]. As was very recently reported for TE^[25], in our study, both TE and PSWE showed excellent negative predictive value for cirrhosis and very good positive predictive value for significant fibrosis. On the contrary, both techniques showed insufficient positive predictive value for cirrhosis and only fair negative predictive value for significant fibrosis.

The values of stiffness obtained using PSWE in healthy subjects were significantly lower than those obtained in patients with nonsignificant fibrosis (F0-F1) based on liver histology. This result indicates that the PSWE technique, which is noninvasive and readily available in ultrasound systems, could be a useful adjunct tool when performing ultrasound examinations of the liver because this method may allow physicians to select patients who need to be further evaluated for chronic liver disease.

Our study has limitations. First, the different stages of fibrosis, particularly advanced fibrosis and cirrhosis, were not equally represented among the patients in our series; almost half of the patients were at the F0-F1 stage, which may have affected the optimal cut-off values obtained with the ROC curves. This uneven distribution of fibrosis stages among consecutive patients reflects what is normally observed in clinical settings. On the other hand, the Obuchowski measure, which was used to minimize the spectrum bias, was good for both

PSWE and TE. Second, our study population had a low prevalence of obesity, which could be a technical limitation; thus, the applicability of these results is limited. Third, the analysis was performed in a relatively small number of patients; thus, these results need to be validated in larger studies.

In conclusion, PSWE is a highly reproducible method for assessing liver stiffness. For staging liver fibrosis, PSWE compares favorably with TE. Healthy volunteers show significantly lower values compared with patients with nonsignificant fibrosis. Further studies in larger series of patients are needed to confirm these results.

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COMMENTS

Background

In the last decade, methods to noninvasively quantify liver fibrosis have been developed. Ultrasound point shear wave elastography is a noninvasive technique that is implemented in an ultrasound system and is able to assess the mechanical properties of tissues. The stiffness of tissues increases under pathological conditions, such as liver fibrosis. Compared with transient elastography, this technique has the advantage of B-mode image guidance, allowing the best acoustic window for correctly performing an examination to be selected in real time.

Research frontiers

The assessment of liver fibrosis is of outmost importance in the management of patients with chronic viral hepatitis. Ultrasound point shear wave elastography is an affordable technique that could help reduce costs compared with more invasive procedures, such as liver biopsy.

Innovations and breakthroughs

The results of this study show that point shear wave elastography values are directly and linearly correlated to the stages of fibrosis identified by histology. Furthermore, the performance of this technique compares with that of transient elastography, which is the most widely accepted method for the noninvasive assessment of liver fibrosis. In our study, both transient elastography and point shear wave elastography showed excellent negative predictive value for cirrhosis and very good positive predictive value for significant fibrosis.

Applications

Point shear wave elastography, which is a noninvasive technique readily available in an ultrasound system, could be a useful adjunct tool when performing ultrasound examinations of the liver because it may allow physicians to select patients who need to be further evaluated for chronic liver disease.

Terminology

Point shear wave elastography is an ultrasound-based technique that generates shear waves inside the liver using the radiation force from a focused ultrasound beam. The ultrasound machine monitors the shear wave propagation and measures the velocity of the shear wave. Assuming that the tissue exhibits very simple behavior, the shear wave velocity is related to stiffness through Young's modulus. The unit of measure of stiffness is the kilopascal (kPa).

Peer review

The authors investigated a point shear wave elastography method for assessing liver stiffness. This paper seems to be important and promising.

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Role of 3DCT in laparoscopic total gastrectomy with spleen-preserving splenic lymph node dissection

Jia-Bin Wang, Chang-Ming Huang, Chao-Hui Zheng, Ping Li, Jian-Wei Xie, Jian-Xian Lin, Jun Lu

Jia-Bin Wang, Chang-Ming Huang, Chao-Hui Zheng, Ping Li, Jian-Wei Xie, Jian-Xian Lin, Jun Lu, Department of Gastric Surgery, Fujian Medical University Union Hospital, Fuzhou 350001, Fujian Province, China

Author contributions: Wang JB and Huang CM designed the research; Zheng CH, Li P and Xie JW performed the research; Lin JX and Lu J contributed to the diagnostic imaging work; Wang JB and Zheng CH analysed the data; Wang JB and Huang CM wrote the paper.

Correspondence to: Chang-Ming Huang, Professor, Department of Gastric Surgery, Fujian Medical University Union Hospital, No. 29 Xinquan Road, Fuzhou 350001, Fujian Province, China. hcm1r2002@163.com

Telephone: +86-591-83320319 Fax: +86-591-83320319

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Abstract

AIM: To investigate whether computed tomography with 3D imaging (3DCT) can reduce the risks associated with laparoscopic surgery.

METHODS: We performed a retrospective case-control study evaluating the efficacy of preoperative 3DCT of the splenic vascular anatomy on surgical outcomes in patients undergoing laparoscopic spleen-preserving splenic hilar lymph node (LN) dissection for upper- or middle-third gastric cancer. The clinical records of 312 patients with upper- or middle-third gastric cancer who underwent laparoscopic total gastrectomy with spleen-preserving splenic lymph node dissection in our hospital from January 2010 to June 2013 were collected, and the patients were divided into two groups (group 3DCT vs group NO-3DCT) depending on whether they underwent 3DCT or not. Clinicopathologic characteristics, operative and postoperative measures, the number of retrieved LNs, and complications were compared between these two groups. Patients were further compared regarding operative and postoperative measures,

the number of retrieved LNs, and complications when subdivided by body mass index (≥ 23 and < 23 kg/m²) and the number of operations performed by their surgeon (≤ 40 vs > 40).

RESULTS: The mean numbers of retrieved splenic hilar LNs were similar in patients in group 3DCT and group NO-3DCT (2.85 ± 2.33 vs 2.48 ± 2.18 , $P > 0.05$). The operation time and blood loss at the splenic hilum were lower in the patients in group 3DCT ($P < 0.05$ each). The postoperative recovery time and complication rates were similar between the two groups ($P > 0.05$ each). Subgroup analysis showed that the operation time at the splenic hilum in patients with a BMI ≥ 23 kg/m² was significantly shorter in patients in group 3DCT than in group NO-3DCT (20.27 ± 5.84 min vs 26.17 ± 11.01 min, $P = 0.003$). In patients with a BMI < 23 kg/m², the overall operation time (171.8 ± 26.32 min vs 188.09 ± 52.63 min, $P = 0.028$), operation time at the splenic hilum (19.39 ± 5.46 min vs 23.74 ± 9.56 min, $P = 0.001$), and blood loss at the splenic hilum (13.27 ± 4.96 mL vs 17.98 ± 8.12 mL, $P = 0.000$) were significantly lower in patients in group 3DCT than in group NO-3DCT. After 40 operations, the operation time (18.63 ± 4.40 min vs 23.85 ± 7.92 min, $P = 0.000$) and blood loss (13.10 ± 4.17 mL vs 15.10 ± 4.42 mL, $P = 0.005$) at the splenic hilum were significantly lower in patients who underwent 3DCT, but there were no significant between-group differences prior to 40 operations.

CONCLUSION: 3DCT is critical for surgical guidance to reduce the risks of splenic LN dissection. This method may be important in safely facilitating laparoscopic spleen-preserving splenic LN dissection.

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Key words: Stomach neoplasms; Spleen preservation; Laparoscopy; Lymph node dissection; Computed to-

mography angiography with three-dimensional imaging

Core tip: The JGCA guidelines recommend splenic hilar lymph node (LN) dissection in patients with upper- and middle-third advanced gastric cancer. However, the surgery is made more difficult by anatomic complications of the vessels around the stomach, particularly the splenic vessels, which are located in a narrow, deep space. The inability to intuitively judge the shape of the splenic vessels increases the likelihood of vascular injury. Preoperative assessment of the splenic vascular anatomy at the splenic hilum is important for the safe and rapid performance of laparoscopic spleen-preserving splenic hilar LN dissection. Computed tomography with 3D imaging can be used for surgical guidance to reduce the risks of splenic LN dissection.

Wang JB, Huang CM, Zheng CH, Li P, Xie JW, Lin JX, Lu J. Role of 3DCT in laparoscopic total gastrectomy with spleen-preserving splenic lymph node dissection. *World J Gastroenterol* 2014; 20(16): 4797-4805 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v20/i16/4797.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4797>

INTRODUCTION

Since the first report of laparoscopic-assisted distal gastrectomy (LADG) for early gastric cancer^[1], laparoscopic surgery for gastric cancer has been shown to be an effective modality, with significant advantages over open surgery, including a smaller surgical incision, reduced intraoperative bleeding, less postoperative pain, faster recovery of bowel function, a shorter hospital stay, faster return to daily activities, and improved quality of life^[2-6]. The indications for laparoscopic gastrectomy have been extended to include patients with advanced gastric cancer (AGC). Laparoscopic surgery, however, still has limitations, including the lack of tactile sensation, a reduced view of the operative field, and the inability of the surgeon to directly manipulate organs and lesions during surgery. Laparoscopy-assisted total gastrectomy (LATG) is more difficult to perform than LADG due to the additional requirement for the dissection of lymph nodes (LNs) at the splenic hilum and the need for esophagojejunostomy. As laparoscopic techniques have improved, the number of patients undergoing LATG has increased annually^[7]. LATG, which has been reported to be technically more feasible than LADG^[2], is increasingly used in the treatment of upper- and middle-third gastric cancer. Japanese Gastric Cancer Association guidelines recommend splenic hilar LN dissection in patients with upper- and middle-third AGC who undergo LATG with D2 LN dissection^[8]. With the regeneration of the surgical treatment concept and the development of laparoscopic surgery, laparoscopic spleen-preserving splenic hilar LN dissection has gradually been applied to upper- or middle-

third gastric cancer patients.

Despite the increased use of laparoscopic spleen-preserving splenic hilar LN dissection in these patients, surgery is made more difficult by anatomic complications of the vessels around the stomach, particularly the splenic vessels, which are located in a narrow, deep space. The inability to intuitively judge the shape of the splenic vessels increases the likelihood of vascular injury and bleeding. Moreover, a lack of knowledge of splenic vascular anatomy, an inability to manipulate tissues, and a limited operative field of view may result in difficult and time-consuming dissections to search for blood vessels and anatomical landmarks, increasing the risk of iatrogenic vascular and visceral injuries.

The preoperative assessment of splenic vascular anatomy at the splenic hilum is important for the safe and rapid performance of laparoscopic spleen-preserving splenic hilar LN dissection. The vascular anatomy can be mapped preoperatively using computed tomography (CT) angiography, followed by processing of the images with rendering software to reconstruct 3D images of the splenic vessels. These models can be rotated and viewed from different angles to identify the course of each splenic vessel and its relationship to other anatomical structures. Determining vascular anatomy by CT with 3D imaging (3DCT) imaging is critical for reducing the risks associated with laparoscopic gastric cancer surgery^[9]. We, therefore, performed a retrospective case-control study evaluating the efficacy of preoperative 3DCT of the splenic vascular anatomy on surgical outcomes in patients undergoing laparoscopic spleen-preserving splenic hilar LN dissection for upper- or middle-third gastric cancer.

MATERIALS AND METHODS

Patients

Between January 2010 and June 2013, 312 patients with upper- or middle-third gastric cancer underwent laparoscopic-assisted total gastrectomy with D2 LN dissection plus spleen-preserving splenic hilar LN dissection in the Department of Gastric Surgery, Fujian Medical University Union Hospital. All subjects were preoperatively confirmed as having upper- or middle-third AGC by analyses of endoscopic biopsy specimens. Preoperative imaging was routinely performed following endoscopic examination, including CT scanning, ultrasonography (US) of the abdomen, and endoscopic US. All patients also underwent intraoperative diagnostic laparoscopy, including a complete examination of the peritoneal cavity and liver. Patients preoperatively diagnosed with T1 and T4b gastric cancer were excluded. Patients with enlarged and integrated splenic hilar LNs were not considered candidates for surgery. The surgical procedure, including its advantages and risks, was explained to all candidates for surgery. The ethics committee of Fujian Union Hospital approved this retrospective study. Written consent was given by the patients for their information to be stored in the hospital database and used for research.

Data collection

All patients underwent abdominal helical CT (Discovery CT750 HD), with scans ranging from the top of the diaphragm to the lower edge of the liver. An average of 100 mL of nonionic contrast agent was infused at a rate of 2 mL/s. CT data acquisition was triggered using the bolus tracking technique, with the region of interest (ROI) placed in the abdominal aorta just below the diaphragmatic dome. The trigger threshold level was set at a CT value of 100 Hounsfield units. Abdominal CT scans were performed at a slice thickness of 5 mm. During each phase, scanning was performed in a single breath-hold. 3DCT images of the splenic vessels were individually reconstructed using the original scanning images. The CT data were downloaded to an offline workstation for image post-processing and analysis, and 3DCT reconstructions were performed by a team of professional radiologists. The patients were assigned to two groups in accordance with their wishes: 231 patients underwent CT with 3D angiography (group 3DCT), and 81 underwent CT without 3D angiography (group NO-3DCT). The following were compared between the two groups: patient demographic and clinical characteristics, including age, sex, BMI, tumour size, depth of invasion, LN metastasis, and TNM stage^[10]; operative data, including operation time, blood loss, number of positive and retrieved splenic hilar LNs, and number of positive and retrieved LNs; and postoperative data, including postoperative hospital stay, day of first flatus, day of first fluid diet, day of first semisolid diet, and postoperative complications. The operation time at the splenic hilum was defined as the time of splenic hilar LN dissection, and the blood loss at the splenic hilum was defined as the volume of blood lost during dissection of the splenic hilar LNs. The patients in each group were further subdivided by BMI (≥ 23 vs < 23 kg/m²) and the number of operations performed by their surgeon (≤ 40 vs > 40 cases). According to patients' wishes, patients in each subgroup were also distributed into 3DCT and No-3DCT groups, and their surgical outcomes were compared. High (≥ 23 kg/m²) and normal (< 23 kg/m²) BMIs were defined according to the World Health Organisation definitions for individuals in the western Pacific region^[11].

Surgical procedures

Splenic hilar LN dissection was performed according to the guidelines of the Japanese Classification of Gastric Carcinoma^[10]. At our institution, laparoscopic spleen-preserving splenic hilar LN dissection has become highly standardised^[12,13], with all operations performed by a senior surgeon (Chang-Ming Huang) who had performed > 500 laparoscopic-assisted gastrectomies before starting to perform this operation.

Patient positioning: The patient was placed in the reverse Trendelenburg position with the head elevated approximately 15-20 degrees and tilted left-side up approximately 20-30 degrees. The surgeon stood between

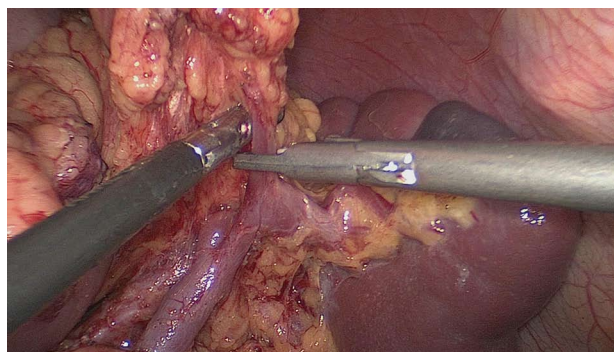


Figure 1 Left gastroepiploic vessels are vascularised at the origin.

the patient's legs, and the assistant and camera operator were both on the patient's right side.

Splenic hilar LN dissection: Before the operation, the assistant placed the greater omentum behind the stomach to keep the visual field clear, pulled the body of the stomach towards the upper right, and tensed the spleno-gastric ligament while the surgeon gently pressed the tail of the pancreas towards the lower left, thus exposing the splenic hilum. The surgeon opened the pancreatic envelope, ultrasonically separated the membrane of the body and tail of the pancreas to reach the posterior pancreas space at the superior border of the pancreas, and opened the vascular envelope at the end of the splenic arteries. The surgeon dissected the lymphatic fatty tissue on the surface of the inferior splenic lobar artery from the lower pole of the spleen. The left gastroepiploic artery issuing from the inferior splenic lobar artery was vascularised and clamped after cutting its origin (Figure 1). The assistant gently pulled the lymphatic fatty tissue on the surface of the inferior splenic lobar artery. Starting from the root of the left gastroepiploic artery, the surgeon, using the non-functioning face of the ultrasonic scalpel, closed the surface of the inferior splenic lobar artery. The surgeon used the ultrasonic scalpel to carefully dissect the lymphatic fatty tissue and vascularise the inferior splenic lobar artery. As the latter was gradually revealed, the two branches of the short gastric arteries issuing from it were skeletonised and divided at their roots, resulting in the complete vascularisation of the inferior splenic lobar artery (Figure 2). The assistant then pulled up the fatty tissues and gastric tissues, and the surgeon dissected the lymphatic fatty tissue on the surface of the superior splenic lobar artery, starting from its root towards the upper pole of the spleen, similar to the procedure used to vascularise the inferior splenic lobar artery. One branch of the short gastric artery issuing from the superior splenic lobar artery was skeletonised and divided at its root, thus concluding the dissection of the LNs at the front of the splenic vessels.

The assistant pulled the root of the inferior splenic lobar artery towards the upper right, revealing the lymphatic fatty tissue behind the splenic hilum, which was pulled by the surgeon towards the lower left to maintain the tension (Figure 3). This lymphatic fatty tissue behind

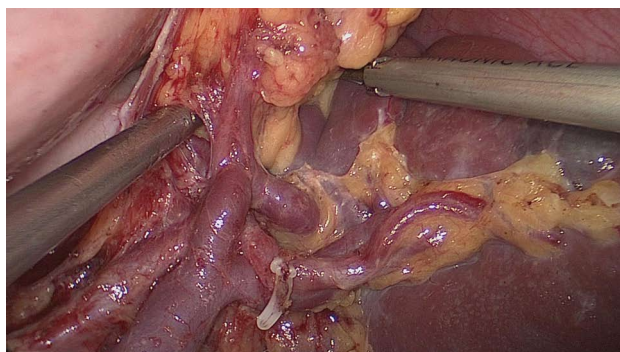


Figure 2 Short gastric vessels are freed, clamped, and cut at the origin.

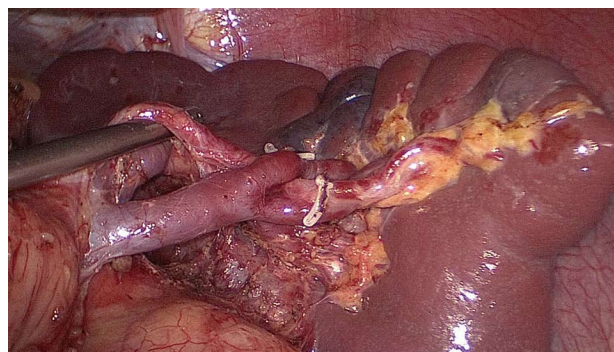


Figure 4 Fatty tissues, including the splenic lymph nodes, are en-bloc removed from the splenic hilum.

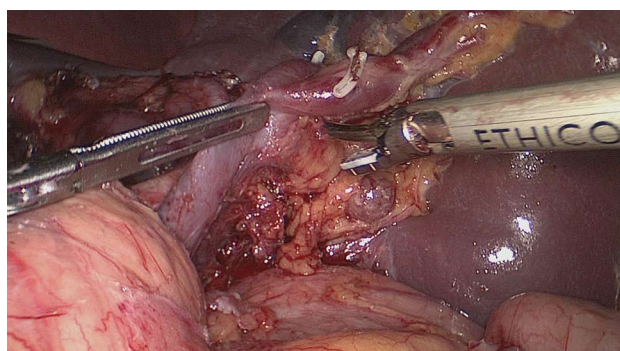


Figure 3 Lymphatic fatty tissues behind the splenic hilum are dissected.

the splenic hilum was dissected. Finally, the splenic hilar LNs were dissected completely (Figure 4).

Statistical analysis

Statistical analyses were performed using the SPSS 18.0 statistical software package. Means were compared using t-tests and categorical data using Chi-square tests. Proportions were compared by the χ^2 test or Fisher's exact test. P values < 0.05 were considered statistically significant.

RESULTS

Between January 2010 and December 2012, 312 patients with upper- or middle-third gastric cancer underwent laparoscopic-assisted total gastrectomy with D2 LN dissection and spleen-preserving splenic hilar LN dissection. Of these, 231 patients underwent CT scans with 3D angiography, and 81 underwent CT scans without 3D angiography. The mean age of the 312 patients was 60.86 ± 10.51 years. The clinicopathologic characteristics were similar among patients in both the 3DCT and NO-3DCT groups (Table 1). The types of splenic lobe vessels observed preoperatively in group 3DCT were in accordance with their intraoperative conditions (Figures 5-7). The mean operation time (173.65 ± 27.12 min *vs* 189.56 ± 48.36 min; $P = 0.007$), mean operation time at the splenic hilum (19.70 ± 5.59 min *vs* 24.47 ± 9.98 min; $P = 0.001$), and mean blood loss at the splenic hilum (13.62 ± 4.50

Table 1 Patient characteristics in group computed tomography with 3D imaging and group NO-computed tomography with 3D imaging

Patient characteristics	Group NO-3DCT	Group 3DCT	<i>P</i> value
No. of patients	81	231	
Age (yr)	59.48 ± 10.82	61.34 ± 10.38	0.542
Gender			0.484
Male	58	178	
Female	23	52	
BMI, kg/m ²	21.67 ± 3.24	21.95 ± 2.74	0.273
Tumour size, mm	57.53 ± 23.66	55.76 ± 27.88	0.251
Histology			0.262
Differentiated	29	98	
Undifferentiated	52	133	
Depth of invasion			0.098
pT1a	5	10	
pT1b	6	9	
pT2	1	22	
pT3	29	87	
pT4a	40	103	
Lymph node metastasis			0.090
pN0	25	45	
pN1	13	37	
pN2	12	43	
pN3	31	106	
TNM stage			0.487
I a	6	15	
I b	4	12	
II a	15	25	
II b	9	27	
III a	12	31	
III B	12	52	
III C	23	69	

3DCT: Computed tomography with 3D imaging; BMI: Body mass index.

mL *vs* 17.92 ± 9.08 mL; $P = 0.001$) were lower in patients who were not evaluated by 3DCT. In contrast, the number of retrieved and positive splenic hilar LNs, number of retrieved and positive LNs, and mean blood loss were similar in the two groups ($P > 0.05$ each). There were also no significant differences between these two groups with regards to days to first flatus, first fluid diet, and first semifluid diet, postoperative hospital stay, and complications ($P > 0.05$) (Tables 2 and 3).

Subgroup analyses of the patients with a BMI ≥ 23 kg/m² showed that those who were evaluated by preop-

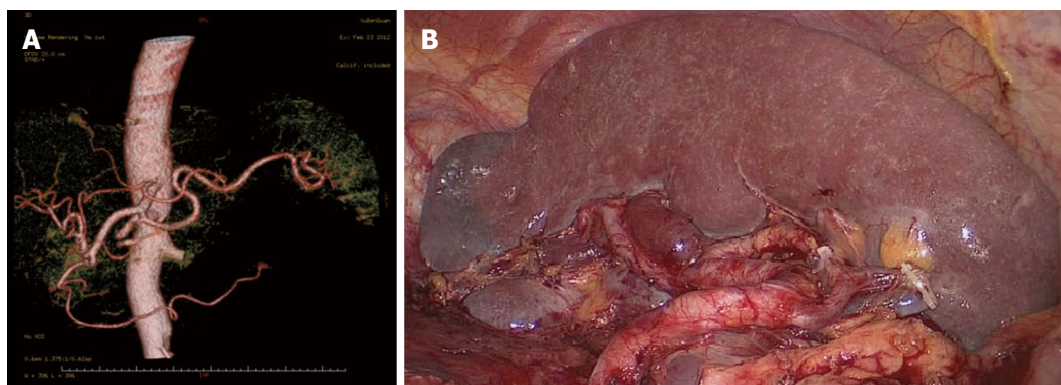


Figure 5 A single-lobe splenic vessel. A: Preoperative assessment of the splenic vascular anatomy using computed tomography with 3D imaging images; B: Operative view after the completion of splenic lymph node dissection.

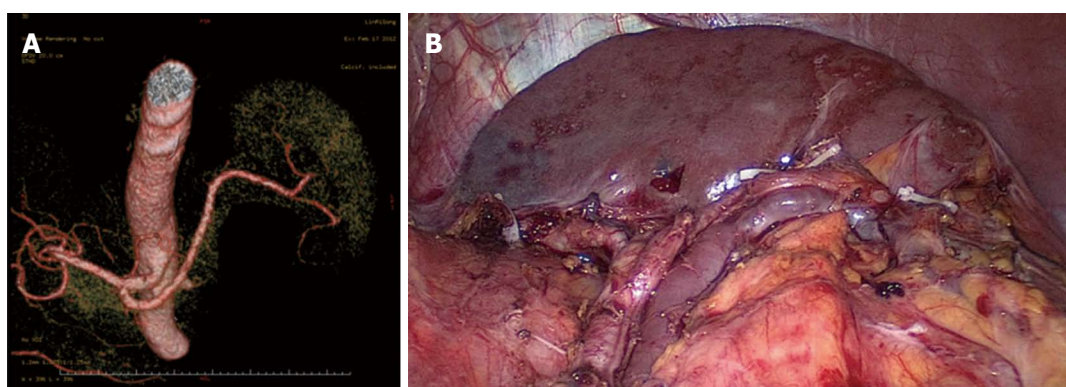


Figure 6 Two-lobe splenic vessels. A: Preoperative assessment of the splenic vascular anatomy using computed tomography with 3D imaging images; B: Operative view after the completion of splenic lymph node dissection.

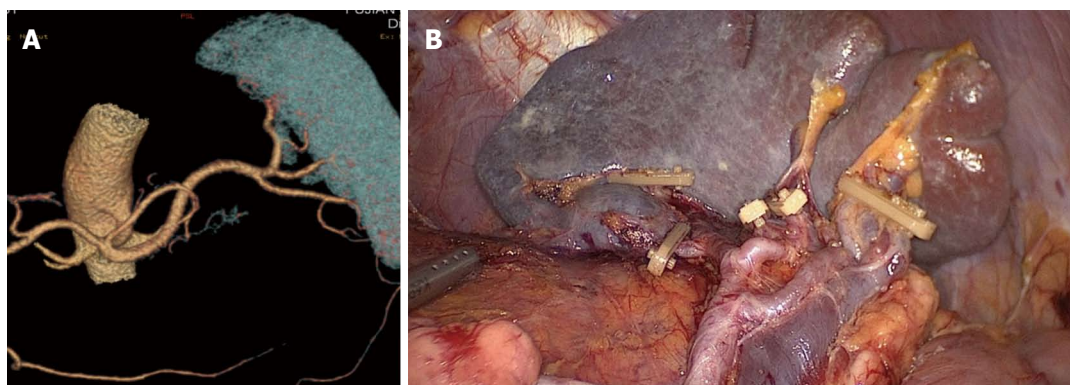


Figure 7 Three-lobe splenic vessels. A: Preoperative assessment of the splenic vascular anatomy using computed tomography with 3D imaging images; B: Operative view after the completion of splenic lymph node dissection.

erative 3DCT had a significantly shorter mean operation time at the splenic hilum (20.27 ± 5.84 min *vs* 26.17 ± 11.01 min; $P = 0.003$). Of the patients with a BMI < 23 kg/m², those who underwent 3DCT had a significantly lower overall operation time (171.8 ± 26.32 *vs* 188.09 ± 52.63 min, $P = 0.028$), operation time at the splenic hilum (19.39 ± 5.46 *vs* 23.74 ± 9.56 min, $P = 0.001$), and blood loss at the splenic hilum (13.27 ± 4.96 *vs* 17.98 ± 8.12 ml, $P = 0.000$) than did the patients in group NO-3DCT (Table 4). To determine the impact of 3DCT on

these parameters, we assessed the effect of the “learning curve” for laparoscopic spleen-preserving splenic hilar LN dissection. A previous report from our department indicated that the learning curve for laparoscopic spleen-preserving splenic hilar LN dissection reached a plateau after 40 operations^[13]. When patients were divided into those treated by surgeons who had performed ≤ 40 or > 40 of these operations, we found that of the patients operated on by surgeons who had performed > 40 operations, those who had undergone 3DCT had a signifi-

Table 2 Operative data in group computed tomography with 3D imaging and group NO-computed tomography with 3D imaging

	Group NO-3DCT	Group 3DCT	<i>P</i> value
Operation time, min	189.56 ± 48.36	173.65 ± 27.12	0.007
Operation time at splenic hilum, min	24.47 ± 9.98	19.70 ± 5.59	0.001
Blood loss, mL	57.34 ± 30.78	50.18 ± 28.89	0.064
Blood loss at the splenic hilum, mL	17.92 ± 9.08	13.62 ± 4.50	0.001
No. of positive No. 10 lymph nodes	0.25 ± 0.79	0.24 ± 0.85	0.889
No. of retrieved No. 10 lymph nodes	2.48 ± 2.18	2.85 ± 2.33	0.225
No. of positive lymph nodes	7.97 ± 10.65	9.10 ± 10.08	0.414
No. of retrieved lymph nodes	39.47 ± 13.51	43.19 ± 15.64	0.067

3DCT: Computed tomography with 3D imaging.

cantly lower operation time (18.63 ± 4.40 min *vs* 23.85 ± 7.92 min, $P = 0.000$) and blood loss (13.10 ± 4.17 mL *vs* 15.10 ± 4.42 mL, $P = 0.005$) at the splenic hilum than patients who had not undergone 3DCT. In contrast, there were no significant differences between these two groups when operated on by surgeons who had performed < 40 such operations (Table 5).

DISCUSSION

Advances in surgical concepts, improvements in anatomical techniques, and progress in organ retention have indicated the increased feasibility of spleen-preserving splenic LN dissection^[14-16]. Laparoscopic splenic LN dissection has also been shown to be safe and feasible compared with open surgery. Its advantages over open surgery include its minimal invasiveness and the ability to leave the spleen intact without mobilising it while similarly dissecting the splenic LNs^[17-19].

The identification of the splenic vessels is often critical in performing laparoscopic spleen-preserving splenic hilar LN dissection. Clinically, the vessels in the splenic hilum are particularly intricate and variable and are covered with much fatty lymphoid tissue; in addition, the spleen often adheres to the omentum or peritoneum. The areas adjacent to the splenic hilum are complex and located in a narrow, but very deep, operating space, making it difficult and time consuming to identify the proper vessels in each patient and to complete splenic regional LN dissection during both open and laparoscopic procedures. Moreover, the laparoscopic technique has significant limitations, being essentially two dimensional with a loss of depth perception and spatial orientation. It also lacks the surgeon's intuitive touch and exposure, with laparoscopic grasping forceps used only for traction and separation in the local area. The inability to manipulate tissue and the limited view of the operative field hinder the identification of vessels and procedure-specific ana-

Table 3 Postoperative data in group computed tomography with 3D imaging and group NO-computed tomography with 3D imaging

	Group NO-3DCT	Group 3DCT	<i>P</i> value
Postoperative hospital stay, d	11.03 ± 2.84	12.29 ± 6.49	0.094
Day of first flatus, d	3.95 ± 1.07	4.19 ± 1.04	0.079
Day of first fluid diet, d	4.29 ± 1.12	4.55 ± 1.70	0.205
Day of first semifluid diet, d	7.85 ± 1.92	8.15 ± 3.52	0.463
Blood transfusion, <i>n</i>	0	0	1.000
Postoperative complications, <i>n</i>	11	35	0.731
Pulmonary infection, <i>n</i>	7	11	0.141
Abdominal infection, <i>n</i>	1	12	0.085
Chylous fistula, <i>n</i>	1	2	0.770
Wound problem, <i>n</i>	0	3	0.303
Anastomotic leakage, <i>n</i>	0	3	0.303
Anastomotic bleeding, <i>n</i>	1	3	0.965
Septicaemia, <i>n</i>	0	1	0.554
Postoperative mortality, <i>n</i>	0	0	1.000

3DCT: Computed tomography with 3D imaging.

tomical landmarks. This difficulty results in longer operating times and an increased risk of visceral and vascular injuries. The latter can cause major complications, such as massive bleeding and bowel ischaemia, particularly for obese patients. In general, laparoscopic spleen-preserving splenic hilar LN dissection is much more difficult in obese patients because of their narrower abdominal cavity, greater accumulation of fatty lymphoid tissue covering the vessels, and limited visualisation of surgical fields^[20]. Preoperative 3DCT evaluation of vascular anatomy may therefore aid the safe and rapid ligation of vessels and dissection of the splenic hilar LNs, particularly in obese patients.

The importance of the preoperative assessment of vessel anatomy has been recognised since the era of interventional percutaneous angiography. Improvements in CT scanner technology have enabled the accurate reconstruction of the images of various vessels^[21]. 3DCT has been shown to be clinically useful in various diagnostic fields, including abdominal surgery, in which 3DCT has been utilised for the preoperative evaluation of aortic aneurysms and pancreatic cancers and for the preoperative assessment and planning of liver and kidney transplants^[22]. Recent advances in 3DCT have also enabled its use in gastric resection. In addition, 3D angiography prior to laparoscopic gastrectomy has been shown to be useful in the detection of perigastric vessels, including the left gastric artery and left gastric vein^[23-26]. 3DCT imaging of the vascular anatomy was found to be of critical importance in reducing the risks associated with laparoscopic gastric cancer surgery^[9] and for reducing blood loss during surgery^[26]. The overlapping of different phase images with 3D reconstruction also helps to identify the anatomical correlations between arteries and veins^[27]. Prior knowledge of the particular gastric vascular anatomy in a patient can aid accurate surgical planning, reducing the risk of complications. To our knowledge, no previous

Table 4 Subgroup comparison according to body mass index

	BMI < 23 kg/m ² (n = 201)			BMI ≥ 23 kg/m ² (n = 111)		
	Group NO-3DCT	Group 3DCT	P value	Group NO-3DCT	Group 3DCT	P value
No. of patients	56	145		25	86	
Age, yr	57.73 ± 11.59	60.95 ± 11.43	0.745	63.40 ± 7.72	61.99 ± 8.37	0.807
Operation time, min	188.09 ± 52.63	171.38 ± 26.32	0.028	187.92 ± 32.00	177.44 ± 28.17	0.121
Operation time at splenic hilum, min	23.74 ± 9.56	19.39 ± 5.46	0.001	26.17 ± 11.01	20.27 ± 5.84	0.003
Blood loss, mL	61.27 ± 35.94	51.83 ± 22.66	0.073	48.33 ± 7.61	49.41 ± 34.73	0.670
Blood loss at splenic hilum, mL	17.98 ± 8.12	13.27 ± 4.96	0.000	17.78 ± 11.28	14.29 ± 3.39	0.054
No. of retrieved lymph nodes	38.38 ± 12.25	46.18 ± 16.86	0.002	42.09 ± 16.15	37.86 ± 11.48	0.172
No. of retrieved No. 10 lymph nodes	2.67 ± 2.27	3.20 ± 2.42	0.162	2.04 ± 1.94	2.23 ± 2.06	0.692
Postoperative hospital stay, d	10.69 ± 2.29	11.62 ± 5.84	0.256	11.79 ± 3.77	13.42 ± 7.36	0.299
Postoperative complications, n	9	18	0.495	2	17	0.169

3DCT: Computed tomography with 3D imaging; BMI: Body mass index.

Table 5 Subgroup comparison according to the number of cumulative laparoscopic spleen-preserving splenic hilar lymph node dissection cases

	Less than 40 cases (n = 40)			More than 40 cases (n = 272)		
	Group NO-3DCT	Group 3DCT	P value	Group NO-3DCT	Group 3DCT	P value
No. of patients	18	22		59	213	
Age, yr	61.05 ± 10.14	59.22 ± 13.46	0.827	63.81 ± 8.03	60.96 ± 10.52	0.053
Operation time, min	205.00 ± 67.36	199.44 ± 46.99	0.769	176.40 ± 27.83	173.39 ± 27.59	0.466
Operation time at the splenic hilum, min	30.27 ± 11.58	28.92 ± 11.61	0.779	23.85 ± 7.92	18.63 ± 4.40	0.000
Blood loss, mL	69.32 ± 37.49	66.35 ± 33.10	0.368	49.18 ± 23.25	50.29 ± 29.97	0.799
Blood loss at the splenic hilum, mL	30.27 ± 8.17	32.38 ± 13.61	0.514	15.10 ± 4.42	13.10 ± 4.17	0.005
No. of retrieved lymph nodes	34.97 ± 10.67	25.33 ± 10.26	0.140	43.54 ± 14.61	43.47 ± 15.56	0.980
No. of retrieved No. 10 lymph nodes	2.81 ± 2.00	2.33 ± 1.87	0.313	2.74 ± 2.33	2.88 ± 2.33	0.719
Postoperative hospital stay, d	12.87 ± 1.78	11.91 ± 2.59	0.112	11.93 ± 5.27	12.15 ± 6.37	0.809
Postoperative complications, n	3	3	0.789	8	32	0.779

3DCT: Computed tomography with 3D imaging.

study has assessed the detectability of the splenic vessels or analysed their anatomic variety to properly plan laparoscopic spleen-preserving splenic hilar LN dissection. 3DCT not only produces high-quality images, which provide excellent visualisation of the vessel anatomy, but can also detect subtle vascular abnormalities. 3D reconstructions have enabled the planning of dissections and the localisation of topographical landmarks for the identification of the splenic vessels, helping to avoid lengthy and harmful dissections while searching for splenic blood vessels or those with non-typical courses and even simplifying their identification in obese patients. These 3D images can be rotated, magnified, and examined in different planes to evaluate the anatomical relationships and anomalies of the splenic vessels and their collateral vessels, as well as their presence or absence^[28].

The types of splenic lobe vessels preoperatively detected by 3DCT were in accordance with intraoperative conditions. Intraoperative complications, such as vascular and visceral injuries and bleeding, as well as the mean operation time and blood loss at the splenic hilum, were significantly reduced in patients who underwent 3DCT compared to those who did not. 3DCT enabled surgeons to determine the distribution of the splenic vessels preoperatively, avoiding intraoperative searching for missing vessels and helping to identify those that were present.

The subgroup analysis showed that the operation time at the splenic hilum was significantly lower in patients with a BMI ≥ 23 kg/m² who had undergone 3DCT than in those who had not. Moreover, both the operation time at the splenic hilum and blood loss were significantly lower in patients with a BMI < 23 kg/m² who had undergone 3DCT. In patients with a higher BMI, visualisation of the surgical fields is limited, the anatomical level of the splenic vessels is unclear due to excessive fat accumulation, and the blood supply is rich in soft tissues. All of these factors can result in uncontrollable bleeding and make surgical fields unclear, increasing the risks and operation time during laparoscopic splenic hilar LN dissection. Thus, precise preoperative knowledge of the splenic vascular anatomy can reduce stress and operation time and avoid unnecessary injury to blood vessels. In patients with a normal BMI without obesity, the variation of the splenic hilar blood vessels is the most important influencing factor when undergoing splenic hilar LN dissection. With the ability to preoperatively confirm the splenic vascular anatomy variation by 3DCT, surgical difficulties and operation time would be dramatically decreased, as would splenic vascular injuries. 3DCT presents more advantages in patients with a normal BMI.

We also performed a subgroup analysis based on the experience of the surgeon in performing laparoscopic

spleen-preserving splenic hilar LN dissection for upper- and middle-third gastric cancer. Twenty-two patients underwent 3DCT and had surgeons who had completed less than 40 operations; 59 patients did not undergo 3DCT and had surgeons who had completed more than 40 operations. Both sets of patients were allocated into the appropriate subgroup according to their wishes. Both the operation time and blood loss at the splenic hilum in patients operated on by surgeons who had performed more than 40 such operations were significantly lower with 3DCT than those in patients without 3DCT. Although the operation time and blood loss were also lower with versus without 3DCT during operations performed by surgeons with less surgical experience, those differences were not statistically significant. Operative technique has been shown to affect the operation time and blood loss at the splenic hilum but not vessel reconstruction. However, surgeons gain additional experience with the improvement of surgical technology, and splenic vascular anatomic variation has gradually become the essential factor affecting operation time and blood loss. 3DCT can have significant effects. Without sufficient knowledge of splenic anatomy, surgeons may injure splenic vessels during LN dissection, increasing operation time and blood loss. By contrast, with the wide application of 3DCT, all these injuries could be avoided during an operation performed by an informed surgeon. Therefore, 3DCT could be beneficial for splenic hilar LN dissection.

In conclusion, preoperative 3DCT analysis of splenic vessels is precise and informative for surgeons, enabling laparoscopic total gastrectomy with spleen-preserving splenic LN dissection to be performed more easily and safely.

COMMENTS

Background

The JGCA guidelines recommend splenic hilar lymph node (LN) dissection in patients with upper- and middle-third advanced gastric cancer. However, the surgery is made more difficult by anatomic complications of the vessels around the stomach, particularly the splenic vessels, which are located in a narrow, deep space. The inability to intuitively judge the shape of the splenic vessels increases the likelihood of vascular injury and bleeding. Preoperative assessment of the splenic vascular anatomy at the splenic hilum is important for the safe and rapid performance of laparoscopic spleen-preserving splenic hilar LN dissection.

Research frontiers

Vascular anatomy can be mapped preoperatively using computed tomography (CT) angiography, followed by processing of the images with rendering software to reconstruct 3D images of the splenic vessels. These models can be rotated and viewed from different angles to identify the course of each splenic vessel and its relationship to other anatomical structures.

Innovations and breakthroughs

The results of the current study demonstrate that with the help of computed tomography with 3D imaging (3DCT), reduced operation time and blood loss at the splenic hilum can be achieved. Furthermore, the operation time at the splenic hilum in patients with a body mass index (BMI) ≥ 23 kg/m² was also shorter in group 3DCT than in group NO-3DCT, as was the overall operation time, operation time at the splenic hilum, and blood loss at the splenic hilum in 3DCT group patients with a BMI < 23 kg/m². After 40 operations, the operation time and blood loss at the splenic hilum were significantly lower in patients who underwent 3DCT.

Applications

Preoperative 3DCT analysis of splenic vessels is precise and informative for surgeons, enabling laparoscopic total gastrectomy with spleen-preserving splenic LN dissection to be performed more easily and safely. Preoperative 3DCT can be used for surgical guidance to reduce the risks of splenic LN dissection.

Terminology

BMI: Body mass index was used as an objective index to indicate massive obesity. The cutoff value was chosen according to the World Health Organisation Western Pacific Region: The Asia-Pacific Perspective.

Peer review

This is an interesting study in which the authors evaluate the efficacy of 3DCT for assessing the distribution of splenic vessels in patients with upper- and middle-third gastric cancer. This finding has important clinical implications for gastrointestinal surgeons in spleen-preserving splenic LN dissection, particularly for beginners.

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Nasogastric tube as protection for recurrent oesophageal stricture: A case report

Marek Woynarowski, Maciej Dądalski, Violetta Wojno, Mikołaj Teisseyre, Marek Szymczak, Anna Chyżyńska, Leszek Hurkała, Emil Płowiecki, Jakub Kmiołek

Marek Woynarowski, Maciej Dądalski, Violetta Wojno, Mikołaj Teisseyre, Jakub Kmiołek, Department of Gastroenterology, Hepatology and Feeding Disorders, Children's Memorial Health Institute, Warsaw 04-730, Poland

Marek Szymczak, Anna Chyżyńska, Department of Pediatric Surgery and Organ Transplantation, Children's Memorial Health Institute, Warsaw 04-730, Poland

Leszek Hurkała, Emil Płowiecki, Balton Ltd., Warsaw 03-152, Poland

Author contributions: Woynarowski M, Dądalski M, Wojno V, Teisseyre M, Szymczak M, Chyżyńska A and Kmiołek J provided medical care to the patient and performed the endoscopic therapy; Woynarowski M, Dądalski M, Wojno V and Teisseyre M developed the idea for the perforated nasogastric tube; Hurkała L and Płowiecki E prepared the prototype of the perforated nasogastric tube; Woynarowski M wrote the paper.

Correspondence to: Marek Woynarowski, MD, PhD, Department of Gastroenterology, Hepatology and Feeding Disorders, Children's Memorial Health Institute, Al. Dzieci Polskich 20, Warsaw 04-730, Poland. m.woynarowski@czd.pl

Telephone: +48-22-8157386 Fax: +48-22-8157382

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Abstract

This report presents the case of an 8.5-year-old boy with Down syndrome after experiencing extensive caustic injury to the oesophagus and stomach resulting from the accidental ingestion of concentrated sulphuric acid. The patient had undergone 32 unsuccessful endoscopic oesophageal stricture dilatations and stenting procedures performed over a period of 15 mo following the accident. Surgical reconstruction of the oesophagus was not possible due to previous gastric and cardiac surgeries for congenital conditions. Before referring the patient for salivary fistula surgery, the patient received a nasogastric tube with perforations located above

the upper margin of the oesophageal stenosis for the passage of saliva and fluid. The tube was well tolerated and improved swallowing; however the backflow of gastric contents caused recurrent infections of the respiratory tract. To overcome these problems, we developed a double lumen, varying diameter, perforated tube for protection of the oesophageal closure. This nasogastric tube was found to be safe and decreased the need for hospitalization and further endoscopic procedures. This newly developed tube can thus be considered as a treatment option for patients with recurrent oesophageal stenosis and contraindications for surgical oesophageal reconstruction.

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Key words: Corrosive oesophageal stenosis; Oesophageal dilatation; Oesophageal stenting; Nasogastric tube

Core tip: This report presents the design and use of a perforated nasogastric tube for passage of saliva and fluids in a paediatric patient who was unsuitable for oesophageal reconstructive surgery. The perforated tube was safe, well tolerated and reduced the need for hospitalization and endoscopic oesophageal dilatation. Therefore, this newly developed perforated nasogastric tube can be used as an alternative method for corrosive oesophageal stenosis therapy in patients who cannot undergo reconstructive surgery.

Woynarowski M, Dądalski M, Wojno V, Teisseyre M, Szymczak M, Chyżyńska A, Hurkała L, Płowiecki E, Kmiołek J. Nasogastric tube as protection for recurrent oesophageal stricture: A case report. *World J Gastroenterol* 2014; 20(16): 4806-4810 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4806.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4806>

INTRODUCTION

Corrosive agents can result in stricture of the oesophagus and/or stomach, requiring endoscopic treatment to restore the easy passage of at least semi-solid food through the oesophagus^[1,2]. Alternative therapies for treatment of these strictures include the use of stents or reconstructive surgery with an intestinal loop. This report presents the case of a paediatric patient with massive chemical injury of the upper gastrointestinal tract who underwent several methods of endoscopic and stenting therapy and could not be treated surgically. The patient was finally stabilized by the implementation of a newly developed perforated nasogastric tube for the protection of a complete oesophageal closure.

CASE REPORT

An 8.5-year-old boy with trisomy 21 was transferred to our hospital 1 mo after accidentally ingesting concentrated sulphuric acid (battery electrolyte). The accident resulted in massive chemical injury of the oesophagus and stomach, with extensive multi-level oesophageal stricture and critical pyloric stenosis. The patient's medical history included a total colon resection for aganglionosis and cardiac surgery for a congenital heart defect. The patient required surgical pyloric bypass and gastrostomy with endless thread placement for further oesophageal stricture dilatations. The patient had undergone 18 procedures of thread-guided oesophageal dilatation and several endoscopies with Savary-Gillard dilatation over the next 10 mo. The effect of dilatation was short, with oesophageal re-stenosis present within 7-10 d of the procedure.

Eleven months after the initial chemical injury, the patient underwent implantation of a coated, metal oesophageal stent (HANAROSTENT; M.I. Tech, Gyeonggi-do, South Korea). This stent migrated to the stomach 3 d after placement and was replaced by another coated, metal stent (WallFlex; Boston Scientific, MA, United States). The patient complained of pain and a foreign body sensation in the chest. The patient tolerated solid food until week 6 and subsequently developed dysphagia related to mucosal hypertrophy and stent orifice occlusion. Removal of the stent resulted in a complete restoration of the oesophageal lumen. However, oesophageal re-stenosis occurred within 2 wk and the patient was returned to endoscopic dilatation treatment. The effects of these procedures were very short and symptoms of re-stenosis were present within a few days of the dilatation.

The lack of effective treatment and severe symptoms of oesophageal stenosis (*i.e.*, the patient could not swallow saliva) prompted the insertion of a 16 Fr nasogastric tube with several perforations above the stricture level (Figure 1). The nasogastric tube resulted in an improvement in swallowing, and the patient's mother reported that his clothing was no longer moistened during the day, and his pillow remained dry during sleeping. Although

Nasogastric tube with perforation located above the upper margin oesophageal stricture

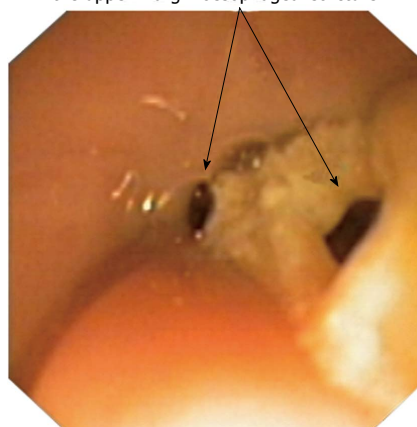


Figure 1 Insertion of nasogastric tube with perforations located above the stricture margin.

Table 1 Comparison of treatments

	Oesophageal dilatation and stenting (Sep 2010-Nov 2011)	Perforated tube protecting oesophageal closure (Nov 2011-Apr 2013)	Tube developed at our institution (Apr 2013-Jan 2014)
Duration of therapy, mo	15	18	9
Hospitalizations, <i>n</i>	27	8	1
Duration of hospitalization (d)	112	32	3
Procedures, <i>n</i>	32	8	1

the patient could easily swallow saliva and liquids, a regurgitation of gastric contents through the perforations located above the stricture was frequently observed. The tube was removed after 6 wk. The oesophageal mucosa was white and fragile and bled easily, but did not have deep ulcerations. An endoscope could be introduced into the stomach without forced traction; although, the effect was not long-lasting and oesophageal re-stenosis reappeared after 2 wk.

A new 16 Fr nasogastric tube was inserted, which was replaced 3 times and remained in place for 16 mo. Within this time period, the patient's weight and height increased. The patient's condition was satisfactory, and he was able to swallow saliva and liquid food. The method was generally well accepted by the patient, except for complaints regarding the thick tube extending from the nose and the frequent backflow of gastric contents to the mouth, which caused coughing and recurrent mild infections of the respiratory tract. This therapy decreased the number and duration of hospitalizations, as well as the number of endoscopic procedures (Table 1).

The improvement in the patient's condition prompted the development at our institution of a specific double lumen, varying diameter, perforated, oesophageal closure protection tube (Figure 2). The closure protection part of the tube was built coaxially over a thin (8 Fr) naso-

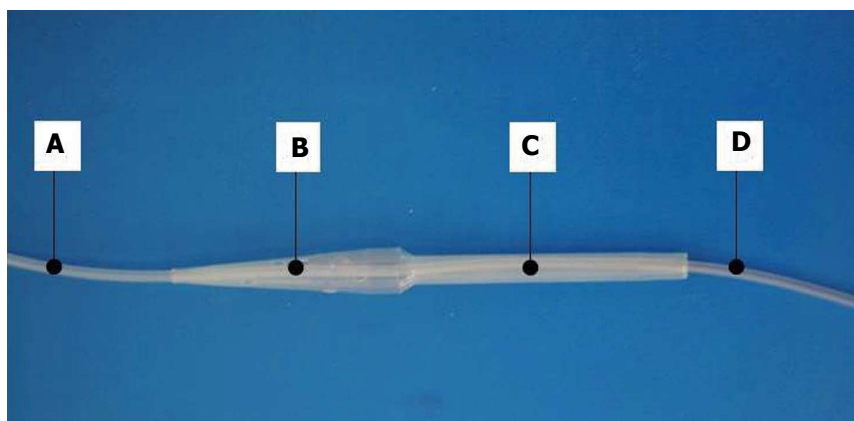


Figure 2 Newly developed nasogastric tube. A perforated nasogastric oesophageal closure protecting tube made of a polyamide polymer, with a double lumen and varying diameter, was developed at the Children's Memorial Health Institute. A: Proximal part of the 8 Fr nasogastric tube; B: Conical, perforated segment setting the tube above the stenosis; C: Portion of the tube located within the stenosis and preventing it from narrowing; D: Distal end of the 8 Fr nasogastric tube to be introduced into the stomach. Liquid diet can be administered into the stomach directly by the nasogastric tube, while oral liquids and saliva can drain through the perforation in the conical portion (B) into the portion located within the stenosis (C), and further into healthy oesophagus below the stenosis. The technology of tube construction, manufacturing and prototype tube production were performed by Balton Ltd. The tube has been registered at the Polish Patent Office (No. P.399031)^[9].

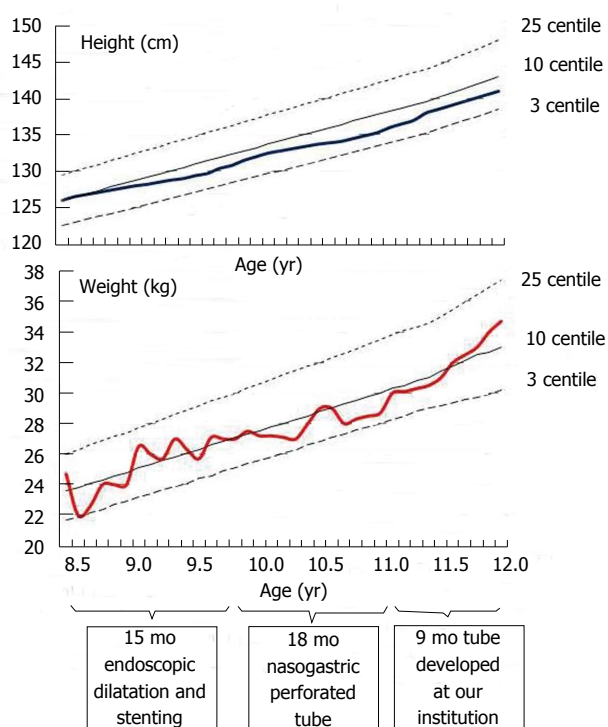


Figure 3 Patient's grow charts. Height and weight of the patient during three phases of therapy: oesophageal dilatation and stenting (15 mo); nasogastric tube with perforation for saliva and fluid passage (18 mo); double lumen, varying diameter nasogastric tube developed at our institution (9 mo).

gastric tube. The proximal end of the tube was fixed to the nose and the distal end was located in the stomach. The diameter and length of the portion located within the oesophageal stricture can be adjusted to the stenosis. The conical proximal end is perforated, allowing for the passage of fluids. The newly developed tube was inserted into the patient in April 2013, allowing him to easily swallow saliva and tolerate liquid diet. The coughing and respiratory tract infections related to the backflow of

gastric contents were eliminated. The tube was replaced with a new, identical one after 5 mo due to partial occlusion of the tube perforations by food. The patient has continued to do well and has been gaining weight (Figure 3). During the 18 mo of therapy with a perforated nasogastric tube and 9 mo of therapy with the tube developed at our institution, there have been no complications that required hospitalization and the patient did not develop pneumonia or local complications related to the presence of the tube in the oesophagus. The implementation and removal of the tube developed at our institution was easy and not traumatic. Moreover, there was no hypertrophy of the oesophageal mucosa or tube migration.

DISCUSSION

The patient described in this report had massive post-corrosive injury of the oesophagus and stomach and did not respond to oesophageal dilatation therapy. Such patients are usually referred for surgical replacement of the disintegrated oesophagus with an intestinal loop^[3,4]. However, surgical replacement was not possible in this case, as the patient had previously undergone a total colectomy for treatment of Hirschsprung's disease. For these reasons, alternative methods of therapy were attempted.

Oesophageal stenting was performed using commercially available coated metal stents designed for use in adults. The stent implementation was uncomplicated, but importantly caused discomfort to the patient, and migrated or caused mucosal hypertrophy and stent orifice occlusion. The oesophageal stenting did not bring long-term benefits to our patient, who frequently returned for repeated oesophageal dilatations. Thus, the patient was considered for a salivary fistula. A nasogastric tube with perforations for saliva and liquid passage was inserted to postpone referring the patient for salivary fistula surgery,

with good results. The tube remained in the oesophagus for several months, decreasing the need for repeated dilatation procedures. Although the patient could easily swallow, the large diameter of the tube extending from the nose and reflux of gastric contents to the mouth caused discomfort.

These experiences prompted us to develop a tube with varying diameters, allowing the dilatation of selected parts of the oesophagus. The purpose of the tube is to provide long-term, artificial conditions allowing oesophageal wall remodelling and final scar formation. The advantages of such a tube are the lack of margins that cause mucosal irritation and ease of removal. A similar approach has already been described in the literature^[5-8]. Atabek *et al*^[5] constructed a semi-tube oesophageal stent secured with a 4 Fr urethral catheter with the proximal end fixed to the nose and the distal end fixed to a gastrostomy tube, which was successfully used in 8 of 11 children. The margins of the semi-tube stent extended approximately 1 cm out of stricture border. Mutaf described a series of 69 children who received stenting after two unsuccessful post-injury dilatations^[7]. These stents had an increasing diameter of 5 to 10 mm and grooves allowing oral liquid diet feeding. The stents were left in place for 1 year with no serious complications, and 69% of the treated children regained the ability to tolerate oral feeding without the need for further dilatation. Foschia *et al*^[8] built a coaxial silicon stent over a 12-14 Fr nasogastric tube, the ends of which were tailored to allow food bolus passage. The stents were used after at least five unsuccessful post-injury dilatations and were effective in 70/79 patients.

The stent described in this case report is built over the nasogastric feeding tube fixed to the nose. Unlike the other stents described above, it has an additional soft, conical portion fixing the stent above the upper stricture margin. This conical portion has perforations that allow food passage. The length of the stent can be adjusted to the length of the oesophageal stricture, and thus there is no irritation or hypertrophy of the oesophageal wall mucosa. The stents used by other authors have been implemented relatively shortly after the corrosive injury and were successful in the majority of subjects. Our patient had stent implementation after a long period of unsuccessful therapy. We plan to gradually increase the diameter of the stent and to leave it in place for many months prior to attempting permanent removal.

The method described in this report decreased the need for repeated oesophageal dilatation, allowed for oral liquid diet feeding, and eliminated the need for saliva fistula surgery. Implementation and removal of this newly developed tube were easy, and no tube migration or complications related to the prolonged stay of the tube in the oesophagus were observed. This therapy can be considered an alternative treatment for patients with difficult corrosive oesophageal stricture, for whom oesophagus reconstruction cannot be performed.

COMMENTS

Case characteristics

An 8.5-year-old patient with extensive upper gastrointestinal tract chemical injury and contraindications for surgical oesophageal reconstruction.

Clinical diagnosis

Recurrent dysphagia not responding to endoscopic therapy.

Differential diagnosis

Congenital oesophageal ring, gastro-oesophageal reflux disease, achalasia, oesophageal tumour.

Laboratory diagnosis

All laboratory tests were within normal ranges.

Imaging diagnosis

Radiography and endoscopy showed extensive oesophageal stricture.

Pathological diagnosis

Post-chemical injury scarification of oesophageal mucosa.

Treatment

Patient underwent several unsuccessful sessions of endoscopic oesophageal stricture dilatation and stenting.

Related reports

The patient markedly improved after implementation of a nasogastric tube with perforations located above the stricture margin, which allowed for saliva and fluid passage. However, the large diameter of the tube extending from the nose was inconvenient for the patient and backflow of gastric contents to the mouth was observed.

Term explanation

The newly developed coaxial, double lumen, varying diameter tube protects the oesophageal closure and provides easy passage of saliva and fluid through the stricture; the design of the tube protects against the backflow of gastric contents to the mouth.

Experiences and lessons

The report presents a difficult to treat post-corrosive injury of the oesophagus.

Peer review

This report demonstrates the successful use of a newly developed nasogastric tube that may be an alternative therapy option in selected cases.

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L- Editor: A **E- Editor:** Wu HL



Overlap syndrome consisting of PSC-AIH with concomitant presence of a membranous glomerulonephritis and ulcerative colitis

Odile Warling, Christophe Bovy, Carla Coimbra, Timothée Noterdaeme, Jean Delwaide, Edouard Louis

Odile Warling, Department of Internal Medicine, CHU de Liège, University of Liège, 4000 Liege, Belgium

Christophe Bovy, Department of Internal Medicine, Nephrology, CHU de Liège, University of Liège, 4000 Liege, Belgium

Carla Coimbra, Department of Abdominal Surgery and Transplantation, CHU de Liège, University of Liège, 4000 Liege, Belgium

Timothée Noterdaeme, Department of Cardiovascular Surgery, German Heart Center, 80636 Munich, Germany

Jean Delwaide, Edouard Louis, Department of Internal Medicine, Gastroenterology, CHU de Liège, University of Liège, 4000 Liege, Belgium

Author contributions: Warling O wrote the paper and collected and analyzed data; Noterdaeme T helped write the paper, as well as translating, proof-reading, and applied any necessary corrections to it; Coimbra C was involved in the medical treatment; Bovy C, Delwaide J and Louis E were the physicians involved in the medical treatment of the mentioned patients and also supervised the article; all authors reviewed and agreed upon the manuscript.

Correspondence to: Odile Warling, MD, Department of Internal Medicine, CHU de Liège, University of Liège, Avenue de l'Hôpital 1, Domaine universitaire du Sart-Tilman, Batiment B35, 4000 Liege, Belgium. owarling@alumni.ulg.ac.be

Telephone: +32-4-94828383 Fax: +32-4-3667889

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PSC-AIH, with the concomitant presence of a membranous glomerulonephritis.

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Key words: Primary sclerosing cholangitis; Autoimmune hepatitis; Overlap syndrome; Ulcerative colitis; Membranous glomerulonephritis

Core tip: This case reports highlights a particular kind of hepatic overlap syndrome. To our knowledge it would be the first case to be described in the literature concerning 4 different diagnoses; all being autoimmune related. These autoimmune diseases were never described together, yet their association in this case is probably not a sheer coincidence. Auto-immune diseases will play an ever more important role in the future; yet the current knowledge and understanding concerning these pathologies is certainly only a fraction of what is necessary to offer the best treatment to our patients.

Warling O, Bovy C, Coimbra C, Noterdaeme T, Delwaide J, Louis E. Overlap syndrome consisting of PSC-AIH with concomitant presence of a membranous glomerulonephritis and ulcerative colitis. *World J Gastroenterol* 2014; 20(16): 4811-4816 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4811.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4811>

Abstract

The association of primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH) is known as an overlap syndrome (OS). OS can also be described in the setting of concomitant presence of AIH and PSC. These diseases can in some cases be associated with ulcerative colitis. In this case report we describe, to our knowledge, the first case in the literature of a young Caucasian male suffering from ulcerative colitis and an overlap syndrome consisting of an association between

INTRODUCTION

The term “primary sclerosing cholangitis-autoimmune hepatitis (PSC-AIH) overlap syndrome” is used to describe different varieties of AIH and/or PSC in the setting where an unspecific number of characteristics of the other disease are present^[1].

Herein we report a particular kind of hepatic overlap

syndrome. To our knowledge it would be the first case to be described in the literature concerning a patient with ulcerative colitis, PSC, auto-immune hepatitis, and extra-membranous glomerulonephritis. These autoimmune diseases were never described together, yet their association in this case is probably not a sheer coincidence.

CASE REPORT

We report the case of a 29-year-old Caucasian male suffering from ulcerative colitis (UC) since 2000 and associated with PSC since 2003. The initial diagnosis of UC was based on concordant endoscopic and histological findings, and PSC diagnosis was established by means of a liver biopsy. The patient was treated using 1 g/d of ursodeoxycholic acid.

In September 2005, the patient was hospitalized for jaundice and fatigue without any indication of pain. Besides a lean physique and conjunctival jaundice, the clinical examination was inconspicuous.

Initial blood analysis showed impaired liver function characterized essentially by the presence of cholestasis and cytolysis (Table 1). Further investigation showed increased IgG [25.2 (normal: 6.9-14) g/L] and IgM [2.36 (normal: 0.3-2.1) g/L] titers. Subanalysis for IgG4 and anti-nuclear antibodies remained negative. Anti-mitochondrial antibodies type 2, anti-myeloperoxidase, as well as anti-LKM were also negative. However, anti-neutrophil cytoplasmic antibodies (determined by indirect immunofluorescence assay) and anti-proteinase 3 antibodies turned out to be positive. Serotype analysis confirmed the presence of HLA-DR 3 in our patient. Further analyses concerning tumor markers (alpha fetoprotein, carcinoembryonic antigen, and CA19-9) as well as hepatitis serology (A, B, and C) were negative. Urinary copper levels were normal and no Keiser Fleischer rings were noted at clinical examination. Abdominal ultrasound only demonstrated the presence of dilated intrahepatic bile ducts. This picture was confirmed by endoscopic retrograde cholangiopancreatography (ERCP), which showed some minor irregularities of the intrahepatic portion. Cholangio-magnetic resonance imaging (MRI) proved to be non-contributive and only confirmed the results obtained by the ERCP. The overall appearance was compatible, but not specific for sclerosing cholangitis. A liver biopsy performed shortly afterwards showed the presence of chronic hepatitis with predominantly centrilobular necrosis (Figure 1), evoking a toxic origin or possibly AIH. Owing to the increased IgGs with concomitant cytolysis and the exclusion of any other causes (*i.e.*, toxics) the diagnosis of AIH associated with PSC was retained, delimitating the picture of an overlap syndrome. The patient was promptly started on 32 mg/d of methyl-prednisolone. Seven days after the initiation of the treatment, there was a marked improvement in the patient's overall condition. Total bilirubin dropped to 53 mg/L. Alanine aminotransferase and IgGs were significantly decreased to 228 U/L and 15.4 g/L, respectively.

Table 1 Initial biology at first presentation

	Value	Range
Hemoglobin (g/dL)	12.1	14.0-17.4
Gamma glutamyltransferase (UI/L)	157	8-61
C-reactive protein (mg/dL)	11.5	< 10
GOT (AST, UI/L)	920	0-35
TGP (ALT, UI/L)	701	3-36
Total bilirubin (mg/dL)	123.7	< 15
Direct bilirubin (mg/dL)	93.8	< 4
Amylase (UI/L)	94	< 160
Lipase (UI/L)	77	< 140
Alkaline phosphatase (UI/L)	208	35-100
Total low density lipoprotein (UI/L)	429	95-195
Albumin (g/dL)	33.4	35-50

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GOT: Glutamate oxaloacetate transaminase; TGP: Glutamate pyruvate transaminase.

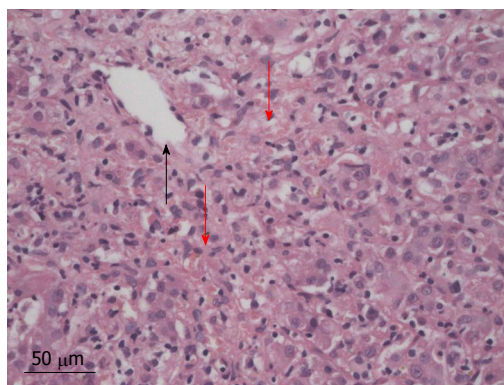


Figure 1 Hematoxylin eosin. Centrilobular necrosis (disorganized appearance) (red arrow) centrilobular vein (black arrow) (original magnification $\times 40$).

However, the IgGs titers fluctuated for the next four years before reaching a normal level again. Two months later, corticosteroids were reduced by half (16 mg/d). Nonetheless we were forced to start the patient on 50 mg/d of azathioprine shortly after this step, as liver function degraded. This medication was so poorly tolerated by the patient that he was switched to 6-mercaptopurine (6-MP). The dosage was steadily increased to 75 mg/d. *TPMT* gene variant genotyping didn't show any abnormal activity, therefore excluding a genetic predisposition to bone marrow toxicity. Further withdrawal of corticosteroids proved to be difficult as clinical jaundice reoccurred as soon as the dosage was reduced 8 mg/d or less. As liver function [total bilirubin: 61 mg/L (range < 4 mg/L), TGP 72 UI/L (range: 3-36 UI/L)] as well as clinical evolution of the patient stagnated, we temporarily interrupted the administration of ursodeoxycholic acid to exclude any possible drug interaction. The titers for 6-MP were in normal range and another cholangio-MRI proved to be non-contributive. We therefore suspected a new episode of AIH, which led us to increase corticosteroids to 12 mg/d. This decision promoted into an excellent clinical and biological evolution with almost a complete normalization of liver function. Following this good evolution,

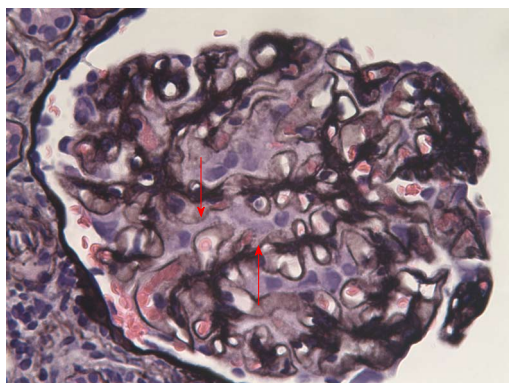


Figure 2 Optic microscopy: Jones' staining. Presence of spikes in a diffuse manner (arrows) (silver impregnation of the basement membrane, original magnification $\times 400$).

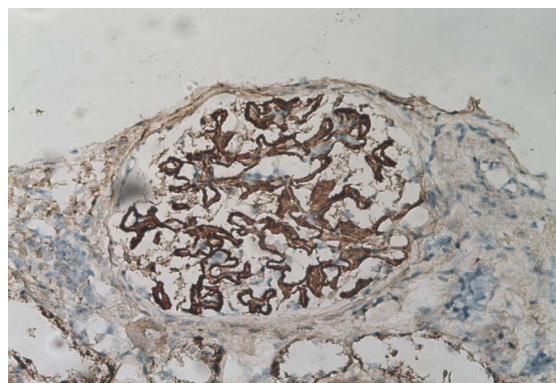


Figure 3 Immunoperoxidase (anti-IgG Ab). Binding of the antibody in the sub-epithelial region of the basal membrane. The immunofixation for C3d and C4d are positive in the same locations, original magnification $\times 400$.

corticosteroids were decreased progressively to 8 mg/d, which would be the minimum dose required for the next several years.

In October 2009, four years after the initial diagnosis of overlap syndrome, the patient presented himself to the emergency department with severe edema of the lower extremities.

Besides a raised blood pressure of 155/105 mmHg, clinical examination was unremarkable. At that moment the patient treatment consisted of 75 mg/d of 6-mercaptopurine, 8 mg/d of methyl-prednisolone, 1 g/d of ursodeoxycholic acid, and 2 g/d of mesalazine. Blood work performed at the emergency department showed hypoalbuminemia [17 (range: 35-50) g/L] with normal creatinine level [1.15 (range: 0.6-1.3) mg/dL]. A 24 h urine sample confirmed the presence of severe nephrotic syndrome with a proteinuria of 23400 mg/L, or 10400 mg/g of creatinine with a total of 46.3% of albumin. The protein fractions were normal. An abdominal ultrasound was unremarkable. On the next day kidney biopsies (Figures 2 and 3) were performed showing the presence of an extra-membranous glomerulonephritis. Immunohistology showed the presence of subepithelial deposits of IgG, C3d, and C4d. C1q, IgA, and IgMs were not detected. Despite the absence of renal failure, the presence of massive proteinuria pushed us to increase the corticosteroids to 64 mg/d. Cyclophosphamide, as well as calcineurin inhibitors, were not available as alternatives due to the patient receiving 6-MP at that time. This treatment was supplemented with bumetanide 0.5-1 mg/d, lisinopril 20 mg/d, CaCO_3 1250 mg/d, and omeprazole 20 mg/d. One month after initiation of treatment, the nephrotic syndrome went into complete remission. Diuretics were stopped and corticosteroids were progressively reduced. Unfortunately, the nephrotic syndrome reemerged as soon as corticosteroids were weaned, and after two consecutive episodes we decided to convert the treatment regimen to cyclophosphamide 2 mg/kg per day. 6-MP was stopped during the next 6 mo. The patient responded well to this alteration so that corticosteroids could again be reduced to 16 mg/d. Liver function re-

mained stable.

In August 2011, the clinical and biological evolution stabilized so that the patient was finally weaned from systemic corticosteroids. His final treatment consisted of 5 mg/d of beclomethasone, 75 mg/d of 6-MP, 2 g/d of mesalazine, and 1 g/d of ursodeoxycholic acid. The administration of cyclophosphamide was stopped indefinitely.

DISCUSSION

The term "PSC-AIH overlap syndrome" is used to describe different varieties of AIH autoimmune and/or PSC in a setting where an unspecific number of characteristics of one disease are present^[1].

Other forms of overlap syndromes have been described as combining the simultaneous presence of different diseases like AIH and primary biliary cirrhosis. A precise clinical and pathological definition is still missing. Concerning PSC-AIH overlap syndrome, Czaja^[2] suggested that it is possibly an atypical manifestation of a classic disease, rather than a completely new entity. However, the International Auto-Immune Hepatitis Group (IAIHG) takes no clear position on the matter, leaving the door open to a wide variety of possibilities: consecutive presentation, concomitant presentation, existence of a continuum between the two diseases, or presence of a disease that also has one or more other features of the other^[3]. The PSC-AIH overlap syndrome is most often described in children, adolescents, or young adults^[1,3-5]. Before confirming the diagnosis of an overlap syndrome it is of the utmost importance to exclude liver toxicity of the pre-existing treatment.

Most large series published in the literature^[5-7] concerning overlap syndrome present patients with an initial diagnosis of PSC to whom the diagnostic criteria for auto-immune hepatitis were applied to diagnose or postulate the presence of an overlap syndrome. This situation is comprehensible in the light that well-defined inclusion criteria, although needed, are still absent. The IAIHG recalls, however, that clear and strict criteria are not in-

tended to diagnose an overlap syndrome^[3].

A study published in 2005^[5] estimated that 17% of patients with PSC have an overlap syndrome (PSC + AIH according to the criteria of IAIHG 1999^[8]). On behalf of the IAIHG^[8], Boberg estimated a prevalence of 1.8% of AIH (according to the diagnostic criteria of the IAIHG) and 8.8% of probable AIH (according to the criteria of the IAIHG) in a sample of 114 patients suffering from PSC. If one would consider that the diagnosis of overlap syndrome doesn't necessitate a complete coverage of both diagnostic criteria of each disease, these figures would be much higher.

The patient mentioned in this case report meets both diagnostic criteria for PSC^[9,10], as well as for AIH, according to the IAIHG criteria^[8], giving us the confidence to diagnose our patient with overlap syndrome. It is important to note that centrilobular necrosis has been described in cases of AIH, particularly in young patients as well as in hepatic transplants^[11,12]. No antibody were detected in our patient, but this is the case in almost 10% of AIH^[3].

A prospective study^[5] of 41 patients comparing those with PSC ($n = 37$) alone *vs* those associated with autoimmune hepatitis ($n = 7$) showed that patients in the latter group were generally younger, had higher IgGs, and higher levels of transaminases. The authors believe that there is a benefit of treating such patients with immunosuppressive and ursodeoxycholic acid. Patients with overlap seemed to have a better survival than those with PSC alone^[5]. However, this study didn't take into account the possibility that the difference in survival may be due to the fact that patients in the overlap group generally presented with less severe forms of PSC. These findings are nonetheless in sharp disagreement with the fact that patients with overlap syndrome have a lower response rate than those with PSC. Statistical analysis methods are not detailed in this study, therefore rendering correct interpretation of results difficult.

Given the lack of specific diagnostic criteria, it is difficult to establish a line of scientifically proven conduct. The guidelines published by the European Association for the Study of the Liver (EASL) recommend treating patients with AIH-PSC overlap by ursodeoxycholic acid and immunosuppressive agents, even though this is not "evidence-based" *per se*^[9]. Note that the American Association for the Study of Liver Diseases also recommends the use of corticosteroids and immunosuppressive agents in these patients^[3]. This is in agreement with the usual treatment of patients with PSC associated with ulcerative colitis, since it appears that the treatment of PSC with ursodeoxycholic acid reduces not only the risk of dysplasia^[4], but also the prevalence of colorectal carcinomas^[4,13], and should be given to every patient with ulcerative colitis and PSC. However, the EASL puts forward that no study could demonstrate any positive effects on the prognosis of patients with PSC, and that the treatment is therefore not "evidence-based"^[9]. Culver and Chapman published a review in 2011^[4] on the management of the treatment of sclerosing cholangitis and its variants, and concluded that many studies included patients with advanced stages of

disease, and that the impact of ursodeoxycholic acid may be different if used much earlier. However, this statement has not been fully evaluated^[4]. The notion of overlap syndrome is important because it has been shown^[2] that in cases of PSC-AIH overlap there is a weakened response to corticosteroids. It has been equally shown^[2,3] that patients, like the one presented in this case report, with AIH, PSC, and ulcerative colitis, have a reduced probability of remission and higher treatment failure rates than patients with normal cholangiography.

The IAIHG noted several working groups that mention a benefit of steroids, but also mentions another group who found a reduced response in these particular patients^[8]. Several reviews^[4,14] also suggest that liver transplantation is indicated for advanced stages of this disease.

Extra-intestinal manifestations of inflammatory bowel disease, like glomerulonephritis (GN), are considered rare^[15] in cases of overlap syndrome. In such instances, GN can have a wide spectrum from minimal change to rapidly progressive (crescent) GN, and may also be accompanied by active tubular-interstitial nephritis^[15].

However, we only found 6 cases of membranous glomerulonephritis associated with ulcerative colitis^[16-21] and only one published case of PSC associated with membranous glomerulonephritis by Verresen *et al*^[22] in 1988. Furthermore, there are 3 described cases in which an extra-membranous glomerulonephritis is associated with autoimmune hepatitis^[23-25].

Lastly, in our review of the literature we found one case similar to the one mentioned in here (*i.e.*, suffering from UC alongside with PSC and membranous glomerulonephritis)^[26]. To our knowledge, this case report seems to be the only one presenting a patient with UC, an overlap syndrome involving PSC and AIH, and membranous glomerulonephritis.

Glomerulonephritis caused by aminosalicylates, cyclosporine^[15,27-29], and tumor necrosis factor- α inhibitor^[27,28] are well known, and described even with a delayed presentation after several years of continuous treatment^[30]. 6-mercaptopurine shows no significant direct nephrotoxicity^[28]. In the group of six patients described with membranous glomerulonephritis associated with ulcerative colitis, 4 patients^[17,18,20,21] did not receive potentially nephrotoxic treatment before the onset of nephrotic syndrome. So it seems that the treatment may not be incriminated in the association of these diseases blindly.

Currently, the research concerning the pathophysiology of glomerulonephritis is in full progress. The recent discovery of the phospholipase A2 receptor as a target antigen in 70% of membranous glomerulonephritis^[31] validates these efforts. Nonetheless, until now no study has been able to link certain forms of membranous glomerulonephritis and ulcerative colitis, even though the autoimmune context seems inevitable.

COMMENTS

Case characteristics

Jaundice, fatigue, and edema in a patient with ulcerative colitis and primary

sclerosing cholangitis.

Clinical diagnosis

Overlap syndrome [autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC)] and membranous glomerulonephritis in a patient with ulcerative colitis.

Differential diagnosis

PSC exacerbation, primary biliary cirrhosis, Wilson disease, and neoplasia are all valid differential diagnoses for hepatic alteration should nephritic syndrome due to treatment toxicity be excluded.

Laboratory diagnosis

Elevation of liver enzymes and IgG for the diagnosis of AIH. Proteinuria and hypoproteinemia for the membranous nephritis.

Imaging diagnosis

For the liver, magnetic resonance imaging and computer tomography were not contributive; the diagnosis is based on histological findings. Such methods are nonetheless necessary to exclude other causes (*i.e.*, neoplastic). For the kidney, the diagnosis is made by means of a urine analysis and kidney biopsy.

Pathological diagnosis

Ulcerative colitis was diagnosed by endoscopy and histology, primary sclerosing cholangitis by magnetic resonance imaging and histology, auto-immune hepatitis by histology, and the criteria of the International Auto-Immune Hepatitis Group. Extra-membranous glomerulonephritis was diagnosed by histology (with Jones' staining).

Treatment

Corticosteroids are used in treatment in cases of auto-immune exacerbation. Maintenance treatment could be azathioprine, ursodeoxycholic acid for auto-immune hepatitis and primary sclerosing cholangitis, or cyclophosphamide for the treatment of membranous nephritis.

Term explanation

The term "PSC-AIH overlap syndrome" is used to describe different varieties of autoimmune hepatitis autoimmune and/or primary sclerosing cholangitis in a setting where an unspecific number of characteristics of the other disease are present. The term overlap just designs an association of disease. It's not very clear if it's just a concomitant presence of the disease, the potential, or if the overlap design are a variant of the diseases.

Experiences and lessons

It's very important not to forget that clear diagnostic criteria are sometimes more of a theoretical concept and rarely apply to the circumstances found in a specific patient. Therefore a correct knowledge and interpretation of the different sub-characteristics of each disease is the key to allowing tailored therapeutic approaches.

Peer review

This paper reports associated "autoimmune" diseases in a single patient. The addition of membranous glomerulonephritis to ulcerative colitis and a PSC-AIH overlap is new information. A short note on this new association could be of interest and useful for the future.

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Gastrointestinal stromal tumor of the ampulla of Vater: A case report

Masayoshi Kobayashi, Nobuto Hirata, So Nakaji, Toshiyasu Shiratori, Hiroyuki Fujii, Eiji Ishii

Masayoshi Kobayashi, Nobuto Hirata, So Nakaji, Toshiyasu Shiratori, Hiroyuki Fujii, Eiji Ishii, Department of Gastroenterology, Kameda Medical Center, Chiba 296-0041, Japan

Author contributions: Kobayashi M designed the study and wrote the manuscript; Hirata N and Nakaji S contributed to the manuscript revision; Shiratori T, Fujii H and Ishii E contributed to the literature review and manuscript editing.

Correspondence to: Masayoshi Kobayashi, MD, Department of Gastroenterology, Kameda Medical Center, 929 Higashi-cho, Kamogawa City, Chiba 296-0041, Japan. k-masayoshi@hotmail.co.jp

Telephone: +81-4-70922211 Fax: +81-4-70991131

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Abstract

Gastrointestinal stromal tumors (GISTs) usually develop in the stomach and small intestine and only rarely occur at the ampulla of Vater, with only 11 cases reported in the literature. We report a case of a GIST of the ampulla of Vater. A 36-year-old, previously healthy man presented with a loss of consciousness lasting a few minutes. A gastroduodenal endoscopy revealed a submucosal tumor with central ulceration at the ampulla of Vater. The enhanced computed tomography scan revealed a smooth-outlined hypervascular solid mass (24 mm × 30 mm) in the second part of the duodenum. Neither lymphadenopathy nor metastasis was observed. Magnetic resonance cholangiopancreatography and endoscopic retrograde cholangiopancreatography showed normal bile and pancreatic ducts. Biopsies were collected from the ulcerative lesion, and the tumor was diagnosed as a GIST. A submucosal tumor with central ulceration may be a characteristic form of GISTs of the ampulla of Vater, and biopsy studies are useful for the diagnosing such tumors. The patient underwent pancreatoduodenectomy, and the operative specimen revealed a 2.2-cm GIST with 1 mitosis per 50 high-power fields. The gold standard for treatment of GISTs

is surgical resection without rupture of a capsule. If technically possible, local resection may be considered. However, when the location of the lesion presents challenges, a pancreatoduodenectomy should be performed for GIST of the ampulla of Vater.

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Key words: Gastrointestinal stromal tumor; Ampulla of Vater; Submucosal tumor; Bleeding; Biopsy; Pancreatoduodenectomy

Core tip: Gastrointestinal stromal tumor (GIST) usually develops in the stomach and small intestine, and GIST of the ampulla of Vater is extremely rare, with only 11 cases reported in the literature. We report a case of GIST of the ampulla of Vater in a 36-year-old, previously healthy man who presented with a brief loss of consciousness. A gastroduodenal endoscopy revealed a submucosal tumor with central ulceration at the ampulla of Vater. Biopsies were collected from the ulcerative lesion, and the tumor was diagnosed as a GIST. The patient underwent pancreatoduodenectomy. The operative specimen revealed a 2.2-cm GIST with 1 mitosis per 50 high-power fields.

Kobayashi M, Hirata N, Nakaji S, Shiratori T, Fujii H, Ishii E. Gastrointestinal stromal tumor of the ampulla of Vater: A case report. *World J Gastroenterol* 2014; 20(16): 4817-4821 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4817.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4817>

INTRODUCTION

The ampulla of Vater can exhibit a variety of neoplasms, such as carcinoma, adenoma, neuroendocrine tumor, gangliocytic paraganglioma and gastrointestinal



Figure 1 A submucosal tumor with central ulceration at the ampulla of Vater.



Figure 2 Endoscopic ultrasonography revealed a round, low-echoic mass.

stromal tumor (GIST). GISTs are mesenchymal tumors of the gastrointestinal tract that express the tyrosine kinase receptor and originate from the interstitial cells of Cajal. The majority of GISTs are located in the stomach (60%-70%) and the small intestine (20%-25%), with only 4% occurring in the duodenum^[1,2]. Cases of GIST affecting the ampulla of Vater are extremely rare, with only eleven cases described in the literature^[1-11]. We report a case of GIST of the ampulla of Vater that was discovered because of loss of consciousness.

CASE REPORT

A 36-year-old, previously healthy man presented with loss of consciousness lasting a few minutes. On admission, his blood pressure was 116/59 mmHg, heart rate was 85 beats/min, and temperature was 36.5 °C. A physical examination revealed mild anemia of the palpebral conjunctivae. The peripheral blood cell count indicated anemia with a hematocrit of 24.5%. The liver and renal function tests were normal. The levels of serum tumor markers, including carcinoembryonic antigen and carbohydrate antigen 19-9, were within normal limits.

A gastroduodenal endoscopy showed a submucosal tumor with central ulceration at the ampulla of Vater (Figure 1). Because there were small amounts of blood around the tumor, we considered that the patient had lost

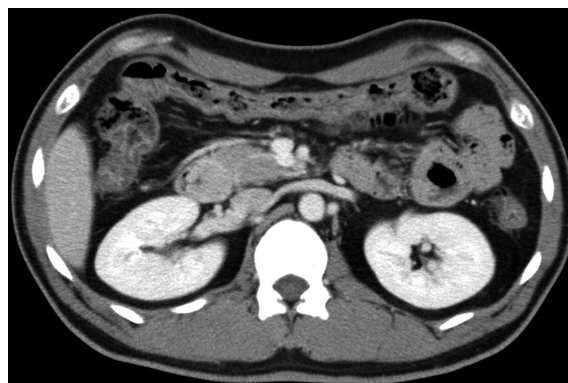


Figure 3 Enhanced computed tomography scan revealed a smooth-outlined hypervascular solid mass.

consciousness because of the bleeding from the tumor, which had already ceased spontaneously.

Endoscopic ultrasonography demonstrated a round, low-echoic mass originating from the muscularis propria (Figure 2). The enhanced computed tomography (CT) scan revealed a smooth-outlined hypervascular solid mass (24 mm × 30 mm) in the second part of the duodenum. Neither lymphadenopathy nor metastasis was observed (Figure 3). We additionally performed magnetic resonance imaging (MRI) and endoscopic retrograde cholangiopancreatography (ERCP) to obtain more information about the tumor, especially the relative position of the mass and the biliopancreatic duct because the tumor was located at the ampulla of Vater. On MRI, the mass showed low signal intensity on T1-weighted images and high signal intensity on T2-weighted images (Figure 4). Magnetic resonance cholangiopancreatography and ERCP showed normal bile and pancreatic ducts.

Biopsies were collected from the ulcerating lesion. The microscopic examination revealed a spindle-cell neoplasm, with the tumor cells positive for c-kit and CD34 (Figure 5). Thus, the tumor was diagnosed as a GIST.

The patient then underwent pancreatoduodenectomy. The operative specimen revealed a 2.2-cm GIST with 1 mitosis per 50 high-power fields, which classified the patient in the low-risk group, according to the National Institutes of Health (NIH) consensus criteria for risk stratification of GISTs. There was no lymph node metastasis. The patient was discharged 18 days after an uneventful postoperative course and has been doing well, with no recurrence, during the one-and-a-half years since the operation.

DISCUSSION

GISTs are mesenchymal tumors of the gastrointestinal tract that express the tyrosine kinase receptor and originate from the interstitial cells of Cajal. The majority of GISTs are located in the stomach (60%-70%) and the small intestine (20%-25%), and only 4% of GISTs occur in the duodenum^[1,2]. GIST of the ampulla of Vater is extremely rare. There are only eleven cases described

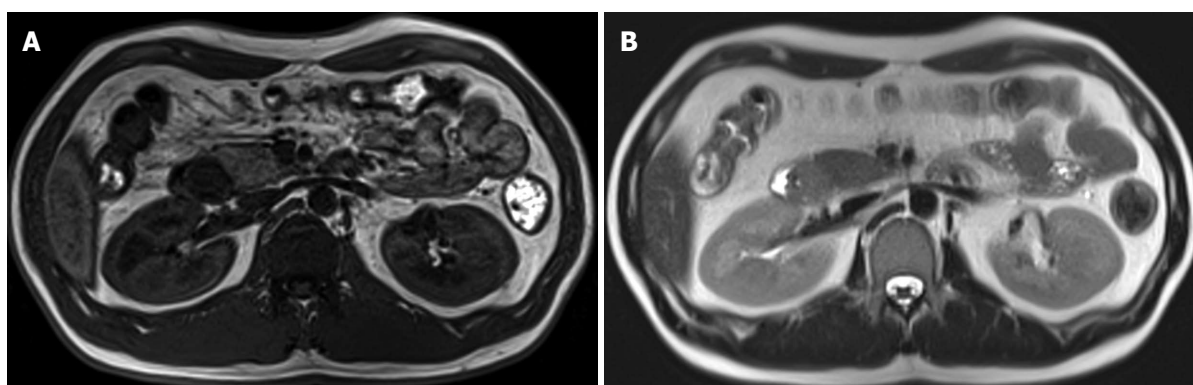


Figure 4 Mass showed low signal intensity on T1-weighted images (A) and high signal intensity on T2-weighted images (B).

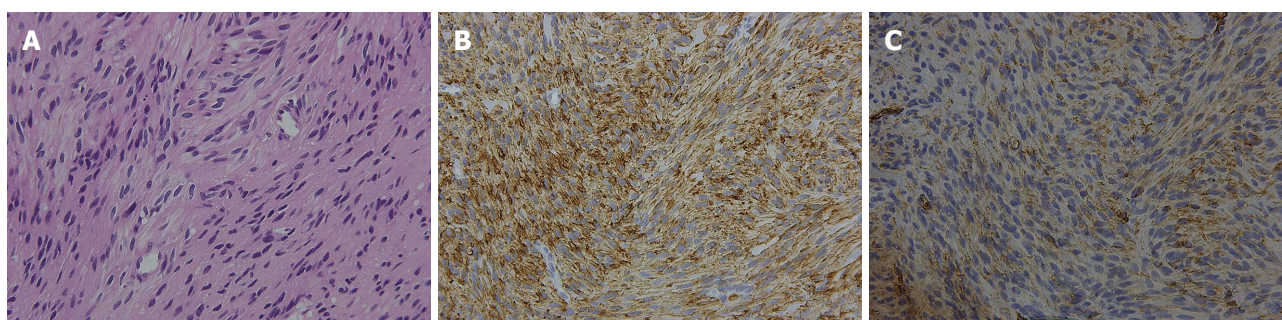


Figure 5 Microscopic examination revealed a spindle-cell neoplasm (A, hematoxylin and eosin, $\times 200$), and the tumor cells were positive for c-kit (B, $\times 200$) and CD34 (C, $\times 200$).

Table 1 Reported cases of gastrointestinal stromal tumor of the ampulla of Vater

Age (yr)	Gender	Symptoms	Size (cm)	Biopsy	Surgery	Ref.	Year
83	Female	-	3.2	-	PD	[11]	2012
59	Male	Jaundice	7.6	GIST	PD	[10]	2010
55	Female	Epigastric pain	4.5	-	PD	[9]	2009
57	Male	Melena	2.6	GIST	Local resection	[8]	2007
69	Male	Melena	3.0	Negative	PD	[7]	2007
44	Male	Weight loss	9.0	GIST	PD	[6]	2007
65	Female	Jaundice	6.0	GIST	Local resection	[5]	2006
44	Male	Jaundice	8.0	Mesenchymal tumor	-	[2]	2005
68	Female	Weight loss	4.5	-	PD	[4]	2004
37	Female	Melena	5.5	GIST	PD	[1]	2004
77	Male	Pallor	4.0	GIST	PD	[3]	2001

GIST: Gastrointestinal stromal tumor.

in the literature according to a Medline search (Table 1) using the key words “ampulla of Vater” and “gastrointestinal stromal tumor”. The patients in the published reports were seven men and four women between 37 and 83 years of age. Our case was the youngest among these. Almost all cases exhibited certain symptoms, such as abdominal pain, jaundice, and melena. The tumor size ranged from 2.6-9 cm. Eight of the eleven cases showed a submucosal tumor with central ulceration, which may be a characteristic form of GISTs of the ampulla of Vater. In six cases, the diagnosis of GIST was made by the study of biopsies collected endoscopically from the ulcerating lesions. Although GISTs are submucosal tu-

mors, biopsy studies are useful for GIST of the ampulla of Vater because the tumor cells are exposed at the ulcerating lesions.

The differential diagnosis may include neuroendocrine tumor, gangliocytic paraganglioma, and intra-ampullary-type carcinoma, and there are also a few case reports of leiomyoma and leiomyosarcoma. In cases of neuroendocrine tumor, gastroduodenal endoscopy typically reveals a yellowish submucosal tumor with dilated vessels and erosion on the surface. Endoscopic ultrasonography demonstrates a low-echoic mass originating from the second/third layer. The diagnostic rate of biopsy is as low as 14%^[12], and some reported cases were diagnosed

by endoscopic ultrasonography fine needle aspiration^[13]. In cases of gangliocytic paraganglioma, gastroduodenal endoscopy reveals a submucosal tumor with erosion and ulceration on the surface, located near the ampulla of Vater. By endoscopic ultrasonography, the tumor can typically be visualized as well circumscribed, located in the submucosal layer, and involving the muscularis propria. In cases of intra-ampullary-type carcinoma, gastroduodenal endoscopy reveals an enlarged papilla, and endoscopic ultrasonography shows an irregularly shaped low-echoic mass. Given these characteristics, a diagnosis can be reached by performing gastroduodenal endoscopy and endoscopic ultrasonography.

We cite this case as GIST of the ampulla of Vater because the GIST was macroscopically located at the ampulla of Vater. By definition, the ampulla of Vater is a field that is anatomically surrounded by the sphincter of Oddi. Therefore, GISTs of the ampulla of Vater should arise from the sphincter of Oddi based on the anatomical definition. In the case presented in this report, the GIST pathologically arose from the duodenal muscularis; of the reported ten cases, none of the reports mentioned the sphincter of Oddi. It is noteworthy to consider whether GISTs arising from the sphincter of Oddi actually exist. When GISTs of the ampulla of Vater are encountered, the relationship between the GISTs and the sphincter of Oddi is important to assess.

Among the ten patients who underwent surgery, eight cases involved pancreatoduodenectomy, and two were local resections. GISTs rarely metastasize to regional lymph nodes. There is only one reported GIST case with lymph nodes metastasis, and this particular patient also had liver metastasis^[2]. The gold standard for GIST treatment is surgical resection without rupture of the tumor capsule. If technically feasible, local resection may be considered. However, when the location of the lesion presents associated difficulties, a pancreatoduodenectomy should be performed for GIST of the ampulla of Vater.

In general, adjuvant therapy with a tyrosine kinase inhibitor is recommended for patients with high-risk tumors, i.e., a tumor size > 10 cm, mitotic count > 10/50 HPF, and tumor rupture. However, no clear consensus exists regarding the cutoff that should be used to select patients for adjuvant therapy. In our case, the patient underwent no adjuvant therapy because he was in the low-risk group according to the NIH consensus criteria for risk stratification of GISTs. However, GISTs located at nongastric sites are associated with less favorable outcomes than are stomach GISTs. The patient in this case report has been doing well without recurrence during the one-and-a-half years since surgery, and we will continue to monitor him with a strict follow-up schedule.

COMMENTS

Case characteristics

A 36-year-old, previously healthy man presented with a loss of consciousness lasting a few minutes.

Clinical diagnosis

Physical examination showed mild anemia of the palpebral conjunctivae.

Differential diagnosis

Gastrointestinal bleeding.

Laboratory diagnosis

The peripheral blood cell count showed anemia with a hematocrit of 24.5%; the liver and renal function tests were normal.

Imaging diagnosis

Gastroduodenal endoscopy showed a submucosal tumor with central ulceration at the ampulla of Vater.

Pathological diagnosis

Biopsies collected from the ulcerating lesion revealed a spindle-cell neoplasm and tumor cell positivity for c-kit and CD34.

Treatment

The patient underwent pancreatoduodenectomy.

Related reports

Gastrointestinal stromal tumor (GIST) of the ampulla of Vater is extremely rare, with only 11 cases reported in the literature.

Experiences and lessons

This case report illustrates one of the rare causes of gastrointestinal bleeding; moreover, GIST of the ampulla of Vater can cause certain symptoms, such as abdominal pain, jaundice, and melena.

Peer review

This article reports a rare case of a GIST of the ampulla of Vater.

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Solitary Peutz-Jeghers-type appendiceal hamartomatous polyp growing into the terminal ileum

Chang In Choi, Dae Hwan Kim, Tae Yong Jeon, Dong Heon Kim, Na Ri Shin, Do Youn Park

Chang In Choi, Dae Hwan Kim, Tae Yong Jeon, Dong Heon Kim, Department of Surgery, Medical Research Institute, Pusan National University Hospital, Seo-Gu, Busan 602-739, South Korea

Na Ri Shin, Do Youn Park, Department of Pathology, Medical Research Institute, Pusan National University Hospital, Seo-Gu, Busan 602-739, South Korea

Author contributions: Choi CI designed the report; Choi CI and Kim DH were attending doctors for the patient; Choi CI performed the surgery; Shin NR and Park DY performed the pathological examinations; Kim DH, Jeon TY and Kim DH organized the report; Choi CI wrote paper.

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Correspondence to: Dae Hwan Kim, MD, PhD, Department of Surgery, Pusan National University Hospital, 179 Gudeok-Ro, Seo-Gu, Busan 602-739, South Korea. dh2-kim@hanmail.net
Telephone: +82-51-2407238 Fax: +82-51-2471365

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Key words: Peutz-Jeghers syndrome; Hamartomatous intestinal polyposis; Neoplasms; Intussusception; Intestinal obstruction

Core tip: Intussusception arising from various leading points can cause intestinal obstruction. We experienced a case of solitary Peutz-Jeghers-type hamartomatous polyp in the appendix. This is an extremely rare condition with an unusual ingrowth characteristic. To the best of our knowledge, ours is the first case of a solitary appendiceal hamartomatous polyp with an ingrowing pattern. Although there are limitations, this case could serve as a reference for diagnostic and management decisions in future similar cases.

Choi CI, Kim DH, Jeon TY, Kim DH, Shin NR, Park DY. Solitary Peutz-Jeghers-type appendiceal hamartomatous polyp growing into the terminal ileum. *World J Gastroenterol* 2014; 20(16): 4822-4826 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4822.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4822>

Abstract

Solitary Peutz-Jeghers type hamartomatous polyp is rare. It is considered to be related to a variant Peutz-Jeghers syndrome (PJS) and may be a separate disease entity. A 50-year-old man was referred to our hospital with a diagnosis of intussusception in the terminal ileum and underwent segmental ileal resection with appendectomy. We identified a 3.5-cm diameter polyp arising from the appendix with ingrowth into the terminal ileum. The polyp was confirmed to be a hamartomatous polyp of Peutz-Jeghers-type, histologically. However, the patient had no characteristic manifestations of PJS such as mucocutaneous pigmentation and family history. There are few reports of appendiceal hamartomatous polyp in PJS patients and solitary appendiceal hamartomatous polyp is even rarer. Also, rather than telescoping, ours is the first reported intussuscepted lesion, to the best of our knowledge.

INTRODUCTION

Hamartomatous polyp is often associated with Peutz-Jeghers syndrome (PJS), which is a rare inherited autosomal dominant disorder characterized by mucocutaneous pigmentation^[1]. Solitary hamartomatous polyp has been considered a variant or a separate disease entity without the features of PJS^[2]. Such hamartomatous polyps occur predominantly in the small bowel, colon, and stomach, in decreasing frequency, and they rarely arise from the appendix^[3]. We report an experience of a solitary appendiceal hamartomatous polyp without the characteristic features of PJS, that led to bowel obstruction by ingrowing



Figure 1 Preoperative abdominal computed tomography findings. "Target sign", which can be shown from intussusception, was identified in the terminal ileum (black arrow). A: Horizontal plane; B: Coronal plane.

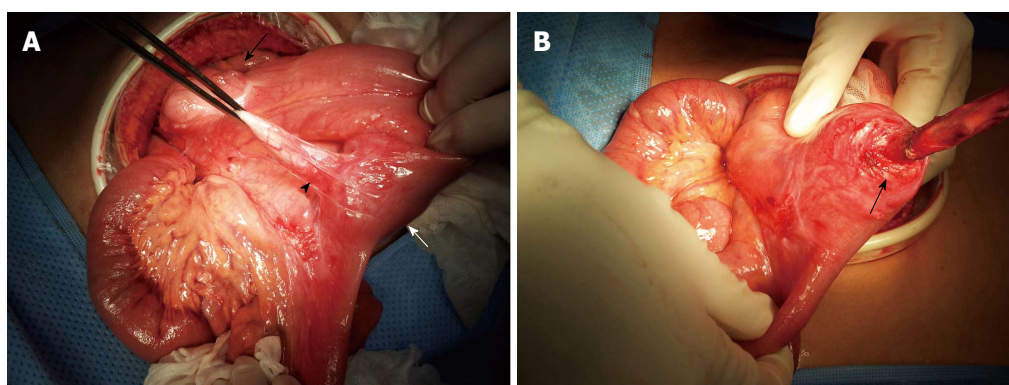


Figure 2 Intraoperative findings. A: The appendix was attached to the terminal ileum and a firm mass was palpable within the terminal ileum (ileocec valve, black arrow; appendiceal tip, black arrowhead; palpable mass, white arrow); B: The appendix base was divided and dissection was performed to the lesion to the entry of the appendiceal tip into the terminal ileum (entry site, black arrow).

through direct adhesion to the terminal ileum rather than by telescoping. This is the first case, to our knowledge, of solitary Peutz-Jeghers-type hamartomatous polyp with ingrowth into the terminal ileum.

CASE REPORT

A 50-year-old man presented to our emergency room complaining of right lower quadrant pain, abdominal discomfort, and bilious vomiting for 3 d. He was diagnosed with intussusception at a local medical clinic. A colonoscopy performed 4 years previously showed no abnormal findings, the patient had no past history of disease, and his vital signs were normal on arrival. On physical examination, mild peristaltic pain and tenderness was observed in the right lower quadrant of the abdomen without rebound tenderness. Mucocutaneous pigmentation of the perioral region, buccal mucosa, hands and feet was absent. Standard laboratory tests were unremarkable except for a mildly elevated white blood cell count of 11480/ μ L. Abdominal computed tomography (CT) revealed a 2.4-cm diameter intussusception due to a polypoid mass and multiple lymph nodes surrounding the lesion (Figure 1). We didn't perform esophagogastroduodenoscopy.

Emergent exploratory laparotomy with midline incision was performed under general anesthesia. Intraoperatively, a solitary firm mass of approximately 4 cm diameter was palpable within the terminal ileum 20

cm proximal to the ileocecal valve. The appendix was attached to the ileal serosa below the mass lesion (Figure 2A). We attempted to dissect the appendix tip but because the appendix tip entered the ileum, it was not entirely separable from the terminal ileum (Figure 2B). Therefore, we divided the appendix base from the cecum and performed an *en-bloc* resection, which included segmental resection of 15 cm of the terminal ileum. An end-to-end anastomosis of the ileum completed the procedure.

The mass was pathologically identified as a hamartomatous polyp of 3.5 cm \times 3.0 cm with surface ulceration. Grossly, the appendix was intussuscepted into the terminal ileum. Tissue cross section revealed invagination of the appendix into the resected terminal ileum, from the ileal external surface (Figure 3). Microscopic examination of the polyp revealed extensive smooth-muscle proliferation with an arborized pattern in the lamina propria (Figure 4). These smooth-muscle bundles were covered with hyperplastic mucosa and we were able to identify the appendiceal lumen penetrating the ileal polyp under microscopy. There were eight regional lymph nodes involved, which showed reactive hyperplasia without tumor metastasis, histopathologically.

Patient resumed an oral liquid diet on postoperative day 3 and he was discharged at day 6 postoperatively without any complications. There was no recurrence or remarkable findings during 2 years of postoperative

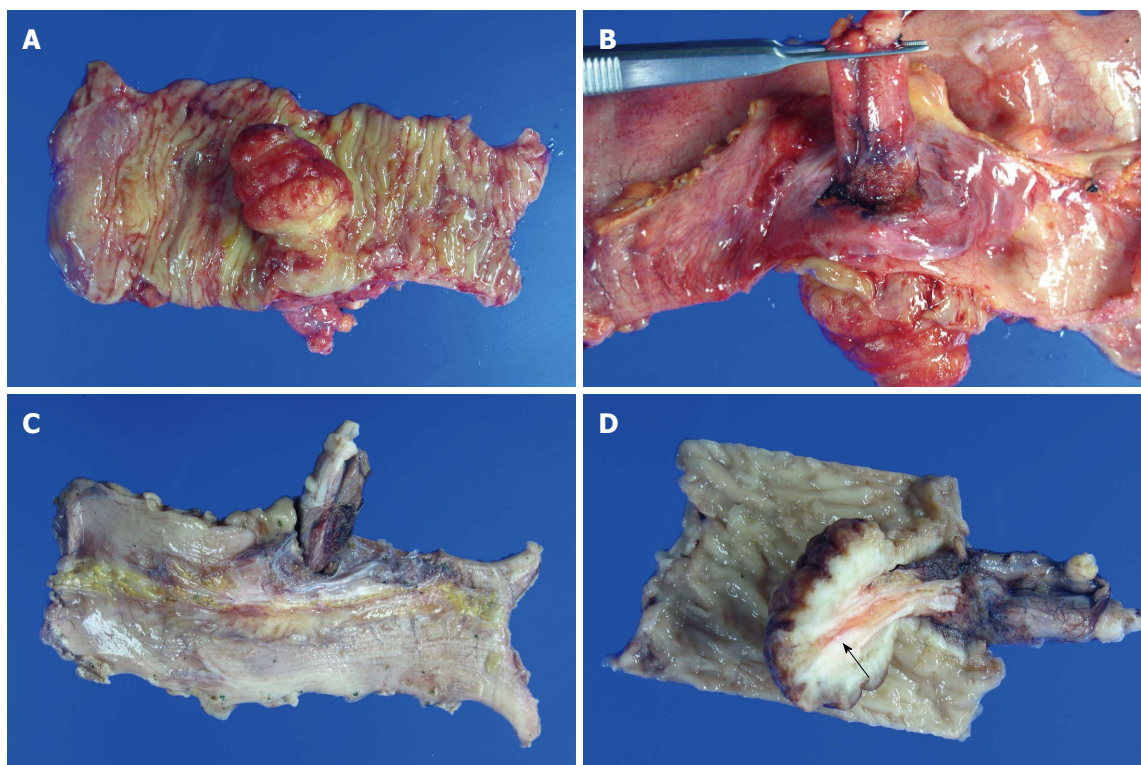


Figure 3 Postoperative gross findings. A: The polyp size was 3.5 cm × 3.0 cm; B, C: The appendiceal tip was continuous from external to the ileum into the ileal lumen; D: Tissue cross section revealed that the appendix penetrated into the polyp inside the ileum. The appendiceal lumen was identified within the ileum (black arrow).

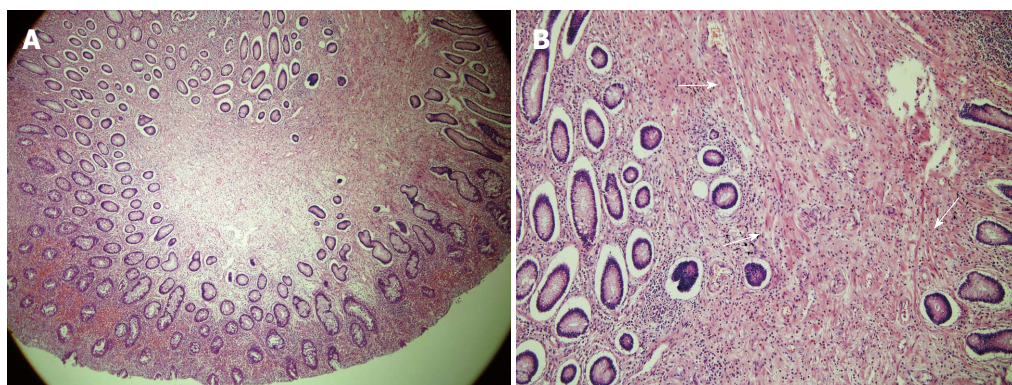


Figure 4 Histopathologic findings (hematoxylin and eosin staining). A: It was shown hyperplastic epithelium. But there was no dysplasia (× 40); B: Demonstrating the arborizing pattern of smooth-muscle proliferation (white arrow). And these smooth muscle bundles were originated from the muscularis mucosa (× 100).

follow-up.

DISCUSSION

PJS is an inherited, autosomal dominant polypoid syndrome with a prevalence of one in 100000 individuals. The disease is characterized by gastrointestinal hamartomatous polyps and mucocutaneous pigmentation on perioral buccal mucosa, and hands and feet^[1]. Hamartomatous polyps can occur throughout the gastrointestinal tract, but the small intestine is the most commonly affected site at 64%. These polyps cause abdominal pain, intestinal bleeding, and obstruction^[3]. Our patient,

who had no family history or juvenile operation history, visited our hospital because of acute onset abdominal pain in the right lower quadrant as a result of intestinal obstruction due to intussusception. Characteristic mucocutaneous pigmentation was not observed in this case, although it is present in more than approximately 90% of patients^[4]. Solitary hamartomatous polyp was reported by Kuwano *et al*^[5] in 1989. It is considered to be either an incomplete variant of PJS or a separate disease entity because of the absence of external features and family history. This solitary lesion has been termed Peutz-Jeghers-type polyp^[2,5,6].

Hamartomatous polyps are often diagnosed incident-

tally on endoscopy. Although abdominal CT findings in PJS patients are nonspecific, in cases with intestinal obstruction by intussusception or a large polyp, abdominal CT or gastrointestinal series can be diagnostic. Because hamartomatous polyps are grossly difficult to distinguish from other hamartomatous polyposis syndromes on endoscopy, diagnostic confirmation depends on histopathology as well as the various clinical manifestations^[1].

Microscopically, Peutz-Jeghers-type polyp shows extensive smooth-muscle proliferation and an elongated arborized pattern of polyp formation, and it is distinguishable from adenomatous polyps that can be seen in familial adenomatous polyposis syndrome^[7]. In our patient, preoperative abdominal CT revealed a “target sign” at the terminal ileum and we were able to identify the intestinal obstruction due to intussusception by the polypoid mass. The postoperative histopathologic results confirmed a hamartomatous polyp.

Miyahara *et al.*^[8] reported a case of intussusception arising from an appendiceal hamartomatous polyp in a PJS patient with anemia and described the appendix invaginating into the cecum in their case^[8]. In general, bowel intussusception is caused by telescoping of the proximal bowel (intussusceptum) into the adjacent distal bowel segment (intussusciptens)^[9]. Appendiceal intussusceptions occur by intraluminal or intramural irritation caused by space-occupying lesions such as appendiceal fecalith, lymphoid hyperplasia, endometriosis, carcinoid tumor, adenoma, or adenocarcinoma. The most common type of appendiceal intussusception is appendico-cecal or ceco-colic^[8,10]. The symptoms are very similar to those of acute appendicitis.

Our case differs from previous cases in that the intussusception was caused by direct ingrowth of the appendiceal tip into the terminal ileal lumen, rather than by a typical telescoping mechanism. To the best of our knowledge, ours is the first case with this rare presentation. Although it is difficult to explain the exact mechanism of the ingrowth in this case, in considering the macro- and microscopic findings, we assume that the hamartomatous polyp arose from the appendiceal tip attached to the serosa of the terminal ileum, and growth of the polyp led to forced entry into the ileal lumen. If it was an ileal hamartomatous polyp, the normal appendix would not be expected to react with an ileal mass to the degree seen in this case. Also, we observed that the appendiceal lumen ran continuously from the external appendix to within the hamartomatous polyp and this was confirmed, microscopically. If the normal appendix was intussuscepted into ileal polyp, we could separate the appendix from the polyp with comparative ease. But appendix and polyp were inseparable. We think that strong tissue interaction between appendix and distal ileum is one of evidence which the polyp is originated from the appendix. Therefore, the primary lesion in our case ought to be the appendix.

Appendiceal tumors are rare, reportedly occurring in < 2% of all appendectomies, and hamartomatous polyps in the appendix are even rarer^[11,12]. There have been spo-

radic reports of appendiceal hamartomatous polyps confirmed in PJS patients; however, there are only two case reports of solitary Peutz-Jeghers-type hamartomatous polyps in the appendix without PJS, to our knowledge^[13,14].

Hamartomatous polyp is considered a benign lesion, but when it is associated with PJS, the risk of malignancy is increased in gastrointestinal and extra-intestinal sites^[15]. Treatment for hamartomatous polyp can include endoscopic resection, polypectomy via enterotomy, and bowel resection. But in patients with PJS, short bowel syndrome can occur due to repeated bowel resection because 30% of these patients require laparotomy and 50% require more than two abdominal surgeries^[16,17]. Therefore, less invasive treatment had to be chosen in those patients. In asymptomatic patients, observation is sufficient, but close follow-up is required because of complications such as multiple cancer, intestinal obstruction, and bleeding. In our case, preoperative abdominal CT definitively revealed the leading point causing the intussusception at the terminal ileum. Because the lesion was not considered to be in spontaneous remission, we performed exploratory laparotomy.

Preventive appendectomy during surgery for other disease is controversial but we believe that if an appendiceal mass is identified pre- or intraoperatively, resection is essential to determine additional treatment after histologic confirmation^[18]. The final histopathological result in this patient occurred following discharge. We attempted to identify mucocutaneous pigmentation and family history to find out an association with PJS, but there were no such characteristic findings of PJS in this patient. For that reason we did not perform postoperative esophagogastroduodenoscopy or colonoscopy. This is able to be a limitation of our follow-up strategy. Surveillance of PJS often follows experts' opinion because of the lack of randomized controlled trials, and in 2006, European experts established age-specific guidelines for the management of these patients^[19].

Appendiceal hamartomatous polyp in our patient had the distinctive aspect of morphogenesis. Because it did not correspond with the conventional concept of the mechanism of intussusception, it was difficult to determine grossly whether this was a case of intussusception or ingrowth. Because definite diagnosis depends on the histopathological finding after resection, the patient with symptomatic mass may needs the resection through the laparotomy or endoscopic approach. Based on histology, periodic surveillance or additional treatment can be required. We believe that ours are a very rare case, clinically, and the first report of its kind to the best of our knowledge.

COMMENTS

Case characteristics

Mild peristaltic pain and tenderness was observed as main symptoms in the right lower quadrant of the abdomen without rebound tenderness.

Clinical diagnosis

Main clinical diagnosis was acute appendicitis.

Differential diagnosis

Authors' had to rule out intestinal obstruction because of abdominal discomfort and bilious vomiting.

Laboratory diagnosis

Blood sample tests were unremarkable except for a mildly elevated white blood cell count of 11480/ μ L.

Imaging diagnosis

Intussusception due to 2.4-cm diameter polypoid mass was identified in distal ileum through the abdominal computed tomography.

Pathological diagnosis

Histopathologic finding (hematoxylin and eosin staining) revealed a hamartomatous polyp of 3.5 cm \times 3.0 cm with surface ulceration.

Treatment

The patient underwent the segmental resection of distal ileum with appendectomy.

Term explanation

The solitary Peutz-Jegher-type polyp is considered to be either an incomplete variant of Peutz-Jegher syndrome or a separate disease entity because of the absence of characteristic external features and family history.

Experiences and lessons

So based on histology, periodic surveillance or additional treatment can be required because hamartomatous polyp has malignant potential when it is associated with Peutz-Jegher syndrome.

Peer review

Among the case reports, this paper is impressive and informative.

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Choledochoduodenal fistula caused by migration of endoclip after laparoscopic cholecystectomy

Tao Hong, Xie-Qun Xu, Xiao-Dong He, Qiang Qu, Bing-Lu Li, Chao-Ji Zheng

Tao Hong, Xie-Qun Xu, Xiao-Dong He, Qiang Qu, Bing-Lu Li, Chao-Ji Zheng, Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

Author contributions: Hong T and Xu XQ designed the report; Hong T, Xu XQ, He XD, Li BL, and Zheng CJ were the patient's attending doctors; Xu XQ and Hong T performed the surgery; Xu XQ and Hong T organized the report; Xu XQ wrote the paper.

Correspondence to: Dr. Xie-Qun Xu, Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Dongcheng District, Beijing 100730, China. xiequnxu@gmail.com

Telephone: +86-10-69152610 Fax: +86-10-6915260

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Key words: Biliary obstruction; Laparoscopic cholecystectomy; Choledochoduodenal fistula

Core tip: Choledochoduodenal fistula caused by endoclip migration; an extremely rare complication after the introduction of laparoscopic cholecystectomy which can occur from days to years after laparoscopic cholecystectomy.

Hong T, Xu XQ, He XD, Qu Q, Li BL, Zheng CJ. Choledochoduodenal fistula caused by migration of endoclip after laparoscopic cholecystectomy. *World J Gastroenterol* 2014; 20(16): 4827-4829 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4827.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4827>

Abstract

The wide use of surgical endoclips in laparoscopic surgery has led to a variety of complications. Post-cholecystectomy endoclips migrating into the common bile duct after laparoscopic cholecystectomy is rare. A migrated endoclip can cause obstruction, serve as a nidus for stone formation, and cause cholangitis. While the exact pathogenesis is still unknown, it is probably related to improper clip application, subclinical bile leak, inflammation, and subsequent necrosis, allowing the clips to erode directly into the common bile duct. We present a case of endoclip migrating into the common bile duct and duodenum, resulting in choledochoduodenal fistula after laparoscopic cholecystectomy and a successful reconstruction of the biliary tract by a hepaticojejunostomy with a Roux-en-Y procedure. This case shows that surgical endoclips can penetrate into the intact bile duct wall through serial maceration, and it is believed that careful application of clips may be the only way to prevent their migration after laparoscopic cholecystectomy.

INTRODUCTION

Since the introduction of the laparoscopic technique, laparoscopic cholecystectomy is considered the gold standard for the management of symptomatic disease with a less than 3% overall complication rate^[1]. Most abnormal biliary-enteric communications are the result of perforation caused by gallstones from the gallbladder or common bile duct into the duodenum, with the remainder being the result of peptic ulcer, tumor, trauma, or other local abnormalities^[2] which often occur before laparoscopic cholecystectomy. Choledochoduodenal fistula caused by endoclip migration is an extremely rare complication after the introduction of laparoscopic cholecystectomy, and can occur from days to years after the procedure. We present a rare case of an endoclip migrating into the common bile duct and duodenum, resulting in choledochoduodenal fistula after the laparoscopic cholecystectomy 10 years prior.



Figure 1 Plain abdominal radiograph showed metal endoclips (arrow) in the right upper quadrant area.

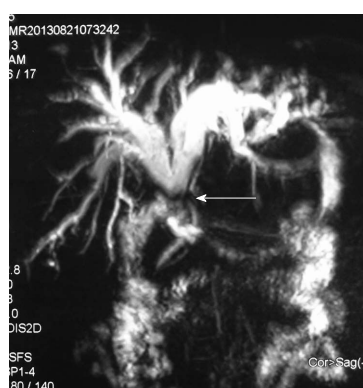


Figure 2 Magnetic resonance cholangiography showed marked dilatation of biliary duct and stenosis of the common bile duct at the hepatic duct confluence (arrow), which was close to the duodenum.

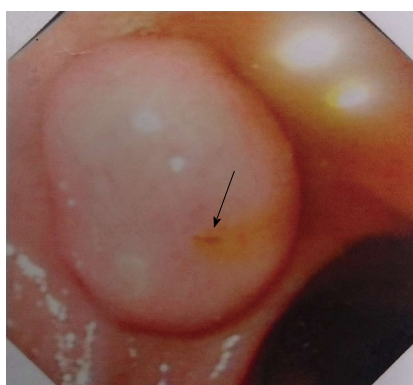


Figure 3 Endoscopic image of the duodenum showed yellowish bile acid (arrow) leaking from a papillary orifice at the first part of duodenum wall.

CASE REPORT

A 48-year-old woman was referred to our hospital with the chief complaint of intermittent epigastric pain, fever, and jaundice for about 3 mo. The patient underwent laparoscopic cholecystectomy (LC) 10 years previously without any intraoperative or postoperative complications. She was diagnosed as suffering acute cholangitis at a rural community hospital, with all symptoms being re-



Figure 4 Computed tomography showed a mass on the duodenal wall (arrow) and linear, highly dense lesions both in the mass (A, arrow) and in the hepatic duct confluence (B, arrow) with dilated hepatic ducts.

lieved after one week of anti-infection treatment. Physical examination at admission revealed no fever, icteric sclera, or jaundice. There was no tenderness at the epigastric area. Laboratory tests revealed white blood cells of 5470/mm³, and elevated levels of alanine aminotransferase (59 U/L, 5-40), gamma glutamyl aminotransferase (300 U/L, 0-50), and total/direct bilirubin (15.1/9.0 μmol/L, 1.7-22.5/0.0-6.0 μmol/L). Tumor markers showed high levels of CA19-9 (326 U/mL), but the levels of carcino-embryonic antigen and alpha-fetoprotein were within the normal range. A plain abdominal radiograph showed metal endoclips in the right upper quadrant area (Figure 1). Magnetic resonance imaging showed marked dilatation of the biliary duct and stenosis of the common bile duct at the hepatic duct confluence, which was close to the duodenum (Figure 2). An endoscopic image of the duodenum (Figure 3) showed yellowish bile acid leaking from a papillary orifice at the first part of duodenum wall. Computed tomography (CT) showed a mass on the duodenal wall, and linear, highly dense lesions both in the mass (Figure 4A) and in the hepatic duct confluence (Figure 4B) with dilated hepatic ducts. The patient's clinical manifestation and imaging studies revealed a choledochoduodenal fistula caused by an injury to the common bile duct by a migrated metal endoclip. Partial resection of the common bile duct and fistula, as well as repair of the duodenum, were performed, followed by reconstruction of the biliary tract by a hepaticojejunostomy with a Roux-en-Y procedure. An endoclip was found in the duodenal

portion of the choledochoduodenal fistula.

DISCUSSION

Surgical endoclips are widely used during LC as substitute ligation materials. Raoul *et al*^[1] first reported the migration of surgical endoclips into the biliary tract acting as a nidus for stone formation after laparoscopic cholecystectomy. A variety of endoclip related complications, such as biliary leaks, endoclip migration into the common bile duct with stone formation, acute pancreatitis, cholangitis, benign stricture, obstructive jaundice, and endoclip embolism have been reported^[3]. Choledochoduodenal fistula is even rarer. Biliary-enteric fistula is a known complication of chronic gallbladder disease which has a reported incidence of 0.06%-0.14%^[4]. However, they usually happen before cholecystectomies, and there are no accurate data for the biliary-enteric fistula, especially for the choledochoduodenal fistula. To the best of our knowledge, this is the first report on a choledochoduodenal fistula caused by an endoclip migrating into the common bile duct and duodenum after LC.

With regard to the pathogenesis of endoclips migration after laparoscopic cholecystectomy, the first possibility is an incomplete closure of the cyst duct caused by an ineffective clip, which then brought on biloma with bile leakage. The second possibility is erosion of the bile duct wall or adjacent adhered duodenal or colonic wall because of localized inflammation around the endoclips. The eroded and inflamed common bile duct and duodenal or colonic wall would develop perforation or scar constriction, resulting in choledochoduodenal fistula or bile duct stenosis^[5].

For the evaluation of choledochoduodenal fistula after LC, magnetic resonance cholangiography, endoscopic retrograde cholangiography, or CT with three-dimensional reconstruction of the biliary tract could be helpful. For the complicated structure around the fistula caused by tissue inflammation and adherence, open surgery is a safe option for reconstructing the biliary tract and repairing the defect in the duodenum due to defects in both the common bile duct and duodenum. Endoclip migration could be potentially avoided by the use of absorbable endoclips, or alternatively ultrasonic dissection without clipping^[6].

In conclusion, we offer a rare case of an endoclip migrating into the common bile duct and duodenum, result-

ing in choledochoduodenal fistula after LC. This situation can be managed by reconstructing the biliary tract via a hepaticojejunostomy with a Roux-en-Y procedure, and could be potentially avoided by using absorbable endoclips or performing ultrasonic dissection without clipping.

COMMENTS

Case characteristics

Choledochoduodenal fistula caused by migration of an endoclip after laparoscopic cholecystectomy.

Differential diagnosis

It should be considered in the differential diagnosis of patients with obstructive jaundice or cholangitis after laparoscopic cholecystectomy.

Diagnostic imaging

Diagnostic imaging must include magnetic resonance cholangiography, endoscopic retrograde cholangiography, or 3D-computed tomography with reconstruction of the biliary tract.

Treatment

Surgical intervention is mostly required to reconstruct the biliary tract and to repair the defect in the duodenum due to defects in both the common bile duct and duodenum.

Peer review

This is an interesting case report owing to its rarity.

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ARC syndrome with high GGT cholestasis caused by *VPS33B* mutations

Jian-She Wang, Jing Zhao, Li-Ting Li

Jian-She Wang, Jing Zhao, Li-Ting Li, Center for Pediatric Liver Diseases, Children's Hospital of Fudan University, Shanghai 201102, China

Jian-She Wang, Department of Pediatrics, Jinshan Hospital Fudan University, Shanghai 201508, China

Author contributions: Wang JS contributed to the patient's medical management, supervision of the genetic study and manuscript drafting; Zhao J contributed to manuscript drafting; Li LT contributed to the performance of genetic study; all authors contributed to analysis and interpretation of data, revising the manuscript and final approval of the version ready for publication.

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Correspondence to: Jian-She Wang, Professor, Department of Pediatrics, Jinshan Hospital Fudan University, Shanghai 201102, China. jshwang@shmu.edu.cn

Telephone: +86-21-64931171 Fax: +86-21-61143167

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Abstract

Arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome (OMIM 208085) is an autosomal recessive disorder that is caused by mutations in 2 interacting genes *VPS33B* and *VIPAS39*. Mutations in *VPS33B* gene account for most cases of ARC. As low or normal gamma-glutamyl transpeptidase (GGT) activity has been described in all patients with ARC syndrome identified so far, ARC syndrome is a possible diagnosis for low GGT cholestasis. Here we describe a Chinese patient with neonatal cholestasis and a high GGT level in three consecutive tests. She had other typical manifestations of ARC syndrome, including arthrogryposis multiplex congenita, renal involvement and ichthyosis. Genetic study of the *VPS33B* gene further confirmed the diagnosis by identification of compound heterozygosity of two known disease-causing mutations, c.403+2T > A and c.1509-1510insG. The mechanism of high GGT in this patient is unclear. Nevertheless, this case indicates

that ARC syndrome cannot be excluded from the differential diagnosis of neonatal cholestasis even if high GGT activity is found.

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Key words: Arthrogryposis, renal dysfunction and cholestasis syndrome; Cholestasis; Gamma-glutamyl-transpeptidase; *VPS33B*; Renal dysfunction; Glucosuria

Core tip: Neonatal cholestasis with low or normal gamma glutamyl transpeptidase (GGT) activity was regarded as a characteristic feature of arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome. Here we describe a patient who presented with neonatal cholestasis and high GGT activities. She had all other typical clinical manifestations of ARC syndrome. The diagnosis was finally confirmed by the presence of compound heterozygosity of two known *VPS33B* disease-causing mutations. Our case indicates that ARC syndrome cannot be excluded in neonatal cholestasis even with unexpected high GGT activity.

Wang JS, Zhao J, Li LT. ARC syndrome with high GGT cholestasis caused by *VPS33B* mutations. *World J Gastroenterol* 2014; 20(16): 4830-4834 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v20/i16/4830.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4830>

INTRODUCTION

Arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome (OMIM 208085) is an autosomal recessive disorder that typically presents with neonatal cholestasis, renal tubular dysfunction and arthrogryposis multiplex congenita^[1]. Mutations in 2 interacting genes *VPS33B* and *VIPAS39* have been identified. Mutations in *VPS33B*

Table 1 Biochemistry of the proband and her past elder sister at different age of days

Age (d)	TBIL (mmol/L)	DBIL (mmol/L)	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	TBA (mmol/L)	TP (g/L)	ALB (g/L)
The proband									
17	80.5	28.1	12	22	694	216	27.2	50.3	31.2
21	55.9	27.5	13	22	486	150	34.2	49.0	24.0
26	107.5	36.5	40	29	558	202	90.7	NA	NA
The past elder sister									
30	263.4	122	22	NA	576	48	NA	48.9	29.0
32	334.7	210.5	16	30	454	43	NA	46.4	30.2
34	271.3	162.9	17	40	419	37	NA	57.1	31.5
Reference range	5-21	0-6	0-40	0-40	0-500	3-50	0-10	60-85	35-55

TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl-transpeptidase; TBA: Total bile acid; TP: Total protein; ALB: Albumin.

gene account for most cases of ARC^[2-5].

As low or normal gamma-glutamyl transpeptidase (GGT) activity has been described in all patients with ARC syndrome identified so far, ARC syndrome is one of the differential diagnosis for low GGT cholestasis^[6,7]. Recently we diagnosed a case with ARC syndrome caused by *VPS33B* mutations, but an unexpectedly high GGT level was noticed.

CASE REPORT

The proband is a female patient, the second child of a non-consanguineous *han* couple. Oligohydramnios, ascites and enhanced echo of the kidneys of the fetus were demonstrated by ultrasound in the 7th mo of pregnancy. She was born in good condition with a birth weight of 3400 g by a cesarean section at 39 wk of gestation, due to breech presentation. Her weight dropped to 2900 g by day 8 while on mixed formula and breast feeding. Hearing screening tests performed on day 7 yielded no definitive results.

Jaundice was first noticed on day 3 after birth and resolved spontaneously on day 10. It recurred from day 14 after birth and dark urine and light yellow colored stools were noticed thereafter. The investigations at the local hospital revealed mild cholestasis so the child was transferred to a children's hospital in Beijing at 21 d of age, when her body weight was 2900 g. She received blood transfusion because of anemia with a hemoglobin level of 71 g/L at 28 d of age, and was then referred to a hepatology centre at age 30 days for investigations of the cause of her cholestasis.

Family history revealed that the mother was healthy and the father had polycystic kidney disease. The mother's first pregnancy produced a full-term girl weighing 3000 g, delivered by cesarean section for breech presentation and II° contaminated amniotic fluid. Enhanced echoes of the fetal kidneys were demonstrated by ultrasound at the 4th mo of pregnancy and oligohydramnios and ascites at the 7th mo of pregnancy. The limbs and skin of this elder sibling looked similar to those of the proband. Jaundice persisted from birth and stool color became lighter after 20 d of age. Laboratory tests at 1 mo of age revealed

persistent positive glucose and protein in the urine and moderate anemia. Liver function tests were listed in Table 1. Ultrasound of the abdomen revealed polycystic kidneys, but CT scan of the brain was normal. This first baby died at 8 mo from infection, anorexia, jaundice and poor weight gain.

On examination of the proband, obvious arthrogryposis multiplex, exfoliative skin (ichthyosis), mild jaundice and simian lines on the right palm were seen. A weak response to surrounding stimulus and no response to sound were noted. Liver was palpable 2 centimeters below the costal edge with normal texture. The spleen was not palpable.

Laboratory investigations of the proband showed mildly elevated conjugated bilirubin, raised alkaline phosphatase, elevated GGT and total bile acids, hypoalbuminemia but normal ALT and AST (Table 1). Proteinuria and glucosuria were present. There was a mildly elevated lactate level. The following results were normal or unremarkable: blood urea, creatinine, electrolytes, free T4 and thyroid stimulating hormone, ammonia, α 1-antitrypsin level, blood tandem mass spectrometry (MS/MS) study of amino acid and carnitine profile, serology for hepatitis A to E, blood immunoglobulin M antibodies to toxoplasma, rubella, cytomegalovirus, herpes simplex virus, Epstein-Barr virus, blood cytomegalovirus DNA and chromosome G bands.

The proband's previous X-ray revealed pneumonia. Cardiac ECHO revealed patent foramen ovale. Abdominal ultrasound showed normally sized kidneys with multiple cysts of various sizes in both kidneys with the largest in the left kidney of 0.8 cm × 0.6 cm, and the largest in the right kidney, 0.6 cm × 0.5 cm. Granular high-echo spots in the medulla of kidneys were revealed. Ultrasound of the hip showed no sign of dislocation. Brain CT scan showed symmetric, bilateral hypodense white matter of the cerebral hemispheres with a CT number of about 14 HU on the Hounsfield scale. CT also revealed swollen bilateral frontal and temporal lobes, narrowed bilateral lateral and third ventricles, and basal ganglia of heterogeneous density.

Management

From the clinical manifestations and previously per-

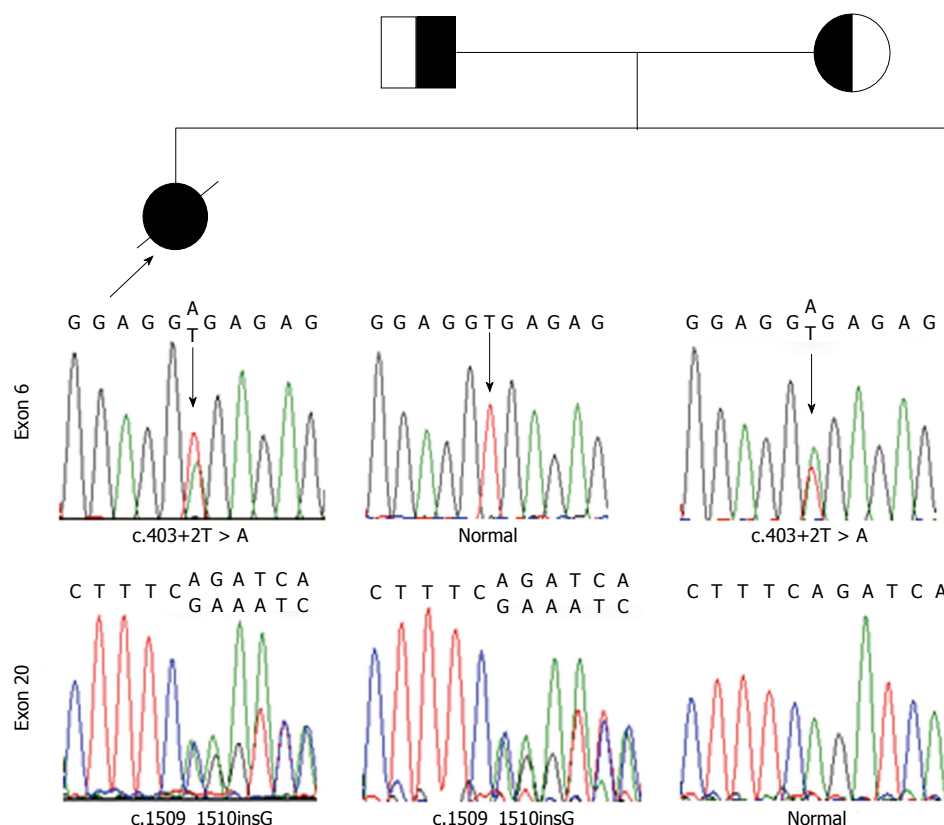


Figure 1 Genomic DNA sequences of the exons 6 and 20 of *VPS33B* gene from the proband and her parents. The arrows in exon 6 indicate T/A heterozygous (c.403+2T > A) in the proband and her mother, but normal sequence (T) in her father. The arrows in exon 20 indicate heterozygous insertion of G (c.1509-1510insG) in the proband and her father, but no change in her mother. These confirmed the proband was a compound heterozygote for c.403+2T > A and c.1509-1510insG in *VPS33B* gene.

formed investigations, the diagnosis of ARC syndrome was suspected. In view of the bad prognosis, the parents were not willing to allow the child to undergo any further tests. However they consented to genetic analysis to help with future prenatal diagnosis. Ursodeoxycholic acid and fat-soluble vitamins were prescribed and follow-up was made over the telephone or by email. Her ichthyosis got much better with olive oil massage after bathing and the patient attained her birth weight at 67 d of age. Facial eczema developed from about 5 mo of age and she died at 7.5 mo of age.

Molecular genetic techniques

The study protocol conforms to the ethical guidelines of the Declaration of Helsinki of 1975. With the approval by the Ethics Committee on human research of the Children's Hospital of Fudan University and informed consent of the parents, 1 mL of whole blood was drawn from the proband and her parents. DNA was extracted routinely and all the coding exons together with adjacent intronic sequence of the *VPS33B* gene were amplified and sequenced according to Gissen P *et al* with modifications (detailed primers, PCR and sequencing condition are available upon request)^[2,3].

Result of molecular genetic studies

In the proband, compound heterozygosity for c.403+2T

> A and c.1509-1510insG mutations was revealed. The parents were found to be heterozygous (Figure 1).

DISCUSSION

ARC syndrome is known to be caused by *VPS33B* and *VIPAS39* mutations and has been reported to occur in many ethnic groups^[3,8]. Normal or low GGT is one of the characteristics of neonatal cholestasis in ARC syndrome and it was listed as one of the four diagnostic features of the syndrome (arthrogryposis, renal tubular dysfunction and cholestasis with a low GGT activity)^[3]. By reviewing the literature to date, none of the cases reported manifested cholestasis with significantly high GGT. Therefore, the proband case here is the first report that ARC syndrome could present as neonatal cholestasis with significantly high GGT activities.

The patients had three major diagnostic features: arthrogryposis multiplex congenita, renal involvement and cholestasis. The genetic study of *VPS33B* gene of the proband further confirmed the diagnosis by identification of two mutations previously reported in the East Asians^[8]. c.1509-1510insG is a frame-shift mutation. c.403+2T > A mutation disrupts the original donor site following new donor site creation and therefore, a 16 bp intronic sequence that contains a stop codon is inserted into the mRNA sequence and results in a truncated

Table 2 Gamma-glutamyl transpeptidase level in arthrogryposis, renal dysfunction and cholestasis patients previously reported

Ref.	GGT level
Di Rocco <i>et al</i> ^[10] (1995)	Normal (3 patients)
Franceschini <i>et al</i> ^[11] (1997)	Normal (3 patients)
Papadia <i>et al</i> ^[12] (1996)	Normal (1 patient)
Coleman <i>et al</i> ^[13] (1997)	Normal (2 patients, 60-70 U/L)
Abdullah <i>et al</i> ^[14] (2000)	Normal (3 patients)
Denecke <i>et al</i> ^[15] (2000)	Normal (2 patients), mildly elevated (1 patient 78 U/L)
Eastham <i>et al</i> ^[16] (2001)	Normal (4 patients)
Howells <i>et al</i> ^[17] (2002)	Normal (1 patient)
Gissen <i>et al</i> ^[2] (2004)	Normal (29 patients)
Abu-Sa'Da <i>et al</i> ^[18] (2005)	Normal (2 patients)
Choi <i>et al</i> ^[19] (2005)	Normal (1 patient)
Tekin <i>et al</i> ^[20] (2005)	Normal (2 patients)
Bull <i>et al</i> ^[21] (2006)	Normal (1 patient)
Gissen <i>et al</i> ^[3] (2006)	Normal (9 patients)
Taha <i>et al</i> ^[22] (2007)	Normal (1 patient)
Hershkovitz <i>et al</i> ^[23] (2008)	Normal (2 patients, 35-83 U/L)
Arhan <i>et al</i> ^[24] (2009)	Normal (1 patient)
Jang <i>et al</i> ^[8] (2009)	Normal (6 patients)
Kim <i>et al</i> ^[25] (2010)	Normal (10 patients)

GGT: Gamma-glutamyl transpeptidase.

VPS33B protein^[9]. Based on this, a diagnosis of ARC syndrome caused by *VPS33B* mutations in the proband case could be confirmed.

An interesting finding is the parallel increase in serum levels of GGT and total bile acids in the proband. Her elder sister demonstrated a typical neonatal cholestasis with low GGT, who should have same genetic background of *VPS33B*, indicating that the high GGT in the proband could not be explained by the specific mutations.

The normal reference range of GGT is age dependent^[7]. It could be quite high in newborns and then decreased to adult range. In Mainland China, because of the lack of age specific data, 50 U/L is widely used as the upper normal limit regardless of age. 93 U/L is defined as the upper normal limit of GGT in infants less than 6 mo of age in National Taiwan University Hospital^[6]. One feature of this proband is that she went to see a doctor much earlier than her elder sister, so the elevation of GGT might be explained as an age-related evolution of normal GGT activity. The limitation of this case report is the lack of follow-up of her liver function test beyond neonatal stage. As a result, we do not know whether the elevated GGT activity would reduce as age advances. However, all cases with available data on GGT activity reported until now had GGT activity labeled normal or with a peak level no more than 83 U/L (Table 2). Her elder sister's GGT activity tested at 30 days of age was also below 50 U/L (Table 1). However, the proband had a GGT activity over 200 U/L at 26 days of age, making it unlikely that it can be fully explained by the specific age.

This case shows that the presence of high GGT activity cannot exclude ARC if the diagnosis is strongly suspected due to the presence of other cardinal features such as ichthyosis, arthrogryposis, agranular platelets, fail-

ure to thrive, and renal tubular acidosis. It indicates that ARC syndrome should be considered as a diagnostic possibility in various populations and a cholestasis with significantly high GGT activity, especially in the early stage after birth, should not exclude the diagnosis.

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COMMENTS

Case characteristics

Arthrogryposis multiplex congenita and ichthyosis were found in a cholestatic infant with high gamma-glutamyl transpeptidase (GGT) activity.

Clinical diagnosis

Arthrogryposis multiplex congenita, renal dysfunction and cholestasis.

Differential diagnosis

Biliary atresia, progressive familial intrahepatic cholestasis, citrin deficiency, idiopathic neonatal cholestasis, etc., should be considered.

Laboratory diagnosis

Genetic study revealed compound heterozygote with known disease-causing mutations in *VPS33B*.

Imaging diagnosis

Multiple cysts in kidneys and patent foramen ovale were revealed by ultrasound.

Treatment

Ursodeoxycholic acid and fat-soluble vitamins were prescribed.

Related reports

Cholestasis of arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome has never been associated with significantly high GGT activities.

Term explanation

ARC refers to arthrogryposis multiplex congenita, renal dysfunction and cholestasis.

Experiences and lessons

ARC syndrome should not be excluded from the list of differential diagnoses in a cholestatic infant with high GGT activity, especially in the first months after birth.

Peer review

This article indicated that ARC syndrome cannot be excluded from the differential diagnosis of neonatal cholestasis based on serum levels of GGT activity.

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Laparoscopic ligation of proximal splenic artery aneurysm with splenic function preservation

Yun-Hai Wei, Jie-Wei Xu, Hua-Ping Shen, Guo-Lei Zhang, Harsha Ajoodhea, Ren-Chao Zhang, Yi-Ping Mou

Yun-Hai Wei, Jie-Wei Xu, Hua-Ping Shen, Guo-Lei Zhang, Department of General Surgery, the Central Hospital of Huzhou, Huzhou 313000, Zhejiang Province, China

Harsha Ajoodhea, Ren-Chao Zhang, Yi-Ping Mou, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China

Author contributions: Wei YH, Xu JW, Shen HP, Zhang GL and Mou YP performed the operation; Wei YH, Xu JW, Shen HP and Zhang GL collected case data; Wei YH, Ajoodhea H and Zhang RC wrote the manuscript; Mou YP proofread and revised the manuscript; all authors approved the version to be published. Supported by Key Disciplines of Medicine, Zhejiang Province, China, No. 11-CX21

Correspondence to: Yi-Ping Mou, MD, FACS, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, 3 East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. mouyp@srsh.com

Telephone: +86-571-86006952 Fax: +86-571-86044817

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Laparoscopic ligation; Splenic function preservation

Core tip: An aneurysm in the proximal splenic artery is rare. Few cases of an aneurysm in the proximal splenic artery treated by laparoscopic techniques have been reported in the literature. We present a case of splenic artery aneurysm located in the proximal splenic artery which was treated by laparoscopic ligation with long-term preservation of splenic function. This is the first case of laparoscopic ligation of a proximal artery aneurysm with preservation of splenic function to be reported in the English literature.

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Abstract

Splenic artery aneurysm is one of the most common visceral aneurysms, and patients with this type of aneurysm often present without symptoms. However, when rupture occurs, it can be a catastrophic event. Although most of these aneurysms can be treated with percutaneous embolization, some located in uncommon parts of the splenic artery may make this approach impossible. We present a patient with an aneurysm in the proximal splenic artery, close to the celiac trunk, which was treated by laparoscopic ligation only, without resection of the aneurysm, and with long-term preservation of splenic function.

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Key words: Laparoscopy; Splenic artery; Aneurysm;

INTRODUCTION

Splenic artery aneurysms (SAAs) account for 46%-60% of all visceral artery aneurysms^[1]. Most occur in the distal third of the splenic artery (75%) followed by the middle third (20%)^[2]. Aneurysms in the proximal splenic artery are uncommon. Treatment of a SAA includes laparotomy, laparoscopy or endovascular techniques. In recent years, open aneurysm repair of SAAs has been largely replaced by minimally invasive surgery, such as endovascular procedures, which result in less surgical trauma and faster postoperative recovery. However, only selected aneurysms are suitable for these procedures, as marked tortuosity of the artery or SAA in the proximal splenic artery may not be suitable for endovascular management. Thus, in these rare cases, laparoscopy may be a challenging alternative, not only to open surgery, but also to endovascular procedures.

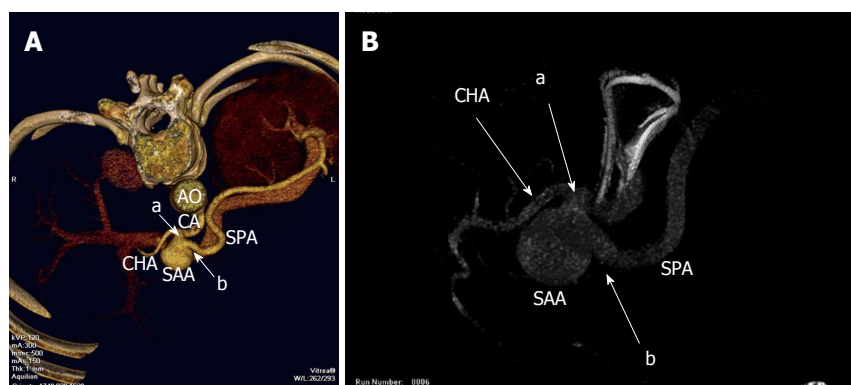


Figure 1 Three-dimensional computed tomography reconstruction. A: A 3-cm splenic artery aneurysm in the proximal splenic artery with afferent (a) and efferent (b) artery; B: Angiography demonstrating a 3 cm splenic artery aneurysm at the same location with afferent (a) and efferent (b) artery. AO: Abdominal aorta; CA: Celiac artery; CHA: Common hepatic artery; SAA: Splenic artery aneurysm; SPA: Splenic artery.

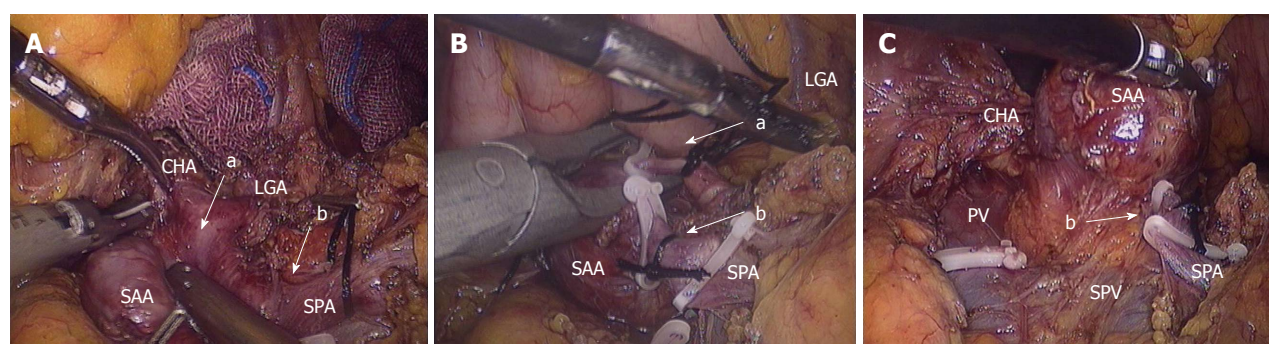


Figure 2 Intraoperative imaging. A: The position of the splenic artery aneurysm with its afferent (a) and efferent (b) artery; B: Both afferent (a) and efferent (b) arteries of the aneurysm were occluded by the ligatures; C: Reverse side of the splenic artery aneurysm showing no collateral vessels connecting with the aneurysm. CHA: Common hepatic artery; SAA: Splenic artery aneurysm; SPA: Splenic artery. LGA: Left gastric artery; PV: Portal vein; SPV: Splenic vein.

To our knowledge, few cases of SAA in the proximal splenic artery treated by laparoscopic techniques have been reported in the literature. The authors present a patient with an aneurysm located in the proximal splenic artery, which was treated by laparoscopic ligation with long-term preservation of splenic function.

CASE REPORT

An asymptomatic 49-year-old woman was found to have a splenic artery aneurysm on medical examination. Computed tomography scan revealed a 3 cm SAA. Both three-dimensional reconstruction and angiography demonstrated the presence of a 3 cm aneurysm in the proximal splenic artery, approximately 0.8 cm from the celiac trunk (Figure 1). Considering the anatomic location of the aneurysm, endovascular treatment was not proposed due to recanalization and coil migration. Thus, laparoscopic surgery was deemed the optimal treatment option. It was decided to attempt simple laparoscopic ligation without resecting the aneurysm.

The patient was placed in the supine position. Five ports were inserted and a pneumoperitoneum was created. First, we divided the gastrocolic ligament, revealing the pancreatic edge, then identified the splenic artery and exposed the aneurysm. We separated its proximal and distal parts, and clips were used to ligate the aneurysm proximally and distally (Figure 2). The collateral vessels of the aneurysm were also completely dissected, thus isolating the aneurysm with no retrograde filling. The

whole spleen appeared dusky, but returned to normal at the end of the procedure. The abdominal cavity was irrigated with normal saline and a drain was placed under the aneurysm to drain the fluid and prevent contingent complications caused by pancreatic leakage.

The operative time was 50 min and blood loss was 10 mL. Three days later, abdominal vascular ultrasound showed a hypoechoic mass between the proximal part of the splenic artery and celiac trunk with no color flow signals (Figure 3A), and the blood flow into the splenic vein was affluent (Figure 3B). The patient had a smooth recovery with no complications and was discharged on postoperative day 5. During the postoperative follow-up period, she recovered very well with no abdominal pain or pancreatic insufficiency. The three-dimensional computed tomography reconstruction (two months after surgery) revealed no recurrence of the aneurysm and her splenic function was well maintained (Figure 3C).

DISCUSSION

Despite an extremely low incidence and almost no symptoms of SAA, death can occur if the aneurysm ruptures^[3]. The available evidence suggests that active treatment should be initiated if the aneurysm is larger than 2 cm in patients at high risk of rupture, such as during pregnancy, patients of childbearing age or following liver transplantation.

Transcatheter embolization is currently considered to be the first-line treatment in most patients with SAAs,

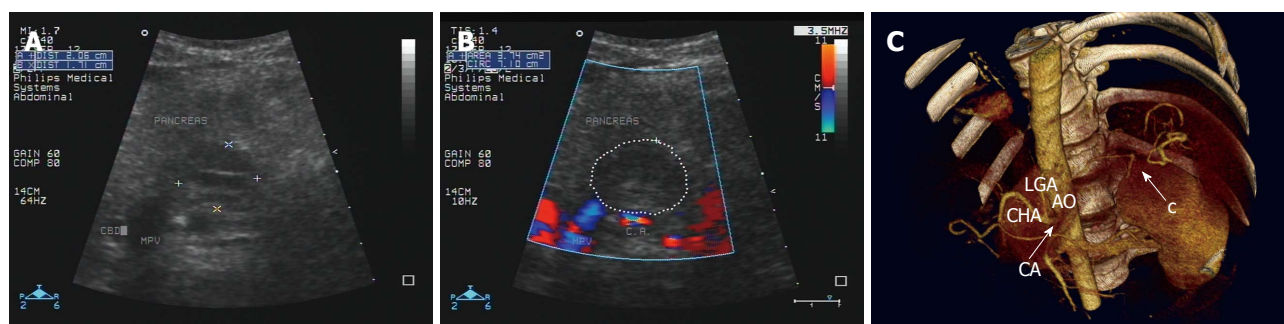


Figure 3 Postoperative imaging. A: Ultrasound showing a 3 cm hypoechoic mass between the proximal splenic artery and the celiac trunk; B: Ultrasound showing no color flow signals in the hypoechoic mass; C: Three-dimensional CT reconstruction revealing the proximal splenic artery without aneurysm recurrence and without collateral vessels supplying the spleen (c). AO: Abdominal aorta; CA: Celiac artery; CHA: Common hepatic artery; LGA: Left gastric artery.

since the procedure can be carried out under local anesthesia with minimal trauma to the patient^[4]. However, this technique may not be proposed if the SAA is located at the hilum of the spleen or if the splenic artery is particularly tortuous. In addition, it presents some critical points: recanalization, coil migration and distal infarction with abscess formation, and the post-treatment splenic flow can be compromised due to related splenic malfunction and infarction^[5].

Stent grafting also requires careful patient selection as the size and tortuosity of the splenic artery as well as the location of the aneurysm may have limitations in the deployment of the stent graft^[6]. Furthermore, complications related to stent migration and arterial occlusion also have to be considered^[7]. Moreover, following stent grafting, life-long oral antiplatelet drugs are required and the long-term durability and patency of these grafts are unknown.

In the present case, the SAA was in the proximal splenic artery, close to the celiac trunk, and the splenic artery was tortuous in this location. Thus, transcatheter embolization and stent grafting were considered inappropriate treatment choices. Laparoscopic management was deemed to be the best treatment option, and was to include either resection of the SAA or just simple ligation of the SAA. Laparoscopic resection of orthotopic SAAs has been espoused by a number of authors as a less invasive alternative to open surgery^[8]. However, it was performed only for aneurysms of small diameter, 2–2.5 cm, affecting the middle or distal third of the artery. In addition, this technique is controversial as it leaves a vascular stump exposing it to pulsatile arterial pressure and the possibility of recurrence^[9].

To date, laparoscopic ligation of aneurysms has only been reported in two cases, and both were located in the middle or distal third of the splenic artery^[10,11]. An SAA in the proximal splenic artery treated by laparoscopic ligation has not yet been reported. In our case, the location of the SAA was in the proximal part of the splenic artery, close to the celiac trunk, and the splenic artery was tortuous in this location. Therefore, we chose laparoscopy as the optimal treatment. As an SAA is a non-solid tumor, it was not necessary to resect the aneurysm. Preoperative

evaluation is essential and blocking the collateral circulation of the SAA is the most important step in the procedure, which ultimately leads to no retrograde filling of the SAA. The risk associated with laparoscopic ligation of the SAA was deficient residual blood flow to the spleen, thus leading to splenic infarction and possible evolution into a splenic abscess. Therefore, intraoperative ultrasound may contribute to determination of the residual blood flow. To the best of our knowledge, this is the first case of laparoscopic ligation of a splenic artery aneurysm in the proximal part of the splenic artery. This approach greatly reduces blood loss and results in a shorter operative time compared to laparoscopic resection. In addition, spleen function was completely preserved. The patient had a faster postoperative recovery and a good long-term outcome associated with preservation of splenic function. Therefore, we believe that laparoscopic ligation is suitable for aneurysms located in the proximal splenic artery.

In conclusion, laparoscopic ligation of a SAA in the proximal splenic artery is safe and effective. It offers good postoperative recovery with a good long-term outcome due to preservation of splenic function. Therefore, laparoscopic ligation may be the preferred treatment in cases in which other treatments are unfeasible.

COMMENTS

Case characteristics

Incidental finding of a 3-cm aneurysm in the proximal splenic artery in an asymptomatic 49-year-old female patient on routine medical examination.

Clinical diagnosis

Incidental finding of a 3-cm aneurysm in the proximal splenic artery on computed tomography (CT).

Differential diagnosis

To exclude a tortuous splenic artery and calcified lymph nodes, three-dimensional CT reconstruction and angiography were performed.

Laboratory diagnosis

The laboratory tests were all within normal ranges.

Imaging diagnosis

Three-dimensional CT reconstruction and angiography revealed a 3-cm aneurysm in the proximal splenic artery, 0.8 cm from the celiac trunk.

Treatment

Laparoscopic ligation of the proximal splenic artery aneurysm, without resection of the aneurysm.

Related reports

To date, laparoscopic ligation of aneurysms has only been reported in two cases, and both were located in the middle or distal third of the splenic artery.

Experiences and lessons

Laparoscopic ligation of a splenic artery aneurysm is safe and effective, and should be considered when other treatment modalities are not feasible.

Peer review

The authors presented their successful experience of laparoscopic ligation of asymptomatic proximal splenic artery aneurysm in 49-year-old woman. The paper is very well presented with very nice intraoperative figures. It is an interesting rare case report with nice laparoscopic views.

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Room 903, Building D, Ocean International Center,

No. 62 Dongsihuan Zhonglu, Chaoyang District,

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Telephone: +86-10-59080039

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Telephone: +86-10-85381892

Fax: +86-10-85381893

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8226 Regency Drive,

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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 Moorselaar 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 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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