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AIMS AND SCOPE

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The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

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FLYLEAF

EDITORS FOR THIS ISSUE

Hepatitis C infected patients need vitamin D3 supplementation in the era of direct acting antivirals treatment

Yasuteru Kondo

Yasuteru Kondo, Department of Hepatology, Sendai Kousei Hospital, Aoba, Sendai City 980, Miyagi, Japan

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Correspondence to: Yasuteru Kondo, MD, PhD, Chief Director, Department of Hepatology, Sendai Kousei Hospital, 4-15 Hirosemachi, Aoba, Sendai City 980, Miyagi, Japan. yasuteru@ebony.plala.or.jp
Telephone: +81-22-2226181
Fax: +81-22-7138013

Abstract

It has been reported that the serum level of vitamin D3 (VitD3) could affect the natural course of chronic hepatitis C (CH-C) and the response to treatment with pegylated interferon (Peg-IFN) and ribavirin. Although several mechanisms for the favorable effects of VitD3 supplementation were reported, the total effect of VitD3 supplementation remains unclear. Previously, we reported that supplementation with 1(OH)VitD3 could enhance the Th1 response inducing not only a favorable immune response for viral eradication but also HCC control. Recently, the main treatment of CH-C should be direct acting antivirals (DAAs) without Peg-IFN. Peg-IFN is a strong immune-modulator. Therefore, an immunological analysis should be carried out to understand the effect of VitD3 after treatment of DAAs without Peg-IFN. The induction of a favorable immune response by adding VitD3 might be able to suppress the hepatocarcinogenesis after achieving SVR, especially in children and elderly patients with severe fibrosis lacking sufficient amounts of VitD3.

Key words: Vitamin D; Hepatitis C virus; Direct acting antivirals; Hepatocarcinogenesis; Immune response

Core tip: Although several mechanisms for the favorable effects of vitamin D3 (VitD3) supplementation were reported, the total effect of VitD3 supplementation remains unclear. Recently, the main treatment of chronic hepatitis C should be direct acting antivirals (DAAs) without pegylated interferon (Peg-IFN). Peg-IFN is a strong immune-modulator. Therefore, an immunological analysis should be carried out to understand the effect of VitD3 after treatment of DAAs without Peg-IFN. The induction of a favorable immune response by adding VitD3 might be able to suppress the hepatocarcinogenesis after achieving SVR, especially in children and elderly patients with severe fibrosis lacking sufficient amounts of VitD3.

Kondo Y. Hepatitis C infected patients need vitamin D3

INTRODUCTION

It has been reported that the serum level of vitamin D3 (VitD3) could affect the natural course of chronic hepatitis C (CH-C) and the response to treatment with pegylated interferon (Peg-IFN) and ribavirin (RBV)[1,2]. Although several mechanisms for the favorable effects of VitD3 supplementation were reported, the total effect of VitD3 supplementation remains unclear. It has been reported that VitD3, as synthesized in the skin by photolysis from 7-dehydrocholesterol, is transported in the blood to the liver where it is hydroxylated at the C-25-position. Then, it is hydroxylated at the C-1α-position to form the active metabolite $1,25(OH)_2$VitD3 in the kidney. $1,25(OH)_2$VitD3 is known to regulate calcium and phosphorus metabolism in skeletal homeostasis. Moreover, $1,25(OH)_2$VitD3 could affect various kinds of immune cells *via* vitamin D receptor[3,4]. Several groups reported that the amount of 25(OH)VitD3 affects the progression of CH-C and response to Peg-IFN/RBV treatment. Moreover several mechanisms for the favorable effects of VitD3 supplementation in CH-C patients have been reported[5]. Dr. Azza reported that the serum level of 25(OH)VitD3 in CH-C children was significantly lower than that in healthy children. In addition to the treatment response, the deficiency of VitD3 could affect bone density. Therefore, we should consider supplementation with VitD3 for CH-C patients even in the era of direct acting antivirals (DAAs).

DISCUSSION

After a sustained virological response, the risk of hepatocarcinogenesis remains. Previously, we reported that supplementation with 1(OH)VitD3 could enhance the Th1 response inducing not only a favorable immune response for viral eradication but also HCC control[5]. The induction of a favorable immune response by adding VitD3 might be able to suppress the hepatocarcinogenesis after achieving SVR, especially in children and elderly patients lacking sufficient amounts of VitD3. Another group reported that $1,25(OH)_2$VitD3 could inhibit HCC development through reducing secretion of inflammatory cytokines from immune-related cells[6]. Moreover, it has been reported that reduced 25(OH)VitD3 serum levels were found to be associated with HCV-related HCC[7]. In addition to the risk of HCC development, 25(OH)VitD3 deficiency could be associated with advanced stages of HCC and it could be a prognostic indicator for a poor outcome[8]. In Japan, hepatocarcinogenesis after achieving SVR is an important issue since many CH-C patients are old and have severe fibrosis. Especially, CH-C patients with severe fibrosis might not have sufficient VitD3 since hepatocytes are necessary to metabolize VitD3. Moreover, it has been reported that there might be a relationship between carcinogenesis and insufficient VitD3[6,9]. Therefore, we should analyze the effect of VitD3 supplementation on hepatocarcinogenesis after achieving SVR[7]. Additionally, the immunological effect of VitD3 might differ between DAAs with and without Peg-IFN.

CONCLUSION

Recently, the main treatment of CH-C should be DAAs without Peg-IFN. Peg-IFN is a strong immune-modulator. Therefore, an immunological analysis should be carried out to understand the effect of VitD3 after treatment of DAAs without Peg-IFN.

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REVIEW

Pediatric gastrointestinal bleeding: Perspectives from the Italian Society of Pediatric Gastroenterology

Claudio Romano, Salvatore Oliva, Stefano Martellossi, Erasmo Miele, Serena Arrigo, Maria Giovanna Graziani, Sabrina Cardile, Federica Gaiani, Gian Luigi de'Angelis, Filippo Torroni

Claudio Romano, Unit of Pediatrics, Department of Human Pathology in Adulthood and Childhood "G. Barresi", University of Messina, 98125 Messina, Italy

Salvatore Oliva, Department of Pediatrics and Infantile Neuropsychiatry, Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome, 00161 Rome, Italy

Stefano Martellossi, Institute for Maternal and Child Health IRCCS 'Burlo Garofalo', 34137 Trieste, Italy

Erasmo Miele, Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", 80131 Naples, Italy

Serena Arrigo, Gastroenterology and Endoscopy Unit, G. Gaslini Children's Hospital-IRCCS, 16147 Genoa, Italy

Maria Giovanna Graziani, Gastroenterology and Endoscopy Unit, "San Giovanni Addolorata" Hospital, 00184 Rome, Italy

Sabrina Cardile, Hepatology, Gastroenterology and Nutrition Unit, Bambino Gesù Children's Hospital, 00165 Rome, Italy

Federica Gaiani, Gian Luigi de'Angelis, Gastroenterology and Endoscopy Unit, University of Parma, 43126 Parma, Italy

Filippo Torroni, Department of Surgery and Transplantation, Digestive Surgery and Endoscopy Unit, Bambino Gesù Children's Hospital, IRCCS, 00165 Rome, Italy

Abstract

There are many causes of gastrointestinal bleeding (GIB) in children, and this condition is not rare, having a reported incidence of 6.4%. Causes vary with age, but show considerable overlap; moreover, while many of the causes in the pediatric population are similar to those in adults, some lesions are unique to children. The diagnostic approach for pediatric GIB includes definition of the etiology, localization of the

bleeding site and determination of the severity of bleeding; timely and accurate diagnosis is necessary to reduce morbidity and mortality. To assist medical care providers in the evaluation and management of children with GIB, the "Gastro-Ped Bleed Team" of the Italian Society of Pediatric Gastroenterology, Hepatology and Nutrition (SIGENP) carried out a systematic search on MEDLINE *via* PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) to identify all articles published in English from January 1990 to 2016; the following key words were used to conduct the electronic search: "upper GIB" and "pediatric" [all fields]; "lower GIB" and "pediatric" [all fields]; "obscure GIB" and "pediatric" [all fields]; "GIB" and "endoscopy" [all fields]; "GIB" and "therapy" [all fields]. The identified publications included articles describing randomized controlled trials, reviews, case reports, cohort studies, case-control studies and observational studies. References from the pertinent articles were also reviewed. This paper expresses a position statement of SIGENP that can have an immediate impact on clinical practice and for which sufficient evidence is not available in literature. The experts participating in this effort were selected according to their expertise and professional qualifications.

Core tip: This review provides a practical diagnostic guide for clinicians for the diagnosis and management of gastrointestinal bleeding (GIB) in children. Clinical presentation can be variable and bleeding can occur in any area of the gastrointestinal tract. The differential diagnosis is important to define the sequence of management. Upper endoscopy and colonoscopy are the mainstay of initial investigations. Best outcomes are possible by a multidisciplinary approach including clinicians with skills in pediatric gastroenterology, radiology and surgery. For cases of major GIB, stabilization of the patient's condition precludes any diagnostic examination.

INTRODUCTION

Gastrointestinal bleeding (GIB) is a common condition in children and can occur in any area of the gastro-intestinal tract, from the mouth to the anus. Fortunately, mortality for acute gastrointestinal bleeding (AGIB) is low in the pediatric population.

Over the last 10 years, there have been a number of improvements in diagnosis and management of GIB in general. Increased involvement has been seen in the management of AGIB and resuscitation and in the correct usage of diagnostic and therapeutic endoscopy. In addition, GIB cases have benefited from advances in diagnostic and therapeutic radiology techniques and equipment, as well as development of more selective and less invasive surgical approaches and of more efficacious, tolerable and safe ulcer-healing drugs. These changes have modified the diagnostic and treatment strategies for patients presenting with non-variceal and variceal upper GIB (UGIB) and those with colonic bleeding.

The major objectives of GIB management are to reduce mortality and the need for major surgery. A secondary objective is to prevent unnecessary hospital admission for patients presenting with minor or self-limited bleeding. This position paper provides recommendations based on current evidence for best practice in the management of acute UGIB and lower GIB (LGIB) in children; management of patients over the age of 18 is not covered by this statement. This statement will be of interest for generalist and specialized pediatricians, as well as general medical professionals who may encounter pediatric patients among their patient population, such as acute physicians, gastroenterologists, gastrointestinal surgeons, endoscopists, pharmacists, anesthesiologists and nurses.

The statement presented herein resulted from a first-phase systematic literature search and review by experts comprising the "Gastro-Ped Bleed Team" of the Italian Society of Pediatric Gastroenterology, Hepatology and Nutrition (SIGENP). The preliminary draft was first circulated among the panel and a subsequent meeting was held, in which a consensus was reached on the points touched, resulting in the final statement that is presented herein. It is important to note that this position paper is not intended to be construed or to serve as a standard of care. Standards of care are determined on the basis of all clinical data available for an individual case and are subject to change as scientific knowledge and technology advances and patterns of care evolve.

DEFINITIONS

UGIB is that originating proximal to the ligament of Treitz, and, in practice, from the esophagus, stomach and duodenum. LGIB is defined as bleeding distal to the ligament of Treitz. Hematemesis (and coffee-ground vomitus) is vomiting of blood from the upper gastrointestinal tract or, occasionally, after swallowing blood from a source in the nasopharynx[1]. Bright red hematemesis usually implies active hemorrhage from

Table 1 Definitions	
Upper gastrointestinal bleeding	GI bleeding originating proximal to the ligament of Treitz (esophagus, stomach and duodenum)
Lower gastrointestinal bleeding	GI bleeding originating distal to the ligament of Treitz (small bowel and colon)
Occult gastrointestinal bleeding	GI bleeding that is not visible to the patient or physician, resulting in either a positive fecal occult blood test or iron-deficiency anemia
Hematemesis	Vomiting of blood or coffee-ground-like material
Hematochezia	Passage of fresh blood per anus
Melena	Passage of black, tarry stools per anus

GI: Gastrointestinal.

Table 2 Causes of upper gastrointestinal bleeding based on age			
	Infants	**2-5 years**	**Older**
Esophagus		Esophagitis	Esophagitis
		Esophageal varices	Mallory-Weiss syndrome
		Mallory-Weiss syndrome	Esophageal varices
Stomach	Gastritis from stress	Gastritis	Dieulafoy lesion
		Gastric ulcer	PHG
		Gastric varices	Hemobilia
Duodenum		Duodenitis	
		Duodenal ulcer	
Variable location	Vitamin K deficiency	Caustic ingestions	Polyps
	Sepsis	Foreign bodies	Crohn's disease
	Trauma (NG tubes)	NSAIDs use	Telangiectasia
	CMPA		Aortoenteric fistula
			Coagulation disorders
			Caustic ingestions
			Foreign bodies
			NSAIDs use

NG: Nasogastric; CMPA: Cow's milk protein allergy; NSAIDs: Non-steroidal anti-inflammatory drugs; PHG: Portal hypertensive gastropathy.

the esophagus, stomach or duodenum. Coffee-ground vomitus refers to the vomiting of black material, which is assumed to be blood. Melena is the passage of black tarry stools, usually due to acute UGIB but occasionally from bleeding within the small bowel or right side of the colon. Hematochezia is the passage of fresh or altered blood *via* rectum, usually due to colonic bleeding[2].

Shock is circulatory insufficiency, resulting in inadequate oxygen delivery that leads to global hypoperfusion and tissue hypoxia; in the context of GIB, shock is most likely to be hypovolemic (due to the inadequate circulating volume resulting from acute blood loss). Varices are abnormal distended veins, most frequently occurring in the esophagus (esophageal varices) and less frequently in the stomach (gastric varices) or other sites (ectopic varices), and usually occurring as a consequence of liver disease; variceal bleeding is characteristically severe and may be life-threatening[3]. Endoscopy is the visualization of the inside of the gastrointestinal tract accomplished by means of videoscope. Examination of the upper gastrointestinal tract (esophagus, stomach and duodenum) is known as gastroscopy or upper gastrointestinal endoscopy. Examination of the colon (large bowel) is referred to as colonoscopy. A list of definitions is provided in Table 1.

UGIB

In children, UGIB is an uncommon but potentially serious, life-threatening clinical condition. From an anatomical perspective, the UGIB tract encompasses the gastrointestinal region from the esophagus to the ligament of Treitz[4]. A study by Cleveland *et al*[5], involving 167 patients, showed the common signs and symptoms of UGIB at presentation to be hematemesis (73%), melena (21%) and coffee-ground emesis (6%); however, patients may also experience epigastric pain, abdominal tenderness or dizziness.

The worldwide mortality rate for UGIB in children can range from 5% to 15%, reflecting the diverse populations that differentially experience conditions associated with UGIB, such as acute variceal hemorrhage[4,6]. The causes of UGIB have been classified based upon variceal bleeding and non-variceal bleeding (Table 2)[7]. Case series reported from Asia and developing countries show a higher incidence of variceal bleeding[8].

The etiology of UGIB can be categorized by age groups, but causative disorders overlap considerably between these[4]. In newborns, the predominant causes include coagulation disorders, such as vitamin K deficiency, cow's milk protein allergy (CMPA)[9], stress-related gastritis, sepsis, and trauma from placement of nasogastric tubes. In infants (1 mo to

History
↓
Signs/symptoms
↓
Evaluate vital signs
Ensure vascular access
Perform baseline tests
NG lavage

Minor bleeding
Stable/well-looking child
↓
Monitor of life-parameters
Monitor hemoglobin

Major/ongoing bleeding
↓
Resuscitation if necessary
Consider EGD within 24 h
after admission

Figure 1 Diagnostic approach of upper gastrointestinal bleeding in infants and children. NG: Nasogastric; EGD: Upper endoscopy.

1 year of age), the most prevalent causes are caustic ingestions, duplication cysts, foreign body ingestion, and medication-induced. In toddlers and young children (1 year to 5 years of age), causes include erosive esophagitis, gastritis, caustic ingestions, peptic ulcer bleeding, varices, and vomiting-induced bleeding (*e.g.*, from a Mallory-Weiss tear). In children and adolescents (ages 5 years to 18 years), bleeding can arise from coagulation disorders, gastritis, Dieulafoy lesions (angiodysplasia), erosive esophagitis, peptic ulcer disease, caustic ingestions, and vomiting-induced bleeding[10].

Crohn's disease is an uncommon cause of UGIB in the pediatric population[11]. Certain foods may create confusion by mimicking the appearance of blood in vomitus [*e.g.*, artificial (red) food-coloring, fruit-flavored drinks, fruit juices, and beets]. All findings suspicious of blood in vomitus should be clinically investigated further[12].

The current diagnostic approach for pediatric UGIB has been mostly extrapolated from studies of adults; the key points are extensive history-taking and examination, laboratory evaluations, and diagnostic procedures[13]. Maternal sources of blood include ingestion of blood during the delivery or from cracked nipples during breastfeeding; infants who ingest maternal blood may present with hematemesis or melena[4]. Historical information includes the presence of abdominal pain, coffee-ground-like emesis, dysphagia, black and tarry stools, bright red blood *via* rectum, hematemesis, and chest pain. In addition, drug use should be elicited, especially any previous use of non-steroidal anti-inflammatory drugs (NSAIDs), aspirin and/or corticosteroids[14]. The physician should also ascertain a history of peptic ulcer bleeding or surgery, as well as any previous episodes of UGIB and previous history of umbilical catheterization[15].

In newborns with suspected UGIB, an alkali denaturation test (*i.e.*, the Apt-Downey test) can differentiate neonatal blood from maternal blood. Gastric lavage *via* nasogastric tube can improve the accuracy of endoscopy[16,17]. Upper endoscopy is the test of choice for evaluating hematemesis. The goals of

endoscopy in UGIB are to identify the site of bleeding and to facilitate initiation of an appropriate therapeutic approach when indicated[5].

A flowchart of the diagnostic approach of UGIB is provided in Figure 1. In summary, UGIB refers to bleeding above the ligament of Treitz and the priority of achieving a differential diagnosis addresses both the clinical presentation and the age of the patient.

LGIB

LGIB in children is a common clinical problem; indeed, it is reportedly the presenting complaint for approximately 0.3% of children in the emergency department[18]. In most cases, the bleeding is self-limiting, with the majority (80%) of LGIB cases in the emergency department undergoing routine discharge[19]. However, conditions such as Meckel's diverticulum, melena by variceal hemorrhages, acute intestinal obstruction or severe attack of ulcerative colitis often present with life-threatening GIB.

The etiology of LGIB is very different between children and adults, and its incidence is age-dependent. The main causes of LGIB in adults are colorectal cancer, colorectal polyps, anorectal disease and inflammatory bowel diseases (IBDs); in children, colorectal polyps, chronic colitis and perianal lesions are the main causes[20]. In infants, allergic colitis and anorectal fissures represent the most common causes, while infectious enteritis and anorectal fissures are the most common causes in older children[21] (Table 3). In young infants (< 1 year of age), the most likely cause of hematochezia with or (more often) without diarrhea is the so-called allergic colitis; although CMPA is usually suspected, the etiology is often uncertain. In breastfed infants, without anemia, who are growing well, hematochezia is usually a benign self-limiting disorder, and a maternal milk-free diet is not necessarily indicated[22].

A valid approach to investigate the causes of LGIB is to classify it according to the child's age, general appearance (ill or well), bleeding rate, and stool characteristics[23]. Meckel's diverticulum should strongly be suspected, at any age, if bleeding is massive and accompanied by both bright and dark red stools. In ill infants, ischemic/surgical causes, such as mid-gut volvulus and intussusception, should be suspected. In older children, other serious medical causes, such as severe attack of ulcerative colitis, Henoch-Schonlein purpura or hemolytic-uremic syndrome, might be the cause of bleeding[24].

In cases of severe LGIB, especially when melena is present or the patient is hemodynamically unstable, the source of bleeding may include the upper gastrointestinal region[25]. In cases with bloody diarrhea that is persistent (> 7 d), recurrent or severe (> 7 bloody stools/d), the child should be seen by a pediatric gastroenterologist with indication to endoscopy. Rectal bleeding with normal stool pattern is suggestive of

Table 3 Causes of lower gastrointestinal bleeding based on age

Infants	2-5 years	Older
Non-specific colitis	Polyps	Anal fissure
Anal fissure	Anal fissure	Infectious Enterocolitis
Milk allergy	Infectious enterocolitis	Polyps
Duplication of bowel	Intussusception	Inflammatory bowel disease
Volvulus	Meckel's diverticulum	Lymphonodular hyperplasia
Hirschsprung's disease	Henoch-Schonlein purpura	Henoch-Schonlein purpura
Necrotizing enterocolitis	Hemolytic-uremic syndrome	Angiodysplasia
Bleeding diathesis	Lymphonodular hyperplasia	Hemolytic-uremic syndrome
	Angiodysplasia	Bleeding diathesis

the presence of juvenile polyp, nodular lymphoid hyperplasia or eosinophilic colitis, as well as IBD and, rarely, vascular malformations.

In a retrospective cross-sectional study, de Ridder *et al*[26] reported data of 137 children undergoing colonoscopy for rectal bleeding (mean duration of 28 wk). The diagnosis rate for first colonoscopy (IBD and colonic polyps) was 80%. No abnormalities were found in 20.4% of the patients, either by colonoscopy or histopathology, and the final diagnosis for these cases was self-limited GIB.

Constipation is commonly associated with the presence of anal fissure and pain on defecation. Visual inspection of the perianal area as well as digital rectal examination are mandatory to detect the possibility of anal fissure, streptococcal cryptitis or rectal polyp. Endoscopy within 6 h after the first evaluation is rarely needed; in cases of severe colitis, a rapid diagnosis and histological evaluation may necessitate a procto-sigmoidoscopy without bowel cleansing[23].

In conclusion, the main priority for the physician in evaluating a patient with LGIB is to identify those patients in whom bleeding is secondary to intestinal obstruction or surgical causes. An algorithm of the diagnostic approach of LGIB is presented in Figure 2.

PRIMARY CLINICAL MANAGEMENT

Stabilization of general conditions should precede any instrumental investigation (usually endoscopy) for children with GIB. The best clinical indicator of blood loss is orthostatic changes in heart rate and blood pressure; defined as an increase in pulse rate by 20 beats/min or a decrease in systolic blood pressure of 10 mmHg or more upon moving the patient from supine to sitting position. For any other emergency situation, the first priority should be to assess the airways, breathing and circulation of the patient[5].

The most important aspect of the initial GIB evaluation is to determine the degree and rapidity of blood loss, and any risk factors (*i.e.*, coagulopathy, sepsis, trauma) or associated signs (*i.e.*, purpuric lesions, hepatosplenomegaly, jaundice, cutaneous hemangiomas, eczema)[7]. In the case of a child with no clinical impairment, it is sufficient to ensure vascular access and perform baseline tests (*i.e.*, blood

count and group, liver and kidney function, blood coagulation) as well as a pre-anesthesia examination. For cases of UGIB, nasogastric aspiration and saline lavage are indicated to confirm the presence of intragastric blood[27], to determine the rate of gross bleeding, to check for ongoing or recurrent bleeding, to clear the gastric field for subsequent endoscopic visualization, to prevent aspiration of gastric contents and to prevent hepatic encephalopathy in patients with cirrhosis. Parenteral vitamin K (1-2 mg/dose) should be administered empirically to infants, even when results of coagulation are pending. The finding of coagulopathy with an international normalized ratio > 1.5 or abnormal partial thromboplastin time should be corrected by administration of fresh frozen plasma (10 mL/kg initially); cryoprecipitate administration may be tried in the presence of severe coagulopathy, especially if the volume of fluid has to be restricted.

In conclusion, supportive measures with stabilization of hemodynamic status, correction of any coagulation or platelet abnormalities are necessary before diagnostic procedures are undertaken.

OBSCURE GASTROINTESTINAL BLEEDING

Obscure gastrointestinal bleeding (OGIB) is defined as bleeding of unknown origin that persists or recurs after negative findings on initial evaluation using bidirectional endoscopy[28]. It can be classified as overt or occult, based on presence or absence of clinically-evident bleeding. Obscure-occult bleeding is generally determined by a positive fecal occult blood test result and/or iron-deficiency anemia[29]. Chronic occult GIB may occur anywhere in the gastrointestinal tract-from the oral cavity to the anorectum. In most cases, the site is identified by upper endoscopy and ileocolonoscopy. Causes depend on age of presentation (*i.e.*, infants, children, adolescents) and location of gastrointestinal tract bleeding. OGIB may be active, as with melena, hematochezia or hematemesis, or it may be inactive, showing intermittent bleeding.

Similar to data from adult patients[30], OGIB accounts for 5% of all pediatric cases of GIB, including both acute overt and chronic occult types of blood loss. In approximately 75% of OGIB cases, the lesions are

Figure 2 Diagnostic approach of lower gastrointestinal bleeding in infants and children. UGIB: Upper gastrointestinal bleeding; GI: Gastrointestinal.

detected in the small bowel (mid-GIB) distal to Vater's papilla and reaching as far as the terminal ileum. The source of mid-GIB is related to age, with children showing a greater likeliness of having small intestinal polyps, Meckel's diverticulum, vascular malformations, Crohn's disease, anastomotic ulcers and intestinal duplications[31].

Diagnostic approaches for OGIB, after negative endoscopy and colonoscopy, can require small bowel endoscopic investigation by video capsule endoscopy (VCE). Balloon-assisted enteroscopy (BAE), with single or double-balloon enteroscopy (DBE), is the second-line technique, having the advantage of therapeutic as well as diagnostic properties. The diagnostic yield is very good (70%-100%), and is significantly higher when BAE is performed after a positive VCE. In a recent pediatric study of 117 children treated with DBE (total of 257 procedures), Yokoyama *et al*[32] found the greatest indication to be OGIB (61.9%) and a low incidence of complications (5.4%), regardless of the associated therapeutic procedures.

Intraoperative enteroscopy, involving insertion of an endoscope through an incision in the mid-small intestine, is currently reserved as a last option, or if small intestinal endoscopy cannot be successfully performed. Laparoscopy and exploratory laparotomy remain important alternative diagnostic tools, for when other measures cannot identify a bleeding source in selected patients[33].

In conclusion, it is reasonable to perform both upper endoscopy and colonoscopy in a patient with OGIB (overt or occult) to identify pathological processes that can explain symptoms or iron-deficiency anemia.

IMAGING STUDIES

Radiological imaging has played an increasingly important role in the diagnosis and management of GIB over the past 30 years. Magnetic resonance imaging has emerged as key pediatric imaging modality, preferred for its lack of ionizing radiation; it is particularly suitable for studying small bowel pathologies, and is currently the first-line modality for such. The exact source of GIB may be localized by means of nuclear scintigraphy, as well as selective angiography. In general, examination by imaging is most commonly requested after negative endoscopy results, or for indeterminate causes or locations of bleeding.

The role of interventional radiology has also in-creased over the past years for the treatment of gastrointestinal hemorrhage, especially in very ill patients who are poor surgical candidates. Nuclear scintigraphy is a sensitive method for detecting GIB (used at a rate of 0.1 mL/min) and the method is more sensitive, but less specific, than angiography[34]. Although arteriographic diagnosis and therapy have been reviewed extensively in the literature describing adult cases, few experiences in children have been reported. In one published pediatric study, which involved 27 children, arteriography had an overall

positive diagnostic rate of 64% and a false-negative rate of 36%. In AGIB, the diagnosis was correct in 71% and falsely negative in 29%, while in chronic or recurrent GIB, it was correct in 55% and falsely negative in 45%[35].

The only angiographic sign that is 100% diagnostic for AGIB is contrast extravasation in the intestinal lumen. However, other angiographic signs can be useful in evaluation of some of the more common pediatric pathologies that cause GIB. One of the main advantages of angiographic diagnosis of GIB is the ability to perform transcatheter treatment after the bleeding site has been located. The two main transcatheter therapies are intraarterial vasopressin infusion and embolization. The most serious complication related to the technique is bowel infarction. Hongsakul *et al*[36] reported the risk factors as being failure to achieve hemostasis, hemoglobin concentration, coagulopathy, UGIB, contrast extravasation, and > 1 embolized vessel.

THERAPY

The pharmacological treatment approach to UGIB and LGIB currently includes 3 classes of drugs: acid suppression drugs, vasoactive drugs, and non-selective β-blockers (NSBBs).

Acid suppression drugs

The proton pump inhibitors (PPIs) have shown benefit in treatment of ulcer-bleeding or UGIB patients and to be superior to the H2-antagonist. There are no differences between the 5 available PPIs: esomeprazole, lansoprazole, omeprazole, pantoprazole and rabeprazole. The recommended administration route is intravenous, as a 1-h infusion at a dose of 1-3 mg/kg to maintain 24-h gastric pH > 6 in active bleeding.

Dosing in children has been extrapolated from the adult literature; although, the available data suggest faster drug clearance and significant interindividual variability in pediatric patients. A meta-analysis of an adult population showed that PPI treatment, with or without endoscopic therapy, compared with placebo or an H2 receptor antagonist, reduced the risk of rebleeding and the need for surgery, but did not affect mortality[37]. Several studies showed that the rate of rebleeding, requirement of blood transfusion, and duration of hospital stay were less in PPI-treated patients[38]. Moreover, PPIs were shown to be effective in the treatment of GIB in children that had developed due to NSAIDs administration[39].

Vasoactive drugs

Vasoactive treatment should be administered as soon as possible when portal hypertension is the suspected cause of GIB. These medications are reported to stop bleeding in 75%-80% of cases[40]. Three vasoactive drugs (terlipressin, somatostatin, and octeotride) control variceal bleeding by reducing portal blood flow

and portal pressure[41,42].

Terlipressin has an important systemic vasoconstrictor effect, which is more noticeable on the splanchnic arteries, causing an increase in systemic vascular resistance and arterial pressure as well as a significant (approximately 20%) and sustained (up to 4 h) decrease in portal vein pressure and flux[43,44]. Several randomized trials and meta-analyses have suggested that terlipressin provides a survival benefit, compared to placebo, to patients with variceal bleeding[45,46]. In adults, terlipressin can be considered as the first choice, with somatostatin or octreotide as the second choice. However, many studies that have compared the clinical efficacies of different types of vasoactive drugs, each administered as monotherapy, have found no differences in mortality rates. Studies in pediatric populations have yet to show the potential superiority of terlipressin over other vasoactive agents; however, Erkek *et al*[47] reported a single-child experience of its use for successful management of severe non-variceal UGIB. Studies have shown that terlipressin has a very good safety profile, compared to vasopressin, although adverse events such as hyponatremia and seizure have been described in children (thus, necessitating monitoring of sodium levels)[48].

Octeotride is a synthetic derivative of somatostatin. It produces selective splanchnic vasoconstriction and decreases portal inflow, thereby indirectly reducing variceal blood flow. In children, intravenously-administered octreotide is effective in decreasing AGIB. Studies of pediatric populations have demonstrated octeotride to be effective at dosages of 2-5 mcg/kg per hour administered by continuous infusion[49], and that initiation with a 1-h bolus may be needed, and to continue the infusion for at least 5 d in patients at risk of rebleeding seems an appropriate and rational choice[50]. However, there is limited evidence regarding the efficacy and safety of octreotide for chronic GIB in children.

NSBBs

NSBBs, such as propranolol, nadolol and carvedilol, have been widely studied in adults with portal hypertension and have been shown to reduce portal pressures by decreasing cardiac output and vasoconstricting the splanchnic vessels *via* blockade of ß-1 and ß-2 receptors; moreover, carvedilol seems to be more effective than the traditional NSBBs in reducing hepatic venous pressure gradient[51].

The pediatric experience described in the literature is limited to primary and secondary prophylaxis of variceal bleeding. No formal randomized controlled trials evaluating safety and efficacy of NSBBs in children have been published. In addition, appropriate dosing of β-blockers has not been established (currently ranging from 2 mg/kg per day to 8 mg/kg per day) and it is unknown whether targeting a change in heart rate of 25% is effective in reducing portal pressures

and the related risk of variceal bleeding in children. Pediatric clinical data supporting use of NSBBs in preventing a first variceal bleed are also limited, likely because there is no indication to use β-blockers to prevent the formation of varices. NSBBs or endoscopic band ligation are recommended, according to the Baveno VI Consensus Workshop, for the prevention of first variceal bleeding of medium or large varices[52].

THERAPEUTIC ENDOSCOPY

The aim of therapeutic endoscopy is to stop bleeding and prevent rebleeding. Endoscopy-based diagnostic and therapeutic management is a goal of physicians treating GIB and should be performed when the patient has been stabilized, and preferably within 24 h of bleeding presentation[4,53]. Several techniques, including injection therapy, ablative therapy and mechanical therapy, have been recommended for AGIB, each of these depending on the bleeding charac-teristics, such as active, oozing or no visible bleeding vessel. In addition, each of these techniques have been adapted to upper and lower endoscopy, as well as to deep endoscopy.

Common therapies for GIB in adults and children include injection therapy with dilute epinephrine and sclerosants, ablation therapy (contact methods, such as thermocoagulation heater probe and elec-trocoagulation; non-contact methods, such as argon plasma coagulation) and mechanical therapy (such as with hemoclips and band ligation)[54,55]. Epinephrine injection arrests about 80% of non-variceal bleeding. Multiple adult meta-analyses have demonstrated that combination therapy (epinephrine injection in conjunction with clipping or ablation therapy) is superior to epinephrine alone in reducing the risk of rebleeding to about 10%[56,57]. The endoscopy laser and argon plasma coagulation methods can be effective therapies for GIB due to vascular abnormalities; indeed, using these, most bleeding from Mallory-Weiss tears stops spontaneously. For Dieulafoy lesions, which are very rare in children, endoscopy therapy is the first choice, using clipping, electrocautery, sclerosant injection, banding methods or laser. Endoclips are currently the preferred mechanical therapy for non-variceal GIB.

In management of acute variceal bleeding, endo-scopic variceal ligation (EVL) is the treatment of choice; a meta-analysis confirmed the superiority of EVL compared with endoscopic sclerotherapy for major outcomes, such as recurrent bleeding, ulceration and stricture[58-60]. For therapeutic colonoscopy, adequate fasting time and appropriate bowel preparation is recommended to facilitate the visualization of mucosal lesions.

CONCLUSION

The diagnostic approach for GIB should include exten-sive history-taking and examination including laboratory evaluations and application of the available and most appropriate diagnostic procedures. Endoscopy is the method of choice for evaluating UGIB and LGIB, after stabilization and resuscitation, and within 24 h of presentation. The goals of endoscopy are to identify the site and etiology of the GIB, as well as to facilitate adequate treatment. Visual inspection of the perianal area and digital rectal examination should always be considered if a bright red blood coating is present on normal or hard stool.

In children, persistent or recurrent iron-deficiency anemia could be considered as a sign of OGIB, for which VCE is the first-line endoscopic investigation. Three vasoactive drugs (terlipressin, somatostatin, and octreotide) play a role in the control of variceal bleeding and all act by reducing portal blood flow and portal pressure. Endoscopy has a therapeutic role for polyps, ulcers, erosions, blue nevi, angiodysplasia, varices, strictures and scalloping.

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ORIGINAL ARTICLE

Basic Study

TGR5 expression in benign, preneoplastic and neoplastic lesions of Barrett's esophagus: Case series and findings

Shivali Marketkar, Dan Li, Dongfang Yang, Weibiao Cao

Shivali Marketkar, Dongfang Yang, Weibiao Cao, Department of Pathology, Rhode Island Hospital and Warren Alpert Medical School of Brown University, Providence, RI 02903, United States

Dan Li, Department of Medicine, Rhode Island Hospital and Warren Alpert Medical School of Brown University, Providence, RI 02903, United States

Abstract

AIM
To examined the bile acid receptor TGR5 expression in squamous mucosa, Barrett's mucosa, dysplasia and esophageal adenocarcinoma (EA).

METHODS
Slides were stained with TGR5 antibody. The staining intensity was scored as 1+, 2+ and 3+. The extent of staining (percentage of cells staining) was scored as follows: 1+, 1%-10%, 2+, 11%-50%, 3+, 51%-100%. A combined score of intensity and extent was calculated and categorized as negative, weak, moderate and strong staining. TGR5 mRNA was measured by real time PCR.

RESULTS
We found that levels of TGR5 mRNA were significantly increased in Barrett's dysplastic cell line CP-D and EA cell line SK-GT-4, when compared with Barrett's cell line CP-A. Moderate to strong TGR5 staining was significantly higher in high-grade dysplasia and EA cases than in Barrett's esophagus (BE) or in low-grade dysplasia. Moderate to strong staining was slightly higher in low-grade dysplasia than in BE mucosa, but there is no statistical significance. TGR5 staining had no significant difference between high-grade dysplasia and EA. In addition, TGR5 staining intensity was not associated with the clinical stage, the pathological stage and the status of lymph node metastasis.

CONCLUSION

We conclude that TGR5 immunostaining was much stronger in high-grade dysplasia and EA than in BE mucosa or low-grade dysplasia and that its staining intensity was not associated with the clinical stage, the pathological stage and the status of lymph node metastasis. TGR5 might be a potential marker for the progression from BE to high-grade dysplasia and EA.

Key words: TGR5; Esophageal adenocarcinoma; Bile acid

Core tip: The expression of a bile acid receptor TGR5 at moderate to strong intensity was significantly higher in high-grade dysplasia and esophageal adenocarcinoma (EA) cases than in BE or in low-grade dysplasia, suggesting that TGR5 may play an important role in the progression from Barrett's esophagus to high-grade dysplasia and EA. TGR5 might be a potential marker for this progression. TGR5 staining intensity was not associated with the clinical stage, the pathological stage and the status of lymph node metastasis, indicating that TGR5 may not be a marker for the prognosis of EA.

INTRODUCTION

Esophageal adenocarcinoma (EA) is a deadly cancer with an increasing incidence[1-3]. It has a poor prognosis with a median survival of less than one year[4,5] and a five-year survival rate of 12.5%-20%[6,7]. Gastro-esophageal reflux disease (GERD) complicated by Barrett's esophagus (BE)[8-10] is a major risk factor for EA. BE carries nearly a 30-125-fold increased risk for the development of EA, with best estimates of cancer incidence of 0.12%-0.8% per year[2,10-14]. However, mechanisms of the progression from BE (intestinal metaplasia) to EA are not fully understood.

Bile acids may play an important role in the progression from BE to EA[15,16] since (1) exposure of the lower esophagus to duodenal juice in animal models leads to EA[17-19]; (2) bile acid causes the production of reactive oxygen species and DNA damage in a non-neoplastic Barrett's cell line BAR-T[20]; (3) bile salts may activate the mitogen-activated protein kinase and NF-κB pathways[21,22] thereby enhancing cell proliferation and preventing cell apoptosis; and (4) Barrett's cells become tumorigenic after long-term exposure to acid and bile acid *in vitro*[23].

A G protein-coupled receptor TGR5 has been shown to mediate bile acids' effects as a cell-surface receptor[24]. The TGR5 receptor is abundantly expressed in human monocytes and macrophages, and participates in the regulation of cell metabolism[25,26]. Primary bile acids (cholic acid, taurocholic acid and glycocholic acid) and secondary bile acids (deoxycholic acid, taurodeoxycholic acid, glycodeoxycholic acid and taurolithocholic acid) have been shown to bind to TGR5 receptors[24]. Primary bile acids are much weaker at inducing cyclic AMP production *via* activation of TGR5 than secondary bile acids. Deoxycholic acid, taurodeoxycholic acid, and glycodeoxycholic acid have similar strengths at inducing cyclic AMP production[24]. TGR5 has been reported to be expressed in human gastric cancers, to promote epithelial-mesenchymal transition in gastric cancer cell lines[27], and to mediate bile acid-induced cholangiocyte proliferation *in vivo* and *in vitro*[28].

We have previously shown that TGR5 receptors are present in EA cells and that TGR5 receptors mediate bile acid-induced increase in cell proliferation[29]. The expression of TGR5 in EA tissues is not well understood. In this study, we examined the bile acid receptor TGR5 expression in squamous mucosa, Barrett's mucosa, dysplasia and EA by immunohistochemistry. We found that TGR5 immunostaining was much stronger in high grade and EA than in BE mucosa or low-grade dysplasia.

MATERIALS AND METHODS

Patients and specimens

Archival cases of BE, low grade dysplasia, high grade dysplasia and EA from 34 patients (18 cases with squamous mucosa, 15 cases with BE, 8 cases with low grade dysplasia, 9 cases with high grade dysplasia and 16 cases with adenocarcinoma) were collected between the years of 2005 and 2013 from the archives of the Department of Pathology at the Rhode Island Hospital (RIH). BE was made based on the histological finding of intestinal metaplasia and the endoscopic finding of column-type mucosa. Patients with previous history of chemoradiation therapy were excluded from the study. Stage was defined according to American Joint Committee on Cancer criteria[30]. This study was approved by the Institutional Review Board at the RIH. All tissue samples were formalin-fixed and paraffin-embedded. The corresponding hematoxylin-eosin slides were reviewed for confirmation of diagnosis and adequacy of material by Dr. Cao W. The detailed clinicopathological features of the study population are given in Table 1.

Immunohistochemistry

Immunohistochemistry for TGR5 was performed

Figure 1 TGR5 mRNA levels in different cell lines. A: TGR5 mRNA level was significantly higher in Barrett's cell line CP-A than in squamous cell line HET-1A; B: Levels of TGR5 mRNA were significantly increased in Barrett's dysplastic cell line CP-D and EA cell line SK-GT-4, when compared with CP-A cells. In addition, TGR5 mRNA was significantly higher in SK-GT-4 cells than in CP-D cells. These data suggest that TGR5 may play an important role in the progression from BE to EA. $n = 3$, t test, [a]$P < 0.02$; ANOVA, [b]$P < 0.01$ *vs* CP-A.

Table 1 Clinical features of the study group	
Number	**34**
Age (yr)	
Range	48-91
Median	65.5
Sex, *n* (%)	
Male	28 (82.4)
Female	6 (17.6)

on 4-μm paraffin sections. Slides were stained with TGR5 antibody (1:1000, Sigma-Aldrich Co., St. Louis, MO) using the DAKO Envision + Dual Link System and the DAKO Liquid 3,3'-diaminobenzidine (DAB+) Substrate Chromagen System (DAKO North America, Inc., Carpinteria, CA). Bile ducts from liver tissue were used as positive controls. Negative controls included replacement of the primary antibody with non-reacting antibodies of the same species. The specificity of TGR5 antibody has been confirmed by Western Blot analysis in our lab[31].

Immunohistochemistry assessment

Cancers and non-neoplastic mucosa that displayed a strong, well-localized, strong staining pattern for TGR5 were scored as +3, moderately intense staining as +2, and weak staining as +1. The extent of staining (percentage of cells staining) was scored as follows: 1+ 1%-10%, 2+ 11%-50%, 3+ 51%-100%. A combined score of intensity and extent was calculated and categorized as follows: weak staining 1-2, moderate staining 3-4, strong staining 5-6. All sections were scored independently by WC and SM without knowledge of the clinicopathologic features or clinical outcome.

Cell culture

Cell culture was similar to those we described previously[29,32,33]. Briefly, human esophageal squamous HET-1A cells were purchased from ATCC, Manassas, VA in 2011 and cultured in the bronchial epithelial cell medium (BEGM BulletKit, Cambrex, East Rutherford, NJ). Human Barrett's cell line CP-A and Barrett's dysplastic cell line CP-D were bought from ATCC (Manassas, VA) and cultured in Barrett's medium containing keratinocyte medium-2 (Cambrex, Rockland, ME), 1.8 mmol/L CaCl$_2$, 5% fetal bovine serum, 400 ng/mL hydrocortisone, 20 ng/ml epidermal growth factor, 0.1 nmol/L cholera toxin, 20 μg/mL adenine, 5 μg/mL insulin, 70 μg/mL bovine pituitary extract, and antibiotics. EA cell line SK-GT-4 was purchased from Sigma and cultured in the Barrett's medium.

Statistical analysis

For immunohistochemical data, statistical differences were determined by χ^2 test. For TGR5 mRNA data, data was expressed as mean ± SE. Statistical differences between two groups were determined by Student's t test. Differences among multiple groups were tested using analysis of variance and checked for significance using Fisher's protected least significant difference test. P values of 0.05 or less were considered statistically significant.

RESULTS

Expression of TGR5 in different cell lines

We have previously shown that the levels of TGR5 mRNA and protein expression are significantly increased in Barrett's mucosal tissue, when compared with normal esophageal mucosa. TGR5 mRNA and protein levels are significantly higher in EA tissue than in normal esophageal mucosa or in Barrett's mucosa[29]. Consistent with our previous findings, TGR5 mRNA level was significantly higher in Barrett's cell line CP-A than in squamous cell line HET-1A (Figure 1A). Levels of TGR5 mRNA were significantly increased in Barrett's dysplastic cell line CP-D and EA cell line SK-GT-4, when compared with CP-A cells. In addition, TGR5 mRNA was significantly higher in SK-GT-4 cells than in CP-D cells (Figure 1B). These data suggest that TGR5 may play an important role in the progression

Squamous mucosa	Barrett's mucosa	Low-grade dysplasia	High-grade dysplasia	EA

Figure 2 Representative images of squamous mucosa, Barrett's esophagus mucosa, low-grade dysplasia, high-grade dysplasia and esophageal adenocarcinoma. Upper panel: Hematoxylin-eosin staining stain, Lower panel: TGR5 immunostaining, magnification × 200. EA: Esophageal adenocarcinoma.

Table 2 TGR5 expression in squamous mucosa, Barrett's esophagus, low-grade dysplasia, high-grade dysplasia and esophageal adenocarcinoma *n* (%)

	Negative	Weak	Moderate	Strong	
Squamous mucosa, *n* = 18	13 (72)	5 (28)	0	0	
Barrett's esophagus, *n* = 15	1 (6.7)	12 (80)	2 (13.3)	0	*P* < 0.001, compared with squamous mucosa
Low grade dysplasia, *n* = 8	0	5 (62.5)	2 (25)	1 (12.5)	*P* > 0.05, compared with BE
High grade dysplasia, *n* = 9	1 (11.1)	0	2 (22.2)	6 (66.7)	*P* < 0.001, compared with BE
					P < 0.05, compared with low grade dysplasia
EA, *n* = 16	0	0	7 (43.7)	9 (56.3)	*P* < 0.001, compared with BE
					P < 0.01, compared with low grade dysplasia

EA: Esophageal adenocarcinoma; BE: Barrett's esophagus.

from BE to EA.

Expression of TGR5 in squamous mucosa, BE mucosa, low grade dysplasia and EA

We have previously shown that TGR5 antibody is relatively specific since only one band was detectable by using TGR5 antibody[31]. The expression of TGR5 in squamous mucosa, BE mucosa, low-grade dysplasia and EA was further examined by immunohistochemistry. We found that 93.3% Barrett's mucosa showed weak to moderate TGR5 staining, which was significantly higher than squamous mucosa (27.8%) (Figure 2 and Table 2). Moderate to strong TGR5 staining was significantly higher in EA cases (100%) than in BE (13.3%, *P* < 0.001) or in low-grade dysplasia (37.5%, *P* < 0.01) (Figure 2 and Table 2). Similarly, moderate to strong TGR5 staining was significantly higher in high-grade dysplasia cases (88.9%) than in BE (13.3%, *P* < 0.001) or in low-grade dysplasia (37.5%, *P* < 0.05) (Figure 2 and Table 2). Moderate to strong staining was slightly higher in low-grade dysplasia (37.5%) than in BE mucosa (13.3%), but there is no statistical significance. TGR5 staining had no significant difference between

high-grade dysplasia and EA.

Expression of TGR5 in different stages of EA

Next, we compared the expression of TGR5 in different clinical and pathological stages of EA tissues. We found that 100% stage Ⅲ and Ⅳ cases showed moderate to strong staining, which was the same as stage Ⅰ and Ⅱ cases (100%; Table 3), indicating that the degree of TGR5 expression might not be associated with clinical stages. T3 and T4 cancers had 63.6% strong TGR5 staining, which was not different from T1 and T2 cancers (*P* > 0.05; Table 4). In addition, TGR5 expression had no significant difference between tumors with and without lymph node metastasis (Table 5).

DISCUSSION

GERD complicated by BE[8-10] is a major risk factor for EA. There is a progression from BE, to dysplasia and to EA. The mechanisms of progression from BE to EA are not fully understood. Many genetic and epigenetic alterations, chromosomal gains and losses, and hypermethylation of gene promoters may be involved

Table 3 TGR5 expression in different clinical stages of esophageal adenocarcinoma n (%)

Clinical stage	Negative	Weak	Moderate	Strong	
I and II, *n* = 9	0	0	4 (44.4)	5 (55.6)	
III and IV, *n* = 7	0	0	3 (42.9)	4 (57.1)	*P* > 0.05

Table 4 TGR5 expression in different pathological stages of esophageal adenocarcinoma n (%)

Pathological stage	Negative	Weak	Moderate	Strong	
T1 and T2	0	0	2 (40)	3 (60)	
T3 and T4	0	0	4 (36.4)	7 (63.6)	*P* > 0.05

Table 5 TGR5 expression in patients with or without lymph node metastasis of esophageal adenocarcinoma n (%)

Lymph node metastasis	Negative	Weak	Moderate	Strong	
Positive	0	0	2 (33.3)	4 (66.7)	*P* > 0.05
Negative	0	0	5 (50)	5 (50)	

in this progression[13,34]. Bile acids have also been indicated to be involved in this progression[15,16].

We have previously shown that TGR5 receptors are present in EA cells and that TGR5 mediates bile-acid-induced increase in cell proliferation, suggesting that TGR5 may be important in the development of EA[29]. We have also reported that moderate to strong TGR5 staining is associated with decreased patient survival in all gastric adenocarcinomas, suggesting that TGR5 may be a negative prognostic marker in gastric cancer[31]. The histological expression of TGR5 in EA has not been reported.

In this study, we examined TGR5 mRNA expression in different cell lines and found that Barrett's cells CP-A had significantly higher levels of TGR5 mRNA than squamous cells HET-1A. Moreover, Barrett's dysplastic cells CP-D had significantly higher levels of TGR5 mRNA than CP-A cells. EA cells SK-GT-4 had much higher levels of TGR5 mRNA than CP-A or CP-D. These data suggest that TGR5 may be involved in the progression from BE to EA.

Next, we examined the TGR5 expression in squamous mucosa, Barrett's mucosa, dysplasia and EA. We found that moderate to strong TGR5 staining was significantly higher in high-grade dysplasia and EA cases than in BE or in low-grade dysplasia. Moderate to strong staining was slightly higher in low-grade dysplasia than in BE mucosa, but there is no statistical significance. TGR5 staining had no significant difference between high-grade dysplasia and EA. These data further support our above results that TGR5 may play an important tant role in the progression from BE to EA. How TGR5 is involved in this progression is not clear. Recently we found that TGR5 mediates

bile acid-induced activation of cyclic AMP response element binding protein (CREB) and NADPH oxidase NOX5-S, which produces reactive oxygen species and causes DNA damage[35]. TGR5 is present in human gastric cancers and promotes epithelial-mesenchymal transition in gastric cancer cell lines[27]. It also mediates bile acid-induced cholangiocyte proliferation *in vivo* and *in vitro*[28]. Therefore, we speculate that in Barrett's patients bile acids may activate TGR5 receptors, which activate CREB and NOX5-S. NOX5-S-derived ROS may increase cell proliferation and cause DNA damage, thereby contributing to the progression from BE to EA. TGR5 might be a potential marker for the progression from BE to high-grade dysplasia and EA. In addition, we compared the expression of TGR5 in different clinical stages of EA tissues. We found that 100% stage III and IV cases showed moderate to strong staining, which was the same as stage I and II cases, indicating that the degree of TGR5 expression might not be associated with clinical stages. Moreover, TGR5 expression had no significant difference between tumors with and without lymph node metastasis, indicating that the degree of TGR5 expression may not be related to the status of lymph node metastasis. These data suggest that TGR5 may not be a marker for the prognosis of EA.

In conclusion, TGR5 immunostaining was much stronger in high-grade dysplasia and EA than in BE mucosa or low-grade dysplasia. Its staining intensity was not associated with the clinical stage, pathological stage and the status of lymph node metastasis.

ACKNOWLEDGMENTS

COMMENTS

Background
Gastroesophageal reflux disease complicated by Barrett's esophagus (BE) is a major risk factor for esophageal adenocarcinoma (EA). However, mechanisms of the progression from BE (intestinal metaplasia) to EA are not fully understood. Recent data suggest that bile acids may play an important role in the progression from BE to EA. We have previously shown that TGR5 receptors are present in EA cells and that TGR5 receptors mediate bile acid-induced increase in cell proliferation. The expression of TGR5 in EA tissues is not well understood. In this study, we examined the bile acid receptor TGR5 expression in squamous mucosa, Barrett's mucosa, dysplasia and EA by immunohistochemistry.

Research frontiers
Bile acids may contribute to the progression from BE (intestinal metaplasia) to EA. The role of a bile acid receptor TGR5 in this progression is not clear.

Innovations and breakthroughs
The expression of a bile acid receptor TGR5 at moderate to strong intensity was significantly higher in high-grade dysplasia and EA cases than in BE or in low-grade dysplasia, suggesting that TGR5 may play an important role in the progression from BE to high-grade dysplasia and EA. TGR5 might be a potential marker for this progression. However, TGR5 may not be a marker for the prognosis of EA.

Applications
TGR5 might be a potential marker for the progression from BE to EA.

Terminology
TGR5 is a G protein-coupled bile acid receptor.

Peer-review
This article is of tremendous importance in highlighting the different expression of TGR5 among different stages of EA.

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Basic Study

Autoantibody profiles in autoimmune hepatitis and chronic hepatitis C identifies similarities in patients with severe disease

Kawa Amin, Aram H Rasool, Ali Hattem, Taha AM Al-Karboly, Taher E Taher, Jonas Bystrom

Kawa Amin, Department of Medical Science, Respiratory Medicine and Allergology, Clinical Chemistry and Asthma Research Centre, Uppsala University and University Hospital, SE-751 85 Uppsala, Sweden

Kawa Amin, Department of Microbiology/Immunology, School of Medicine, University of Sulaimani, Sulaimani 334, Iraq

Aram H Rasool, Laboratory Department, General Hospital of Derbandixan, Derbandixan 332, Iraq

Ali Hattem, Department of Community Health, Sulaimani Polytechnic University, Sulaimani 334, Iraq

Taha AM Al-Karboly, Department of Medicine, School of Medicine, Faculty of Medical Sciences, University of Sulaimani; Kurdistan Center for Gastroenterology and Hepatology, Sulaimani 334, Iraq

Taher E Taher, Jonas Bystrom, Experimental Medicine and Rheumatology, William Harvey Research Institute, Barts and the London, Queen Mary, University of London, Charterhouse Square, EC1M 6BQ London, United Kingdom

Abstract

AIM
To determine how the auto-antibodies (Abs) profiles overlap in chronic hepatitis C infection (CHC) and autoimmune hepatitis (AIH) and correlate to liver disease.

METHODS
Levels of antinuclear Ab, smooth muscle antibody (SMA) and liver/kidney microsomal-1 (LKM-1) Ab and markers of liver damage were determined in the sera of 50

patients with CHC infection, 20 AIH patients and 20 healthy controls using enzyme linked immunosorbent assay and other immune assays.

RESULTS

We found that AIH patients had more severe liver disease as determined by elevation of total IgG, alkaline phosphatase, total serum bilirubin and serum transaminases and significantly higher prevalence of the three non-organ-specific autoantibodies (auto-Abs) than CHC patients. Antinuclear Ab, SMA and LKM-1 Ab were also present in 36% of CHC patients and related to disease severity. CHC cases positive for auto-Abs were directly comparable to AIH in respect of most markers of liver damage and total IgG. These cases had longer disease duration compared with auto-Ab negative cases, but there was no difference in gender, age or viral load. KLM-1^{+} Ab CHC cases showed best overlap with AIH.

CONCLUSION

Auto-Ab levels in CHC may be important markers of disease severity and positive cases have a disease similar to AIH. Auto-Abs might have a pathogenic role as indicated by elevated markers of liver damage. Future studies will unravel any novel associations between these two diseases, whether genetic or other.

Core tip: This paper aims to determine what patients with chronic hepatitis C (CHC) and autoimmune hepatitis (AIH) produce autoantibodies. Disease duration in CHC is linked to disease severity and autoantibodies. Patients with severe CHC resemble AIH.

INTRODUCTION

Antibodies are a vital part of the immune response for recognition and elimination of invading organisms. However, when the immune system is dysfunctional, it can develop antibodies that react to self. The development of autoantibodies (auto-Abs) generally occurs during auto-immune disease, but their induction can also be a consequence of a chronic infection in

susceptible individuals. A number of auto-Abs with different specificities have been identified. As some auto-Abs occurrence in plasma is disease specific, they can be used in the diagnosis and classification of autoimmune diseases[1-3]. The hepatitis C virus (HCV) infection causes liver damage by inducing cirrhosis and can also lead to hepatocellular carcinoma. Recent studies have demonstrated that the virus may be involved in loss of tolerance to self-antigens and thereby promotion of auto-Ab production[4-6]. In particular, non-organ-specific auto-Abs (NOSAs) including smooth muscle ab (SMA), anti-nuclear ab (ANA) and liver/kidney microsomal-1 (LKM-1) ab are common and frequently found in sera of patients with chronic HCV (CHC)[7,8]. NOSA in HCV-infected patients correlate with the severity of necro-inflammation, fibrosis development, markers of liver damage: aspartate transaminase (AST) and alanine transaminase (ALT), alkaline phosphatase (AP) and levels of IgG[9].

Clinical and laboratory features of CHC can sometimes lead to a mistaken diagnosis of autoimmune hepatitis (AIH). AIH is characterized by a liver-specific autoimmune response, infiltrating immune cells, auto-Abs in circulation, elevated immunoglobulin and serum transaminase level, and a favourable response to immunosuppression[10,11]. In AIH, ANA, SMA, and LKM-1 Abs can differentiate the severity of the disease. The existence of detectable hepatitis C viral load with or without circulating antibodies specific to HCV can often be used to differentiate CHC from AIH[12]. These two conditions, CHC and AIH involve different management strategies; chronic HCV infection has until recently often been treated with interferon-α (IFN-α) which can provoke liver auto-immunity. The HCV infection can, in a few cases, develop into AIH, suggesting that the liver cells are damaged not only by the infection but also by an immune reaction to self[13,14]. AIH on the other hand requires immunosuppression, a treatment that could induce viral replication in cases of co-infection[11,14].

In this study, we have assessed the prevalence of ANA, SMA and LKM-1 Abs in CHC and AIH patients and correlated this with markers of liver disease to determine any overlapping features. In the study, disease severity, immunoglobulin levels and disease duration were assessed. We found that the auto-Ab profile was directly associated with severity of disease in both groups of patients and that subgroups within the patients showed a significant overlap in respect to the laboratory markers assessed.

MATERIALS AND METHODS

A total of 70 patients and 20 healthy controls were recruited during the duration of this study (Table 1). These included 50 patients diagnosed with CHC and 20 patients with AIH. Approval of the study was received from the Ethics board and the Office of the Vice President for Scientific Affairs and Postgraduate

Table 1 Distribution of study group according to age and gender

Parameter	CHC (*n* = 50)	AIH (*n* = 20)	HC (*n* = 20)
Age (yr), mean ± SD			
Range	10-65	16-69	16-69
mean ± SD	33 ± 2.38	37 ± 3.22	37 ± 3.22
Gender, *n* (%)			
Female	24 (48)	15 (75)	15 (75)
Male	26 (52)	5 (25)	5 (25)
Age (yr), mean ± SD			
Female	34 ± 2.28	35 ± 3.42	35 ± 3.42
Male	33 ± 2.38	47 ± 3.12	47 ± 3.12

CHC: Chronic hepatitis C; AIH: Autoimmune hepatitis; HC: Hepatitis virus.

Table 2 Distribution and comparison of autoantibodies between the chronic hepatitis C and the autoimmune hepatitis patient group *n* (%)

Autoantibodies	CHC (*n* = 50)	AIH (*n* = 20)	HC (*n* = 20)	HCV-AIH *P* value
SMA	4 (8)	0 (0)	0 (0)	
LKM-1	11 (22)	9 (45)	0 (0)	
ANA	16 (32)	13 (65)	4 (20)	
Total	31 (36)	23 (75)	4 (20)	0.0031[1]

[1]Indicates significant difference on the 0.01 level. Statistically significant differences were determined using analysis of variance followed by Duncan's test. CHC: Chronic hepatitis C; AIH: Autoimmune hepatitis; HC: Hepatitis virus; HCV: Hepatitis C virus.

Studies at the Sulimani University. CHC patients were diagnosed based on anti-HCV antibody positivity and by assessment of their HCV RNA viral load. Patients with liver damage due to excessive alcohol consumption, hepatotoxic drugs, or human immunodeficiency virus infections were excluded from the study. Patients diagnosed positive for hepatitis B surface antigen were also excluded. The diagnosis of AIH was based on the criteria established by the international AIH group. This includes predominant elevation of serum aminotransferase and IgG, exclusion of viral hepatitis, toxic or alcoholic liver injury and with a liver biopsy confirming lymphocyte infiltration indicative of autoimmune disease[15]. Some patients were diagnosed for the first time when enrolled in the study while others had been undergoing treatment for between two and 11 years. Information of age and gender was recorded for each patient. Twenty healthy gender- and age-matched blood donors served as controls.

Enzyme linked immunosorbent assay and other assays
SMA, ANA and LKM-1 Ab in serum were assayed by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (CUSABIO, Wuhan, China). Total IgG and IgM was estimated quantitatively using a biochemical assay according to the manufacturer's instructions (VITAL Diagnostics, Puteaux, France). Serum AP, ALT, AST, serum albumin and total serum bilirubin (TSB) was quantitatively determined using a biochemical assay (BIOLABO, Maizy, France).

Statistical analysis
Analysis of data was performed by using software package SPSS (Statistical Package for Social Science) version 21. Normal distribution of the data was determined using D'Agostino & Pearson omnibus normality test. Results are expressed as mean ± SD. Statistical differences were determined by Duncan's test for multiple comparisons after analysis of variance. Significant differences between groups were determined using the χ^2 test. A *P* value less than 0.05, 0.01 or 0.001

respectively, were considered statistically significant at the different levels.

RESULTS
In the aim of discovering overlapping features comparing CHC and AIH cases such patients were recruited and sera gathered for analysis of auto-Ab levels and levels of markers of liver damage. Table 1 shows the demographics of the CHC-, the AIH- and the healthy control-group (HC). The CHC group included 50 patients (48% female) and the mean age were 33.4 ± 2.4 years. In agreement with other studies, AIH was more common among females (gender ratio 3:1) with males being older when diagnosed (47.0 ± 3.1 years). There were no significant differences in age or gender comparing patients and HC.

One or more of the auto-Abs was detected in 36% of CHC patients' serum (9 male, 9 female, Table 2). Reactivity for ANA was the most frequent (32%, 7 male, 9 female) while LKM-1 Ab was detectable in 22% (4 male, 7 female) and SMA in 8% (1 male, 3 female) of the cases. Also 20% of the healthy controls were positive for ANA (2 male, 2 female) but not for LKM-1 or SMA. A statistical analysis using the Chi-square test showed that there was no significant difference in the level of ANA comparing the control group and the CHC group. In AIH patients, 75% (3 male, 12 female) had at least one type of auto-Ab. ANA was also the most frequently detected, 65% (3 male, 10 female). LKM-1 Ab was identified in 9 cases (45%) all of which were female. SMA was not detected in any of the AIH patient's serum. A statistical analysis showed significant differences between the AIH and CHC groups of patients regarding auto-Ab prevalence (AIH patients were more often positive for the auto-Abs, *P* = 0.0031, Table 2). The level of ANA and LKM-1, however, tended to be highest in CHC patients plasma (for ANA level in CHC: 4.6 ± 2.7 pg/mL and in AIH, 3.3 ± 1.8 pg/mL, for LKM-1, level in CHC: 4.9 ± 2.8 and in AIH: 4.0 ± 1.4 pg/mL).

Next, we assessed the level of IgG in the patients' serum. As expected, the highest level was found in the serum from the AIH patients (Table 3). There was

Table 3 Estimation of IgG and IgM and comparison between study groups (mean ± SD)

Groups	IgG (mg/dL)	IgM (mg/dL)
CHC	1841 ± 66.44	176 ± 5.56[1]
AIH	2054 ± 152.62	228 ± 5.56[1]
HC	1098 ± 57.69[1]	127 ± 4.80[1]

[1]Denotes significant difference comparing with the other two groups, *P* < 0.05. Statistically significant differences were determined using analysis of variance followed by Duncan's test. CHC: Chronic hepatitis C; AIH: Autoimmune hepatitis; HC: Hepatitis virus.

no significant difference in IgG level comparing CHC and AIH, but the levels were significantly higher than the healthy controls (Table 3). In contrast, although significantly higher than the healthy controls, the mean concentration of IgM remained within the normal range in the CHC and AIH groups. The AIH patients IgM level was significantly higher than the CHCs' and the HCs'.

All of the markers of liver damage (AP, ALT AST, and TSB) except serum albumin assessed in the serum were elevated above the level of the HC's for both the AIH patients and the CHC patients (Table 4). However, in the AIH group, the levels of these markers were significantly higher than in the CHC group (Duncan's test, *P* < 0.05). Only the level of TSB was at a similar level in both patient groups.

To determine whether the presence of auto-Abs in the CHC group was associated with worse disease progression we compared the level of liver damage markers from patients with and without auto-Abs. We found that the duration of infection was twice as long for patients with auto-Abs compared to without. Furthermore, patients with auto-Abs had significantly higher levels of IgG, AP, AST, ALT and TSB (Table 5). This finding is in agreement with that of a previous study[9]. The age and gender of the patients, viral load, IgM, and serum albumin did, however, not differ in the two CHC subgroups.

The CHC patients with auto-Abs, the longest duration of infection and the highest level of markers of liver damage were directly comparable with the AIH patients (Table 5). These two groups were remarkably similar in respect to IgG levels and most markers of liver injury (Table 6). Only the levels of IgM and TSB were higher in the AIH patients (*P* < 0.05).

Further, we found that the LKM-1 Abs present only in sera of female patients in the AIH group. LKM-1 Ab positive AIH patients are often young females with worse prognosis[16,17]. In our study, 22% of the CHC patients were positive for LKM-1 Ab. However, such positivity was found among both male and female subjects (5 male, 6 female). Importantly, our immunological and biochemical analysis revealed that there were no significant differences between these two subgroups of LKM-1+ CHC and AIH in respect of IgG, AP, AST, ALT, albumin or TSB. The only difference

was the concentration of IgM (Table 7).

DISCUSSION

In this study, we have shown that certain patients with chronic hepatitis C (CHC) infection develop an auto-Ab profile similar to that of AIH. This group of patients had longer disease duration and more extensive liver damage, thereby sharing many features of AIH. The comparison of LKM-1 Ab positive patient emphasised the similarity between severe cases of CHC and the AIH group. Only IgM levels discriminated these two groups of patients. Such knowledge is important when interpreting biomarker results from patients with liver disease. Furthermore, the findings suggest an underlying similarity in disease aetiology in these cases of auto-Ab positive CHC and AIH patients.

It is important to differentiate between CHC and AIH as their treatment is completely different[18]. Thus although auto-Abs and elevated liver enzymes might suggest a diagnosis of AIH, patients require further investigation to exclude CHC. When comparing NOSA production from the CHC and AIH patients in our study there was a significant difference, underscoring the overall more severe progression of the disease in the patients with autoimmune disease. In other studies of CHC 6%-41% was positive for ANA, 5%-66% for SMA, and up to 86% for LKM-1 Abs[12,19-22]. There are several reasons to explain why such a variation in levels of auto-Abs has been observed. ANA measured by indirect immune-fluorescence of Hep-2 cells or by different ELISAs produce variable results dependent on the method used[23]. Regional differences in prevalence of autoimmune manifestations in HCV might influence the results[5,24]. Furthermore, differences in auto-Ab levels might be due to the local ethnic background. Hence, in HCV infected patients from Crete or Sweden, very few showed positivity for LKM-1[25,26].

It is not known why 36% of the CHC infected patients in our study develop auto-Abs. Liver damage in CHC cases results in release of auto-antigens to which the immune system can react[27]. As stated above, infection duration might be one factor in the development of auto-Abs. Other studies have, however, failed to identify an association between infection duration and NOSA levels[9,28]. Other factors such as presence of the HLR-DR3 genotype might be more important for auto-Ab development[29].

AIH is known to induce liver damage[11]. CHC patients with auto-Abs and long-standing disease had liver damage at the same level as the AIH cases. The appearance of auto-Ab positive CHC patients can show such high similarity to AIH that they can be misdiagnosed. This is especially the case for patients with extrahepatic symptoms[10,11]. The presence of circulating antibodies specific to HCV is chief for correct diagnosis[12]. The liver damage experienced during AIH is induced by infiltrated inflammatory cells which can

Table 4 Comparison of the markers of liver injury among study groups (mean $\pm$ SD)

Groups	AP (UI/L)	ALT (UI/L)	AST (UI/L)	ALB (g/dL)	TSB (mg/dL)
CHC	296 ± 19.90[1]	30 ± 1.32[1]	45 ± 1.71[1]	3.8 ± 0.11[2]	1.0 ± 0.27[2]
AIH	373 ± 32.10[1]	37 ± 3.20[1]	56 ± 4.10[1]	3.3 ± 0.15[1]	4.0 ± 1.57[1]
HC	171 ± 8.30[1]	19 ± 1.00[1]	28 ± 1.28[1]	4.0 ± 0.06[2]	0.6 ± 0.05[2]

[1]Denotes significant difference comparing with the other two groups, *P* < 0.05; [2]Indicate significant difference comparing with the group indicated with an [1]only. Statistically significant differences were determined using analysis of variance followed by Duncan's test. CHC: Chronic hepatitis C; AIH: Autoimmune hepatitis; HC: Hepatitis virus; ALB: Albumin; AP: Alkaline phosphatase; AST: Aspartate transaminase; TSB: Total serum bilirubin; ALT: Alanine transaminase.

Table 5 Comparison of different parameters in chronic hepatitis C with and without autoantibodies (mean $\pm$ SD)

Parameters	CHC with autoantibodies M/F (9/9)	CHC without autoantibodies M/F (17/15)	*P* value
Mean age (yr)	33 ± 16	34 ± 14.3	0.36 NS
Viral load (copy/mL)	$8.3 \times 10^5 \pm 10.4 \times 10^6$	$3 \times 10^6 \pm 19 \times 10^5$	0.52 NS
Duration of infection (mo)	21.3 ± 12.3	11 ± 7.3	0.002[2]
IgG (mg/dL)	2109 ± 462.4	1630 ± 323	0.001[1]
IgM (mg/dL)	190 ± 25	172 ± 45.3	0.3 NS
AP (U/L)	428 ± 91.5	221 ± 104	0.001[1]
AST (U/L)	52 ± 12.7	41 ± 9.81	0.007[1]
ALT (U/L)	36 ± 9.7	27 ± 7.2	0.007[1]
S. Albumin (g/dL)	3.8 ± 0.87	3.9 ± 0.72	0.47 NS
TSB (mg/dL)	1.5 ± 3.1	0.8 ± 0.58	0.001[1]

[1,2]Indicate significant difference on the 0.01 and the 0.001 level respectively, NS indicates non-significance. Statistically significant differences were determined using analysis of variance followed by Duncan's test. CHC: Chronic hepatitis C; AIH: Autoimmune hepatitis; AP: Alkaline phosphatase; AST: Aspartate transaminase; TSB: Total serum bilirubin; ALT: Alanine transaminase.

Table 6 Autoantibody positive chronic hepatitis C and autoimmune hepatitis, comparison of different parameters (mean $\pm$ SD)

Parameters	Autoantibody positive CHC (*n* = 18)	Autoantibody positive AIH (*n* = 20)	*P* value
IgG (mg/dL)	2109 ± 249	2053 ± 710.5	0.42 NS
IgM (mg/dL)	190 ± 26.8	227 ± 63.1	0.027[1]
AP (U/L)	428 ± 37.9	373 ± 166.1	0.33 NS
AST (U/L)	52 ± 8.42	57 ± 18.64	0.42 NS
ALT (U/L)	36 ± 5.5	37 ± 15.6	0.81 NS
TSB (mg/dL)	1.5 ± 4.1	4.0 ± 7.0	0.0033[2]
Albumin (g/L)	3.8 ± 0.78	3.3 ± 0.69	0.12 NS

[1,2]Indicate significant difference on the 0.05 and the 0.01 level respectively, NS indicates non-significance. Statistically significant differences were determined using analysis of variance followed by Duncan's test. CHC: Chronic hepatitis C; AIH: Autoimmune hepatitis; AP: Alkaline phosphatase; AST: Aspartate transaminase; TSB: Total serum bilirubin; ALT: Alanine transaminase.

Table 7 Comparison between liver/kidney microsomal-1 positive patients in chronic hepatitis C and autoimmune hepatitis groups (mean $\pm$ SD)

Parameters	Groups		*P* value
	LKM-1[+] in AIH M/F (0/9)	LKM-1[+] in CHC M/F (5/6)	
Mean age (yr)	31 ± 10	35 ± 19	0.51 NS
IgG (mg/dL)	2205 ± 566.1	2522 ± 295.6	0.34 NS
IgM (mg/dL)	262 ± 41.21	185 ± 26.26	0.001[1]
AP (U/mL)	404 ± 146.7	472 ± 3	0.57 NS
AST (U/mL)	64 ± 12.71	57 ± 37.92	0.22 NS
ALT (U/mL)	41 ± 9.64	40 ± 5.41	0.45 NS
S. Albumin (g/dL)	3.4 ± 0.8	3.9 ± 0.9	0.5 NS
TSB (mg/dL)	3.4 ± 4.97	1.9 ± 3.90	0.06 NS

[1]Indicates significant difference on the 0.01 level, NS indicates non-significance. Statistically significant differences were determined using analysis of variance followed by Duncan's test. CHC: Chronic hepatitis C; AIH: Autoimmune hepatitis; AP: Alkaline phosphatase; AST: Aspartate transaminase; TSB: Total serum bilirubin; ALT: Alanine transaminase; LKM-1: Liver/kidney microsomal-1.

We speculate that the severe disease seen in AIH and CHC with auto-Abs is mediated by similar mechanisms and that the auto-Abs can contribute to liver damage in these cases.

Sixty-five percent of the AIH patients had any of the three auto-Abs investigated in this study. As previously reported, ANA was the most prevalent auto-Ab[9]. LKM-1 Ab is a serological marker for one subtype of the autoimmune disease, AIH-2 which is more prevalent among adolescent women and young girls[16,17]. In this study LKM-1 Ab was detected in 45% of samples which is an unusually high proportion. This might be explained by that the study contained many young women. It is also possible that the genotype associated with the development of LKM-1 antibodies is more prevalent in the area from where patients were recruited[31]. LKM-1 has been shown to recognize CYP2D7 which is expressed on the surface of hepatocytes. Such self-recognition, unique for LKM-1 among NOSA, could explain the worse disease in these patients[32]. Possibly of importance, epitopes within CYP2D7 share homology with HCV proteins[33]. CYP2D6 epitopes can induce both poly-reactive B cells and T cells[34]. It has been proposed that polymorphisms in the *CYP2D6* gene lead to altered amino acids sequences and more immunogenic epitopes[35]. It is not known whether CYP2D6 polymorphisms could differentiate

be visualized by a liver biopsy[11]. Indeed a liver biopsy is necessary for confirmation of AIH. Immune cells are, however, present in the liver albeit to a lesser extent also in CHC patients with serum auto-antibodies[30].

AIH-1 and AIH-2. There are, however, other diffe-rences between the AIH subtypes; AIH-2 share an antibody profile with autoimmune polyendocrine syndrome type 1 which is caused by a mutation in the *AIRE* gene, leading to a break in tolerance[31]. In this study the levels of both ANA and LKM-1 tended to be higher in CHC than in AIH. Other studies have detected higher level of LKM-1 in AIH-2, than in CHC[36]. Further studies are required to explain why LKM-1 and ANA is higher in sera from the CHC patients than from AIH patients in this study. High level of LKM-1 Ab in CHC has previously been reported in paediatric cohorts[37]. Similar to our findings, LKM-1 Ab have previously been reported in HCV where the titre is associated with disease severity[38]. It is not known whether there is any relationship between patients with CHC, AIH and LKM-1 Ab or whether these CHC cases are more prone to develop AIH. Although no longer commonly used, it has been proposed that IFNα therapy for CHC, can induce autoimmune symptoms in LKM-1^{+} individuals[39].

SMA was detected in none of the patients in the AIH group, which is in disagreement with previous studies. As many as 70% to 80% of AIH cases have been described as SMA positive[40]. The successful treatment of our patients might be one explanation for the lack of SMA[+] patients as this is this is associated with the disappearance of serum ANA and SMA[41]. It should be pointed out that seronegativity in AIH have been described in 1%-34% of cases (in our study 25%) underscoring the heterogeneity of this autoimmune disease[42].

High IgG levels are a distinctive feature of AIH[43-45]. In our study, however, there was no significant diffe-rence in levels comparing AIH and CHC. Other studies have reported elevated levels of IgG in both AIH and CHC patients[38]. The level of IgG has previously been associated with severity of disease among chronic HCV infected patients which is in line with our findings. Both diseases are characterized by activation of B cells and a large number of plasma cells[6]. We speculate that many of these released auto-Abs are of the IgG subclass which can explain the elevated level of these Abs in serum from both patient groups. This polyclonal activation is likely taking place either as a consequence of chronic antigen stimulation or due to loss of immune regulation[46,47]. Our interest is in Th17 cells that have an established pathogenic function in autoimmune diseases[48]. Th17 cells are present in the liver of both AIH and CHC patients[30]. Further studies will determine whether these cells could contribute to B cell activation or generation of an inflammatory environment that promotes auto-Ab production[49,50].

In conclusion, we found AIH-related auto-Abs associated with HCV infection. The emergence of auto-Abs in CHC might be infection duration dependent, but it is not related to gender, the age of patients and serum viral load. Auto-Abs, especially LKM-1, in CHC cases might have pathogenic role leading to more severe disease which is indicated by an alteration of liver function tests and elevation of total IgG. It is not known whether auto-Ab prevalence is due to prolonged disease or whether certain patients are more susceptible for their development. We conclude that auto-Ab levels in CHC may be important markers of disease severity and that these patients have a disease similar to AIH. Future studies will unravel any further associations between these two diseases, whether genetic or other.

COMMENTS

Background

Worldwide, 130-200 million individuals are infected with hepatitis C. Although current therapies control the disease rather well, 80% of the infected patients develop chronic hepatitis C (CHC). It is not known why some of the patients develop more severe disease. Autoimmune hepatitis (AIH) is a disease of the liver that has a prevalence of 10-20/100000 individuals. This disease can be controlled by using immunosuppressive therapies. In this paper the authors show that the presence of non-organ specific autoantibodies (NOSA) in both CHC and AIH is associated with severe disease. Although overall CHC is less damaging to the liver than AIH cases, a subgroup can be defined with more severe pathology. This CHC subgroup is defined by liver/kidney microsomal-1 (LKM-1) positivity.

Research frontiers

In the field of autoimmunity, novel therapies have recently been developed as well as better understanding of the aetiology of disease. Recent discoveries of novel immune cells and their dysregulation in the autoimmunity have increased the knowledge of disease aetiology and provided prospect for development of novel therapies. Novel genome-, transcriptome- and epigenome-sequencing techniques have given important insight of autoimmune associations to certain genomic regions genomic and the immunological heterogeneity underlying disease. As some subpopulations of CHC patients have antibodies that react to self, we speculate that the analysis of novel immune cells and comparison of immune cells from CHC patients with AIH using novel genomic and epigenomic tools can provide disease related knowledge useful for novel future treatment-strategies of these patients.

Innovations and breakthroughs

Th17 cells have recently been implicated in the development of AIH and response to hepatitis C virus infection in the liver. Although it is not known how these cells confer pathology, studies from other autoimmune diseases has suggested that the cells support B cell germinal centre formation and production of auto-antibodies. This can be both through IL-21 production by Th17 cells and through transdifferentiation of Th17 cells to follicular T cells. Studies have shown that polymorphisms in the TNFα gene are associated with development of AIH. We are currently analysing how TNFα is suppressing Th17 cell expansion in rheumatoid arthritis patients. Future studies by authors would aim to determine the role of Th17 cells and possibly TNFα in the development of autoantibodies (auto-Abs) in AIH and LKM-1^{+} CHC.

Applications

The current study has characterized the immune response in CHC and AIH in homogenous patient cohorts. The findings differentiated CHC and AIH patients into different subgroups. These findings open the way for future studies of immune cell mediated induction of autoimmunity in CHC and AIH. We aim to characterize immune cells present in the liver and the peripheral blood of the patient cohorts. Further, genome wide association studies, analysis for expression quantitative trait loci and whole genome epigenome will be undertaken to gain better understanding of the autoimmune disease process which is regulating the immune cells. Findings from these studies will be correlated to disease severity, auto-Ab levels and other markers of liver disease. Importantly the knowledge gained will provide the possibility to discover overlapping genomic/epigenomic features between AIH and the

subgroup of LKM-1 positive CHC patients with severe disease.

Peer-review

The manuscript by Amin *et al* compares the incidence of auto-Abs associated with AIH, immunoglobulin levels and markers of liver disease in groups of age-matched subjects with autoimmune hepatitis, CHC infection and healthy controls.

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ORIGINAL ARTICLE

Basic Study

Anti-inflammatory intestinal activity of *Combretum duarteanum* Cambess. in trinitrobenzene sulfonic acid colitis model

Gedson Rodrigues de Morais Lima, Flavia Danniele Frota Machado, Larissa Lucena Périco, Felipe Meira de Faria, Anderson Luiz-Ferreira, Alba Regina Monteiro Souza Brito, Cláudia Helena Pellizzon, Clélia Akiko Hiruma-Lima, Josean Fechine Tavares, José Maria Barbosa Filho, Leônia Maria Batista

Gedson Rodrigues de Morais Lima, Flavia Danniele Frota Machado, Josean Fechine Tavares, José Maria Barbosa Filho, Leônia Maria Batista, Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Laboratório de Farmacologia do Trato Gastrintestinal, Universidade Federal da Paraíba (UFPB), Cidade Universitária, João Pessoa, Paraíba, CEP 58051-970, Brazil

Larissa Lucena Périco, Clélia Akiko Hiruma-Lima, Departamento de Fisiologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo 18618-970, Brazil

Felipe Meira de Faria, Alba Regina Monteiro Souza Brito, Departamento de Biologia Estrutural e Funcional, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo 13083-970, Brazil

Anderson Luiz-Ferreira, Departamento de Ciencias Biológicas, Instituto de Biotecnologia, Universidade Federal de Goiás, Catalão, Goiás, 75704-020, Brazil

Cláudia Helena Pellizzon, Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo 18618-970, Brazil

Author contributions: de Morais Lima GR, Machado FDF, Périco LL, de Faria FM, Luiz-Ferreira A, Souza Brito ARM, Pellizzon CH, Hiruma-Lima CA, Tavares JF, Barbosa Filho JM and Batista LM contributed to the conception and design of the study, performed most of the experiments, coordinated the study, participated in writing the manuscript and approved the final version of the article to be published.

Institutional review board statement: The protocol was approved by the Committee for Ethics in Animal Experimentation, Laboratory of Pharmaceutical Technology, Federal University of Paraíba (CEPA/LTF/UFPB), João Pessoa, Paraíba, Brazil.

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Correspondence to: Leônia Maria Batista, PhD, Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Laboratório de Farmacologia do Trato Gastrintestinal, Universidade Federal da Paraíba (UFPB), Cidade Universitária, s/n - Castelo Branco, João Pessoa, Paraíba, CEP 58051-970, Brazil. leoniab@uol.com.br
Telephone: +55-83-32167307
Fax: +55-83-32167502

Abstract

AIM

To evaluate the anti-inflammatory intestinal effect of the ethanolic extract (EtOHE) and hexane phase (HexP) obtained from the leaves of *Combretum duarteanum* (*Cd*).

METHODS

Inflammatory bowel disease was induced using trinitrobenzenesulfonic acid in acute and relapsed ulcerative colitis in rat models. Damage scores, and biochemical, histological and immunohistochemical parameters were evaluated.

RESULTS

Both *Cd*-EtOHE and *Cd*-HexP caused significant reductions in macroscopic lesion scores and ulcerative lesion areas. The vegetable samples inhibited myeloperoxidase increase, as well as pro-inflammatory cytokines TNF-α and IL-1β. Anti-inflammatory cytokine IL-10 also increased in animals treated with the tested plant samples. The anti-inflammatory intestinal effect is related to decreased expression of cyclooxygenase-2, proliferating cell nuclear antigen, and an increase in superoxide dismutase.

CONCLUSION

The data indicate anti-inflammatory intestinal activity. The effects may also involve participation of the antioxidant system and principal cytokines relating to inflammatory bowel disease.

Key words: *Combretum duarteanum*; Medicinal plants; Combretaceae; Inflammatory bowel disease; Anti-inflammatory intestinal activity

Core tip: Inflammatory bowel diseases are chronic inflammatory disorders that include Crohn's disease and ulcerative colitis (UC). Genetic, immunologic and environmental factors are postulated as possible etiologic agents. Their conventional treatment is centered in reducing inflammation and abnormal symptom relief. A variety of herbal medicines have been demonstrated to produce promising results in the treatment of those diseases. *Combretum duarteanum* is a species popularly used in folk medicine to treat inflammation. Thus, the present study was designed to evaluate the intestinal anti-inflammatory effect in an UC rat model, contributing to the safe use and collaborating with the scientific knowledge of natural products.

de Morais Lima GR, Machado FDF, Périco LL, de Faria FM, Luiz-Ferreira A, Souza Brito ARM, Pellizzon CH, Hiruma-Lima CA, Tavares JF, Barbosa Filho JM, Batista LM. Anti-inflammatory intestinal activity of *Combretum duarteanum* Cambess. in trinitrobenzene sulfonic acid colitis model. *World*

INTRODUCTION

The inflammatory bowel diseases (IBDs) are chronic disorders of the gastrointestinal (GI) tract characterized by alternating periods of remission and relapse[1]. These diseases represent a large group of inflammatory disorders, the most common being Crohn's disease (CD) and ulcerative colitis (UC)[2,3].

CD can affect any part of the GI tract and has the classic symptoms of fatigue, prolonged diarrhea (with or without bleeding), abdominal pain, weight loss, and fever[4,5]. UC is a type of chronic inflammation restricted to the colon; however, the entire large intestine may be affected[6]. Affected patients show symptoms such as rectal bleeding, frequent bowel movements, tenesmus, rectal mucus discharge, and abdominal pain[5].

The etiology of IBDs is still not fully understood; however, it is believed that environmental, genetic and immunologic factors have important roles in their occurrence and progression[4,7].

Emerging models in the study of IBD pathogenesis suggest three key disease development factors: (1) breaking the intestinal barrier function; (2) lamina immune cell exposure to luminal contents; and (3) exacerbation of immune response. However, the factors responsible for initiation and perpetuation of the cycle leading to exacerbation of the disease are still unclear[8,9].

A combination of genetic and environmental factors may foment changes in the intestinal mucosal barrier function; this allows translocation of luminal antigens (commensal bacteria or microbial products) into the intestinal wall, and consequent immune cell activation and excessive production of cytokines, causing the acute phase of inflammation. If the acute inflammatory process is not resolved by anti-inflammatory mechanisms and suppression of pro-inflammatory cytokines, chronic intestinal inflammation develops. This can lead to tissue destruction and complications of the disease[10].

Conventional treatments are aimed at reducing inflammation and consequent abnormal symptom relief. Patients with UC are treated with amino-salicylates, corticosteroids, and immunomodulatory drugs[11].

Natural products have become the most attractive source of new drugs for the treatment and prevention of diseases and their use is constantly expanding worldwide. A variety of herbal medicines have been shown to produce promising results in the treatment of peptic ulcer and IBD[11-14].

Combretum duarteanum (*C. duarteanum*, *Cd*) Cambess., the species selected for this study, is popularly known as "mufumbo, cipiúba, cipaúba". This

shrubby species is exclusive to South America with registrations in Bolivia, Paraguay, and Brazil. It occurs in the northern and northeastern regions of Brazil, being associated with the "caatinga" biome[15,16].

In folk medicine, *C. duarteanum* is used to treat pain, inflammation and GI tract disorders, which justifies its selection, using ethno-pharmacological indication as the criterion of choice. Phytochemical studies suggest the presence of flavonoids and triterpenes, whose pharmacological effects have been attributed[16-18].

C. duarteanum has presented *in vitro* and *in vivo* anti-inflammatory, anti-nociceptive, and antioxidant capacities[15]. Quintans *et al*[17] demonstrated orofacial nociceptive activity as promoted by the hexane phase and Fridelin terpenes, isolated from the species studied.

de Morais Lima *et al*[16,19] demonstrated gastro-protective and antiulcer activity in *C. duarteanum* in different models of acute ulcer induction (acidified ethanol, ethanol, nonsteroidal anti-inflammatory drugs (commonly known as NSAIDs), stress, pylorus ligature, acetic acid) in animals. Previous studies demonstrated low toxicity and no change of liver enzymes in animals treated with the tested plant sample for 15 d in acid acetic-induced gastric ulcer model[19].

Given the need for new IBD therapies, this study aimed to evaluate the intestinal anti-inflammatory activity promoted by the species *C. duarteanum*, validating its popular use and contributing to the search for new therapies for diseases that affect the GI tract.

MATERIALS AND METHODS

Reagents

The drugs and reagents were prepared immediately before use. The following drugs were used: trinitro-benzenesulfonic acid (TNBS) (Sigma-Aldrich), ketamine 5% (Vetanarcol), xilazine 2% (Dorcipec), ethanol (Merck®), Tween 80 (Merck®), sodium chloride (Sigma-Aldrich). TNF-α, IL-1β and IL-10 in enzyme-linked immunosorbent assay (ELISA) kits were provided by R&D Systems Inc.

Plant material

Plant samples used in intestinal anti-inflammatory activity research experiments in rats were obtained from the leaves of *C. duarteanum* Cambess., collected at Serra Branca City, Paraíba State, Brazil, in 2010. The species was identified by Dr. Maria Fatima Agra and a voucher specimen (No. 6767) was deposited in the Herbarium Prof. Lauro Pires Xavier (JPB) at the Universidade Federal da Paraíba.

The ethanolic extract (*Cd*-EtOHE) and the hexane phase (*Cd*-HexP) obtained from the leaves of *C. duarteanum* Cambess. were provided by Dr. Josean Fechine Tavares and his group, all of PgPNSB/UFPB.

The dried leaves (5 kg) were powdered, extracted with ethanol, stirred, and macerated at room temperature for approximately 48 h, with the procedure being repeated three times. The solvent was fully evaporated under reduced pressure, and the extract (yield of 200 g) was concentrated. The *Cd*-EtOHE was subjected to liquid-liquid partition with the following solvents: hexane, chloroform (CHCl₃), and ethyl acetate (EtOAc), obtaining their respective phases. This step was repeated to secure the required quantity for the study.

Pharmacological assays

Investigation of *Cd*-EtOHE and *Cd*-HexP effects on acute phase intestinal inflammation (TNBS)-induced in rats: The intestinal anti-inflammatory activity of *Cd*-EtOHE and *Cd*-HexP was assessed in rats using the Morris *et al*[20] method. Male Wistar rats (n = 5-8) fasted for 24 h were divided into four groups: non-colitic, colitic, *Cd*-EtOHE and *Cd*-HexP. The animals were anesthetized for rectal administration of TNBS (2,4,6-trinitro-benzene sulfonic acid) - 10 mg solubilized in 0.25 mL of 50% v/v ethanol. The induction of inflammation was performed with the aid of a probe (2-mm diameter), which was inserted about 8 cm into the rectum of the animal. After TNBS administration, animals were maintained upside down for 15 min to enable total absorption of the administered inducing agent. The non-colitic group underwent the same procedures but they did not receive TNBS.

Each group of rats was pretreated with vehicle (12% Tween 80), *Cd*-EtOHE (31.25, 62.5, 125, 250 mg/kg) or *Cd*-HexP (31.25, 62.5, 125, 250 mg/kg), at 48, 24 and 1 h prior to administration of TNBS/50% ethanol, and at 24 h after colitis induction. At 48 h after inducing inflammation, the animals were euthanized and colonic segments were removed, opened, washed, and photographed for quantification of ulcerative lesion area (ULA) and macroscopic score evaluation of the intestinal inflammatory process. Analysis of the extent of intestinal injury was performed according to the scale described previously by Bell *et al*[21]. General parameters such as diarrhea and the colon weight/length ratio were also evaluated. The most effective doses obtained in this model were used in the chronic model with relapse of UC in rats.

Investigation of *Cd*-EtOHE and *Cd*-HexP effects in the chronic phase with intestinal inflammation relapse induced by TNBS in rats: Male Wistar rats (n = 7-9) were divided into non-colitic, colitic, *Cd*-EtOHE and *Cd*-Hexp groups. After 24 h of fasting, induction of intestinal inflammation was performed with TNBS (10 mg/0.25 mL ethanol 50% v/v, rectally)[20]. At 24 h after initial induction, the animal groups were treated orally with 12% Tween 80 solution (non-colitic and colitic), *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg). On day 14 after the first induction, the second administration

(relapse) was performed with TNBS (10 mg/0.25 mL ethanol 50% v/v, rectally) to mimic recurrent relapses in IBDs in humans.

General parameters such as diarrhea, water and food intake, and body weight were recorded daily throughout the treatment period. At day 21 all animals were euthanized, the colon removed, opened, and washed for macroscopic lesion analysis and evaluation of the intestinal inflammatory process[21]. Collection of material for biochemical and histological analysis was also performed. The samples were stored at -80 ℃ for evaluation of myeloperoxidase (MPO) and cytokines involved in intestinal inflammation.

Histological analysis

Colonic segments intended for light microscopy were collected. For this, the tissues were fixed in Alfac solution for 24 h at room temperature. Afterwards, the pieces were kept in 80% alcohol until the block assembly time. The pieces were dehydrated and embedded in paraplast forming blocks, and then cut to 10-mm thickness for mounting on slides. These were stained with hematoxylin and eosin for morphological analysis[22].

Quantification of MPO activity

Colon segments, stored at -80 ℃ were used with dosages of MPO and pro-inflammatory and anti-inflammatory cytokines. The samples were homogenized in hexadecyltrimethylammonium bromide buffer (HTAB) (0.5% in 50 mmol/L sodium phosphate buffer, pH 6.0) that acts as a detergent, lysing granules of neutrophils containing MPO, which is released. The sample was centrifuged for 10 min at 4 ℃. The homogenate was subjected to a three-fold freezing and thawing process to facilitate the rupturing of cell structures and the consequent release of the enzyme. On ELISA plates were placed 50 μL of supernatant from each sample and 150 μL of reaction buffer[23].

The results were expressed as MPO units per gram of tissue, where 1 U of MPO activity is defined as that degrading 1 μmol of hydrogen peroxide per min at 25 ℃.

Assessment of the involvement of pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10)

TNF-α, IL-1β and IL-10 levels were determined from colonic specimens, frozen in -80 ℃, and collected in the UC relapse model. For this, we used PBS buffer pH 7.4 (1:5) to homogenize the samples. The homogenate tubes were centrifuged at 12000 rpm for 10 min. The supernatants were frozen at -80 ℃ until assay. Subsequently, the samples were shaken in water bath at 37 ℃ for 20 min and then centrifuged at 10000 rpm for 5 min at 4 ℃. The supernatant was collected and the cytokines TNF-α, IL-1β and IL-10 were quantitated

using ELISA assay kits (DuoSet®; R & D Systems Inc.). The concentrations of the cytokines in relation to the amount of total protein was quantified by bicinchoninic acid method[24].

Immunohistochemical analysis cyclooxygenase-2, proliferating cell nuclear antigen, and superoxide dismutase expression

Histological samples were incubated with anti-cyclo-oxygenase-2 (COX-2) secondary antibody (marker for assessing anti-inflammatory effect), anti-proliferating cell nuclear antigen (PCNA) (cell division marker to assess potential for regeneration), and anti-superoxide dismutase (SOD) (marker to evaluate the antioxidant effect). The positively stained cells were counted for the various immunohistochemical reactions in a fixed number of fields by means of an image analyzer (Q-Win Standard Version 3.1.0; Leica) coupled to the Leica DM microscope. They were photographed and analyzed by AVSoft program Bioview Spectra and Seeker 4.0.

Animal care and use statement

The experimental protocols were approved by the Committee for Ethics in Animal Experimentation (CEPA/LTF/UFPB) under number 1112/10. Male Wistar albino rats (180-250 g) from the "Prof. Thomas George Vivarium" of LTF/UFPB were fed a certified Presence® diet, with free access to water under fixed conditions of illumination (12/12 h light/dark cycle), humidity (60% ± 1.0%), and temperature (21.5 ± 1.0 ℃). Fasting was used prior to all assays since standard drugs were administered orally (by gavage) or by intra-rectal route, using a 12% solution of Tween 80 (10 mL/kg) as the vehicle. The animals were kept in cages with raised, wide-mesh floors to prevent coprophagy.

Statistical analysis

Results with parameter values (inflammatory bowel lesion area and weight/length ratio) were subjected to analysis of variance (ANOVA) followed by Dunnett's or Tukey test, and expressed as mean ± SD of the average. In quantitation assays of antioxidant enzymes, cytokines and MPO values obtained were presented as mean ± standard error of mean (SEM).

For nonparametric values (score of intestinal inflammation), the Kruskal-Wallis test (ANOVA, Dunn's post-test) was used. The results were expressed as median (minimum-maximum). Data were analyzed using the software GraphPad Prism 6.0, and the significance level was set at $P < 0.05$.

RESULTS

Investigation of Cd-EtOHE and Cd-HexP effect on induced acute phase intestinal inflammation (TNBS) in rats

A significant reduction in the intestinal ULA for rats

Table 1 Effects of oral administration of *Cd*-EtOHE or *Cd*-HexP in acute phase of intestinal inflammation in trinitrobenzenesulfonic acid-induced ulcerative colitis in rats

Group	Dose (mg/kg)	ULA (mm^2)	Inhibition (%)	Lesion score	Weight/length (mg/cm)	Diarrhea (%)
Non-colitics	-	-	100		110 ± 7.7	0
Colitics	-	107 ± 38	-	6.0 (5-7)	148 ± 17[f]	100
Cd-EtOHE	31.25	79 ± 31	26	5.0 (3-7)	152 ± 19[f]	100
	62.5	46 ± 12[b]	57	4.0 (1-5)[a]	146 ± 19[f]	57
	125	19 ± 8[c]	82	3.0 (2-5)[b]	134 ± 5[d]	14[b]
	250	76 ± 23	29	6.0 (4-7)	152 ± 14[f]	86
Non-colitics	-	-	-	-	102 ± 14	0
Colitics	-	101 ± 45	-	7.0 (5-8)	154 ± 27[f]	87
Cd-HexP	31.25	52 ± 18[b]	49	5.0 (1-6)[a]	142 ± 20[e]	87
	62.5	21 ± 7[c]	79	5.0 (4-5)[b]	129 ± 20[a]	29[a]
	125	95 ± 33	7	6.0 (5-6)	154 ± 10[f]	71
	250	80 ± 12	22	5.0 (4-7)	155 ± 16[f]	86

Results expressed as mean ± SD or median (minimum-maximum) of the parameters analyzed (n = 5-8). For the parametric data, mean ± SD was used, with ANOVA and *a posteriori* Dunnett's test. For the non-parametric data, median (minimum-maximum) was used, with Kruskal-Wallis test and *a posteriori* Dunn. [a]$P < 0.05$, [b]$P < 0.01$ and [c]$P < 0.001$ *vs* colitic group; [d]$P < 0.05$, [e]$P < 0.01$ and [f]$P < 0.001$ *vs* non-colitic group. *Cd*: *Combretum duarteanum*; EtOHE: Ethanolic extract; HexP: Hexane phase.

treated with *Cd*-EtOHE at doses of 62.5 and 125 mg/kg (46 ± 12, $P < 0.01$ and 19 ± 8, $P < 0.001$ respectively) was observed compared to the colitic group (107 ± 38). In the experimental evaluation of the effect of *Cd*-HexP, a significant reduction was observed at doses of 31.25 and 62.5 mg/kg (52 ± 18, $P < 0.01$ and 21 ± 7, $P < 0.001$ respectively), when compared to the colitic animals (101 ± 45) (Table 1).

For the lesion score, *Cd*-EtOHE at doses of 62.5 and 125 mg/kg significantly reduced the amounts of lesion to 4.0 (1-5) ($P < 0.05$) and 3.0 (2-5) ($P < 0.01$) respectively, compared to the colitic control of 6 (5-7). *Cd*-HexP 31.25 and 62.5 mg/kg significantly reduced lesion to 5.0 (1-6) ($P < 0.05$) and 5.0 (4-5) ($P < 0.01$) respectively, compared to the colitic group of 7 (5-8) (Table 1).

A significant increase in weight/length for the colitic group (148 ± 17, $P < 0.001$, 154 ± 27, $P < 0.001$) was also observed when compared to the non-colitic group (110 ± 8, 102 ± 14 respectively). Treatment with different doses of *Cd*-EtOHE (31.25, 62.5, 125 and 250 mg/kg) did not reduce the weight/length ratio (152 ± 19, 146 ± 19, 134 ± 5 and 152 ± 14 respectively) for the parameter compared to the colitic group (148 ± 17). However, treatment with *Cd*-HexP at a dose of 62.5 mg/kg significantly reduced the ratio to 129 ± 20 ($P < 0.05$), compared to their respective colitic group (154 ± 27) (Table 1).

The administration of TNBS resulted in a diarrhea rate of 100% in the colitic animals. *Cd*-EtOHE at a dose of 125 mg/kg significantly reduced the diarrhea involvement to 14%. For *Cd*-HexP, treatment at dose of 62.5 mg/kg significantly reduced diarrhea to 29% ($P < 0.05$) when compared to their respective colitic control (87%) (Table 1).

Intestines of colitic, non-colitic and treated rats with different tested doses of *Cd*-EtOHE or *Cd*-HexP in the model can be seen in Figures 1 and 2 respectively.

Investigation of Cd-EtOHE and Cd-HexP effects in chronic phase intestinal inflammation with induced relapse using TNBS in rats

A significant reduction in macroscopic damage scores was observed for both *Cd*-EtOHE (125 mg/kg) and *Cd*-HexP (62.5 mg/kg), to 1.0 (1-4) ($P < 0.05$) and 1.0 (1-4) ($P < 0.01$) respectively, compared to the colitic control 4 (3-6). Moreover, the tested plant sample reduced the onset of diarrhea by 56% when compared to colitic animals (94%) (see Table 2).

The weight/length ratio significantly increased in the colitic, *Cd*-EtOHE and *Cd*-HexP groups (143 ± 14, $P < 0.001$; 132 ± 11, $P < 0.001$; 122 ± 12, $P < 0.01$, respectively) compared to the non-colitic group (97 ± 9). However, *Cd*-HexP 62.5 mg/kg caused a significant reduction (122 ± 12, $P < 0.01$), compared to the colitic group (143 ± 14). These results are shown in Table 2, and can be seen best in Figure 3.

A significant decrease in water (28 ± 3, $P < 0.01$) and food (19 ± 2, $P < 0.01$) intake was observed in the colitic group, compared to the non-colitic animals (31 ± 4 and 22 ± 2 respectively). Only treatment with *Cd*-HexP increased water (31 ± 2, $P < 0.05$) and food (22 ± 3, $P < 0.01$) intake, compared to colitic animals (28 ± 3, $P < 0.01$ and 19 ± 2, $P < 0.01$ respectively) (Table 3).

As an additional parameter to the data described above, we evaluated the effect of repeated administrations of *Cd*-EtOHE (125 mg/kg) and *Cd*-HexP (62.5 mg/kg) on the body weights of animals affected with intestinal inflammation. At the end of the experiment, there was a significant reduction in mean body weight for the colitic group (215 ± 21, $P < 0.001$) when compared to the non-colitic group (261 ± 35). However, when the treatments were performed with *Cd*-HexP (62.5 mg/kg), a significant increase in mean body weight (239 ± 17, $P < 0 05$) was observed compared to the colitic animals (215 ± 21) (Table 4).

A significant increase was observed in spleen weight

Figure 1 Representative macroscopic images of rat colonic mucosa in non-colitics (A), colitics (B), *Cd*-EtOHE at 31.25 mg/kg (C), *Cd*-EtOHE at 62.50 mg/kg (D), *Cd*-EtOHE at 125 mg/kg (E), and *Cd*-EtOHE at 250 mg/kg (F). *Cd*: *Combretum duarteanum*; EtOHE: Ethanolic extract.

in the colitic group (2.8 ± 0.5, $P < 0.05$) compared to the non-colitic group (2.1 ± 0.2). Analyzing the organs of animals treated with *Cd*-EtOHE or *Cd*-HexP, a significant increase in spleen weight for animals treated with *Cd*-EtOHE as compared to the non-colitic group was demonstrated (Table 5). In other evaluations (heart, liver and kidneys) no significant changes, compared to the non-colitic group, were observed.

Histological analysis

Histological examination of the colon of non-colitic rats showed normal histological structure, highlighting the structure of the mucosal straight intestinal glands with large numbers of goblet cells and lamina propria classical (or normal). The animals belonging to the colitic group had transmural inflammation, necrosis of the mucosa with disruption of glands, and loss of goblet and epithelial cells. The presence of granulation, highlighting neutrophilic and lymphocytic infiltration, was also observed.

Treatment with *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) maintained some areas of the mucosal structure and epithelium intact, reducing the reducing the Inflammatories cells in lamina propria as compared to the colitic group, suggesting the re-epithelialization of the animals treated with the vegetable samples (Figure 4).

Quantification of MPO activity

According to the results obtained, there was a significant increase in MPO to 40.270 ± 3.077 ($P < 0.001$) in the colitic group when compared to non-colitics (10.120 ± 1.672). When compared to the colitic controls (40.270 ± 3.077), treatments with *Cd*-EtOHE (15.187 ± 1.158, $P < 0.001$) or *Cd*-HexP (17.620 ± 2.395, $P < 0.001$) significantly reduced MPO (Figure 5).

Figure 2 Representative macroscopic images of rat colonic mucosa in non-colitics (A), colitics (B), *Cd*-HexP at 31.25 mg/kg (C), *Cd*-HexP at 62.50 mg/kg (D), *Cd*-HexP at 125 mg/kg (E), and *Cd*-HexP at 250 mg/kg (F). *Cd*: *Combretum duarteanum*; HexP: Hexane phase.

Assessment of the involvement of pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10)

The results showed a significant increase in TNF-α levels in colitic animals (2.0 ± 0.2, $P < 0.01$) compared to non-colitics (1.3 ± 0.1). However, the treatment of *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) resulted in TNF-α level reduction (1.2 ± 0.2 and 1.4 ± 0.1, $P < 0.01$) respectively, compared to the colitic group (2.0 ± 0.2) (Figure 5).

IL-1β levels increased in colitic animals (63 ± 6, $P < 0.001$) when compared to the non-colitic group (32 ± 4.0). Treatment with *Cd*-EtOHE significantly reduced to 41 ± 5 ($P < 0.01$) IL-1-β, compared to the colitic group (63 ± 6.2). *Cd*-HexP did not cause significant change when compared to the negative control (Figure 5).

The results showed significant reduction of IL-10 in intestinal tissues, comparing the colitic group 3.2 ± 0.3 ($P < 0.05$) to the non-colitic group (5.1 ± 0.8). Treatment with *Cd*-EtOHE or *Cd*-HexP caused significant increases (5.0 ± 0.4, $P < 0.05$, 5.8 ± 0.3, $P < 0.01$,

respectively) when compared to the colitic group ($3 2 \pm 0.3$) (Figure 5).

Immunohistochemical analysis (COX-2, PCNA and SOD expression)

In analyzing the results of COX-2 expression shown in Table 6 and Figure 6, we observed a significant increase in COX-2 expression in the colitic group animals to 505 (100-1450) ($P < 0.05$) when compared the non-colitic group of 230 (110-700). However, treatment with *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) significantly reduced the expression of COX-2 to 90 (10-2590) ($P < 0.001$) and 205 (20-790) ($P < 0.001$) respectively, compared to the colitic group of 505 (100-1450).

A significant increase in PCNA expression was observed in the animals of the colitic group of 5380 (850-15960) ($P < 0.001$) when compared to the non-colitic group of 1425 (60-7890). However, treatment with *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) significantly reduced PCNA expression to 1.630 (90-14790) ($P < 0.001$) and 1.570 (250-9500) ($P <$

Table 2 Effects of oral administration of *Cd*-EtOHE or *Cd*-HexP in chronic phase with relapse of intestinal inflammation in trinitrobenzenesulfonic acid-induced ulcerative colitis in rats

Group	Dose (mg/kg)	Lesion score	Weight/length (mg/cm)	Diarrhea (%)
Non-colitics	-		97 ± 9	0
Colitics	-	4.0 (3-6)	143 ± 14[f]	94
Cd-EtOHE	125	1.0 (1-4)[a]	132 ± 11[f]	56[a]
Cd-HexP	62.5	1.0 (1-4)[b]	122 ± 12[b,e]	56[a]

Results expressed as mean ± SD or median (minimum-maximum) of the parameters analyzed (n = 7-9). For the parametric data, mean ± SD was used, with ANOVA and a posteriori Dunnett's test. For non-parametric data, median (minimum-maximum) was used, with Kruskal-Wallis test and a posteriori Dunn. [a]P < 0.05, [b]P < 0.01 *vs* colitic group; [e]P < 0.01 and [f]P < 0.001 *vs* non-colitic group. *Cd*: *Combretum duarteanum*; EtOHE: Ethanolic extract; HexP: Hexane phase.

Table 3 Effect of oral administration of *Cd*-EtOHE or *Cd*-HexP for 21 d on water and food consumption in trinitrobenzenesulfonic acid-induced ulcerative colitis in rats

Group	Dose (mg/kg)	Water intake (mL)	Food intake (g)
Non-colitics	-	31 ± 4	22 ± 2
Colitics	-	28 ± 3[e]	19 ± 2[e]
Cd-EtOHE	125	30 ± 4	20 ± 3[d]
Cd-HexP	62.5	31 ± 2[a]	22 ± 3[b]

Values are expressed as mean ± SD (n = 7-9). One-way ANOVA, followed by Dunnett's test, [a]P < 0.05 and [b]P < 0.01 *vs* colitic group; [d]P < 0.05 and [e]P < 0.01 *vs* non-colitic group. *Cd*: *Combretum duarteanum*; EtOHE: Ethanolic extract; HexP: Hexane phase.

Table 4 Effect of oral administration of *Cd*-EtOHE or *Cd*-HexP for 21 d on body weight on trinitrobenzenesulfonic acid-induced ulcerative colitis in rats

Group	Initial weight (g)	Final weight (g)	Average increase (g)
Non-colitics	202 ± 32	261 ± 35	51 ± 10
Colitics	189 ± 19	215 ± 21[f]	35 ± 12[d]
Cd-EtOHE	178 ± 13	230 ± 7	43 ± 6
Cd-HexP	184 ± 14	239 ± 17[a]	48 ± 10[a]

Values are expressed as mean ± SD (n = 7-9). One-way ANOVA, followed by Dunnett's test, [a]P < 0.05 *vs* colitic group; [d]P < 0.05, [f]P < 0.001, *vs* non-colitic group. *Cd*: *Combretum duarteanum*; EtOHE: Ethanolic extract; HexP: Hexane phase.

Table 5 Effect of oral administration of *Cd*-EtOHE or *Cd*-HexP for 21 d on weight organs in trinitrobenzenesulfonic acid-induced ulcerative colitis in rats

Group	Heart	Liver	Kidneys	Spleen
Non-colitics	4.0 ± 0.2	41 ± 1.5	8.6 ± 0.6	2.1 ± 0.2
Colitics	4.0 ± 0.5	43 ± 5.3	8.6 ± 1.0	2.8 ± 0.5[d]
Cd-EtOHE	4.0 ± 0.3	43 ± 3.1	8.6 ± 0.2	2.8 ± 0.4[e]
Cd-HexP	4.0 ± 0.3	44 ± 1.2	8.4 ± 0.5	2.4 ± 0.2

Values are expressed as mean ± SD (n = 7-9). One-way ANOVA, followed by Dunnett's test, [d]P < 0.05 and [e]P < 0.01 *vs* non-colitic group. *Cd*: *Combretum duarteanum*; EtOHE: Ethanolic extract; HexP: Hexane phase.

0.001) respectively, compared to 5.380 of the colitic group (850-15960) (P < 0.001) (Table 6 and Figure 6).

The results of this analysis demonstrated a significant decrease in the expression of SOD in colitic controls to 165 (10-1000) (P < 0.001) when compared to the non-colitic group of 400 (80-1115). The treatment of the animals with *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) increased the expression of SOD to 400 (50-1480) (P < 0.05) and 435 (20-1650) (P < 0.001) respectively, compared to the colitic control (Table 6 and Figure 6).

DISCUSSION

A promising area for research, many plant species and their chemical constituents exert therapeutic actions. This has led to the development of new, effective and safe drugs for the treatment of various pathological processes. There is also an interest in targeted therapy for diseases derived from oxidative stress, such as IBD[11,25].

IBDs are progressive and destructive chronic disorders of the GI tract, the most common being CD or UC. There is evidence that the pathogenesis of IBD is related to a dysfunctional interaction between the bacteria of the intestinal microflora and mucosal immune system[26,27].

CD and UC are immunologically different diseases. CD is characterized by an exaggerated cellular Th1 response (CD4+) and Th17, characterized by high levels of INF-γ/IL-17 and IL-12/IL-23. UC is characterized by a heightened Th2 response, and excessive IL-5 and IL-13[28-30].

TNBS is a hapten, administered by enema in rats in combination with 50% ethanol to break the mucus barrier and facilitate penetration of the hapten into the intestinal epithelium. TNBS reacts with autologous proteins and stimulates the development of hypersensitivity, leading to the activation of antigen-specific T cells. The immune response induced by the hapten causes severe ulceration of the mucosal and epithelial barrier, characterized by trans-mural infiltration of mononuclear cells[20].

Preventive treatment with *Cd*-EtOHE (62.5 to 125 mg/kg) or *Cd*-HexP (31.25 and 62.5 mg/kg) caused a significant reduction in the severity and extent of injury as reflected in the macroscopic lesion score. The macroscopic/microscopic damage scores and colon weight/length ratio can be considered as sensitive and reliable markers to estimate the severity of the disease, and thus the anti-inflammatory effect promoted by the test drug[31].

A low incidence of diarrhea in animals treated with *Cd*-EtOHE and *Cd*-HexP was also observed. Diarrhea is a major symptom of disease in both animals and humans and indicates loss of the absorptive capacity of the colon, which is impaired in intestinal infla-

Figure 3 Representative macroscopic images of rat colonic mucosa in non-colitics (A), colitics (B), *Cd*-EtOHE at 125 mg/kg (C), and *Cd*-HexP at 62.5 mg/kg (D). *Cd*: *Combretum duarteanum*; HexP: Hexane phase; EtOHE: Ethanolic extract.

Figure 4 Representative histological appearance of rat colonic mucosa in the non-colitic group (A), colitic group (B), *Cd*-EtOHE (C), and *Cd*-HexP (D). Aumento magnification × 40 (*goblet cells; → ulceration region; 50 μm). Colonic tissue sections were stained with hematoxylin and eosin, and observed under light microscope (magnification × 40). *Cd*: *Combretum duarteanum*; HexP: Hexane phase; EtOHE: Ethanolic extract.

mmation[32]. The results suggest that treatment with the plant samples restored the intestinal absorptive capacity.

Cd-HexP at 62.5 mg/kg significantly reduced the weight/length ratio. This effect is possibly related to the scavenging capacity of free radicals caused by treatment at this dose[33]. These results demonstrate, for the first time, anti-inflammatory intestinal activity

for a species belonging to the genus *Combretum*.

The more effective doses of *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) were selected to investigate their effects in the chronic phase with relapse in TNBS-induced UC in rats. This model mimics the disease in humans and can be used to evaluate new treatments potentially applicable in IBD[31].

Cd-EtOHE at 125 mg/kg or *Cd*-HexP at 62.5 mg/kg

Figure 5 Effect of acute administration of *Cd*-EtOHE at 125 mg/kg and *Cd*-HexP at 62.5 mg/kg on myeloperoxidase activity (MPO, U/mg protein) (A), tumor necrosis factor-alpha (TNF-α, pg/mg protein) (B), interleukin-1β (IL-1β, pg/mg protein) (C), interleukin-10 (IL-10, pg/mg protein) (D) in trinitrobenzene sulfonic acid-induced colitis model with relapse in rats (TNBS, 10 mg/animal). Data are expressed as the mean ± SEM. [a]$P < 0.05$, [b]$P < 0.01$ and [c]$P < 0.001$ *vs* colitic group. [d]$P < 0.05$, [e]$P < 0.01$ and [f]$P < 0.001$ *vs* non-colitic group. *Cd*: *Combretum duarteanum*; HexP: Hexane phase; EtOHE: Ethanolic extract.

Table 6 Effect of oral administration of *Cd*-EtOHE or *Cd*-HexP for 21 d on the expression of cyclooxygenase-2, proliferating cell nuclear antigen and superoxide dismutase in trinitrobenzenesulfonic acid-induced ulcerative colitis in rats

Group	Dose (mg/kg)	COX-2 (μm^2)	PCNA (μm^2)	SOD (μm^2)
Non-colitics	-	230 (110-700)	1425 (60-7890)	400 (80-1.115)
Colitics	-	505 (100-1.450)[d]	5380 (850-15.960)[f]	165 (10-1.000)[f]
Cd-EtOHE	125	90 (10-2.590)[c]	1630 (90-14.790)[c]	400 (50-1.480)[c]
Cd-HexP	62.5	205 (20-790)[c]	1570 (250-9.500)[c]	435 (20-1.650)[c]

Results are expressed as median (minimum-maximum) of the analyzed parameters (n = 7-9). For nonparametric data, median (minimum-maximum) was used, with Kruskal-Wallis and Dunn's test *a posteriori*. [c]$P < 0.001$ *vs* colitic group; [d]$P < 0.05$ and [f]$P < 0.001$ *vs* non-colitic group. COX-2: Cyclooxygenase-2; PCNA: Proliferating cell nuclear antigen; SOD: Superoxide dismutase; *Cd*: *Combretum duarteanum*; EtOHE: Ethanolic extract; HexP: Hexane phase.

significantly decreased signs of disease, such as macroscopic lesion (lesion area and score), weight/length ratio and diarrhea, showing the anti-inflammatory effect at 21 d of treatment.

Intestinal inflammation in the TNBS-induced model promoted the loss of 8% to 10% of body weight notably at 1 wk after induction, this is related to reduction in food intake due to abdominal pain and diarrhea during the active phase of the disease[34]. Treatment with *Cd*-HexP at 62.5 mg/kg reversed low water and food intake (and body weight loss) caused by disease.

The spleen recycles and acts as a reserve of red blood cells. The organ is the center of reticulo-endothelial system activity, and is an essential part of the immune system[35]. A significant increase in spleen weight of colitic animals when compared to non-colitic animals was demonstrated. UC and CD are mentioned in lists of factors that can cause increased spleen size, which can occur through lymphoid cell accumulation in immune functions[35,36].

MPO is an enzyme found in neutrophils and has been used as a quantitative index of neutrophil influx into inflamed intestines. The recruitment and activation of neutrophils results in a significant increase in free radical production, capable of overcoming antioxidant protections, and resulting in oxidative stress and inflammation[37,38]. *Cd*-EtOHE at 125 mg/kg and *Cd*-HexP at 62.5 mg/kg significantly decreased MPO activity when compared to colitic animals. This may

Figure 6 Photomicrograph of colonic samples from rats submitted to trinitrobenzene sulfonic acid-induced colitis model with relapse in rats after 21 d of treatment. A: Non-colitic group; B: Colitic group; C: *Cd*-EtOHE at 125 mg/kg; D: *Cd*-HexP at 62.5 mg/kg. Immunohistochemical localization of Line Ⅰ: COX-2; Line Ⅱ: PCNA; Line Ⅲ: SOD (→ COX-2, PCNA and SOD respectively). COX-2: Cyclooxygenase-2; PCNA: Proliferating cell nuclear antigen; SOD: Superoxide dismutase; *Cd*: *Combretum duarteanum*; HexP: Hexane phase; EtOHE: Ethanolic extract.

be interpreted as a manifestation of anti-inflammatory effect for *C. duarteanum* species.

The inflammation in the TNBS-induced colitis model is characterized by a Th1 pathway immune response, in which there is an increase in TNF-α, IL-1β, IL-12, IL-17, IL-18 and IL-6[39-41].

Up-regulation of the inflammatory state with increased TNF-α and IL-1β levels in colitic rats was observed, which corroborates the literature findings[42]. The treatment with *Cd*-EtOHE at 125 mg/kg or *Cd*-HexP at 62.5 mg/kg was able to significantly reduce TNF-α levels when compared to the colitic controls, suggesting that TNF-α suppression is related to the anti-inflammatory effect promoted by the vegetable samples studied. *Cd*-EtOHE at 125 mg/kg was able to decrease IL-1β levels, suggesting that compounds of this plant sample may interfere with the synthesis machinery of IL-1β activation by inhibiting production[10,43,44].

IL-10 suppresses production of pro-inflammatory cytokines, such as IL-12, IL-6, IL-1 and TNF-α, in activated macrophages *in vitro,* and blocks the ability of macrophages stimulating the production of interferon by Th1 cells. IL-10 is produced in large amounts by TCD4+ regulatory cells subtype (Tregs). Tregs maintain homeostasis by suppressing the adaptive response of T cells and preventing autoimmunity[45].

A significant elevation in IL-10 levels in animals treated with *Cd*-EtOHE at 125 mg/kg or *Cd*-HexP at 62.5 mg/kg, compared to the colitic group,

was demonstrated. This increase is attributable to compensatory mechanisms against colonic injury, possibly playing a role in reducing mucosal inflammation and preventing it from becoming uncontrolled. IL-10 down-regulates antigen presentation, and thereafter the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6[46,47].

Studies have shown that COX-2 is expressed predominantly in experimental colitis. Colotic humans and animals show considerable improvement in the inflammatory process when COX-2 inhibitors are used[48]. The vegetable samples tested prevented the increase in expression of this enzyme, and suggest that the intestinal anti-inflammatory effect promoted by *Cd*-EtOHE at 125 mg/kg or *Cd*-HexP at 62.5 mg/kg is mediated by a reduction in COX-2 expression.

PCNA is an intra-nuclear protein, whose expression is related to cell proliferation and DNA repair. It is highly expressed during the S phase of the cell cycle[49]. Studies show that PCNA expression is up-regulated during chronic inflammation, inducing the proliferation of epithelial cells to repair the mucous tissue[50,51].

The positive expression of PCNA was increased in colitic animals in this study, while *Cd*-EtOHE at 125 mg/kg or *Cd*-HexP at 62.5 mg/kg treatment significantly decreased expression of this protein. Treatment with the plant samples studied protected against intestinal epithelial cell damage induced by TNBS and the effect was mediated *via* regulation of PCNA.

SOD is a key enzyme that converts superoxide to H_2O_2, a more stable metabolite. During oxidative stress and inflammation, SOD activity is decreased in inflamed tissues as compared to non-inflamed tissues. The decreased SOD activity allows superoxide accumulation and subsequent oxidative effects in the intestinal tissue, as well as increased expression of adhesion molecules[52].

Treatment with *Cd*-EtOHE at 125 mg/kg or *Cd*-HexP at 62.5 mg/kg significantly increased the expression of this enzyme when compared to colitic animals, suggesting involvement of an antioxidant effect in the intestinal anti-inflammatory activity promoted by the vegetable samples.

In conclusion, *C. duarteanum* presents promising anti-inflammatory intestinal effects that are related to reduced levels of the pro-inflammatory cytokines (TNF-α and IL-1β) and increased anti-inflammatory cytokine (IL-10), which feature regulatory effects on the immune response, with reduction in the expression of COX-2, PCNA, and an increase in the antioxidant enzyme SOD.

ACKNOWLEDGMENTS

COMMENTS

Background

A variety of herbal medicines have been shown to produce promising results in the treatment of peptic ulcer and inflammatory bowel disease. *Combretum duarteanum* (*C. duarteanum*, *Cd*), the species selected for this study, is popularly known as "mufumbo, cipiúba, cipaúba". In folk medicine, it is used to treat pain, inflammation and gastrointestinal (GI) tract disorders. Given the need for new inflammatory bowel disease therapies, this study aimed to evaluate, for the first time, the intestinal anti-inflammatory activity promoted by the species *C. duarteanum*, validating its popular use and contributing to the search for new therapies for diseases that affect the GI tract.

Research frontiers

C. duarteanum has presented *in vitro* and *in vivo* anti-inflammatory, antinociceptive and antioxidant capacities. Furthermore, it has demonstrated low toxicity, gastroprotective and antiulcer activity in different models of acute ulcer induction (acidified ethanol, ethanol, nonsteroidal anti-inflammatory drugs, stress, pylorus ligature and acetic acid) in animals.

Innovations and breakthroughs

This study evaluated, for the first time, the intestinal anti-inflammatory activity promoted by the species *C. duarteanum* Cambess.

Applications

This study validated the popular use of *C. duarteanum* and contributes to the search for new therapies for diseases that affect the GI tract.

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ORIGINAL ARTICLE

Basic Study

Pharmacokinetics and pharmacodynamics of Da-Cheng-Qi decoction in the liver of rats with severe acute pancreatitis

Yu-Mei Zhang, Hong-Yu Ren, Xian-Lin Zhao, Juan Li, Jun-Yi Li, Fu-Sheng Wu, Hang Su, Wen-Fu Tang

Yu-Mei Zhang, Hong-Yu Ren, Juan Li, Jun-Yi Li, Wen-Fu Tang, Sichuan Provincial Pancreatitis Center, Department of Integrative Medicine, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Fu-Sheng Wu, Hang Su, School of Acupuncture, Chengdu University of Traditional Chinese Medicine, Chengdu 610075, Sichuan Province, China

Xian-Lin Zhao, Department of Integrative Medicine, Chengdu Integrated TCM and Western Medicine Hospital, Chengdu 610016, Sichuan Province, China

Abstract

AIM

To explore the pharmacokinetics and pharmacodynamics of Da-Cheng-Qi decoction (DCQD) in the liver of rats with severe acute pancreatitis (SAP) based on an herbal recipe tissue pharmacology hypothesis.

METHODS

Healthy male Sprague-Dawley rats were randomly divided into a sham operation group (SOG); a model group (MG); and low-, median- and high-dose treatment groups (LDG, MDG, and HDG, respectively). Different dosages (6, 12 and 24 g/kg for the LDG, MDG, and HDG, respectively) of DCQD were administered to the rats with SAP. The tissue concentrations of aloe-emodin, rhein, emodin, chrysophanol, honokiol, rheo chrysophanol, magnolol, hesperidin, naringenin and naringin in the liver of the treated rats were detected

by high-performance liquid chromatography tandem mass spectrometry. Alanine transaminase (ALT) and aspartate transaminase (AST) in serum, inflammatory mediators in the liver and pathological scores were evaluated.

RESULTS

The major components of DCQD were detected in the liver, and their concentrations increased dose-dependently. The high dose of DCQD showed a maximal effect in ameliorating the pathological damages, decreasing the pro-inflammatory mediators tumor necrosis factor-α and interleukin (IL)-6 and increasing anti-inflammatory mediators IL-4 and IL-10 in the liver. The pathological scores in the pancreas for the MG were significantly higher than those for the SOG ($P < 0.05$). DCQD could reduce the pathological scores in the pancreas and liver of the rats with SAP, especially in the HDG. Compared to the SOG, the ALT and AST levels in serum were higher in the MG ($P < 0.05$), while there was no statistical difference in the MG and HDG.

CONCLUSION

DCQD could alleviate liver damage by altering the inflammatory response in rats with SAP based on the liver distribution of its components.

Key words: Pharmacokinetics; Pharmacodynamics; Da-Cheng-Qi decoction; Acute pancreatitis; Acute liver injury

Core tip: Our study group had raised the herbal recipe tissue pharmacology hypothesis, which assumed that the effect of herb formula is related to its target tissue distributions or concentrations of the effective components in target tissues. This study was to investigate the mechanism by which Da-Cheng-Qi decoction (DCQD) ameliorates acute liver injury complicated with severe acute pancreatitis in rats by detecting the tissue distributions of the components from DCQD in the liver and the inflammatory mediators as well as the pathological scores.

INTRODUCTION

Acute pancreatitis (AP) is a sudden inflammation of the pancreas and remote tissues. Approximately 20% of AP patients develop a severe form with systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), which can affect the lung, kidney, intestine, liver and heart[1,2]. One retrospective study showed that hepatic failure associated with a high mortality rate was present in 83% of patients with severe acute pancreatitis (SAP) (among the 178 consecutive patients with SAP admitted to the surgical department from 1994 to 1998, 113 treated in the general intensive care unit were included in the study)[3]. The liver triggering massive inflammatory response during AP was first described in 1996[4]. Inflammatory mediators from the pancreas can directly injure liver cells *via* the portal vein and can also stimulate Kupffer cells to express tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6) and other cytokines that are involved in the development of local and systemic inflammatory responses associated with SAP[5]. Additionally, the activation of Kupffer cells mediates lung injury in acute hemorrhagic necrotizing pancreatitis[6,7]. Inhibitors of Kupffer cell activation, such as gadolinium chloride (GdCl$_3$), can decrease serum TNF-α levels and relieve remote organ injury in AP[6]. In the early stage of SAP, the liver stimulates TNF-α generation. However, with the progression of the disease, excessive TNF-α was released into the blood circulation, which led to remote organ damages; this indicated that liver injury plays a central role in the development of MODS in SAP[8]. Therefore, it is important to improve therapies for liver injury to decrease the severity of SAP.

Da-Cheng-Qi decoction (DCQD), a famous traditional Chinese medicine (TCM) prescription, has been widely used for the treatment of AP for over 30 years in China[9]. DCQD is composed of *Rheum palmatum* L. (Dahuang, dried marshmallow root or rhizome, bitter), *Magnolia henryi* Dunn (Houpu, tree bark, bitter), *Citrus aurantium* L. (Zhishi, dried immature fruit, bitter), and *Natrii Sulfas* (Mangxiao, Na$_2$SO$_4$·10H$_2$O, salty)[9]. It has been reported that DCQD can promote gastrointestinal motility, inhibit cytokine activity and inflammatory response, and relieve acute lung injury in AP[10]. Furthermore, our previous study confirmed that the effects of DCQD on the pancreas, intestine and lung were associated with the tissue distribution of its potential target components[11].

We hypothesized that the tissue pharmacology of the herbal recipe is related to its target tissue distribution or the concentration of its effective components in target tissues[12]. This study aimed to explore the relationship between the effects of DCQD and the distribution/concentration of its absorbed components in the liver of rats with SAP.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats ($n = 30$) weighing 220 ±

15 g were purchased from the Experimental Animal Center of West China Hospital (Chengdu, China). All of the animals were fed and handled according to the Guide for the Care and Use of Laboratory Animals of Sichuan University (Chengdu, China) and the Animal Ethics Committee Guidelines of the Animal Facility of the West China Hospital (protocol number: 2016001A, Chengdu, China). After one week of acclimation, the rats were fasted for 24 h before the induction of SAP and were kept under food-free conditions throughout the experiment.

DCQD preparation
The Dahuang, Houpu, Zhishi and Mangxiao spray-dried drug powders were purchased from Chengdu Green Herbal Pharmaceutical Co. Ltd. (Chengdu, China). The crude formula components were extracted twice by refluxing with boiling distilled water (1:12, g/mL) for 1 h, and the obtained solution was concentrated and spray-dried. The dry powder was stored at 4 ℃ until use. DCQD comes from Shang-Han-Lun, a classic TCM book in China, in which the described dosages are 12 g, 24 g, 12 g and 9 g, respectively, and 57 g of DCQD per person (60 kg body weight) is suggested. According to the Method of Pharmacology, the dosage for rats is adjusted to 1/6-1/20 of the human dosage. We chose 1/6.3 to 1/18.9, meaning that the lowest dosage was 6 g/kg body weight (0.6 g/100 g). As previously described[11], the spray-dried powders were mixed and reconstituted with sterile distilled water according to a standard ratio (12:24:12:9) at different concentrations for the crude drug, giving 0.6 g/mL, 1.2 g/mL and 2.4 g/mL of DCQD.

Induction of AP and intervention
As previously described[11], the rats were randomly divided into a sham operation group (SOG), a model group (MG), a low-dose treatment group (LDG, 6 g/kg BW), a medium-dose treatment group (MDG, 12 g/kg BW) and a high-dose treatment group (HDG, 24 g/kg BW). After anesthetization with 10% chloral hydrate injected into the abdominal cavity at 3 mL/kg body weight, SAP in rats was induced by retrograde injection of 3.5% sodium taurocholate (Sigma, St. Louis, MO, United States) into the biliopancreatic duct (1 mL/kg body weight) at a rate of 0.2 mL/min with a micro-infusion pump[11]. The SOG received a similar injection procedure but with saline. After the rats recovered from the anesthesia, DCQD was administered intragastrically to rats 2 h after operation at the corresponding dosages. Rats in the SOG and MG were given equal volumes of saline.

Pancreatic and hepatic histopathology
At 24 h after operation, the rats were sacrificed and pancreas and liver samples were collected for pathological analysis. These tissue samples were fixed with 10% neutral formalin, embedded in paraffin, cut into sections and stained with hematoxylin and eosin. All of the pathological sections were scored in a blinded fashion by two independent pathologists using a scoring system for the extent and severity of tissue injury (0-4, representing edema, neutrophil infiltration, necrosis, and hemorrhage)[11,13].

Measurement of inflammatory mediator levels in the liver and ALT and AST levels in serum
The homogenate tissue levels of inflammatory mediators, including TNF-α, IL-6, IL-4 and IL-10, were measured using a Milliplex MAP Rat Cytokine/Chemokine magnetic bead immunoassay kit (Millipore Corporation, Billerica, MA)[11]. The values were read with a MAGPIX Luminex xMAP instrument (Luminex Corp, Austin, TX) and analyzed with the MILLIPLEX Analysis software version 3 (Millipore Corporation, Billerica, MA)[11]. Blood samples (5 mL) were collected for centrifugation at 3000 rpm for 7 min at low temperature, and then, the supernatants were obtained for the alanine transaminase (ALT) and aspartate transaminase (AST) detection with an Automatic Biochemical Analyzer (AU5400, SIEMENS, Munich, Germany).

Measurement of DCQD component concentrations in the liver
The concentrations of the 10 major components of DCQD (aloe-emodin, rhein, emodin, chrysophanol, honokiol, rheo chrysophanol, magnolol, hesperidin, naringenin and naringin) in liver tissue homogenates (10%) were measured by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) for the tissue distribution analysis[11]. The HPLC-MS/MS system consists of an SIL-HTc auto-sampler (Shimadzu, Kyoto, Japan), an LC-10ADvp pump (Shimadzu, Kyoto, Japan), and an API3000 triple-quadrupole LC-MS system (Applied Biosystems, Foster City, CA, United States). The conditions for this system were described previously[11,14]. The mean contents of the components of DCQD, which were detected three times in our previous study, were as follows: rhein, 0.86 mg/g; emodin, 2.48 mg/g; aloe-emodin, 1.73 mg/g; chrysophanol 0.55 mg/g; rheochrysidin, 2.61 mg/g; naringin, 3.83 mg/g; naringenin 4.16 mg/g; hesperidin, 11.06 mg/g; honokiol, 1.26 mg/g; and magnolol, 1.11 mg/g[15].

Preparation of standard and quality control samples
Our study group had detected the ten components in a previous study by HPLC-MS/MS[14]. Quality control (QC) samples were prepared to obtain the following plasma concentrations for the examined components: (1) 3750, 625, 156.25 and 39.06 ng/mL for rhein; (2) 100, 25 and 6.25 ng/mL for emodin; (3) 600, 100, 25 and 6.25 ng/mL for aloe-emodin, chrysophanol, naringin, naringenin, hesperidin, magnolol and honokiol; and (4) 120, 20, 5 and 1.25 ng/mL for rheochrysidin. The spiked plasma samples (standard and QC samples)

Figure 1 Liver distribution of the ten major components of Da-Cheng-Qi decoction in rats with severe acute pancreatitis. Rats (*n* = 6 per group) were orally administered with different dosages of Da-Cheng-Qi decoction (DCQD) (0.6 g/mL for the low-dose group, 1.2 g/mL for the medium-dose group, and 2.4 g/mL for the high-dose group) 2 h after operation. After 24 h, the livers were collected to examine the DCQD component concentrations using a sensitive HPLC-MS/MS method. The results are represented as the mean ± SD.

Figure 2 Effect of different dosages of Da-Cheng-Qi decoction on inflammatory mediators in liver tissues from rats with severe acute pancreatitis. Rats (*n* = 6 per group) were orally administered with different doses of Da-Cheng-Qi decoction (DCQD) (6 g/kg for the LDG, 12 g/kg for the MDG, and 24 g/kg for the HDG) 2 h after operation. After 24 h, the livers were collected to examine pro-inflammatory cytokine (IL-6 and TNF-α) and anti-inflammatory cytokine (IL-4 and IL-10) levels. The inflammatory cytokines were measured by ELISA. The results are represented as the mean ± SD. [a]*P* < 0.05 *vs* SOG and [b]*P* < 0.05 *vs* MG. SOG: Sham operation group; MG: Model group; LDG: Low-dose group; MDG: Medium-dose group; HDG: High-dose group.

were pretreated and detected in each analytical batch along with the unknown samples[14]. The detected DCQD samples were stored in the Public Experiment Platform at West China Hospital (Chengdu, China).

Data collection, peak integration, and calibration were all calculated with the Analyst 1.4.2 software. Calibration curves were plotted according to the peak ratios of the analytes to the internal standards (ibuprofen), and the linear regression between the tissue concentration and the peak area ratio was determined by $1/\chi^2$. The concentrations of QC and unknown samples were measured by interpolation from the calibration curves[14].

Statistical analysis
All of the data were processed with statistical software PEMS 3.1. All of the values are expressed as the mean ± SD. One-way repeated-measure ANOVA, followed by multiple pair-wise comparisons using

the Student-Neuman-Keuls procedure, was used to detect differences among the groups. *P* < 0.05 was considered significantly different.

RESULTS

Major components of DCQD distributed in liver tissues
The ten major components of DCQD were measured in liver tissues. Chrysophanol, emodin, magnolol, rhein, aloe-emodin and rheo chrysophanol increased dose-dependently with the DCQD dosage. The concentration of naringenin was the highest (Figure 1).

In hepatic tissue, the pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) displayed a significant increase in the MG compared with the SOG (*P* < 0.05). Compared to the MG, the expression of pro-inflammatory cytokines in the liver tissues decreased in the DCQD-treated groups with the lowest levels (*P* < 0.05), while the levels of anti-inflammatory cytokines were significantly increased and their levels were the highest in the HDG (*P* < 0.05) (Figure 2).

DCQD alleviates the pathological damage in the pancreas and liver of rats with AP
The pancreas of rats in the SOG was slightly edematous without obvious acinar tissue hemorrhage and necrosis and the liver had no obvious changes. However, the pancreas of rats in the MG showed distinct necrosis, interstitial edema, infiltration of neutrophil and mononuclear cells, and hemorrhage in the tissues. The liver tissues of rats in the MG showed mainly edema and infiltration of neutrophil without necrosis. After treatment with DCQD, we found a significant reduction in inflammatory cell infiltration, hemorrhage, necrosis and interstitial edema in the tissues, and the effects were the best in the HDG. DCQD could reduce the pathological scores in the pancreas and liver of rats with SAP, especially in the HDG (Figure 3).

DCQD has no obvious influence on the ALT and AST levels in serum
Based on the highest distribution in the liver tissues of the major components of DCQD in the HDG, the ALT and AST levels were detected in the SOG, MG and HDG. Compared to the SOG, the ALT and AST levels in serum were higher in the MG (*P* < 0.05), while there was no statistical difference between the MG and HDG (Figure 4).

DISCUSSION

In our study, we found that the components of DCQD in liver tissues were similar to those in plasma and pancreas or intestine tissues as detected by HPLC-MS/MS[11,14]. As shown above, the concentration of naringenin in the liver was the highest of the components, which was also true in the intestine[11]. How-

Figure 3 Da-Cheng-Qi decoction alleviates pancreas and liver damages in rats with severe acute pancreatitis. Rats (*n* = 6 per group) were orally administered with different doses of Da-Cheng-Qi decoction (DCQD) (6 g/kg for the LDG, 12 g/kg for the MDG, and 24 g/kg for the HDG) 2 h after operation. At 24 h after operation, pancreas and liver samples were collected for pathological analysis and stained with hematoxylin and eosin (HE); A: Pathological pictures of the pancreas (the left) and liver tissues (the right, HE, × 200); B: Pathological scores for the pancreas and liver damages. The results are represented as the mean ± SD. [a]*P* < 0.05 *vs* SOG and [b]*P* < 0.05 *vs* MG. SOG: Sham operation group; MG: Model group; LDG: Low-dose group; MDG: Medium-dose group; HDG: High-dose group.

Figure 4 Da-Cheng-Qi decoction has no obvious effect on the alanine transaminase and aspartate transaminase levels in serum in rats with severe acute pancreatitis. Rats (*n* = 6 per group) were orally administered with different doses of Da-Cheng-Qi decoction (DCQD) (24 g/kg for the HDG) 2 h after operation. At 24 h after administration, blood samples were collected for detection. The results are represented as the mean ± SD. [a]*P* < 0.05 *vs* SOG. SOG: The sham operation group; MG: Model group; HDG: High-dose group; ALT: Alanine transaminase; AST: Aspartate transaminase.

ever, there were still differences in the distribution of these components in the liver. In the pancreas, the concentration of rhein was the highest in the HDG[11], while naringenin had the highest concentration in the liver, followed by aloe-emodin at 24 h after induction of AP (Figure 1), indicating that naringenin and aloe-emodin might be the major target components of DCQD associated with liver damage in SAP. As described previously, the target tissues of aloe-emodin might be the liver and kidney[16]. In combination with our studies[11,14], the distribution of these DCQD components includes different target tissues, which is consistent with the hypothesis regarding the tissue pharmacology in the herb recipe[12]. For example, the concentration of rhein was highest in the pancreas, the

concentration of emodin was highest in the lung, and that of naringenin was highest in the intestine and liver at 24 h after induction of AP[11]. Blood-tissue barriers may contribute to these phenomena[11]. To date, most studies mainly focused on the blood-brain barrier and gut barrier, with research on the blood-pancreas juice barrier (BPJB) and the liver barrier being rare. It has been reported that the BPJB might lead to the selective excretion of some antibiotics, such as chloramphenicol in dogs with chronic pancreatic fistula[17] and anti-tumor drugs including 5-fluorouracil and mitomycin (MMC) during pancreatic adenocarcinoma chemotherapy[18]. We know that the liver barrier consists of a cell-cell barrier, a hepatic cell-blood barrier (the liver sinusoidal cell populations (LSECs) of the reticulo-endothelial

system) and a blood-gall barrier. The LSECs of the reticulo-endothelial system could clear the pro-inflammatory molecules derived from the gut, such as lipopolysaccharide (LPS), in the absence of any signs of inflammation[19,20]. Future research could pay more attention to the BPJB and liver barrier, which may play important roles in the treatment of AP with herbal medicine[3].

AP is a potentially lethal acute inflammatory disease mediated by pro- and anti-inflammatory mediators released from the pancreas and other sources[21,22]. During SAP, the pro- and anti-inflammatory responses occurred early and persisted in the systemic circulation for several days[23]. Their main sources are tissue macrophages, and they attract neutrophils and additional macrophages and induce the production of proteases, elastases, and phospholipases, which can cause tissue damage[24]. The pro-inflammatory serum mediator IL-6 increased early and sensitively predicts severity in patients with AP[25]. The release of IL-6 in inflamed pancreatic tissue is associated with the development of remote organ dysfunction[26]. Furthermore, the induction of the hepatic production of acute phase proteins contributes to the pathophysiological roles of IL-6 in the acute phase response[27]. TNF-α is the most important factor involved in inflammatory response and shock, not only inducing apoptosis and necrosis but also entering blood circulation to induce the expression of interleukins, such as IL-6, which can cause inflammatory response waterfall cascades and lead to SIRS[28]. However, the anti-inflammatory cytokines IL-4 and IL-10 can inhibit macrophage function[29]. IL-10 is a marker of Th2 lymphocyte activity[30] and shows a positive effect on the proliferation and differentiation of B lymphocytes, which promote the production of immunoglobulins[22]. IL-10 can reduce the free oxygen radicals affecting macrophages and T-helper lymphocytes[22] and lower necrosis and mortality in AP[31]. IL-10 can also inhibit the secretion of pro-inflammatory cytokines, including TNF-a, IL-1β, IL-6, and IL-8[29]. It appears that balancing pro-inflammatory and anti-inflammatory cytokines is crucial in the progression of inflammation in AP[32].

In this study, we examined the levels of IL-4, IL-6, IL-10 and TNF-α as predictors of inflammatory changes in AP. Our results showed that IL-6 levels were higher than those of other inflammatory mediators, while TNF-α levels were lowest in the liver. The role of pro-inflammatory IL-6 in the prediction of AP severity seems more valuable than TNF-α, which is in line with other studies[11,25]. We found that the pro-inflammatory cytokines IL-6 and TNF-α and the anti-inflammatory cytokines IL-4 and IL-10 displayed a relative balance (Figure 2). DCQD treatment could increase the expression of IL-4 and IL-10 and inhibit the expression of TNF-α and IL-6, which ameliorated SAP-associated liver damages; better effects were observed at higher DCQD dosages (Figure 2). These data provided evidence for the regulatory effect of

DCQD on the balance of pro-inflammatory and anti-inflammatory mediators to ameliorate liver damages and reduce AP severity. This effect was similar to the effects of DCQD on lung, pancreas and intestine damages[11]. Similar results can be found in other studies. It has been reported that DCQD could alleviate liver injury by decreasing the levels of TNF-α, IL-6 and NO[33]. DCQD might promote the synthesis of hepatic cell RNA to maintain the structure and function of hepatic cells[34]. Purgative therapy with DCQD improved hepatocyte apoptosis in rats with acute hepatic injury induced by LPS/D-galactosamine by down-regulating the caspase-2 and BAX protein expression, up-regulating BCL-2 expression and adjusting the BCL-2/BAX balance[35]. As mentioned above, naringenin might be the major DCQD target component in the liver of rats with SAP. It has been reported that naringenin increases resistance to oxidative stress and inflammation and protects against multiple organ injury in various animal models[36]. Furthermore, naringenin reduces macrophage infiltration by significantly decreasing hepatic pro-inflammatory mediators and the expression of relevant genes including TNF-α, IL-6, IL-1β, inducible nitric oxide synthase, matrix metalloproteinases (MMP-2 and -9), and EGF-like module-containing mucin-like hormone receptor-like 1[36]. Our study found that aloe-emodin might be another effective target component of DCQD in the liver. Hu *et al*[37] showed that aloe-emodin conferred anti-inflammatory effects through a likely mechanism involving a decrease in pro-inflammatory cytokine production *via* inhibition of the nuclear factor κB (NF-κB), MAPK, and PI3K pathways. Of course, other components could also cause anti-inflammatory effects. For example, rhein could inhibit NF-κB activation and sequentially suppress its downstream targets, including inducible nitric oxide synthase, IL-6, and TNF-α, by inhibiting IKKβ in LPS-activated macrophages[38]. Emodin could enhance peritoneal macrophage (p$M\Phi$) phagocytosis and apoptotic cell clearance by altering intercellular adhesion molecule-3 expression in SAP or SIRS[39]. Based on the close relationship between DCQD or its components and inflammatory mediators, future studies should focus on the molecular mechanism of the components and their target tissues.

According to our results, the effect of DCDD on the liver damages was mainly dose-dependently improving the inflammatory injuries[11]. As early as 1998, Zhao *et al*[40] had determined that the inhibitory effect of DCQD on acute phase protein levels was dose-dependent in MODS. It was recently reported that DCQD improved the pathological scores and decreased serum amylase and lipase dose-dependently in a study showing that DCQD attenuates inflammatory responses by inhibiting the high mobility group box 1 -mediated NF-x03BA, B and P38 MAPK signaling pathways in SAP[41]. Additionally, the concentrations of nine major components of DCQD, all except for

naringenin, were increased in a dose-dependent manner (Figure 1). The concentrations of the ten major components of DCQD increased dose-dependently in the intestine[11]. Therefore, a DCQD dose-concentration effect may exist for the treatment of AP, which would support the tissue pharmacology herbal recipe. However, studies substantiating these results remain rare. In this study, DCDD showed no obvious effect in ameliorating the ALT and AST levels. We considered that the action time of the single dose of DCQD might be too short or the dosage of DCQD and the concentrations of the components in the tissues was not enough to reduce the ALT and AST levels. This issue needs to clarify in further studies.

In conclusion, most of the DCQD components could be absorbed into the liver of rats with SAP. Additionally, DCQD may ameliorate histopathological damages and inflammatory responses by increasing the expression of anti-inflammatory cytokines and inhibiting the expression of pro-inflammatory cytokines, which may be associated with the DCQD intake dosages.

COMMENTS

Background
Acute lung injury and acute liver injury are the determinants of morbidity and mortality at early stage of sever acute pancreatitis (SAP). The Chinese herbal formula Da-Cheng-Qi decoction (DCQD) may ameliorate acute lung injury complicated with SAP by improving gastrointestinal function and reducing the inflammatory response. However, the mechanism of DCQD in protecting against liver injury during the course of SAP remains unclear.

Research frontiers
Authors tested the herbal recipe tissue pharmacology hypothesis, which proposes that the effect of the herbal formula is related to its target tissue distributions or the concentrations of the effective components in the target tissues. This hypothesis may be important for screening the effectively absorbed components of an herbal formula.

Innovations and breakthroughs
Based on the herbal recipe tissue pharmacology hypothesis, this study confirmed that the tissue distributions of emodin, rhein, aloe-emodin, chrysophanol, rheochrysidin, naringin, naringenin, hesperidin, magnolol and honokiol in the liver of rats treated with high dose DCQD showed a maximal effect in ameliorating the pathological damages. DCQD may ameliorate the liver injury complicated with acute pancreatitis (AP) by alleviating the inflammatory response.

Applications
This study provides insights for the implementation and further screening for effective components of Chinese herbs in ameliorating acute liver injury complicated with AP.

Terminology
A high-performance liquid chromatography tandem mass spectrometry method with a long analytical column and negative MRM mode was confirmed as a specific, sensitive, accurate and reproducible method to successfully identify the 10 major components of DCQD in dog plasma after oral administration.

Peer-review
Overall this is a well conducted animal study and the authors did a very good job to investigate the mechanism by which DCQD ameliorates acute liver injury complicated with AP in rats. It is greatly appreciated.

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ORIGINAL ARTICLE

Basic Study

Hydrogen-rich water protects against inflammatory bowel disease in mice by inhibiting endoplasmic reticulum stress and promoting heme oxygenase-1 expression

Nai-Ying Shen, Jian-Bin Bi, Jing-Yao Zhang, Si-Min Zhang, Jing-Xian Gu, Kai Qu, Chang Liu

Nai-Ying Shen, Jian-Bin Bi, Jing-Yao Zhang, Si-Min Zhang, Jing-Xian Gu, Kai Qu, Chang Liu, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Nai-Ying Shen, Department of General Surgery, Shaanxi Nuclear Geology 215 Hospital, Xianyang 712000, Shaanxi Province, China

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Correspondence to: Chang Liu, MD, PhD, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, No. 277 Yanta West Road, Xi'an 710061, Shaanxi Province, China. liuchangdoctor@163.com
Telephone: +86-29-85323900
Fax: +86-29-85324642

Abstract

AIM

To investigate the therapeutic effect of hydrogen-rich water (HRW) on inflammatory bowel disease (IBD) and to explore the potential mechanisms involved.

METHODS

Male mice were randomly divided into the following four groups: control group, in which the mice received equivalent volumes of normal saline (NS) intraperitoneally (ip); dextran sulfate sodium (DSS) group, in which the mice received NS ip (5 mL/kg body weight, twice per day at 8 am and 5 pm) for 7 consecutive days after IBD modeling; DSS + HRW group, in which the mice received HRW (in the same volume as the NS treatment) for 7 consecutive days after IBD modeling; and DSS + HRW + ZnPP group, in which the mice received HRW (in the same volume

as the NS treatment) and ZnPP [a heme oxygenase-1 (HO-1) inhibitor, 25 mg/kg] for 7 consecutive days after IBD modeling. IBD was induced by feeding DSS to the mice, and blood and colon tissues were collected on the 7th d after IBD modeling to determine clinical symptoms, colonic inflammation and the potential mechanisms involved.

RESULTS
The DSS + HRW group exhibited significantly attenuated weight loss and a lower extent of disease activity index compared with the DSS group on the 7th d ($P < 0.05$). HRW exerted protective effects against colon shortening and colonic wall thickening in contrast to the DSS group ($P < 0.05$). The histological study demonstrated milder inflammation in the DSS + HRW group, which was similar to normal inflammatory levels, and the macroscopic and microcosmic damage scores were lower in this group than in the DSS group ($P < 0.05$). The oxidative stress parameters, including MDA and MPO in the colon, were significantly decreased in the DSS + HRW group compared with the DSS group ($P < 0.05$). Simultaneously, the protective indicators, superoxide dismutase and glutathione, were markedly increased with the use of HRW. Inflammatory factors were assessed, and the results showed that the DSS + HRW group exhibited significantly reduced levels of TNF-α, IL-6 and IL-1β compared with the DSS group ($P < 0.05$). In addition, the pivotal proteins involved in endoplasmic reticulum (ER) stress, including p-eIF2α, ATF4, XBP1s and CHOP, were dramatically reduced after HRW treatment in contrast to the control group ($P < 0.05$). Furthermore, HRW treatment markedly up-regulated HO-1 expression, and the use of ZnPP obviously reversed the protective role of HRW. In the DSS + HRW + ZnPP group, colon shortening and colonic wall thickening were significantly aggravated, and the macroscopic damage scores were similar to those of the DSS + HRW group ($P < 0.05$). The histological study also showed more serious colonic damage that was similar to the DSS group.

CONCLUSION
HRW has a significant therapeutic potential in IBD by inhibiting inflammatory factors, oxidative stress and ER stress and by up-regulating HO-1 expression.

Key words: Hydrogen; Inflammatory bowel disease; Oxidative stress; Endoplasmic reticulum stress; Heme oxygenase-1

Core tip: Inflammatory bowel disease (IBD) is a chronic and relapsing disease primarily caused by the production of pro-inflammatory cytokines and leukocyte infiltration, resulting in structural and functional damage to the bowel. Hydrogen has obvious anti-oxidative and anti-inflammatory effects. We launched a study to investigate the protective role of hydrogen-rich water (HRW) on IBD in mice. The present study found that HRW has a significant therapeutic potential in IBD by inhibiting inflammatory factors, oxidative stress and endoplasmic reticulum stress and by up-regulating heme oxygenase-1 expression.

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic and relapsing disease primarily caused by the production of pro-inflammatory cytokines and leukocyte infiltration, resulting in structural and functional damage to the bowel. It is associated with environmental factors, genetics, microbial factors and so on[1-3]. The major symptoms of IBD include inflammation of the colon, abdominal pain, altered visceral sensation, diarrhea, rectal bleeding, weakness and weight loss[4]. CD is often located in the terminal ileum and/or the colon and characterized by formation of non-caseating granulomas, which are involved in transmural and discontinuous inflammation in the mucosa. In contrast, UC is a colon disorder in which inflammation is restricted to the mucosal and submucosal areas, initially affecting the rectum, but it may extend continuously and diffusely throughout the colon[5].

The major therapeutic goals in IBD patients are the alleviation of inflammation and the attenuation of IBD symptoms, mainly abdominal pain and altered bowel movements. The current range of treatments for IBD covers both conventional and biological therapies. Conventional therapy includes the use of anti-inflammatory drugs, immunosuppressive agents, antibiotics, and probiotics; biological therapy mainly includes the use of different anti-TNF-α agents, and a plethora of other novel biological agents[6]. Dextran sulfate sodium (DSS)-induced IBD in mice is a classical mouse IBD model that is accepted worldwide. The mechanism of DSS-induced colitis is mainly due to the direct toxicity to the colonic epithelial cells, subsequently increasing the permeability of the intestinal mucosa and allowing the transport of luminal bacterial products from the bowel lumen to the submucosal tissue[7,8].

Molecular hydrogen, which has been explored as a new medical gas over the last ten years, is a potent anti-oxidative, anti-apoptotic, and anti-inflammatory agent and an ideal therapy for many diseases[9]. The benefit of hydrogen as a novel anti-oxidant is that it can penetrate cell membrane, diffuse into the cytosol

and target organelles easily, and selectively reduce hydroxide radicals and peroxynitrite without affecting physiological reactive oxygen species (ROS) involved in normal cell signaling[10]. Moreover, hydrogen therapy has been proven to be safe and effective in many clinical trials[11,12]. With respect to intestinal diseases, previous studies have shown that hydrogen may alleviate intestinal ischemia-reperfusion injury, UC, and colon inflammation[13-15]. However, the detailed mechanism responsible for this effect is not yet well illustrated. Hydrogen-rich water (HRW) is an effective, convenient way to deliver molecular hydrogen, which has the same effectiveness as inhaled hydrogen gas and is more suitable for clinical applications. Therefore, the main aim of our study was to assess the protective effect of HRW on IBD in mice and explore the detailed mechanisms involved.

MATERIALS AND METHODS

Experimental animals and preparation of HRW

This study was conducted using male C57BL/6J mice (4-5 wk old, 21-26 g) (Animal Feeding Center of Xi'an Jiaotong University Medical School). The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark cycle, 50% humidity, and *ad libitum* access to food and water) for one week prior to experimentation. All mice were housed (5 per cage) in clear, pathogen-free polycarbonate cages in the animal care facility, and they were fed a standard animal diet (No. 120161128007, Jiangsu Xietong Pharmaceutical Bio-technology Co., Ltd.) and water *ad libitum* under controlled temperature conditions with 12-h light-dark cycles. They were cared in accordance with the Ethical Committee, Xi'an Jiaotong University Health Science Center. The study was reviewed and approved by the Xi'an Jiaotong University Health Science Center Institutional Review Board. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Xi'an Jiaotong University Health Science Center. The animal protocol was designed to minimize pain and discomfort to the animals. All animals were euthanized with isoflurane gas for tissue collection. HRW was produced by Naturally Plus Japan International Co., Ltd. and was stored under atmospheric pressure at 4 ℃ in an aluminum bag with no dead volume, as performed in our previous studies[16-18].

Induction of IBD

IBD was induced by DSS feeding. Male C57BL/6J mice were provided with drinking water containing 5% (wt/vol) DSS (35-50 kDa, Sigma-Aldrich, Steinheim, Germany) *ad libitum* from day 0 to day 5. On days 6 to 7, the animals received tap water (without DSS). Control animals received tap water throughout the entire experiment.

Study design

Mice in the present study were divided into the following four groups: (1) control group, in which the mice received equivalent volumes of normal saline (NS) intraperitoneally (ip); (2) DSS group, in which the mice received NS i.p (5 mL/kg body weight, twice per day at 8 a.m. and 5 p.m.) for 7 consecutive days after IBD modeling; (3) DSS + HRW group, in which the mice received HRW (in the same volume as the NS treatment) for 7 consecutive days after IBD modeling; and (4) DSS + HRW + ZnPP group, in which the mice received HRW (in the same volume as the NS treatment) and ZnPP [a heme oxygenase-1 (HO-1) inhibitor, 25 mg/kg] for 7 consecutive days after IBD modeling. Six mice were used per group in this study. The weight, presence of blood in stool, and gross stool consistency of all mice were monitored daily. Each score was determined as follows: (1) change in weight (0: < 1%, 1: 1%-5%, 2: 5%-10%, 3: 10%-15%, 4: > 15%); (2) blood in stool (0-1: negative; 2-3: hemoccult positive; 4: gross bleeding); and (3) stool consistency (0: normal; 1-2: loose stools; 3-4: diarrhea). The disease activity index was determined by combining the scores from these three categories and dividing that number by three (Supplementary Table 1)[19].

Euthanasia

Mice were sacrificed after being anesthetized with isoflurane gas on the 7th d after IBD modeling, and blood samples were collected from the periorbital plexus. The serum was separated by centrifugation at 3000 *g* for 15 min at 4 ℃. The colon without the cecum was removed immediately from each mouse and stored at -80 ℃ until further analysis.

Macroscopic and microscopic scoring and histological studies

After the mice in all groups were sacrificed, the colon from each mouse was rapidly isolated and weighed with fecal content. The colon was then opened along the mesenteric border, and the fecal material removed. The total macroscopic damage score was calculated for each animal based on the following parameters: fecal blood (0: absence; 1: presence), presence of diarrhea (0: no diarrhea; 1: loosely shaped moist pellets; 2: amorphous, moist, sticky pellets; 3: diarrhea), the extent of colon damage (0: no inflammation; 1: reddening, mild inflammation; 2: moderate or widely distributed inflammation; 3: severe and/or extensively distributed inflammation), colon length (0: < 5% shortening; 1: 5%-14%; 2: 15%-24%; 3: 25%-35%; 4: > 35%) and weight (0: < 5% weight loss; 1: 5%-14%; 2: 15%-24%; 3: 25%-35%; 4: > 35%) (Supplementary Table 2)[20].

Samples from the distal colon were fixed in 10% formalin solution for 24 h, dehydrated and embedded in paraffin. Serial sections of 5-μm thickness were

obtained and stained with hematoxylin and eosin to evaluate the morphology. Two researchers examined the results in a blinded fashion. The microscopic total damage score was assessed using the following parameters: the depletion of goblet cells (0: absence; 1: presence), crypt abscesses (0: absence; 1: presence), the destruction of mucosal architecture (1: normal; 2: moderate; 3: extensive), the extent of muscle thickening (1: normal; 2: moderate; 3: extensive), and the presence and degree of cellular infiltration (1: normal; 2: moderate; 3: transmural) (Supplementary Table 3)[21].

Measurements of cytokines in murine serum

The levels of serum TNF-α, IL-6 and IL-1β were measured with commercial ELISA kits according to the instructions from the manufacturer (Dakewe, Shenzhen, China).

Measurement of colonic oxidative stress

The concentrations of malonaldehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) in colon tissue were measured as markers of oxidative stress of colon tissue. Colon tissues were homogenized on ice in 10 volumes (w/v) of NS. The homogenates were centrifuged at 4000 rpm at 4 ℃ for 15 min for MDA, SOD and GSH detection by using assay kits purchased from Nanjing Jiancheng Corp., China. MDA levels in the supernatants were determined by measurement of thiobarbituric acid (TBA)-reactive substance levels using an MDA assay kit according to the manufacturer's instructions. The samples were heated with TBA under acidic conditions and the pink color formed was read at 532 nm. The results are calculated as nmol/mg protein. SOD activity in the supernatants of colon tissue was evaluated by inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/xanthine oxidase system in accordance with the manufacturer's instructions. The rate of NBT reduction was measured at 560 nm. The results were expressed as U/mg protein. For GSH assay, 5,5'-Dithiobis-2-nitrobenzoicacid (DTNB) was used to develop color. The development of yellow color was monitored at 412 nm on a spectrophotometer. The results are expressed as mg/g protein. For MPO assay, colon samples were homogenized in 5 volumes (w/v) of phosphate buffered saline containing 0.5% hexadecyltrimethylammonium hydroxide. Samples were measured on a spectrophotometry at 460 nm absorbance. One unit of MPO activity is defined as degrading 1 μmol of hydrogen peroxide at 37 ℃, and MPO activity of tissue is expressed as U/g protein.

Western blot analysis

Proteins were extracted from the colon according to the manufacturer's instructions. BCA protein assay kit was used to detect the concentration of extracted proteins. Equal amounts of protein were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then the proteins were transferred onto poly-vinylidene difluoride (PVDF) membranes, which were immunoblotted with the appropriate primary antibody at 4 ℃ overnight. Then the membranes were incubated with secondary antibodies. The anti-p-eIF2α, ATF4, XBP1s, CHOP, HO-1 and β-actin monoclonal antibodies were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. The protein concentration was determined by the BCA method. Western blot analysis was performed as previously described[22].

Statistical analysis

Measurement data are expressed as the mean ± standard error of mean (SEM). Differences between the experimental and control groups were assessed by either the analysis of variance or *t* test, as applicable, using SPSS 18.0 (SPSS, 165 Inc.). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Treatment with HRW significantly alleviates the symptoms of DSS-induced IBD in mice

To investigate the effect of HRW treatment on IBD, the weight change and disease activity index were assessed on the 7th d after IBD modeling (Figure 1). The results showed that the weight of the mice showed a downward trend on day 7 after IBD induction. However, the DSS + HRW group had significantly less weight loss compared with the DSS group on the 7th d (*P* < 0.05). Considering the change in weight, blood in stool, and stool consistency, the disease activity index was calculated. The disease activity index observably increased after DSS treatment, and the DSS + HRW group exhibited a lower extent of disease than the DSS group (*P* < 0.05).

Treatment with HRW markedly ameliorates colonic damage in DSS-induced IBD

Mice were sacrificed, and the colons were assessed on the 7th d after IBD modeling. We discovered that the average length of the colons exhibited a significant reduction after DSS administration. More importantly, HRW exerted a protective effect against the shortening of the colon in the DSS + HRW group, in which the colon was markedly longer than that in the DSS group (*P* < 0.05). In addition, the colonic wall thickening was alleviated in the DSS + HRW group in comparison to the DSS group (*P* < 0.05). The macroscopic damage score assessed by diarrhea, colon damage and colon length showed that the DSS + HRW group also received a lower score than the DSS group (*P* < 0.05) (Figure 2).

For the further study of the alterations of the colon,

Figure 1 Hydrogen-rich water decreases the weight loss and achieves a lower disease activity index score in dextran sulfate sodium-induced inflammatory bowel disease. The changes in weight and disease activity index were assessed on the 7th d after inflammatory bowel disease (IBD) modeling. *n* = 6, mean ± SEM, [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* dextran sulfate sodium (DSS) group. HRW: Hydrogen-rich water.

Figure 2 Hydrogen-rich water alleviates the changes in colon length, thickness and macroscopic score in dextran sulfate sodium-induced inflammatory bowel disease. Mice were sacrificed, and the colons were rapidly removed and processed for analysis on the 7th d after inflammatory bowel disease (IBD) modeling. A: The representative pictures of the colons in each group; B-D: The macroscopic score, length, and the thickness of the colons from each group. *n* = 6, mean ± SEM, [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* dextran sulfate sodium (DSS) group. HRW: Hydrogen-rich water.

we conducted the experiments in microcosmic aspect. The histological study revealed that the mice in the DSS group developed severe colonic inflammation including mucosal hyperemia, inflammatory cell infiltration, formation of crypt abscesses, destruction of the mucosal architecture, and the depletion of goblet cells. Conversely, the DSS + HRW group exhibited mild inflammation that was much closer to normal. The microcosmic scores based on the goblet cell depletion, crypt abscesses, destruction of the mucosal architecture, muscle thickening, and cellular infiltration in the DSS + HRW group were also lower than those in the DSS group (*P* < 0.05) (Figure 3). This evidence indicated that HRW could improve colonic damage in DSS-induced IBD.

HRW inhibits oxidative stress and inflammatory factors in DSS-induced IBD

Oxidative stress and inflammation play an initial and crucial role in the process of IBD. The oxidative stress parameters in the colon, including MDA and MPO, were significantly decreased in the DSS + HRW group compared with the DSS group (*P* < 0.05). The protective indicator, SOD, was markedly increased with the use of HRW. Additionally, HRW also reversed the depletion of GSH caused by DSS administration (Figure 4). These facts demonstrated that HRW could indeed inhibit oxidative stress.

To explore the anti-inflammatory mechanism of HRW, inflammatory factors were assessed on the 7th d after IBD modeling by determining the plasma

A

B

Figure 3 Hydrogen-rich water alleviates the changes in histological studies and microscopic score in dextran sulfate sodium-induced inflammatory bowel disease. Mice were sacrificed, and the colons were rapidly removed and processed for analysis on the 7th d after inflammatory bowel disease (IBD) modeling. A: Hematoxylin-eosin staining of colon tissues (magnification × 100, 200); B: The microscopic score of the colon in each group. *n* = 6, mean ± SEM, [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* dextran sulfate sodium (DSS) group. HRW: Hydrogen-rich water.

A

B

C

D

Figure 4 Hydrogen-rich water decreases the oxidative stress in dextran sulfate sodium-induced inflammatory bowel disease. On the 7th d after inflammatory bowel disease (IBD) modeling, the colon tissues were harvested to evaluate the oxidative stress. The levels of MDA, MPO, SOD and GSH in the colon tissues were measured. *n* = 6, mean ± SEM, [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* dextran sulfate sodium (DSS) group. HRW: Hydrogen-rich water; MDA: Malonaldehyde; SOD: Superoxide dismutase; GSH: Glutathione.

Figure 5 Hydrogen-rich water decreases inflammatory factors in dextran sulfate sodium-induced inflammatory bowel disease. On the 7th d after inflammatory bowel disease (IBD) modeling, the blood samples were harvested to evaluate the oxidative stress. HRW reduced the serum TNF-α, IL-6 and IL-1β concentrations (A-C). n = 6, mean ± SEM, [a]P < 0.05 *vs* control group; [b]P < 0.05 *vs* dextran sulfate sodium (DSS) group. HRW: Hydrogen-rich water.

Figure 6 Hydrogen-rich water inhibits endoplasmic reticulum stress in DSS-induced inflammatory bowel disease. On the 7th d after inflammatory bowel disease (IBD) modeling, the colon tissues were harvested to evaluate the endoplasmic reticulum stress. Western blot analysis was conducted to evaluate the protein contents of p-eIF2α, ATF4, XBP1s and CHOP. β-actin was used as an internal control. n = 6, mean ± SEM, [a]P < 0.05 *vs* control group; [b]P < 0.05 *vs* dextran sulfate sodium (DSS) group. HRW: Hydrogen-rich water.

levels of TNF-α, IL-6 and IL-1β. A marked increase in TNF-α, IL-6 and IL-1β secretion was observed after DSS treatment. Moreover, the DSS + HRW group had significantly reduced levels of TNF-α, IL-6 and IL-1β compared with the DSS group (P < 0.05) (Figure 5).

HRW inhibits endoplasmic reticulum stress in DSS-induced IBD

Endoplasmic reticulum (ER) stress participating in a cellular process triggered by a variety of conditions that disturb the folding of proteins in the ER also aggravates the progress of IBD. The pivotal proteins involved in ER stress, including p-eIF2α, ATF4, XBP1s and CHOP, were detected to assess the effect of HRW on ER stress in DSS-induced IBD (Figure 6). The results demonstrated that the expression of

these proteins was significantly increased after DSS administration. Moreover, the p-eIF2α, ATF4, XBP1s and CHOP proteins were dramatically reduced after HRW treatment in contrast to the control group. These findings indicated that HRW may ameliorate the manifestation of IBD by inhibiting the process of ER stress.

HRW up-regulates HO-1 expression to alleviate IBD

HO-1 has anti-inflammatory and anti-oxidative effects protecting against many diseases. On the 7th d after IBD modeling, the mice were sacrificed, and the colon tissues were obtained to detect the HO-1 expression (Figure 7). The results revealed that HRW treatment markedly accelerated HO-1 expression compared with the DSS group. For the further study of the role that

A

B

C

Figure 7 Hydrogen-rich water up-regulates expression of heme oxygenase-1 in dextran sulfate sodium-induced inflammatory bowel disease. On the 7th d after inflammatory bowel disease (IBD) modeling, the colon tissues were harvested and Western blot analysis was conducted to evaluate the level of heme oxygenase-1 (HO-1). n = 6, mean ± SEM, [a]$P < 0.05$ vs control group; [b]$P < 0.05$ vs dextran sulfate sodium (DSS) group. HRW: Hydrogen-rich water.

HO-1 played in IBD, ZnPP, an HO-1 inhibitor, was used. We discovered that colon length was visibly reduced in the DSS + HRW + ZnPP group compared with the DSS + HRW group ($P < 0.05$). The colonic wall in the DSS + HRW + ZnPP group was also thicker than that in the DSS + HRW group ($P < 0.05$). In addition, the DSS + HRW + ZnPP group got a higher macroscopic damage score in comparison to the DSS + HRW group ($P < 0.05$). The histological study showed that the DSS + HRW + ZnPP group exhibited more serious colonic damage that was similar to that observed in the DSS group (Figure 8). Based on these findings, we confirmed that HO-1 plays a key role in the mechanisms for HRW to alleviate IBD.

DISCUSSION

Hydrogen has anti-oxidant, anti-inflammatory, anti-apoptotic and other protective effects, and great progress has been achieved in the research of hydrogen therapy for diseases such as metabolic disorders, tissue ischemia reperfusion injury, myo-cardial injury, and hepatic injury[23-26]. In this study, a model of IBD was established in mice by DSS feeding, and the therapeutic role of HRW was assessed. We demonstrated that treatment with HRW significantly alleviated the symptoms and colonic damage in DSS-induced IBD. The mechanisms by which HRW alleviates DSS-induced IBD may include the following: (1) inhibiting the secretion of inflammatory factors, such as TNF-α, IL-6 and IL-1β, to ameliorate the inflammatory response; (2) inhibiting oxidative stress, such as reducing MPO and ROS as well as increasing SOD and GSH; (3) inhibiting ER stress such as de-

creasing the expression of p-eIF2α, ATF4, XBP1s and CHOP; and (4) up-regulating HO-1 expression to ease oxidative stress and decrease inflammation. All the evidence revealed that HRW is a potential new method for the treatment of IBD.

IBD is an enteric disorder characterized by acute and chronic intestinal inflammation. The etiology and precise pathogenesis of IBD are still unclear. However, several possible causes, including genetic, infectious, immunological factors and dysfunction of the adaptive and innate immune systems in response to the fecal microbiome, have been recognized[27-30]. DSS is a physical agent with an intrinsic capacity to disrupt the epithelial barrier and activate macrophages, causing inflammation and tissue damage[8]. The related histological changes include ulceration and inflammation of the intestinal mucosa with leukocyte infiltration. The clinical presentation includes weight loss, blood in stool, and diarrhea. In the present study, we chose to evaluate changes in weight, the disease activity index, colon shortening, colonic wall thickening, histological study, and macroscopic and microcosmic scores to assess the severity of the DSS-induced IBD.

Oxidative stress is an imbalance of oxidation and anti-oxidation systems in the body and may be caused by excessive detrimental ROS, depletion of GSH, *etc.* In IBD, the production of ROS and MPO exceeds anti-oxidant defenses and leads to a state of oxidative stress that fuels inflammation and causes direct mitochondrial damage[31,32]. Hydrogen selectively quenches detrimental ROS, such as hydroxyl radicals and peroxynitrite, but it does not damage physiological ROS, such as superoxide anion radicals, hydrogen peroxide, and nitric oxide[33]. In this study, we found

Figure 8 Lack of heme oxygenase-1 reverses the protective role of hydrogen-rich water in dextran sulfate sodium-induced inflammatory bowel disease. Mice were sacrificed, and the colons were rapidly removed and processed for analysis on the 7th d after inflammatory bowel disease (IBD) modeling. A: The representative pictures of the colon in each group; B: The macroscopic score of the colon in each group; C: The length of the colon in each group; D: The thickness of the colon in each group; E: Hematoxylin-eosin staining of colon tissues (magnification ×100). *n* = 6, mean ± SEM, [a]*P* < 0.05 *vs* dextran sulfate sodium (DSS) group; [b]*P* < 0.05 *vs* DSS + HRW group. HO-1: Heme oxygenase-1; HRW: Hydrogen-rich water; HE: Hematoxylin and eosin.

that HRW significantly reduced the levels of MDA and MPO and facilitated the protective indicators SOD and GSH. Furthermore, we measured the levels of inflammatory factors, and the results revealed that HRW markedly inhibited the release of TNF-α, IL-6 and IL-1β. TNF-α is one of the most important pro-inflammatory cytokines, and it stimulates the production of downstream cytokines such as IL-6 and IL-8 and plays a significant role in activating the cytokine cascade[34,35]. The researchers reported that anti-TNF-α monoclonal antibodies and other drugs had dramatically improved the treatment of IBD[36,37]. In summary, our study revealed that HRW could quench detrimental oxidative stress and exert an anti-TNF-α role to alleviate IBD.

ER stress participates in a cellular process triggered by a variety of conditions that disturb the folding of proteins in the ER. ER stress further triggers the unfolded protein response (UPR) by activating the PKR and PERK signals and phosphorylating eIF2α, which is required by the initiation phase of polypeptide chain synthesis[38,39]. ATF4 is a UPR-dependent transcriptional factor, and its sustained expression may up-regulate CHOP expression and induce apoptosis[40]. XBP1s is also a UPR-dependent transcriptional factor induced by AFT6[41]. ER stress exerts important roles in many diseases such as ischemia/reperfusion injury in the liver, diabetes, and cardiac myocyte injury[42-44]. A previous study also proved that epithelial ER stress participated in CD and UC[45]. Moreover, studies have

found that hydrogen has anti-apoptotic and anti-inflammatory functions[46,47]. In this study, we discovered that HRW dramatically reduced the expression of p-eIF2α, ATF4, XBP1s and CHOP proteins and conclude that HRW protects against IBD by inhibiting ER stress.

To determine the deeper mechanism of the protective effect of HRW against IBD, we focused on the effect of hydrogen on HO-1 expression. Heme oxygenases (HOs) catalyze the rate-limiting step in heme degradation, which can produce bilirubin, iron, and carbon monoxide (CO). HO-1, increased by stimuli that induce cellular stress, reduces the secretion of inflammatory cytokines in many diseases, such as sepsis and LPS-stimulated macrophages[48,49]. Additionally, HO-1 conferred its cytoprotective effects by increasing anti-oxidative capacity and inhibiting oxidative stress[50,51]. In addition, recent studies have shown that HO-1 was involved in the downstream effect of Treg cells[52]. Based on these facts, we speculated that hydrogen may confer its cytoprotective role by up-regulating HO-1. We measured the level of HO-1 and used ZnPP, an HO-1 inhibitor, for the further study. Not surprisingly, HRW treatment markedly up-regulated HO-1 expression, and the use of ZnPP clearly reversed the protective role of HRW. We verified that HO-1 indeed plays a key role in the mechanisms by which HRW alleviates IBD. The detailed mechanism may be that HO-1 inhibits the secretion of inflammatory cytokines and oxidative stress to alleviate IBD.

In this study, we have proven that HRW has a significant therapeutic potential in the treatment of IBD by inhibiting inflammatory factors and oxidative stress. More importantly, we discovered that HRW could inhibit ER stress to prevent apoptosis and up-regulate HO-1 expression. The high level of HO-1 further exerted anti-oxidative and anti-inflammatory functions in the process of IBD. Additionally, due to its advantageous distribution characteristics, hydrogen can penetrate biomembranes and diffuse into the cytosol, mitochondria, and nucleus, successfully targeting the organelles. All of these effects make HRW a potential new treatment method against DSS-induced IBD. However, our study is based on animal experiments, and prospective clinical studies are needed to evaluate whether HRW is fit for the clinical treatment of IBD.

In conclusion, the results of the present study demonstrate that HRW can alleviate the symptoms and colonic damage in DSS-induced IBD, most likely due to its unique cytoprotective properties such as its anti-oxidant and anti-inflammatory activities. More importantly, HRW can inhibit ER stress and up-regulate HO-1 expression. All of these findings indicate that HRW can be a potential therapy for DSS-induced IBD.

COMMENTS

Background
Inflammatory bowel disease (IBD) is a chronic and relapsing disease, and

therapeutic goals include controlling inflammation and ameliorating clinical symptoms. Hydrogen-rich water (HRW) is a potent anti-oxidative, anti-apoptotic, and anti-inflammatory agent and an ideal therapy for many diseases.

Research frontiers
Effective therapeutic schemes for IBD are lacking. Research of mechanisms and new therapeutic approaches for IBD has received increasing attention from scientists and clinicians. Hydrogen therapy is a new medical approach that has recently gained much appreciation. HRW exerts considerable anti-oxidative, anti-apoptotic, and anti-inflammatory effects. More importantly, drinking HRW is very convenient in the course of daily life. To explore the effect of HRW on different types of diseases and promote its clinical usage is currently an important goal in hydrogen medicine.

Innovations and breakthroughs
The present study concluded that HRW can significantly prevent IBD in mice by inhibiting inflammatory factors, oxidative stress, and endoplasmic reticulum stress and by up-regulating HO-1 expression. Moreover, based on the use of the pharmaceutical inhibition of HO-1, we can conclude that HO-1 may be a key effective protein in HRW function.

Applications
Hydrogen therapy may be a safe and effective treatment for IBD. Moreover, the application of drinking HRW is very convenient and acceptable for usage.

Terminology
Hydrogen is the lightest gas in nature, which has powerful anti-oxidant and anti-inflammatory effects. It has therapeutic effects in many diseases, which is proven by many basic research and clinical studies. HRW is produced by forcing hydrogen gas into water by a specific device under high pressure.

Peer-review
Congratulations. It is a very well designed work with very interesting results. Some suggestions: Was there any examination or histological study of the puncture site? It would have been interesting to know if there is any reaction in that place.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Octogenarian patients with colorectal cancer: Characterizing an emerging clinical entity

Hadar Goldvaser, Noa Katz Shroitman, Irit Ben-Aharon, Ofer Purim, Yulia Kundel, Daniel Shepshelovich, Tzippy Shochat, Aaron Sulkes, Baruch Brenner

Hadar Goldvaser, Irit Ben-Aharon, Ofer Purim, Yulia Kundel, Aaron Sulkes, Baruch Brenner, Institute of Oncology, Davidoff Cancer Center, Beilinson Hospital, Rabin Medical Center, Petach Tikva 4941492, Israel

Hadar Goldvaser, Noa Katz Shroitman, Irit Ben-Aharon, Ofer Purim, Yulia Kundel, Daniel Shepshelovich, Aaron Sulkes, Baruch Brenner, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 64239, Israel

Hadar Goldvaser, Division of Medical Oncology and Hematology, Princess Margaret Cancer Centre and the Department of Medicine, University of Toronto, Toronto M5G 0A4, Canada

Daniel Shepshelovich, Department of Medicine A, Beilinson Hospital, Rabin Medical Center, Petach Tikva 4941492, Israel

Tzippy Shochat, Statistical Consulting Unit, Beilinson Hospital, Rabin Medical Center, Petach Tikva 4941492, Israel

Correspondence to: Hadar Goldvaser, MD, Davidoff Cancer Center, Beilinson Hospital, Rabin Medical Center, 39 Jabotinski Street, Petach Tikva 4941492, Israel. hadar7g@gmail.com
Telephone: +972-3-9378004
Fax: +972-3-9377902

Abstract

AIM
To characterize colorectal cancer (CRC) in octogenarians as compared with younger patients.

METHODS
A single-center, retrospective cohort study which included patients diagnosed with CRC at the age of 80 years or older between 2008-2013. A control

group included consecutive patients younger than 80 years diagnosed with CRC during the same period. Clinicopathological characteristics, treatment and outcome were compared between the groups. Fisher's exact test was used for dichotomous variables and χ^2 was used for variables with more than two categories. Overall survival was assessed by Kaplan-Meier survival analysis, with the log-rank test. Cancer specific survival (CSS) and disease-free survival were assessed by the Cox proportional hazards model, with the Fine and Gray correction for non-cancer death as a competing risk.

RESULTS
The study included 350 patients, 175 patients in each group. Median follow-up was 40.2 mo (range 1.8-97.5). Several significant differences were noted. Octogenarians had a higher proportion of Ashkenazi ethnicity (64.8% *vs* 47.9%, *P* < 0.001), a higher rate of personal history of other malignancies (22.4% *vs* 13.7%, *P* = 0.035) and lower rates of family history of any cancer (36.6% *vs* 64.6%, *P* < 0.001) and family history of CRC (14.4% *vs* 27.3%, *P* = 0.006). CRC diagnosis by screening was less frequent in octogenarians (5.7% *vs* 20%, *P* < 0.001) and presentation with performance status (PS) of 0-1 was less common in octogenarians (71% *vs* 93.9%, *P* < 0.001). Octogenarians were more likely to have tumors located in the right colon (45.7% *vs* 34.3%, *P* = 0.029) and had a lower prevalence of well differentiated histology (10.4% *vs* 19.3%, *P* = 0.025). They received less treatment and treatment was less aggressive, both in patients with metastatic and non-metastatic disease, regardless of PS. Their 5-year CSS was worse (63.4% *vs* 77.6%, *P* = 0.009), both for metastatic (21% *vs* 43%, *P* = 0.03) and for non-metastatic disease (76% *vs* 88%, *P* = 0.028).

CONCLUSION
Octogenarians presented with several distinct characteristics and had worse outcome. Further research is warranted to better define this growing population.

Key words: Colon; Rectum; Elderly; Octogenarian; Age

Core tip: Data regarding octogenarians with colorectal cancer (CRC) are scarce. We compared octogenarians with CRC to younger patients. Octogenarians had a predominance of Ashkenazi ethnicity, a higher rate of personal history of other malignancies and a lower rate of family history of any cancer or of CRC. Their performance status (PS) at presentation was worse and their tumors were more likely to be located in the right colon and to have a poorer differentiation. Octogenarians received less treatment and treatment was less aggressive, regardless of PS. This might contribute to the worse outcome which was found among the octogenarians.

INTRODUCTION
Colorectal cancer (CRC) is the fourth most common cancer and the third leading cause of cancer death globally[1]. CRC carries an approximately 4.4% lifetime risk and accounts for 8% of all new cancer cases[2]. It is classified according to local invasion depth (T stage), lymph node involvement (N stage), and presence of distant metastases (M stage). These classification are combined into an overall stage scoring from 1 to 4[3], which provides the basis for therapeutic decisions and prognosis[1].

CRC is predominantly associated with the elderly, with an increasing incidence with age. The median age for CRC diagnosis is 68 years, with about 35% of the patients diagnosed above the age of 75 years[2]. Since the elderly population in Western countries constantly grows, the incidence of CRC in octogenarians is expected to increase in the coming years[4]. Clearly, octogenarians are becoming a substantial population among CRC patients.

Currently, the impact of older age on tumor biology and outcome remains unclear. While some studies imply that elderly patients with CRC might have unique features[5-7] as well as worse outcome[7-9], these findings are not consistent[10-13].

Elderly patients are considerably underrepresented in clinical trials[14,15]. Hutchins *et al*[15] reported that CRC patients who were older than 65 and 70 years accounted for only 40% and 14% of patients in clinical trials, respectively. Furthermore, the cut-off for "elderly" patients with CRC in not consistent across different studies, starting from 65 years of age. Studies evaluating octogenarians are scarce[5,6,16]. At present, as octogenarians are rarely included in clinical trials, their optimal management is not clearly defined.

The aim of this study was therefore to better define this growing entity of elderly patients with CRC. As the median age at diagnosis of CRC is 68[2], similar to some recent studies[5,6,16] we chose a cut-off of 80 years old in order to emphasize age-related characteristics.

MATERIALS AND METHODS
This was a retrospective, single center cohort study. The study population included all patients who were 80 years old or older at diagnosis of CRC during the years 2008-2013 and were treated at our institute, a large academic tertiary medical center. This group

Table 1 Patient characteristics[1] *n* **(%)**

	Study cohort (*n* = 350)	Older (age ⩾ 80 yr) (*n* = 175)	Younger (age < 80 yr) (*n* = 175)	*P* value
Median age (range)	80 (20-99)	83 (80-99)	63 (20-79)	-
Male gender	155 (44.3)	84 (48)	71 (40.6)	0.162
Ethnicity				
Ashkenazi	193 (56.4)	112 (64.8)	81 (47.9)	< 0.001
Sephardic	120 (35.1)	58 (33.5)	62 (36.7)	
Arab	12 (3.5)	0 (0)	12 (7.1)	
Other	17 (5)	3 (1.7)	14 (8.3)	
2nd malignancy	63 (18.1)	39 (22.4)	24 (13.7)	0.035
Family history of cancer	157 (51.1)	53 (36.3)	104 (64.6)	< 0.001
Family history of CRC	65 (21.2)	21 (14.4)	44 (27.3)	0.006
IBD	1 (0.3)	1 (0.6)	0 (0)	0.335
Polyps[2]	122 (35.4)	55 (33.2)	67 (38.5)	0.233
HNPCC/FAP	9 (2.7)	5 (3)	4 (2.4)	0.695
Smoking history	104 (34.9)	41 (24.6)	74 (44.3)	< 0.001
Diagnosis d/t screening	45 (12.9)	10 (5.7)	35 (20)	< 0.001
Performance status[3] of 0-1	268 (82.5)	115 (71)	153 (93.9)	< 0.001

[1]Valid percentages. Missing data as follows: ethnicity (*n* = 8); 2nd malignancy (*n* = 1); family history of malignancy (*n* = 43); family history of CRC (*n* = 43); IBD (*n* = 5), polyps (*n* = 5); HNPCC/FAP (*n* = 14); smoking history (*n* = 16), performance status (*n* = 25); [2]Polyps- diagnosis of polyps before or during CRC diagnosis; [3]Performance status was determined according to the Eastern Cooperative Oncology Group score during the first encounter with the oncologist. CRC: Colorectal cancer; IBD: Inflammatory bowel disease; FAP: Familial adenomatous polyposis; HNPCC: Hereditary non-polyposis colorectal cancer; d/t: Due to.

was matched by year of diagnosis with a control group of consecutive patients younger than 80 years at diagnosis. We assumed this population to be representative of the average CRC population.

The medical records of all patients were reviewed and detailed data on patient demographics, risk factors for CRC, clinical-pathological parameters, treatment, adverse events and outcome were retrieved. Patients' performance status (PS) at presentation was determined according to the Eastern Cooperative Oncology Group scale. Staging was defined according to the American Joint Committee on Cancer Staging, 7th edition[3]. Grade of toxicity was determined according to the Common Terminology Criteria for Adverse Events version 3.0[17]. The study protocol was approved by the institutional ethics committee.

Statistical analysis

The statistical analysis was generated using SAS software, version 9.4. Fisher's exact test was used for dichotomous variables and χ^2 was used for variables with more than two categories. Overall survival (OS) was assessed by Kaplan-Meier survival analysis, with the log-rank test. Cancer specific survival (CSS) and disease-free survival (DFS) were assessed by the Cox proportional hazards model, with the Fine and Gray correction for non-cancer death as a competing risk. Cox proportional hazard models were also applied for multivariate analysis and hazard ratios estimations. Two-sided *P* values less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

Three hundred fifty patients with CRC were included

in the study, 175 patients in each group. The clinical characteristics of the two groups are detailed in Table 1. Several significant differences were noted. There were more Ashkenazi Jews (64.8% *vs* 47.9%) in the octogenarians group and less Arab patients (0% *vs* 7.1%) or other (1.7% *vs* 8.3%) ethnicities (*P* < 0.001). Octogenarians had a higher incidence of second malignancies (22.4% *vs* 13.7%, *P* = 0.035) but had lower rates of family history of any cancer (36.3% *vs* 64.6%, *P* < 0.001) or CRC (14.4% *vs* 27.3% *P* = 0.006). Smoking was less prevalent in octogenarians (24.6% *vs* 44.3%, *P* < 0.001), while the incidence of other risk factors, including inflammatory bowel disease, history of polyps and familial CRC syndromes, were comparable between both groups.

As expected, there was a remarkable difference in CRC diagnosis following screening, with only 5.7% octogenarians diagnosed by screening compared to 20% in the control group (*P* < 0.001). In addition, octogenarians were less likely to have a PS of 0 or 1 at presentation (71% *vs* 93.9%, *P* < 0.001).

Tumor characteristics

Tumor characteristics are depicted in Table 2. Primary tumor location differed between the groups: tumors were located in the right colon in 45.7% of the octogenarians, compared with 34.3% patients in the control group (*P* = 0.029). At presentation, octogenarians had a higher perforation rate (5.7% *vs* 1.1%, *P* = 0.019), while obstruction rates were similar.

Well differentiated histology (grade 1) was less prevalent in octogenarians (10.4% *vs* 19.4%, *P* = 0.025), while other histological characteristics, as well as tumor stage at presentation, were comparable between the groups. With limited genomic data, no

Table 2 Tumor characteristics[1] *n* (%)

	Study cohort (*n* = 350)	Older (age ≥ 80 yr) (*n* = 175)	Younger (age < 80 yr) (*n* = 175)	*P* value
Tumor location[2]				
Right colon	140 (40)	80 (45.7)	60 (34.3)	0.029
Left colon/rectum	210 (60)	95 (54.3)	115 (65.7)	
Histology				0.201
NOS	260 (76.5)	124 (74.2)	136 (78.6)	
Mucinous	64 (18.8)	38 (22.8)	26 (15)	
Signet ring cell	11 (3.2)	3 (1.8)	8 (4.6)	
Other	5 (1.5)	2 (1.2)	3 (1.78)	
Grade[3]				0.025
1	48 (14.8)	17 (10.4)	31 (19.3)	
2-3	276 (85.2)	146 (89.3)	130 (80.7)	
T				0.27
T1	17 (5.2)	5 (3.1)	12 (7.4)	
T2	57 (17.6)	28 (17.4)	29 (17.8)	
T3	237 (73.2)	123 (76.4)	114 (69.9)	
T4	13 (4)	5 (3.1)	8 (4.9)	
N				0.357
N0	179 (67)	94 (71.2)	85 (63)	
N1	56 (21)	24 (18.2)	32 (23.7)	
N2	32 (12)	14 (10.6)	18 (13.3)	
M1	72 (20.7)	34 (19.8)	38 (21.7)	0.655
TNM stage				0.259
I	65 (19)	30 (17.7)	35 (20.2)	
II	116 (33.8)	66 (38.8)	50 (28.9)	
III	90 (26.2)	40 (23.5)	50 (28.9)	
IV	72 (21)	34 (20)	38 (22)	
LVI	18 (6)	11 (7.5)	7 (4.6)	0.296
VVI	36 (11.9)	15 (10.1)	21 (13.6)	0.348
Obstruction[4]	36 (10.3)	22 (12.6)	14 (8)	0.159
Perforation[4]	12 (3.4)	10 (5.7)	2 (1.1)	0.019
Synchronous CRC	11 (3.1)	4 (2.3)	7 (4)	0.358
Metachronous CRC	2 (0.6)	1 (0.6)	1 (0.6)	0.997
RAS mutated	8 (22.2)	2 (15.4)	6 (26.1)	0.458
BRAF mutated	1 (3.7)	0 (0)	1 (7.7)	0.29
MSI-H	6 (25)	3 (100)	3 (14.3)	0.001

[1]Valid percentages. Missing data as follows: histology (*n* = 10); grade (*n* = 26); T stage (*n* = 26); N status (*n* = 83); M status (*n* = 3); TNM stage (*n* = 7); lymphatic invasion (*n* = 51); vascular invasion (*n* = 48); metachronous tumor (*n* = 1); KRAS (*n* = 314); BRAF (*n* = 323); MSI-H (*n* = 326); [2]Right colon = appendix, ascending colon and transverse colon, Left colon = descending colon and sigma; [3]Grade 1 = well differentiated, grade 2 moderately differentiated, poorly differentiated; [4]Obstruction or perforation at presentation. CRC: Colorectal cancer; NOS: Not otherwise specified; LVI: Lymphovascular invasion; VVI: Venovascular invasion; MSI-H: Microsatellite instability- high.

apparent differences in RAS and BRAF mutation status were noted. Octogenarians were more likely to have MSI-H (Microsatellite instability- high) status (*P* = 0.001), but such information was available for only 24 (6.9%) patients.

Treatment

Significant differences were identified in treatment approach (Table 3). Octogenarians with non-metastatic disease were less likely to receive adjuvant or neo-adjuvant treatment (27.5% *vs* 60.9%, *P* < 0.0001). Even a subset analysis for patients with PS 0-1 demonstrated a lower use of adjuvant/neoadjuvant treatment: 32.6% compared to 61.7% (*P* < 0.0001). Of all patients treated with chemotherapy, the percentage of octogenarians treated with oxaliplatin-based regimes was also lower compared with younger patients (29.7% *vs* 59.5%, *P* = 0.002).

Octogenarians with metastatic disease were treated with fewer chemotherapy lines: 34.6% did not receive any treatment, 42.3% received one line and 23.1% received at least two lines, compared with 8.8%, 38.2% and 53%, respectively, in the control group (*P* = 0.016). This difference persisted for patients with metastatic disease with PS 0-1: 23.5% octogenarians did not receive any chemotherapy compared to only 7.7% in the control group (*P* = 0.045). Moreover, octogenarians with metastatic disease underwent local treatment to metastatic sites (including surgery, chemoembolization, stereotactic body irradiation and radiofrequency ablation) less frequently (9.7% *vs* 65.5%, *P* < 0.0001).

Chemotherapy in both the adjuvant setting and in patients with metastatic disease had comparable rates of grade 3-5 hematologic and non-hematologic adverse events (Table 3).

Outcome

The median follow-up time was 40.2 mo (range 1.8-97.5

Table 3 Treatment[1]

	Study cohort	Older (age ≥ 80 yr)	Younger (age < 80 yr)	*P* value
No. of LN dissected, mean (SD)[2]	14.4 (6.1)	14.3 (5.4)	14.4 (6.8)	0.893
Adjuvant/neoadjuvant Tx[2]	119/271 (43.9)	38/138 (27.5)	81/133 (60.9)	< 0.0001
Oxaliplatin-based adjuvant chemotherapy[2]	58/116 (50)	11/37 (29.7)	47 (59.5)	0.002
No. of chemotherapy lines in stage IV[3]				
0	12 (20)	9 (34.6)	3 (8.8)	0.016
1	24 (40)	11 (42.3)	13 (38.2)	
≥ 2	24 (40)	6 (23.1)	18 (53)	
Type of chemotherapy in stage IV, 1st line[3,4]				0.239
Fluoropyrimidine	11 (21.6)	6 (30)	5 (16.1)	
Fluoropyrimidine+oxaliplatin/irinotecan	40 (78.4)	14 (70)	26 (80.9)	
Local interventions to metastatic sites[5]				< 0.001
None	45 (57)	28 (90.3)	17 (35.4)	
Surgery ± other local intervention	31 (39.2)	2 (6.5)	29 (60.4)	
Other local intervention	3 (3.8)	1 (3.2)	2 (4.2)	
Hematological toxicity, grade ≥ 3	17 (11)	4 (8)	13 (12.5)	0.404
Non-hematological toxicity, grade ≥ 3	37 (23.6)	9 (18)	28 (26.7)	0.261

[1]Valid percentages. Missing data as follows: adjuvant/neoadjuvant treatment (*n* = 4); no. of lymph nodes dissected (*n* = 20); type of chemotherapy (*n* = 7); No. of chemotherapy lines (*n* = 12); local intervention to a metastatic site (*n* = 4), hematological toxicity (*n* = 19); non-hematological toxicity (*n* = 19); [2]Data regarding patients who presented with non-metastatic disease; [3]Data regarding patients who presented with metastatic disease; [4]Chemotherapy was given with or without a biological agent including bevacizumab, cetuximab or panitumumab; [5]Local intervention to a metastatic site included: radiofrequency ablation, chemoembolization, intra-arterial chemotherapy, stereotactic body irradiation, and radiotherapy. LN: Lymph nodes; Tx: Treatment.

mo). During this period, 120 patients died of CRC and 230 remained alive or died of other causes. Octogenarians achieved a status of no evidence of disease (NED) less frequently: 88.8% patients with non-metastatic disease and 5.9% of those with metastatic disease achieved NED, compared to 97.8% and 38.9% in the younger patient group (*P* = 0.003 and *P* = 0.001, non-metastatic and metastatic disease, respectively).

Among patients with non-metastatic disease 5-year DFS rates were 68.7% for octogenarians and 78.7% for younger patients, without reaching statistical significance (*P* = 0.154). The 5-year OS and CSS rates were worse for octogenarians (5-year OS: 38.5% *vs* 74.8%, *P* < 0.0001, 5-year CSS: 63.4% *vs* 77.6%, *P* = 0.009) (Figures 1 and 2). Octogenarians had a lower 5-year CSS rate even when the analysis was limited to patients with non-metastatic disease: 76% *vs* 88% (HR = 2.23, 95%CI: 1.09-4.58, *P* = 0.028). However, patients with non-metastatic disease who received adjuvant or neoadjuvant treatment had comparable 5-year CSS rates (80% *vs* 88% for the octogenarians and the control group respectively, *P* = 0.327). Octogenarians with metastatic disease had a worse 5-year CSS rate: 21% *vs* 43% (HR = 1.86, 95%CI: 1.06-3.25, *P* = 0.03).

Most "classical" CRC prognostic factors were found to correlate with CSS and DFS on univariate analysis, including T, N, TNM stage, histological subtype of signet ring cell carcinoma, and presentation with obstruction (Table 4). Diagnosis through screening, perforation, PS 0-1 at presentation and grade were associated with CSS, but not DFS. In addition, family history of CRC was associated with better DFS and CSS rates. Patients with metastatic disease who underwent local treatment to metastatic sites also had a better CSS. We performed multivariate analysis for DFS and CSS including age, gender and variables which were found significant in the univariate analysis. TNM stage, histology, family history of CRC and presentation with perforation retained statistical significance on multivariate analysis for CSS (Table 4). TNM stage, histology, family history of CRC and presentation with obstruction were associated with DFS on multivariate analysis. Age was not associated with neither CSS nor with DFS on these multivariate analyses.

DISCUSSION

While the burden of elderly patients with CRC is increasing[2,4], current literature regarding characteristics and optimal management of this subpopulation is unclear. Data are even more limited on the very elderly patients, octogenarians with CRC. In this study, we elected a cut-off of 80 for several reasons. First, the growing number of octogenarians emphasizes the need to explore this population. Second, the cut-off for elderly patients in current literature is inconsistent, starting from 65 years of age, which is clearly not well representative of the nowadays elderly population. Lastly, using a relatively high cut-off may better emphasize the differentiation between the two populations and may elucidate age-dependent differences that might have been masked using a lower cut-off.

In this study, we found a variety of significant differences between octogenarians with CRC and younger patients ("average" patients). Octogenarians had a predominance of Ashkenazi ethnicity, a higher rate of personal history of other malignancies and a lower rate of family history of any cancer or of CRC.

Figure 1 Overall survival. OS: Overall survival.

Figure 2 Cancer specific survival. CSS: Cancer specific survival.

In addition, they were less frequently diagnosed by screening; their PS at presentation was worse and their tumors likely to be located in the right colon and to have a poorer differentiation. Moreover, they received less treatment and treatment was less aggressive, both for metastatic and non-metastatic disease. Not surprisingly, their CSS was worse, both for metastatic and for non-metastatic disease. Some of these findings were described before, and some are novel.

In contrast to other studies[5,6], CRC among octogenarians had no female predominance. The ethnic composition was considerably different between the two groups. There were no Arabs in the octogenarians group as opposed to 7.1% in the control group. This finding is consistent with previous studies which reported a high proportion of Arabs in the young CRC population in Israel[18-20]. The reason for the higher prevalence of Ashkenazi Jews in the elderly group is unclear, although it may at least in part reflect the ethnic distribution in Israel in this age group[21].

Younger patients had a higher prevalence of family history of CRC, a well-established risk factor. In addition, a higher rate of family history of other malignancies in the younger population might represent the presence of other risk factors (for example a family history of endometrial cancer) or even undiagnosed actual cancer-related syndromes. Our finding of better outcome in

patients with family history of CRC might be related to better adherence to screening or higher incidence of MMR (mismatch repair) deficiency, which are both associated with better outcome[22-24]. Octogenarians had a higher incidence of secondary malignancies, probably reflecting the increasing incidence of malignancies in the older population[2]. Indeed, most of the other cancer identified in our older group represented other common non-CRC cancers.

As expected, the screening rate was considerably lower among the octogenarians with only 5.7% diagnosed by screening in this group. The higher perforation rate in this population might be related to a lower screening rate. Although the current United States Preventive Services Task Force recommends against routine screening for average risk individuals older than 75[22], current literature regarding the optimal age to discontinue screening is unclear, and it seems that at least some patients might benefit form screening after this age[23,25]. Our findings of low screening rate and worse CSS in octogenarians might further support the need to consider screening for CRC in the elderly, taking into account their life expectancy and comorbidities.

In agreement with previous reports[5-7], we found that octogenarians had clear predilection for right colon tumors. This finding might suggest a distinct pathogenesis of CRC among older patients, such as a higher rate of MMR deficiency or its phenotype, MSI-H tumors. Indeed, we noted, for the first time, that octogenarians had a significantly higher rate of MSI-H tumors. However, as data regarding MSI status were scarce, conclusions from this specific analysis are limited. Since patients with MMR deficiency might benefit from immune check point blockade[26], a routine evaluation of MSI-H status in elderly patients should be considered.

Similarly to earlier studies[16,27,28], octogenarians in the current study were less likely to receive treatment and treatment was less aggressive, both for metastatic and non-metastatic disease. This difference remained statistically significant after adjusting for PS. We could not determine the reason for this finding due to the retrospective nature of this study. As the benefit of both surgery and chemotherapy are well established in CRC[29,30], the worse CSS in the octogenarians in our cohort might be related to the demonstrated avoidance of treatment. Comparable CSS in patients who received adjuvant or neoadjuvant treatment further supports this postulation. Current literature regarding treatment decisions for elderly patients with CRC is conflicting. Alongside reports on the benefit of chemotherapy and surgery in elderly CRC patients[31-38], there are data implying a minimal benefit for oxaliplatin in the adjuvant setting[27,39] and a higher treatment complication rate[28,40] in older patients. In this cohort, there was no difference in chemotherapy associated toxicity. These findings bolster the need for prospective trials aiming to establish the optimal

Table 4 Univariate and multivariate analyses of cancer specific survival and disease free survival

	Univariate analysis CSS[1]		Multivariate analysis CSS[1,2]		Univariate analysis DFS[3]		Multivariate analysis DFS[2,3]	
	5-yr CSS	*P* value	Hazard ratio	*P* value	5-yr DFS	*P* value	Hazard ratio	*P* value
Age (yr)	63%	0.009	1.7 (0.8-3.6)	0.155	71%	0.226	1.2 (0.5-2.9)	0.668
≥ 80	78%				79%			
< 80								
Gender		0.274	0.9 (0.5-1.6)	0.762		0.682	1.1 (0.5-2.2)	0.865
Male	68%				77%			
Female	73%				74%			
Ethnicity		0.293	-	-		0.608	-	-
Ashkenazi	73%				73%			
Sephardic	65%				78%			
Arab	88%				69%			
Other	79%				93%			
Second malignancies		0.497	-	-		0.429	-	-
Yes	75%				81%			
No	70%				74%			
Family hx of cancer		0.068	-	-		0.096	-	-
Yes	78%				82%			
No	68%				71%			
Family hx of CRC		0.024	0.3 (0.1-0.8)	0.015		0.048	0. 2 (0.1-0.8)	0.016
Yes	86%				91%			
No	70%				73%			
Performance status		0.0003	1.2 (0.5-3)	0.62		0.068	2.1 (0.7-6.3)	0.175
0-1	77%				78%			
2-4	53%				60%			
Mode of Diagnosis		0.024	1 (0.3-3.4)	0.973		0.235	-	-
Symptoms	68%				74%			
Screening	90%				85%			
Tumor location		0.102	-	-		0.723	-	-
Right colon	66%				77%			
Left colon/rectum	75%				75%			
Histology		< 0.0001	5.7 (1.5-21.1)	0.009		< 0.0001	6.9 (1.7-28)	0.007
NOS/mucinous/other	74%				77%			
Signet ring cell	33%				48%			
Grade		0.05	-	-		0.561	-	-
1	87%				71%			
2-3	69%				76%			
T			-	-			-	-
T1	92%	< 0.0001			100%	< 0.0001		
T2	90%				82%			
T3	73%				72%			
T4	39%				76%			
N		0.0003	-	-		0.001	-	-
N0	89%				81%			
N1	79%				72%			
N2	56%				57%			
M		< 0.0001	-	-	-	-	-	-
M0	85%							
M1	26%							
TNM stage		< 0.0001		-		0.014		
I	94%		1	0.433	90%		1	-
II	90%		1.9 (0.4-8.7)	0.013	77%		3.5 (0.8-15.6)	0.107
III	73%		6.7 (1.5-30.1)	< 0.0001	63%		8.4 (1.9-36.4)	0.004
IV	26%		20 (4.6-87.6)		-		-	-
LVI		0.073	-	-		0.848	-	-
Yes	57%				79%			
No	75%				75%			
VVI		0.272	-	-		0.791	-	-
Yes	65%				72%			
No	75%				76%			
Obstruction		0.001	2.3 (0.99-5.4)	0.052		0.001	2.95 (1.1-7.9)	0.03
Yes	49%				49%			
No	74%				79%			
Perforation		0.0001	3.5 (1.1-10.9)	0.028		0.262	-	-
Yes	28%				64%			
No	73%				76%			
Adjuvant/neoadjuvant tx			-	-			-	-
Yes	87%	0.137			72%	0.236		
No	78%				80%			

Local Tx to metastatic sites			-	-	-	-	-	-
Yes	41%	0.007						
No	20%							

[1]Univariate and multivariate analysis for CSS were performed for patients with stage I -IV; [2]Multivariate analysis was calculated for the entire follow-up period. The column of multivariate analysis depicts only factors that were included in the model; [3]Univariate and multivariate analysis for DFS were performed for patients with stage I -III. DFS: Disease free survival; CRC: Colorectal cancer; CSS: Cancer specific survival; LVI: Lymphovascular invasion; NA: Not applicable; NOS: Not otherwise specified; Tx: Treatment; VVI: Venovascular invasion.

treatment for octogenarians.

Consistent with earlier studies[7-9], octogenarians in our cohort had worse outcome. Nonetheless, as other studies have indicated similar outcomes across different age groups[10-13], the actual impact of age on the outcome of the disease still remains to be established.

Limitations of this study include the retrospective methodology that may cause bias due to unknown or unrecorded confounders. As this is a single center study, it is more vulnerable to such bias. In addition, patients with CRC treated at our tertiary center might not represent the average population with CRC. An additional possible limitation might be a selection bias. Octogenarians included in our study were those referred to an oncologist. Therefore, our cohort might represent more "fit" octogenarians, as other frail octogenarians might have been undiagnosed or were not referred to an oncologist due to their poor clinical status. Nonetheless, the octogenarians in this cohort, who were potentially more "fit" than the average ones, were considerably under-treated compared with younger patients, thus bolstering the validity of this observation. Moreover, data regarding dose reductions were not documented. Therefore, although toxicity rates were comparable between both groups, prospective randomized trials are needed to determine whether toxicity in the older and younger populations is indeed similar. Last, as octogenarians were more likely to have comorbidities and their life expectancy is shorter, conclusions that can be drawn from the difference in survival are limited. However, the difference in CSS was also substantial, implying that octogenarians may indeed have worse outcome.

This study has several strengths. First, it includes a relatively large patient cohort, with a highly representative control group. We found correlations between outcome and most known prognostic factors of CRC, adding to the reliability and validity of the results. Second, as opposed to some large registry-based studies, which might lack important data, we extracted very detailed clinical data from the patients' individual medical files. Third, in contrast to other studies evaluating elderly patients with CRC which included much lower age cut-off[9,10,12,13,19,27,34,38], this study's cut-off probably might highlighted the differences between older and the younger population better.

Our study indicates that octogenarians with CRC display several differences in clinical and tumor characteristics, supporting the hypothesis of a unique clinical entity in this population, possibly with a distinct pathogenesis. They were less likely to receive treatment despite adequate PS and their outcome was worse. In light of these findings tailoring the management of octogenarians according to their PS and comorbidities should be further studied. Further research is warranted to better clarify the role of screening for the aging population and to determine well defined treatment guidelines for octogenarians with CRC.

COMMENTS

Background

Colorectal cancer (CRC) is predominantly a disease of the elderly. The incidence of octogenarians with CRC is expected to increase in the coming years. Some studies imply that elderly patients with CRC might represent a unique entity and has worse outcome, but data are not consistent. Although the benefit of chemotherapy and surgery in CRC are well establish, elderly patients often receive less treatment. Octogenarians are becoming a substantial population among CRC patients; but they are rarely included in clinical trials. The authors believe more research is desired to better understand the characteristics and the appropriate management of these patients.

Research frontiers

The definition of elderly patients with CRC is inconsistent; some studies used relatively low age cut-off. The authors believe focusing on octogenarians enabled better characterization of the elderly population with CRC and highlighted the differences between elderly patients with CRC compared to the average CRC population. In contrast to registry-based studies, we performed a detailed chart review and extracted data regarding various characteristics, as well as treatment and chemotherapy related adverse events. We found correlation between most known prognostic factors for CRC and outcome, which further supports the validity of this study.

Innovations and breakthroughs

The clinical and pathological differences between octogenarian and the control group suggest CRC in octogenarians might represent a unique clinical entity. Octogenarians were less likely to receive treatment; even if they had good performance status. Chemotherapy treatment was associated with comparable severe adverse events rates. Remarkable worse overall survival and cancer specific survival might imply that avoidance form treatment could contribute to these results.

Applications

Older age by itself should not be a contra-indication for oncological treatment. Lack of difference in severe adverse event rates further supports this

postulation. In addition, as life expectancy is increasing, screening in fit older population should be considered.

Peer-review

The matter studied in the manuscript is important and needed. The overall structure of the manuscript is clear and complete. The language and methods are appropriate.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of smoking habit on surgical outcomes in non-B non-C patients with curative resection for hepatocellular carcinoma

Keita Kai, Hiroki Koga, Shinichi Aishima, Atsushi Kawaguchi, Koutaro Yamaji, Takao Ide, Junji Ueda, Hirokazu Noshiro

Keita Kai, Shinichi Aishima, Department of Pathology, Saga University Hospital, Saga 849-8501, Japan

Hiroki Koga, Koutaro Yamaji, Takao Ide, Junji Ueda, Hirokazu Noshiro, Departments of Surgery, Saga University Hospital, Saga 849-8501, Japan

Shinichi Aishima, Koutaro Yamaji, Pathology and Microbiology, Saga University Hospital, Saga 849-8501, Japan

Atsushi Kawaguchi, Center for Comprehensive Community Medicine, Saga University Faculty of Medicine, Saga 849-8501, Japan

Abstract

AIM

To analyzed the correlation between smoking status and surgical outcomes in patients with non-B non-C hepatocellular carcinoma (NBNC-HCC), and we investigated the patients' clinicopathological characteristics according to smoking status.

METHODS

We retrospectively analyzed the consecutive cases of 83 NBNC-HCC patients who underwent curative surgical treatment for the primary lesion at Saga

University Hospital between 1984 and December 2012. We collected information about possibly carcinogenic factors such as alcohol abuse, diabetes mellitus, obesity and smoking habit from medical records. Smoking habits were subcategorized as never, ex- and current smoker at the time of surgery. The diagnosis of non-alcoholic steatohepatitis (NASH) was based on both clinical information and pathological confirmation.

RESULTS
Alcohol abuse, diabetes mellitus, obesity and NASH had no significant effect on the surgical outcomes. Current smoking status was strongly correlated with both overall survival ($P = 0.0058$) and disease-specific survival ($P = 0.0105$) by multivariate analyses. Subset analyses revealed that the current smokers were significantly younger at the time of surgery ($P = 0.0002$) and more likely to abuse alcohol ($P = 0.0188$) and to have multiple tumors ($P = 0.023$).

CONCLUSION
Current smoking habit at the time of surgical treatment is a risk factor for poor long-term survival in NBNC-HCC patients. Current smokers tend to have multiple HCCs at a younger age than other patients.

Key words: Hepatocellular carcinoma; Non-B Non-C; Smoking; Surgery; Prognosis

Core tip: We retrospectively analyzed the surgical outcomes and clinicopathological characteristics according to smoking habits in consecutive 83 cases with non-B non-C hepatocellular carcinoma (NBNC-HCC) patients who underwent curative surgical treatment for the primary lesion. Current smoking status was strongly correlated with both overall survival and disease-specific survival by multivariate analyses. Subset analyses revealed that current smokers tended to have multiple HCCs at a younger age than other patients. To our knowledge, this is the first report regarding surgical outcomes of NBNC-HCC patients in relation to their smoking status.

INTRODUCTION

Infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are well known risk factors for the development of hepatocellular carcinoma (HCC). As more than 90% of countries around the world have now introduced the HBV vaccine into their national infant immunization schedules, the incidence of HBV-related HCC has been decreasing dramatically[1]. The development of antivirus therapy can also reduce the incidence of HCV-related HCC[2]. The number of HCC patients who are negative for both hepatitis B surface antigen (HBsAg) and hepatitis C antibody (HCVAb), *i.e.*, those who have so-called "non-B non-C (NBNC) HCC" has rapidly increased in recent years. NBNC HCC patients were reported to account for 24.1% of all HCC patients in a 2010 Japanese survey[3]. It is thus very important, toward the prevention of HCC, to establish all of the etiologies of NBNC-HCC and to devise countermeasures for it.

The known etiologies of NBNC-HCC are alcoholic liver disease (ALD)[4], non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH)[5,6], hemochromatosis[7], and Budd-Chiari syndrome[8]; other known etiologies include primary biliary cirrhosis, autoimmune hepatitis, metabolic disease, congestive disease, parasitic disease and unknown etiology[9]. Emerging epidemiologic data suggest that cigarette smoking may increase the risk of developing HCC[10-12], but a smoking habit is generally less recognized as a risk factor of developing HCC compared to other etiologies such as ALD and NASH/NAFLD.

Surgery is one of the most important therapeutic measures for HCC. The correlations between surgical outcomes and each etiology of HCC are very important because knowing these correlations will provide the motivation and strategies for the prevention of each specific etiology. The clinicopathological characteristics and surgical outcomes of patients with HBV-HCC and HCV-HCC have been well investigated[13-15]. There are also many studies comparing the surgical outcomes or clinicopathological characteristics of patients with NBNC-HCC and those with viral-associated HCC or between each etiology of NBNC-HCC[16-29]. However, no study has addressed the impact of smoking habit on surgical outcomes or clinicopathological characteristics according to smoking habits in patients with NBNC-HCC, to our knowledge.

In the present study we analyzed the correlation between smoking status and surgical outcomes in patients with NBNC-HCC, and we investigated the patients' clinicopathological characteristics according to smoking status.

MATERIALS AND METHODS

Patients
The study protocol was approved by the Ethics Committee of the Faculty of Medicine at Saga University (approval no. 28-23). The initial enrollees in the study were 477 consecutive patients with HCC who underwent curative surgical treatment for the primary lesion at Saga University Hospital between 1984 and

Table 1 Clinicopathologic features of the patients with non-B non-C hepatocellular carcinoma (*n* = 83)

Characteristic		*n* (%)
Age, yr (mean ± SD)		66.4 ± 11.6
Gender	Male	66 (79.5)
	Female	17 (20.5)
Alcohol abuse	(+)	19 (22.9)
	(-)	64 (77.1)
Smoking habit	Never	36 (43.4)
	Ex	23 (27.7)
	Current	24 (28.9)
Diabetes mellitus	(+)	29 (34.9)
	(-)	54 (65.1)
Obesity	(+)	26 (31.3)
	(-)	57 (68.7)
BMI (mean ± SD)		22.8 ± 4.50
Tumor size (mean ± SD mm)		65.2 ± 41.4
Solitary/Multiple	Solitary	52 (62.7)
	Multiple	31 (37.3)
Vp	(+)	32 (38.6)
	(-)	51 (61.4)
Background liver fibrosis[1]	F0	16 (19.5)
	F1	21 (25.6)
	F2	10 (12.2)
	F3	18 (22.0)
	F4	17 (20.7)
NASH[1]	(+)	10 (12.2)
	(-)	72 (87.8)

[1]Noncancerous liver tissue was not available in one case (*n* = 82). NASH: Non-alcoholic steatohepatitis. Vp: Portal vein invasion.

December 2012. Of these patients, we retrospectively examined the cases of the 83 patients who were both non-B (HBsAg-negative) and non-C (HCVAb-negative) in serological tests. One patient with Budd-Chiari syndrome and another patient with Dubin-Johnson syndrome were included. Written informed consent was obtained from all patients for the use of their clinical information.

Analyses of alcohol abuse, obesity, diabetes mellitus and smoking status

Alcohol abuse was defined as a daily ethanol intake > 40 g for men and > 20 g for women. Obesity was defined as a body mass index (BMI) > 25 kg/m^2 in both genders. Smoking status was categorized as never smoker, ex-smoker and current smoker at the time of surgery. A current smoker was defined as an individual who regularly smoked and continued to smoke within 1 year prior to the surgery. An ex-smoker was defined as an individual who quit smoking at least 1 year before his or her surgery[30]. Only patients who were clinically diagnosed as having diabetes mellitus were categorized as being in the present diabetes mellitus group. All of this information was collected from medical records.

Histopathological analysis

The histopathological diagnosis and classification were performed by two pathologists (Kai K and Aishima

S). The degree of fibrosis in noncancerous liver tissues was assessed according to the new Inuyama classification system which is widely used in Japan, as follows: F0, no fibrosis; F1, portal fibrosis widening; F2, portal fibrosis widening with bridging fibrosis; F3, bridging fibrosis plus lobular distortion; and F4, liver cirrhosis[31]. In one case the fibrosis could not be assessed because of an insufficiency of noncancerous liver tissue. The diagnoses of NASH were based on both clinical information and pathological confirmation.

Statistical analyses

We used JMP ver. 12.2 software and SAS software ver. 9.4 (SAS Institute, Cary, NC, United States) for the statistical analyses. The comparisons of pairs of groups were performed using Student's *t*-test, the χ^2 test and Fisher's exact test, as appropriate. Disease-free survival (DFS) was determined as the length of time after surgery that the patient survived without new lesions of HCC. Overall survival (OS) was determined from the time of surgery to the time of death or the most recent follow-up. Disease-specific survival (DSS) was determined from the time of surgery to the time of cancer-related death or most recent follow-up.

Cox proportional hazards modeling was applied for uni- and multivariate analyses. The purpose of the multivariate analysis was to adjust potential covariates for the comparison of smoking status; then, age, gender, portal vein invasion, T factor and multiple tumors were always kept in the model and others were selected by the stepwise procedure with the *P* value threshold of 0.2. Postoperative survival curves were calculated using the Kaplan-Meier method. Differences in survival curves were compared using the log-rank test. Values of *P* < 0.05 were considered significant. All statistical analyses were supervised by the statistician co-author (Kawaguchi A).

RESULTS

Clinicopathological features and risk factors of NBNC-HCC

The clinicopathological features of the 83 cases of NBNC-HCC are summarized in Table 1. The patients were 66 men and 17 women with a mean age at the time of surgery of 66.4 years. Nineteen patients (22.9%) were etiologically categorized into the alcohol abuse group; 29 patients (34.9%) had diabetes mellitus, and 26 patients (31.3%) were judged to be obese. Ten patients (12.2%) were pathologically confirmed as having NASH. Thirty-six patients (43.4%) were categorized into the never-smoker group and 23 (27.7%) into the ex-smoker group and the remaining 24 patients (28.9%) were currently smoking at the time of their surgery. The smoking cessation periods of the ex-smokers were as follows: 1-5 years, two patients; 5-10 years, four patients; and > 10 years, 17 patients.

Figure 1 Survival curves of disease-free survival, overall survival and disease-specific survival according to smoking habit (never, Ex and current) or current smoking habit (current and other). A-C: Kaplan-Meier curves according to smoking habit (current, Ex and never) for disease-free survival (DFS), overall survival (OS) and disease-specific survival (DSS); D-F: Kaplan-Meier curves according to smoking habit (current and other) for DFS, OS and DSS.

Univariate analyses for DFS, OS and DSS

The results of the univariate analyses for DFS, OS and DSS by Cox's proportional hazards model are summarized in Table 2. The factors significantly co-rrelated with DFS were smoking (Ex *vs* current, P = 0.0271), smoking (current *vs* other, P = 0.035), portal vein invasion (P = 0.0035), T factor (P = 0.0004), and multiple tumors at the time of surgery (P = 0.0001). No patient had received adjuvant therapy after curative surgery until recurrence.

The factors significantly correlated with OS were smoking (Ex *vs* current, P = 0.0349), smoking (current *vs* never, P = 0.0034), smoking (Ex + current *vs* never, P = 0.0436), smoking (current *vs* other, P = 0.0024), portal vein invasion (P = 0.0362), and multiple tumors at the time of surgery (P = 0.0211). The factors significantly correlated with DSS were smoking [Ex *vs* current, P = 0.0155, smoking (current *vs* never, P =

0.0092)], smoking (current *vs* other; P = 0.0025), T factor (P = 0.0364) and multiple tumors at the time of surgery (P = 0.0065). The survival curves of DFS, OS and DSS according to smoking habit (never, Ex and current) or current smoking habit (current and other) are provided as Figure 1. The current-smoking group showed significantly poor survival curves compared to all other patient groups in each analysis of DFS, OS and DSS.

Multivariate analyses for DFS, OS and DSS

The results of the multivariate analyses for DFS, OS and DSS by Cox's proportional hazards model are summarized in Table 3. The only factor that was significantly correlated with DFS was portal vein invasion (P = 0.0229). The factors significantly correlated with OS were smoking (current *vs* other) and portal vein invasion (P = 0.0058 and P = 0.0061, respectively). The

Table 2 Univariate analyses for disease-free survival, overall survival and disease-specific survival after hepatic resection

	DFS		OS		DSS	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (≤ 70 yr)	0.777 (0.434-1.389)	0.3948	0.959 (0.529-1.739)	0.8915	0.757 (0.360-1.591)	0.4622
Gender (male)	0.742 (0.382-1.443)	0.3796	1.466 (0.653-3.291)	0.3537	1.351 (0.515-3.545)	0.5404
Occult HBV infection	1.052 (0.564-1.961)	0.8736	1.184 (0.628-2.232)	0.6009	1.328 (0.625-2.821)	0.4604
Alcohol abuse	0.700 (0.326-1.503)	0.3605	1.492 (0.749-2.969)	0.2549	1.489 (0.631-3.514)	0.3639
Smoking (Ex *vs* current)	0.405 (0.182-0.903)	0.0271	0.451 (0.215-0.945)	0.0349	0.299 (0.113-0.795)	0.0155
Smoking (Ex *vs* never)	0.680 (0.319-1.453)	0.3197	1.295 (0.597-2.807)	0.5132	0.905 (0.328-2.501)	0.848
Smoking (current *vs* never)	1.680 (0.874-3.230)	0.1199	2.869 (1.417-5.807)	0.0034	3.026 (1.315-6.968)	0.0092
Smoking (Ex + current *vs* never)	1.078 (0.606-1.920)	0.7976	1.926 (1.019-3.640)	0.0436	1.735 (0.804-3.745)	0.1604
Smoking (current *vs* other)	1.926 (1.047-3.544)	0.035	2.569 (1.397-4.724)	0.0024	3.144 (1.497-6.604)	0.0025
Diabetes mellitus	0.894 (0.474-1.685)	0.7285	1.715 (0.941-3.126)	0.0779	1.414 (0.665-3.008)	0.3681
Pathological NASH	1.222 (0.516-2.892)	0.649	1.076 (0.423-2.736)	0.8781	1.342 (0.465-3.873)	0.5863
Obesity	0.989 (0.535-1.828)	0.9714	1.049 (0.556-1.981)	0.8827	1.183 (0.549-2.549)	0.6673
Fibrosis	1.163 (0.699-1.936)	0.5608	1.004 (0.583-1.729)	0.9883	1.407 (0.742-2.670)	0.2956
Vp	2.428 (1.338-4.407)	0.0035	1.892 (1.042-3.436)	0.0362	1.918 (0.919-4.002)	0.0828
T factor (T3/4 *vs* T1/2)	3.220 (1.680-6.169)	0.0004	1.793 (0.984-3.268)	0.0565	2.222 (1.052-4.693)	0.0364
Multiple tumors	3.275 (1.784-6.014)	0.0001	2.009 (1.110-3.636)	0.0211	2.767 (1.329-5.761)	0.0065

Vp: Portal vein invasion; DFS: Disease-free survival; DSS: Disease-specific survival; HBV: Hepatitis B virus; NASH: Non-alcoholic steatohepatitis; OS: Overall survival.

Table 3 Multivariate analyses for current smokers *vs* other (age, gender, Vp, T factor in, *P* = 0.2)

Type	Label	HR (95%CI)	*P* value
DFS	Smoking (current *vs* other)	1.897 (0.888-4.054)	0.0985
	Age (≤ 70 yr)	0.627 (0.330-1.191)	0.1535
	Gender (male)	0.611 (0.279-1.337)	0.2176
	Vp	2.656 (1.145-6.165)	0.0229
	T factor (T3/4 *vs* T1/2)	1.574 (0.537-4.609)	0.4083
	Multiple tumors	1.930 (0.807-4.614)	0.1394
	Alcohol abuse	0.547 (0.228-1.310)	0.1755
OS	Smoking (current *vs* other)	2.807 (1.349-5.840)	0.0058
	Age (≤ 70 yr)	1.189 (0.603-2.346)	0.6177
	Gender (male)	1.362 (0.555-3.343)	0.4999
	Vp	3.069 (1.377-6.839)	0.0061
	T factor (T3/4 *vs* T1/2)	0.532 (0.187-1.512)	0.2364
	Multiple tumors	1.830 (0.760-4.405)	0.1774
DSS	Smoking (current *vs* other)	3.133 (1.307-7.512)	0.0105
	Age (≤ 70 yr)	0.988 (0.424-2.302)	0.9775
	Gender (male)	1.406 (0.463-4.271)	0.5478
	Vp	2.756 (1.095-6.935)	0.0313
	T factor (T3/4 *vs* T1/2)	0.610 (0.167-2.233)	0.4555
	Multiple tumors	2.476 (0.797-7.693)	0.1169
	Pathological NASH	2.320 (0.689-7.811)	0.1741

DFS: Disease-free survival; DSS: Disease-specific survival; OS: Overall survival; Vp: Portal vein invasion.

factors significantly correlated with DSS were smoking (current *vs* other) and portal vein invasion (*P* = 0.0105 and *P* = 0.0313, respectively).

Subset analysis between the current smokers and the other patients

To clarify the characteristics of the current smokers, we further performed subset analyses regarding the clinicopathological factors, treatment and causes of death (Table 4). The current smokers were significantly younger than the Never + Ex patient group (mean age 59.4 year *vs* 69.3 year, *P* = 0.0002) at the time of

surgery. The current smokers group had significantly greater incidences of alcohol abuse (*P* = 0.0188) and multiple tumors (*P* = 0.023). No significant difference was observed in gender, diabetes mellitus, obesity, indocyanine green retention rate at 15 min (ICG R15), tumor size, portal vein invasion, T factor, serum AFP level or liver fibrosis.

Thirteen of the 24 patients (54.2%) in the Current smoking group died of HCC, whereas 17 of the 59 patients (28.8%) in the Never + Ex patient group died of HCC (*P* = 0.0293). Five of the 24 patients (20.8%) in the Current smoking group died of other causes: cerebral hemorrhage, surgical complication for gastric cancer, pneumoniae (two cases) and sepsis due to pseudomembranous colitis.

In the Never + Ex group, 11 of the 59 patients (18.6%) died of other causes: other malignancy, four patients (two cases of bile duct cancer, prostatic cancer, malignant lymphoma); cerebral infarction, two patients; liver failure, two patients; pneumonia, one patient; sudden cardiac death, one patient and renal failure, one patient.

DISCUSSION

To determine the influence of etiological differences on the outcomes of the surgical treatment for HCC, many studies have compared surgical outcomes between patients with NBNC-HCC and those with viral-associated HCC, but the results are controversial. Some studies showed that surgical outcomes in patients with NBNC-HCC were not significantly different compared to those of patients with hepatitis virus-related HCC[17,20,23,29]. Other studies reported that NBNC-HCC patients had significantly better surgical outcomes than HCV-HCC patients[18,24,26]. In a recent Japanese nationwide study of 2738 NBNC-HCC patients, the

Table 4 Comparison of current smoking status and clinicopathological factors (*n* = 83)

	Current (*n* = 24)	Never + Ex (*n* = 59)	*P* value
Age, yr (mean ± SD)	59.4 ± 12.0	69.3 ± 10.2	0.0002
Gender (male/female)	22/2	44/15	0.0803
Alcohol abuse (+/-)	10/14	9/50	0.0188
Diabetes mellitus (+/-)	8/16	21/38	0.8448
Obesity (+/-)	6/18	20/39	0.6024
ICG R15 (%)	11.7 ± 1.8	14.8 ± 1.2	0.1235
Tumor size (mean ± SD mm)	68.4 ± 44.0	63.9 ± 40.6	0.6724
Solitary/Multiple	10/14	42/17	0.023
Vp (+/-)	9/15	23/36	0.8998
T factor (T12/T34)	10/24	33/26	0.3329
AFP (mean ± SD)	4450 ± 14563.6	3235 ± 13042	0.6442
Fibrosis (F12/F34, *n* = 82)	11/13	36/22	0.2224
Recurrence (+/-)	16/8	32/27	0.3362
Therapy for recurrent tumor (+/-)[1]	14/2	27/5	0.7724
TAE only	11	14	
Surgical resection	1	5	
Ablation	1	2	
Chemotherapy	1	3	
Multiple therapy[2]	0	3	
Tumor-related death (%)	13 (54.2)	17 (28.8)	0.0293
Other cause of death (%)	5 (20.8)	11 (18.6)	0.8187

[1]Comparison restricted in recurrent case; [2]Two cases of TAE + operation and one case of TAE + ablation. ICG R15: Indocyanine green retention rate at 15 min; TAE: Transcatheter arteriography embolization; Vp: Portal vein invasion.

DFS of the NBNC-HCC group was significantly better than those of the HBV-HCC and HCV-HCC groups[16]. For the purpose of clarifying the association between smoking status and surgical outcomes, we focused on NBNC-HCC in the present study because the surgical outcomes of viral-associated HCC may be influenced by cirrhosis due to viral-associated hepatitis.

Many studies have been reported regarding surgical outcomes and etiologies of NBNC-HCC. The relationships between the surgical outcomes of NBNC-HCC and metabolic diseases such as obesity, diabetes mellitus and NAFLD/NASH have been extensively investigated. Several investigations indicated favorable surgical outcomes of NBNC-HCC associated with NAFLD/NASH compared to those of viral-associated HCC[21-32]. It was reported that obesity did not affect survival in patients with NBNC-HCC after curative therapy[29]. In contrast, a large retrospective Japanese multicenter cohort study of 5326 patients with NBNC-HCC indicated that patients with BMI values > 22 and ≤ 25 kg/m^{2} showed the best prognoses compared to other BMI categories, after adjusting for age, gender, tumor-related factors, and Child-Pugh score[3]. However, these previous studies regarding the surgical results of NBNC-HCC patients did not involve an analysis of smoking habit despite the recognition of smoking as risk factor for NBNC-HCC. We therefore focused on the smoking habit in the present study.

The major finding of the present study is the strong correlation between smoking habit and surgical outcomes. Although emerging epidemiologic data suggest that cigarette smoking may increase the risk of HCC[10-12], the influence of cigarette smoking on HCC survival has not been well documented. We were able to find 10 studies in the English literature that analyzed the correlation of smoking and HCC mortality[33-42]. Large cohort studies indicating an impact of smoking habit on HCC mortality have been reported form Japan[33,34], the United Kingdom[35], China[36] and Taiwan[37-39]. In contrast, several studies reported a negative correlation between HCC mortality and smoking habit, although those studies analyzed relatively small numbers of HCC cases (262-552 cases)[40-42].

Most of the previous studies regarding HCC mortality and smoking habit did not focus on the surgical outcomes of HCC patients or distinguish surgical cases versus nonsurgical cases. We were able to identify only two reports from China that focused on surgical outcomes of HCC patients: Zhang *et al*[30] analyzed the outcomes of 302 patients with HBV infection who had undergone surgical resection for HCC, and their findings revealed a significant influence of smoking status on both recurrence and mortality. Lv *et al*[43] investigated the outcomes of 425 patients with a predominant population (74%) of HBV infection who were undergoing hepatectomy for HCC, and those authors' analysis revealed that cigarette smoking is an independent risk factor for the development of liver-related and infectious complications. We were unable to find any other study reporting the surgical outcomes of NBNC-HCC patients in relation to their smoking status.

In the present study, the survival of the current smokers was significantly poorer than that of the never-smokers, and no significant difference in the survival of the ex-smokers and never-smokers was revealed. We speculate that the reason for the latter finding is due to the long cessation period (> 10 years) for most of the ex-smokers. Our subset analyses comparing the current smokers and the other patients revealed that the current smokers were significantly younger and were more likely to have multiple tumors at the time of surgery. These findings suggest that a smoking habit may affect multicentric liver carcinogenesis. The underlying mechanism of liver carcinogenesis induced by smoking is as yet unclear and should be investigated in further studies.

Generally, alcoholic abuse is known as a poor-prognosis risk factor in surgical treatment for HCC because of the poor liver function of individuals who abuse alcohol. Indeed, in the present study the proportion of alcohol-abusing patients was higher in the current smoking group, but no significant between-group difference was observed in liver function as represented by the ICG R15 or liver fibrosis. Notably, over half of our study's current-smoker patients died of HCC, and only five of the current smokers died of other causes. Thus, the causes of death in the current-

smoker group were significantly different from those of the other patient groups.

Although current smoking was significantly co-rrelated with DFS in our univariate analysis, no such significance was observed in the multivariate analyses, whereas current smoking showed significant correlations with the OS and DSS in the multivariate analyses. One possible reason for this is our study's small sample size. Another possible reason is that the malignant potential of the recurrent tumors in the current-smoker group may be different from those of the other groups, because the survival after recurrence was significantly different despite the lack of a significant difference in the recurrence rate and the treatments for the recurrent tumors between the current-smoker group and the other groups.

The limitations of the present study are its re-trospective design, the relatively small number of patients, and the long study period for enrollment. In addition, information about our patients' post-surgery smoking status was not available, and thus the effects on survival of a smoking habit after surgery and a smoking habit after recurrence could not be examined. It remains quite regrettable that the previous large case series and multicenter studies did not investigate the HCC patients' smoking status. We hope a larger prospective study verifies the precise influence of smoking on the survival of patients with NBNC-HCC, as its results can be expected to provide further motivation for smoking cessation.

In conclusion, the results of our single-institute retrospective study indicate that current smoking habit is significantly correlated with the surgical outcomes of patients with NBNC-HCC. Our analyses also revealed that the current smokers were significantly younger than the other patient groups and had significantly greater incidences of alcohol abuse and multiple tumors at the time of surgery.

COMMENTS

Research frontiers
No previous study has addressed the impact of smoking habit on surgical outcomes or clinicopathological characteristics according to smoking habits in patients with non-B non-C hepatocellular carcinoma (NBNC-HCC).

Innovations and breakthroughs
The novel findings of this study are (1) current smoking habit at the time of surgical treatment is a risk factor for poor long-term survival in NBNC-HCC patients; and (2) current smokers tend to have multiple HCCs at a younger age than other patients.

Applications
The results of present study can be expected to provide further motivation for smoking cessation.

Terminology
NBNC-HCC is defined as hepatocellular carcinoma that has arisen in an individual who is negative for both hepatitis B surface antigen and hepatitis C antibody. Alcohol abuse was defined as a daily ethanol intake > 40 g for men and > 20 g for women. Obesity was defined as a body mass index > 25 kg/m^2

in both genders. A current smoker was defined as an individual who regularly smoked and continued to smoke within 1 year prior to the surgery. An ex-smoker was defined as an individual who quit smoking at least 1 year before his or her surgery.

Peer-review
An interesting paper about risk factors in patients with NBNC hepatocellular cancer. Results are adequate and conclusions are very clear.

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Retrospective Cohort Study

Hepatic artery infusion with raltitrexed or 5-fluorouracil for colorectal cancer liver metastasis

Jian-Hai Guo, Hang-Yu Zhang, Song Gao, Peng-Jun Zhang, Xiao-Ting Li, Hui Chen, Xiao-Dong Wang, Xu Zhu

Jian-Hai Guo, Hang-Yu Zhang, Song Gao, Peng-Jun Zhang, Hui Chen, MD, Xiao-Dong Wang, Xu Zhu, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Interventional therapy, Peking University Cancer Hospital and Institute, Beijing 100142, China

Xiao-Ting Li, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Radiology, Peking University Cancer Hospital and Institute, Beijing 100142, China

Correspondence to: Xu Zhu, MD, Chief, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Interventional therapy, Peking University Cancer Hospital and Institute, 52 Fucheng Road, Haidian District, Beijing 100142, China. drzhuxu@163.com
Telephone: +86-10-88196330

Abstract

AIM
To evaluate the efficiency and safety of hepatic artery infusion chemotherapy (HAIC) using raltitrexed or 5-fluorouracil for colorectal cancer (CRC) liver metastasis (CRCLM).

METHODS
A retrospective analysis of patients with unresectable CRCLM who failed systemic chemotherapy and were subsequently treated with HAIC at our institute from May 2013 to April 2015 was performed. A total of 24 patients were treated with 5-fluorouracil, and 18 patients were treated with raltitrexed.

RESULTS
The median survival time (MST) from diagnosis of CRC was 40.8 mo in the oxaliplatin plus raltitrexed (TOMOX) arm and 33.5 mo in the oxaliplatin plus 5-fluorouracil (FOLFOX) arm ($P = 0.802$). MST from first HAIC was 20.6 mo in the TOMOX arm and 15.4 mo in the FOLFOX arm ($P = 0.734$). Median progression-free survival (PFS) from first HAIC was 4.9 mo and 6.6 mo, respectively, in the TOMOX arm and FOLFOX arm (P

= 0.215). Leukopenia (P = 0.026) was more common in the FOLFOX arm, and hepatic disorder (P = 0.039) was more common in the TOMOX arm. There were no treatment-related deaths in the TOMOX arm and one treatment-related death in the FOLFOX arm. Analysis of prognostic factors indicated that response to HAIC was a significant factor related to survival.

CONCLUSION
No significant difference in survival was observed between the TOMOX and FOLFOX arms. HAIC treatment with either TOMOX or FOLFOX was demonstrated as an efficient and safe alternative choice.

Key words: Liver metastasis; Hepatic artery infusion chemotherapy; Raltitrexed; Colorectal cancer; FOLFOX

Core tip: Our study shows that hepatic artery infusion chemotherapy (HAIC) with either TOMOX (oxaliplatin plus raltitrexed) or FOLFOX (oxaliplatin plus 5-fluorouracil) was proven to be an efficient and safe alternative choice for patients with chemotherapy refractory colorectal cancer liver metastasis and no significant difference in survival was found between these two treatments. Cox univariate analysis shows that response to HAIC was a significant predictive factor.

INTRODUCTION

Colorectal cancer is the third leading cause of cancer death and has the third leading incidence of new cases in Western countries[1]. The situation in China is similar; there were 376.3 thousand new colorectal cancer cases in 2015, and colorectal cancer was the fifth leading cause of cancer death[2]. Approximately 30%-50% of patients develop liver metastasis, and no more than 20% of liver metastasis patients are candidates for liver resection[3,4]. Chemotherapy is the primary treatment for advanced colorectal cancer. The efficiency and survival benefit of standard first- or second-line systemic therapy have been improved by the combination of targeted therapy[5,6], and the overall survival (OS) after effective first-line therapy is nearly 30 mo[7-9]. However, the survival of chemotherapy refractory patients, who failed previous systemic treatment, is expected to improve. Third-line chemotherapy could result in an OS period of 9.3 mo[10]. Alternative treatments, such as transarterial

chemoembolization (TACE) and hepatic artery infusion chemotherapy (HAIC), are greatly needed.

HAIC with FOLFOX [oxaliplatin plus 5-fluorouracil (5-Fu)] in patients with CRCLM has also been demonstrated as a feasible and low-toxicity treatment, with a local overall disease control rate of 50%-79.2%[11,12]. However, 5-Fu should be administered intra-arterially for approximately 44 h, and a higher incidence of catheter thrombosis and catheter-associated infection is reported[13]. As a specific inhibitor of thymidylate synthase, raltitrexed has been used in CRC patients and could be infused in approximately 1 h. Several previous studies have shown that TOMOX (oxaliplatin plus raltitrexed) showed efficiency similar to other traditional first-line treatments in CRC patients and was associated with less neutropenia and gastrointestinal toxicity and uncommon cardiotoxicity[14-16]. However, studies concerning HAIC with TOMOX are rare. Khouri *et al*[3] examined 17 patients who underwent HAIC with TOMOX, and the treatment was demonstrated as a safe alternative choice. The goal of this retrospective study was to report a head-to-head study comparing the TOMOX and FOLFOX arms in CRCLM patients treated at our center.

MATERIALS AND METHODS

Study design and patient population
From May 2013 to April 2015, 42 patients were treated with oxaliplatin-based HAIC at our center. All of the patients were histologically confirmed with colorectal adenocarcinoma with unresectable liver metastasis and failed two lines of systemic chemotherapy. The treatment criteria for HAIC were: ECOG performance status no more than 2 points; life expectancy ⩾ 3 mo; tumor involvement less than 70% of liver volume; and adequate liver and renal dysfunction (total bilirubin serum levels < 3 mg/dL, serum albumin level > 20 g/L, and serum creatinine level < 2 mg/dL). Patients with extrahepatic metastases were included if their main lesion remained in the liver.

Operative technique
The Seldinger technique was used to access the femoral artery after the achievement of local anesthesia. Then, arteriography was routinely performed prior to chemoembolization to gather information for the abdominal aorta and celiac trunk. Subsequently, a coaxial catheter (Renegade Hi Flo, Boston Scientific, United States/Stride ASAHI INTECC, Japan) was inserted into the hepatic artery and subsegmental arteries. According to tumor stain, Spongostan particles (Jinling, Nanjing, China) and iodized oil (Lipiodol; Laboratoire Andre Guerbet, Aulnaysous-Bois, France) mixed with 20-40 mg epirubicin hydrochloride (Main Luck Pharmaceutical, Shenzhen, China) were injected. The temporary indwelling catheter was inserted into the hepatic artery until the end of HAIC. HAIC was performed *via* the catheter

Table 1 Summary of patient baseline characteristics

	Overall cohort (*n* = 42)	TOMOX (*n* = 18)	FOLFOX (*n* = 24)	*P* value
Gender				0.700
Male	29	13	16	
Female	13	5	8	
Age at first TACE (yr)	59 ± 10.7	60 ± 9.1	58 ± 11.8	0.473
Primary tumor site				0.601
Right hemicolon	10	5	5	
Left hemicolon	32	19	13	
Time to liver metastasis				0.508
Synchronous	28	11	17	
Metachronous	14	7	7	
Primary tumor grade				0.639
Poor	6	3	3	
Well to moderate	36	15	21	
Genetic condition				0.459
KRAS mutation	8	5	3	
KRAS wild type	21	8	13	
Unknown	13	5	8	
Extrahepatic metastasis				0.927
Present	27	12	15	
Absent	15	6	9	
Combined with other local treatments				0.209
Yes	10	6	4	
No	32	12	20	

Table 2 Response evaluation *n* (%)

Response	Treatment group		*P* value
	FOLFOX (*n* = 24)	TOMOX (*n* = 18)	
Partial response	7 (29.2)	2 (11.1)	0.158
Stable disease	14 (58.3)	11 (61.1)	0.856
Progressive disease	3 (12.5)	5 (27.8)	0.734

analyses. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) was used to generate the charts. For all tests, a *P* value < 0.05 was defined as significant. Student's *t*-test was used to analyze continuous variables. These variables were reported as the means ± SD if normally distributed or as a median and range if skewed. The χ^2 test was used to analyze categorical variables. These variables were reported as a proportion (%) of the overall cohort. The Kaplan-Meier method was used to approximate the PFS and OS, and the significance of survival differences between the TOMOX and FOLFOX arms was determined using the log-rank test.

with oxaliplatin (Hengrui Medicine Co., Ltd., Jiangsu, China) administered at 85 mg/m^2 in 4 h, 5-Fu (Jinyao aminoacid Co., Ltd., Tianjing, China) administered at 2000 mg/m^2 in approximately 44 h, CF (Hengrui Medicine Co., Ltd. Jiangsu China) administered at 200 mg/m^2 in 2-4 h *via* the peripheral vein, and raltitrexed (Tianqing Pharmaceutical Co., Ltd., Nanjing, China) administered at 3 mg/m^2 in approximately 1 h. At the end of perfusion, the catheter was removed every cycle.

HAIC was regularly applied every 3 wk, until the patient died or liver function was Child-Pugh C or disease progressed. Enhanced computed tomography or magnetic resonance imaging and laboratory tests were regularly performed, and all patients were followed until death or loss to follow-up. Objective response rate (ORR) was evaluated using Response Evaluation Criteria in Solid Tumor (RECIST) version 1.1, and adverse reactions were evaluated using Common Terminology Criteria for Adverse Events (CTCAE) 2.0. Peripheral neuropathy was graded according to a modified Levi Scale.

Statistical analysis
OS after diagnosis was calculated from the date of diagnosis of CRC to the date of death or last follow-up time, OS after first HAIC was calculated from the date of first HAIC to the date of death or last follow-up time, and PFS was calculated from the date of the initiation of therapy to the date of disease progression. A biomedical statistician conducted the statistical review in the present study. The SPSS software program (version 19; SPSS, Chicago, Illinois) was used for the

RESULTS

Patient characteristics
There were 18 patients in the TOMOX arm and 24 patients in the FOLFOX arm. The baseline characteristics of the patients are shown in Table 1. The baseline demographics were similar between the two treatment groups, with no significant imbalances in sex, age, primary tumor site, time of liver metastasis, KRAS mutation rate, extrahepatic metastasis, or additional radiofrequency ablation. Patients in the TOMOX arm received a median of 2.2 cycles of treatment, and those in the FOLFOX arm received a median of 2.1 cycles of treatment.

Efficacy and toxicity
With a median follow-up period of 18 mo, the OS after the first HAIC in the FOLFOX and TOMOX arms was 15.4 and 20.6 mo (*P* = 0.734), respectively. The PFS in the FOLFOX and TOMOX arms was 6.6 and 4.0 mo (*P* = 0.215), respectively (Figure 1). The response rates of the two different treatment groups are shown in the Table 2. The overall response rate was 29.2% in the FOLFOX arm and 11.1% in the TOMOX arm, and no significant difference was observed between the FOLFOX and TOMOX groups (*P* = 0.158).

Cox univariate analysis (Table 3) showed that the response to HAIC was a predictive factor for prognosis. However, age, histology grade, primary tumor site, serum tumor markers, and extrahepatic metastasis showed no significance as predictive factors.

All patients were evaluated for toxicity. The toxicity of the two groups is shown in Table 4. The most common adverse events were transient elevation of serum liver enzymes and bilirubin and abdominal

Table 3 Predictors of overall survival

Factor	Univariate analysis		
	HR	95%CI	*P* value
TOMOX/FOLFOX	0.877	0.410-1.876	0.736
Male sex	0.915	0.411-2.035	0.827
Age (> 60/60 yr)	0.758	0.353-1.627	0.477
Histology (poor/well and moderate)	1.768	0.686-4.554	0.238
Primary tumor site (left/right hemicolon)	0.715	0.285-1.797	0.476
Serum CA19-9 (high/normal)	1.725	0.803-3.706	0.162
Serum CA72-4 (high/normal)	1.325	0.536-3.278	0.542
Serum CEA (high/normal)	1.339	0.463-3.873	0.590
Extrahepatic metastasis (present/absent)	1.220	0.550-2.706	0.624
Time to liver metastasis (synchronous/metachronous)	1.281	0.560-2.932	0.558
Response to TACE			0.047
PD	1.000	1.000	
SD	0.275	0.081-0.931	
PR	0.272	0.095-0.783	

Figure 1 Kaplan-Meier curves showing the survival data after hepatic artery infusion chemotherapy. The median survival time of the TOMOX arm was 20.6 mo (curve A), and that of the FOLFOX arm was 15.4 mo (curve B).

Table 4 Observed toxicity according to common terminology criteria for adverse events grading *n* (%)

Adverse event	TOMOX (*n* = 18)		FOLFOX (*n* = 24)		*P* value
	All grade	Severe	All grade	Severe	
Hematological					
Anemia	7 (39)		11 (46)		0.212
Leucopenia	3 (16)		12 (50)	1 (4)	0.026
Neutropenia	1 (5)		6 (25)	1 (4)	0.094
Thrombocytopenia	8 (44)		13 (54)	3 (12)	0.533
Nonhematological					
Elevation of liver enzymes	18 (100)	9 (50)	19 (79)	7 (29)	0.039
Elevation of bilirubin	17 (94)	3 (17)	23 (95)	4 (17)	0.834
Nausea/vomiting	14 (78)		17 (71)		0.839
Asthenia	13 (72)		12 (50)		0.414
Neuropathy	5 (28)		7 (29)	1 (4)	0.921
Pain	14 (78)	7 (39)	19 (79)	13 (54)	0.914
Fever	6 (33)		11 (46)		0.558

pain. The transient elevation of serum liver enzymes was more frequent in the TOMOX arm than in the FOLFOX arm (100% *vs* 79%, *P* = 0.039). Hematologic adverse events were more frequent in the FOLFOX arm than in the TOMOX arm (leukopenia: 16% *vs* 50%, *P* = 0.026; anemia: 39% *vs* 46%, *P* = 0.212; and thrombocytopenia: 44% *vs* 54%, *P* = 0.533). No significant differences were observed in fever, asthenia, nausea and vomiting and neuropathy between these two treatment groups. Treatment associated cardiotoxicity was not observed in either group. One treatment-related death, diagnosed as neutropenic sepsis, occurred in the FOLFOX arm. No treatment-related death was observed in the TOMOX arm.

DISCUSSION

Without an efficient treatment, systemic chemotherapy refractory patients show a median OS of 3.5 mo[17]. HAIC has been demonstrated as an alternative choice for advanced CRC patients. Most studies report the efficiency and survival data of HAI with FOLFOX, while reports concerning HAI with TOMOX are rare. Raltitrexed has been demonstrated as a considerable first-line treatment for patients with advanced CRC. Herein, we present the first head-to-head study comparing HAI with TOMOX or FOLFOX in systemic chemotherapy refractory CRC patients.

The median OS after first HAIC in the present study was 15.4 mo in the FOLFOX arm and 20.6 mo in the TOMOX arm, which was favorable compared with that of the third-line systemic chemotherapy, which achieved a median OS of 9.3 mo[10]. When TOMOX was used as a first-line treatment, the ORR was 16%-50%, and the median PFS was 5-11 mo[18-20]. Among all patients in the present study who failed in previous systemic chemotherapy, the ORR (11.1%) and median PFS (4.9 mo) were relatively low. The ORR in the FOLFOX arm was 29.2% with a median PFS of 6.6 mo, consistent previous studies[11,21,22]. Similarly, the median OS of 15.4 mo in the present study is consistent with the 11 and 18.3 mo reported in two previous studies[11,21].

The most common adverse events were the transient elevation of serum liver enzymes and bilirubin and abdominal pain. These common adverse events could be sufficiently controlled by efficient treatments. Similar to previous studies, the incidence of leukopenia grade was significantly higher in the FOLFOX arm, and the elevation of transient hepatic enzymes was significantly higher in the TOMOX arm. The TOMOX arm had no treatment- related deaths, while the FOLFOX arm had one case of neutropenic sepsis. These findings suggest that HAIC with TOMOX could represent tolerable treatments for refractory CRC patients. Survival predictor analysis suggested that early tumor response is a meaningful predictor for patients receiving oxaliplatin-based HAIC. Other factors, including age, primary tumor site, and serum tumor markers, did not show significant difference, partly reflecting the limited sample size in the present study.

The limitation of the present study is a single-center retrospective study with a limited sample size. We could not avoid some bias for the evaluation of clinical outcome and the incomplete patient data. However, the present study was the first to compare the efficiency, survival data, and toxicity of HAIC with TOMOX and FOLFOX in advanced CRC patients, and the results will provide new directions for clinical practice.

COMMENTS

Background

Although liver metastasis develops in approximately 30%-50% of colorectal cancer patients, efficient treatments for advanced colorectal cancer are rare. Third-line chemotherapy confers only a survival period of 9.3 mo. Alternative treatment, such as hepatic artery infusion, is greatly needed. Previous studies have shown that hepatic artery infusion with oxaliplatin and 5-Fu is a safe and efficient choice for these patients; however, 5-Fu should be administered intra-arterially for approximately 44 h and is associated with a higher incidence of catheter thrombosis and infection. Raltitrexed, which could be infused in one hour, is a specific inhibitor of thymidylate synthase and has been reported as an efficient agent in colorectal cancer.

Research frontiers

The authors propose that hepatic artery infusion with raltitrexed and oxaliplatin (TOMOX) is a safe and efficient treatment for patients with colorectal cancer liver metastasis. Herein, we provide support for this hypothesis, showing similar response rates and survival data between the FOLFOX and TOMOX arms.

Innovations and breakthroughs

Previous studies have shown that raltitrexed is a considerable first-line treatment for patients with advanced colorectal cancer. The present study is the first head-to-head study comparing hepatic artery infusion (HAI) with TOMOX or FOLFOX in systemic chemotherapy refractory colorectal cancer patients.

Applications

Patients with colorectal cancer liver metastasis who failed systemic chemotherapy were treated with hepatic artery infusion with TOMOX or FOLFOX.

Terminology

HAI chemotherapy is designed to improve the chemotherapy benefits for liver cancer by increasing the amount of chemotherapy delivered to the site of the tumor. Chemotherapy is dispensed from a specialized infusion system in which a catheter is placed into the hepatic artery to directly deliver the chemotherapy to the liver.

Peer-review

This study, concerning hepatic artery infusion with raltitrexed or 5-fluorouracil for colorectal cancer liver metastasis, is interesting.

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ORIGINAL ARTICLE

Retrospective Study

Do pathological variables have prognostic significance in rectal adenocarcinoma treated with neoadjuvant chemoradiotherapy and surgery?

Luca Reggiani Bonetti, Simona Lionti, Federica Domati, Valeria Barresi

Luca Reggiani Bonetti, Federica Domati, Department of Laboratory Integrated Activities, Anatomic Pathology and Legal Medicine, Section of Pathology, University of Modena and Reggio Emilia, 41124 Modena, Italy

Simona Lionti, Valeria Barresi, Department of Pathology in Evolutive Age and Adulthood, "Gaetano Barresi", University of Messina, 98125 Messina, Italy

Author contributions: Reggiani Bonetti L designed the study, revised the histological slides, analyzed the results and wrote the paper; Lionti S designed the study, performed the statistical analyses and supervised the report; Domati F designed the study, collected the clinical data and supervised the report; Barresi V designed the study, revised the histological slides, analyzed the results and supervised the report.

Institutional review board statement: Ethical issues were discussed with the local ethics committee. Since the study was retrospective, no approval was needed to revise the histological slides.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Correspondence to: Dr. Valeria Barresi, Department of Pathology in Evolutive Age and Adulthood, "Gaetano Barresi", University of Messina, Via Consolare Valeria, 98125 Messina, Italy. vbarresi@unime.it
Telephone: +39-90-2212537

Abstract

AIM
To clarify which factors may influence pathological tumor response and affect clinical outcomes in patients with locally advanced rectal carcinoma treated with neo-adjuvant chemoradiotherapy and surgery.

METHODS
Tumor regression grade (TRG) according to the Dworak system and yTNM stage were assessed and correlated with pre-treatment clinico-pathological variables in 215 clinically locally advanced (cTNM stage II and III) rectal carcinomas. Prognostic value of all pathological and clinical factors on disease free survival (DFS) and cancer specific survival (CSS) was analyzed by Kaplan Meier and Cox-regression analyses.

RESULTS
cN+ status, mucinous histotype or poor differentiation in the pre-treatment biopsy were significantly associated with lower pathological response (low Dworak grade and TNM remaining unchanged/upstaging). Cases showing acellular mucin pools in surgical specimens all had unremarkable clinical courses with no deaths or recurrences during follow-up. Dworak grade had

prognostic significance for DFS and CSS. However, compared to the 5-tiered system, a simplified two-tiered grading system, in which grades 0, 1 and 2 were grouped as absent/partial regression and grades 3 and 4 were grouped as total/subtotal regression, was more reproducible and prognostically informative. The two-tiered Dworak system, yN stage, craniocaudal extension of the tumor and radial margin status were significant independent prognostic variables.

CONCLUSION
Our data suggest that caution should be applied in using a conservative approach in rectal carcinomas with cN+ status, extensive/lower involvement of the rectum and mucinous histotype or poor differentiation. Although Dworak TRG is prognostically significant, a simplified two-tiered system could be preferable. Finally, cases with acellular mucin pools should be carefully evaluated to definitely exclude residual mucinous carcinoma.

Key words: Rectal carcinoma; Dworak; Acellular mucin pools; Downstaging; Mucinous

Core tip: This study evaluates the prognostic significance of clinico-pathological variables in patients with locally advanced rectal carcinoma treated with neo-adjuvant chemo-radiotherapy (CRT) and surgery. Our data show that tumors with cN+ status, extensive/lower involvement of the rectum, mucinous histotype and poor differentiation have a lower response to pre-operative CRT. Dworak tumor regression grade was prognostically informative; however, a simplified two-tiered system was more reproducible and prognostically significant. Acellular mucin pools were found in a percentage of cases with excellent outcomes. Although acellular mucin pools should be considered as complete pathological responses, careful histological examination is mandatory to exclude residual mucinous carcinoma.

INTRODUCTION

Neo-adjuvant chemo-radiotherapy (CRT) currently represents the standard of care for locally advanced (clinical T stage 3/4 or N+) rectal cancer[1,2]. Indeed, CRT improves resectability and sphincter preservation and decreases the probability of local recurrence in patients affected by rectal carcinoma[3,4]. However,

tumor response to CRT is highly heterogeneous and governed by unclear mechanisms[5]. Post-treatment Tumor Node Metastasis (yTNM) stage and Tumor Regression Grade (TRG) are currently used to measure tumor response in surgical specimens obtained after CRT. According to several studies, TRG seems to have prognostic relevance on both disease-free survival (DFS) and overall survival in patients with rectal cancer[6-9]. Several systems have been proposed to score TRG, and all are based on the proportion of residual tumor to stromal fibrosis in the primary tumor site[10-13]. However, none of the systems currently in use is universally accepted, and all may have fair interobserver concordance due to the lack of precise and objective criteria for scoring[14]. Of note, several authors reported on the presence of mucin pools devoid of neoplastic cells in surgical rectal carcinomas pre-treated with CRT with an incidence ranging between 4.8% and 31%[15-19]. One issue in the assessment of TRG of rectal cancer relates to the interpretation of acellular mucin pools[15-19]. Indeed, only the TRG proposed by the Royal College of Pathologists clearly states that acellular mucin pools should be regarded as complete tumor regression[20], while the other grading systems do not give a precise indication on this topic. Additionally, only a few papers addressed the prognostic significance of acellular mucin pools in rectal cancer treated with neo-adjuvant therapy and these studies reported controversial findings[15-19]. Further, some authors suggest that acellular mucin deposits are associated with higher biological aggressiveness of the tumor[16], while others do not find any association with patient prognosis[15,17-19]. Finally, whether development of acellular mucin pools is associated with any of the clinico-pathological characteristics present in the tumor prior to CRT is still to be determined.

In this study, we analyzed a cohort of rectal carcinomas submitted to neo-adjuvant CRT with the aim to investigate: (1) the reproducibility and prognostic significance of Dworak TRG[11]; (2) the prognostic significance of acellular mucin pools; and (3) the possible correlation between TRG, acellular mucin pools or TNM stage variation after therapy and the various clinico-pathological characteristics present in the tumor before treatment.

MATERIALS AND METHODS

A total of 238 rectal adenocarcinomas, treated by neoadjuvant CRT and surgical resection with mesorectal excision, were identified in the Tumor Registry of Colorectal Cancer of the University of Modena and Reggio Emilia, Italy, in the period between 2001 and 2012.

Twenty-three patients were excluded from this study because they had clinical TNM (cTNM) stage Ⅳ. Thus, the final cohort in this analysis was composed of 215 patients (141 males, 74 females; mean age: 66.3 years; age range: 30-85 years) with cT3/T4

or cN+ rectal cancer. cTNM staging workup had been performed by using digital rectal examination, chest radiography, total-body computed tomography (CT), magnetic resonance imaging, endorectal ultrasonography and coloscopy with biopsy.

Clinical records were reviewed to retrieve information on the localization in the rectum (upper, medium, lower or extensive), the circumferential involvement of the rectal wall (one-third, middle or complete), distance from the anal verge (more or less than 1 cm) and craniocaudal extension (more or less than 3 cm) of the tumor. The histological grade and histotype of the tumor were assessed using endoscopic biopsy and according to World Health Organization criteria[21,22].

After the histological diagnosis on endoscopic biopsy, all patients received a total dose of 50 Gy radiotherapy, which was administered in 28 fractions of 1.8 Gy each for five consecutive days per week, and a daily continuous infusion of 225 mg 5-fluorouracil per day and per square meter of body surface for the duration of radiotherapy.

Then, patients were submitted to surgical resection. Data on cancer-specific survival (CSS) and DFS were available for all patients. After surgery, patients were monitored for disease progression by using total-body CT scan, coloscopy and blood tests (including measurement of CEA and CA 19-9). Patients who died of diseases independent of rectal cancer were censored. Both local and distant recurrences were considered in the assessment of DFS. Information on eventual adjuvant therapy was available for 127 patients.

Pathological examination

Surgical specimens were fixed in formalin for 24 h at room temperature and were grossly examined for obvious or presumable tumor remains as a mass, ulcer or fibrotic lesion. At least 3 samples were taken for paraffin embedding from specimens showing an obvious tumor mass. On the other hand, lesions with questionable residual tumors were completely embedded, and if no tumor cells were detected on first paraffin sections, three additional leveled sections were examined from each paraffin block. The total number of paraffin blocks from the primary tumor region ranged between 3 and 25; the average number was 6.4. In each case, at least 12 lymph nodes were retrieved from perirectal fat.

The histological slides of each case were retrieved from the archive of the Unit of Anatomic Pathology of the University of Modena and Reggio Emilia and reviewed by two independent pathologists (L.R.B. and V.B.) to assess the TRG and yTNM staging.

TRG was assessed in the primary tumor, but not in nodal metastases, according to Dworak scale[11]. In detail, cases were defined as follows: grade 0, no regression; grade 1, dominant tumor mass with obvious fibrosis and/or vasculopathy; grade 2, dominant fibrotic

changes with few neoplastic cells or groups (easy to find); grade 3, evidence of very few neoplastic cells in fibrotic tissue; and grade 4, no tumor cells (pathological complete response)[11]. Cases showing acellular mucin pools were initially considered separately and then categorized as grade 4 because of the absence of tumor cells.

yTNM staging was established using the criteria of Union for International Cancer Control (UICC) (TNM 7th edition)[23].

We also evaluated the status of the radial (circumferential resection) margin, which was defined as positive when normal tissue from the edge of the tumor measured 1 mm or less[24].

In the comparison between cTNM and yTNM, we were able to establish the variation rate of T, N and TNM staging after neoadjuvant CRT and define three groups of tumors, as follows: (1) rectal cancer with no change in TNM staging; (2) rectal cancers with downstaging after therapy; and (3) rectal carcinomas with upstaging after therapy.

Statistical analysis

Fleiss-Cohen weighted κ statistics were used to establish interobserver variability in the assessment of TRG.

The χ^2 test was applied to analyze the statistical correlations between Dworak TRG and the various clinico-pathological parameters and to investigate the statistical association between acellular mucin pools and clinico-pathological variables in the subgroup of Dworak grade 4 tumors.

We also used the χ^2 test to establish the statistical correlation between T, N, or TNM stage changing and the other clinico-pathological parameters.

DFS and CSS were assessed by the Kaplan-Meier method, using the date of primary surgery as the entry date. The end point for the DFS analysis was the length of survival to disease progression (either local or distant). CSS was characterized as the length of survival to death from rectal cancer or to the last follow-up date. For DFS and CSS analyses, Dworak grades 0, 1 and 2 were grouped and defined as absent/partial regression, while grades 3 and 4 were grouped together and considered as total/subtotal regression.

The Mantel-Cox log-rank test was applied to assess the strength of the association between DFS or CSS and each of the parameters (age and gender of the patient as well as site, circumferential spread, distance from the anal verge, craniocaudal extension, histological grade, histotype, cT, cN, yT, yN, yM, yTNM stage, T stage variation, N stage variation, TNM stage variation, radial margin, Dworak regression grade, tumor regression, and adjuvant chemotherapy) as a single variable.

Subsequently, a stepwise multivariate analysis (Cox regression model) was utilized to determine the independent effect of each variable on survival. TNM stage variation (and not single T or N stage variation)

Figure 1 Tumor regression in rectal surgical specimens after neo-adjuvant chemo-radiotherapy. A: Dworak grade 2, corresponding to dominant fibrosis with few neoplastic cells or groups; B: Dworak grade 4, showing no tumor cells (pathological complete response); C: Acellular mucin pools.

Figure 2 Distribution of Dworak regression score according to clinical Tumor Node Metastasis stage. Rectal cancers with cTNM stage II had a high proportion of Dworak grade 4 cases while tumors with cTNM stage III mainly had Dworak grade 1 regression.

The Dworak grade could be assessed in all 215 cases. Interobserver concordance in the assessment of Dworak TRG was good (K: 0,74) and increased to very good (K: 0.82) when cases were subdivided into total/subtotal regression (Dworak grades 3 and 4) and absent/partial regression (Dworak grades 0, 1 and 2).

In detail, 34 (16%) rectal carcinomas were classified as Dworak grade 0, 76 (35%) as grade 1, 50 (23%) as grade 2 (Figure 1A), 21 (10%) as grade 3 and 34 (16%) as grade 4 (Figure 1B and Table 1). Lower Dworak grade (0/1/2) was significantly more frequent among cN+ tumors (cTNM stage III) (P = 0.0008) (Figure 2) and was also significantly associated with death from rectal cancer (P = 0.0047) and recurrence (P = 0.0254) (Table 2).

A total of 15 cases in our cohort were classified as mucinous on the endoscopic biopsy; 11 (73%) were classified as Dworak grade 0 or 1; 1 (7%) was classified as Dworak grade 2; 1 (7%) was classified as Dworak grade 3 and 2 (13%) were classified as Dworak grade 4 on the surgical specimen (Table 2). A low Dworak grade (0-1-2) was more frequently observed in rectal carcinomas with mucinous histotype or high histological grade on the endoscopic biopsy, although statistical significance was not achieved (Table 2). Among the patients with mucinous carcinoma, 5 (33%) died of disease, and 6 (40%) experienced disease recurrence (Table 1).

Among the cases with Dworak grade 4, 7 (21%) had acellular mucin pools (Figure 1C); 5 (72%) originated from cTNM stage II cases; and 2 (28%) originated from cTNM stage III tumors. All of these cases were classified as yT0N0M0 stage and having an uneventful clinical course (no evidence of recurrence or death from rectal cancer in a median follow-up period of 71 mo; follow-up ranged between 71 and 183 mo). The presence of acellular mucin pools in Dworak 4 rectal cancer was significantly associated with mucinous histotype (P = 0.004) (Table 3). Indeed, 2/34 cases had been classified as mucinous on endoscopic biopsy, and both had acellular mucin pools in the surgical specimen (Table 3).

and tumor regression (not Dworak regression grade) were included in the multivariate analyses. Adjuvant chemotherapy was not considered in the multivariate analyses because data were available in only a proportion of patients.

A probability (P) value less than 0.05 was considered significant. Statistical analysis was performed using MedCalc 12.1.4.0 statistical software (MedCalc Software, Mariakerke, Belgium).

RESULTS

The clinico-pathological characteristics of rectal cancer in the study are summarized in Table 1. The median follow-up period of the patients was 70 mo (range: 3-183 mo). During the follow-up 73 (34%) patients developed recurrences, and 58 died of disease.

Table 1 Clinico-pathological features of 215 rectal carcinomas treated with neo-adjuvant chemo-radiotherapy *n* (%)

Variables	Category	*n*	Death due to disease	Recurrence
Sex	M	141	35 (25)	43 (30)
	F	74	23 (31)	30 (40)
Age	≤ 67 yr	106	24 (23)	32 (30)
	> 67 yr	109	34 (31)	41 (38)
Site	Upper	93	23 (25)	30 (32)
	Medium	32	10 (31)	13 (41)
	Lower	77	21 (27)	26 (34)
	Extensive	13	4 (31)	4 (31)
Circumferential spread	One-third	94	24 (26)	32 (34)
	One middle	62	17 (27)	21 (34)
	Complete	59	17 (29)	20 (34)
Craniocaudal extension	< 3 cm	51	7 (14)	10 (20)
	≥ 3 cm	164	51 (31)	63 (38)
Distance from the anal verge	≥ 1 cm	204	56 (27)	2 (18)
	< 1 cm	11	2 (18)	71 (35)
Histological grade	1	6	0 (0)	0 (0)
	2	179	47 (26)	61 (34)
	3	30	11 (37)	12 (40)
Histotype	NOS	200	53 (26)	67 (33)
	Mucinous	15	5 (33)	6 (40)
cT	cT2	12	1 (8)	2 (17)
	cT3	170	43 (25)	54 (32)
	cT4	33	14 (42)	17 (51)
cN	cN0	98	21 (21)	28 (28)
	cN+	117	37 (32)	45 (38)
yT	yT0	35	3 (9)	8 (23)
	yT1	19	1 (5)	1 (5)
	yT2	61	13 (21)	16 (26)
	yT3	83	32 (39)	36 (43)
	yT4	17	9 (53)	12 (70)
yN	yN0	146	28 (91)	38 (26)
	yN+	69	30 (43)	35 (51)
yM	yM0	212	56 (20)	70 (33)
	yM+	3	2 (67)	3 (100)
y stage	T0N0M0	33	3 (9)	8 (24)
	1	63	10 (16)	13 (21)
	2	50	15 (30)	17 (34)
	3	66	28 (42)	32 (48)
	4	3	2 (67)	3 (100)
TNM Stage variation	None	80	33 (41)	38 (47)
	Downstaging	119	19 (16)	28 (23)
	Upstaging	16	6 (38)	7 (44)
T stage variation	None	86	34 (40)	41 (48)
	Downstaging	122	22 (18)	29 (24)
	Upstaging	7	2 (29)	3 (43)
N stage variation	None	141	43 (31)	55 (39)
	Downstaging	61	11 (18)	14 (23)
	Upstaging	13	4 (31)	4 (31)
Dworak Regression grade	0	34	16 (47)	19 (56)
	1	76	20 (26)	26 (24)
	2	50	16 (32)	16 (32)
	3	21	3 (14)	4 (19)
	4	34	3 (9)	8 (23)
Regression	Absent/partial	160	52 (33)	61 (38)
	Total/subtotal	55	6 (11)	12 (22)
Radial margin	Negative	201	51 (25)	65 (32)
	Positive	14	7 (50)	8 (57)
Adjuvant chemotherapy	No	47	16 (34)	18 (38)
	Yes	80	24 (30)	31 (39)

However, the remaining five cases with acellular mucin pools showed no evidence of mucin in the endoscopic sample taken prior to CRT and surgery.

In the comparison between cT and yT staging, we noticed that 86 (40%) cases showed no variation of T stage after CRT, 122 (57%) tumors underwent T downstaging, while 3% had T upstaging (Table 2). T remaining unchanged/upstaging was significantly more frequent in female patients (*P* = 0.001) and in tumors with mucinous histotype (*P* = 0.01), cN+ stage

Table 2 Statistical correlations between Dworak regression grade and clinico-pathological variables

Variable	Dworak regression grade					P value
	0	1	2	3	4	
Sex						
M	23	52	29	11	26	0.285
F	11	24	21	10	8	
Age						
≤ 67 yr	14	35	32	9	16	
> 67 yr	20	41	18	12	18	0.202
Site						
Upper	9	37	22	8	17	
Medium	7	11	7	1	6	
Lower	13	23	19	11	11	
Extensive	5	5	2	1	0	0.2506
Circumferential spread						
One-third	14	30	28	8	14	
Middle	12	19	10	9	12	
Complete	8	27	12	4	8	0.31
Craniocaudal extension						
< 3 cm	8	15	13	6	9	
≥ 3 cm	26	61	37	15	25	0.873
Distance from the anal verge						
≥ 1 cm	2	4	2	2	1	
< 1 cm	32	72	48	19	33	0.853
Histological grade						
1	1	3	2	0	0	
2	28	61	36	20	34	
3	5	12	12	1	0	0.0732
Histotype						
NOS	28	71	49	20	32	
Mucinous	6	5	1	1	2	0.0875
cT						
2	2	2	5		3	
3	23	63	37	18	29	
4	9	11	8	3	2	0.219
cN						
cN0	14	28	21	10	25	
cN+	20	48	29	11	9	0.008
Death due to disease						
No	18	56	34	18	31	
Yes	16	20	16	3	3	0.0047
Recurrence						
No	15	50	34	17	26	
Yes	19	26	16	4	8	0.0254

(P = 0.014), high histological grade (P < 0.0001) and low Dworak grade (0-1-2) (P < 0.0001) and was significantly associated with death from rectal cancer (P = 0.002) and development of recurrence (P = 0.002) (Table 4).

Furthermore, 56 (48%) cases with cN+ status showed no change in N stage after CRT, while 61 (52%) had N downstaging. On the other hand, 13 cN0 (13%) rectal carcinomas underwent N upstaging after CRT (Table 3). N remaining unchanged/upstaging was significantly more frequent in female patients (P = 0.0347) and in tumors with extensive involvement of the rectum or localization in the lower rectum (P = 0.0183), and low Dworak grade (0-1-2) (P = 0.0013) and was significantly associated with death from rectal cancer (P = 0.0043) and development of recurrence (P = 0.0013) (Table 5).

On the whole, 80 (37%) cases showed no change in TNM stage, and 119 (55%) exhibited TNM downstaging, while 16 (8%) underwent TNM upstaging (Figure 3). In 13 cases, upstaging was due to development of nodal metastases, while, in 3 cases, upstaging was related to development of liver (2 cases) and peritoneal (1 case) metastases. Unchanged/increased TNM stage was significantly more frequent in cases showing extensive involvement of the rectum (P = 0.036), mucinous histotype (P = 0.04), cN+ stage (P = 0.001), and low Dworak grade (0-1-2) (P < 0.0001) and was significantly associated with death from rectal cancer (P < 0.0001) and development of recurrence (P = 0.001) (Table 6).

Univariate analyses showed that craniocaudal extension (P = 0.0225; P = 0.022) (Figure 4), cT (P = 0.0215; P = 0.021), yT (P < 0.0001; P = 0.0001), yN (P < 0.0001; P < 0.0001) (Figure 5), yM (P = 0.0164; P = 0.005), ystage (P < 0.0001; P = 0.0001), TNM stage variation (P < 0.0001; P = 0.0005), T stage variation (P < 0.0001; P = 0.001), Dworak regression grade (P

Table 3 Statistical correlations between the presence of acellular mucin pools grade and clinico-pathological variables in Dworak 4 rectal carcinomas

Variable	Acellular mucin pools		P value
	Absent	Present	
Sex			
M	20	6	
	7	1	0.523
Age			
≤ 67 yr	13	3	
> 67 yr	14	4	0.805
Site			
Upper	14	3	
Medium	4	2	
Lower	9	2	
Extensive	0	0	0.695
Circumferential spread			
One-third	11	3	
Middle	10	2	
Complete	6	2	0.898
Craniocaudal extension			
< 3 cm	7	2	
≥ 3 cm	20	5	0.889
Distance from the anal verge			
≥ 1 cm	1	0	
< 1 cm	26	7	0.61
Histotype			
NOS	27	5	
Mucinous	0	2	0.004
cT			
2	2	1	
3	23	6	
4	2	0	0.664
cN			
cN0	20	5	
cN+	7	2	0.889
Death due to disease			
No	24	7	
Yes	3	0	0.362
Recurrence			
No	19	7	
Yes	8	0	0.1047

Table 4 Statistical correlations between T stage variation and clinico-pathological variables

Variable	T stage variation			P value
	None	Downstaging	Upstaging	
Sex				
M	58	83	0	
F	28	39	7	0.001
Age				
≤ 67 yr	40	64	2	
> 67 yr	46	58	5	0.375
Site				
Upper	33	56	4	
Medium	12	19	1	
Lower	35	41	1	
Extensive	6	6	1	0.692
Circumferential spread				
One-third	41	51	2	
Middle	22	37	3	
Complete	23	34	2	0.792
Craniocaudal extension				
< 3 cm	21	29	1	
≥ 3 cm	65	93	6	0.832
Distance from the anal verge				
≥ 1 cm	5	6	0	
< 1 cm	81	116	7	0.789
Histotype				
NOS	77	118	5	
Mucinous	9	4	2	0.01
cN				
cN0	29	66	3	
cN+	57	56	4	0.0145
Histological grade				
1	0	4	2	
2	68	107	4	
3	18	11	1	< 0.0001
Dworak grade				
0	23	7	0	
1	38	35	7	
2	23	27	0	
3	2	19	0	
4	0	34	0	< 0.0001
Death due to disease				
No	52	100	5	
Yes	34	22	2	0.0027
Recurrence				
No	45	93	4	
Yes	41	29	3	0.0014

= 0.0023; P = 0.023) (Figure 6), tumor regression (P = 0.0006; P = 0.01) (Figure 7) and radial margin (P = 0.0069; P = 0.025) were significant prognostic factors for CSS and DFS, respectively. Craniocaudal extension, yN status, radial margin status and tumor regression were independent variables in the multivariate analyses for CSS. On the other hand, craniocaudal extension (P = 0.0189), yT stage (P = 0.0327) and yN stage (P = 0.001) were significant prognostic parameters for DFS.

DISCUSSION

In this study, we aimed to identify clinico-pathological variables, which may have significance for predicting recurrence risk and outcome in patients with rectal cancer submitted to neo-adjuvant CRT. Our findings can be summarized as follows. First, we confirmed previous evidence[7-9] that Dworak TRG is a reproducible histological parameter able to discriminate rectal carcinomas at the increased risk of recurrence or adverse outcome. However, the comparison of Kaplan-Meier curves showed that DFS and CSS were only slightly different between cases classified as Dworak 4 and 3 and among Dworak 0, 1 and 2 rectal carcinomas. These results suggested that a two-tiered rather than a five-tiered system could be used to grade tumor regression in surgical specimens. Accordingly, the use of a simplified dichotomized system, in which Dworak 0, 1 and 2 were considered as absent/partial regression and Dworak 3 and 4 as total/subtotal regression not only raised the inter-observer reproducibility but also increased the prognostic relevance of TRG; furthermore, two-tiered TRG was an independent and significant

Figure 3 cTNM stage II rectal cancers mainly showed downstaging to yT0N0M0 or yTNM stage I. On the other hand, the majority of cTNM stage III cases had unchanged TNM stage after therapy, and some even showed upstaging to yTNM stage IV after therapy.

Figure 4 Kaplan Meier curves showing cancer specific survival according to craniocaudal extension. Compared to tumors with craniocaudal extension lower than 3 cm rectal carcinomas with craniocaudal extension with higher cms had significantly shorter cancer specific survival (CSS).

Figure 5 Kaplan Meier curves showing cancer specific survival according to yN status. Compared to tumors with the presence of nodal metastases, rectal carcinomas with the absence of nodal metastases at histological examination of surgical specimen after chemo-radiotherapy (CRT) had significantly longer cancer specific survival (CSS).

prognostic variable for CSS in the multivariate analysis. Moreover, the advantages of a simplified Dworak system were already demonstrated by Elezkurtaj *et al*[25] who showed that compared to Dworak's five tier system, dichotomized TRG has higher correlations with nodal disease and UICC stage. Finally, grouping

Figure 6 Kaplan Meier curves showing cancer specific survival according to Dworak grade. Compared to tumors with Dworak grades 0, 1 or 2, rectal carcinomas with Dworak grades 3 and 4 had significantly longer cancer specific survival (CSS).

Figure 7 Kaplan Meier curves showing ancer specific survival according to tumor regression. Compared to tumors with absent/partial regression (Dworak 0, 1 and 2), rectal carcinomas with subtotal/total regression (Dworak 3 and 4) had significantly longer cancer specific survival (CSS).

Dworak grades 3 and 4 together would avoid the need to determine complete pathological response by using step sections, which is highly time consuming and whose benefits appear to be questionable[26]. Of note, a lower response to CRT (corresponding to Dworak TRG 0, 1 or 2) was observed in tumors showing mucinous histotype or poor differentiation in pre-treatment biopsy. Some authors recently suggested a "watch and wait" approach to avoid the side-effects of surgery in patients with rectal cancer showing complete clinical tumor regression after CRT[27]. Our findings indicate that caution should be used when applying a "watch and wait" policy to rectal cancer with mucinous or poorly differentiated histology, as these features are associated with a higher possibility of absent /incomplete tumor regression and T/TNM remaining unchanged/upstaging after CRT.

Seven (3.2%) rectal surgical specimens in our cohort showed acellular mucin pools upon histological examination. Two of those cases were derived from tumors with a mucinous histotype in the pre-treatment endoscopic biopsy. In those cases, acellular mucin deposits could be interpreted as a complete tumor response of mucinous rectal carcinoma. However, in 5 cases we could not find extracellular mucin in the

Table 5 Statistical correlations between N stage variation and clinico-pathological variables

Variable	N stage variation			*P* value
	None	Downstaging	Upstaging	
Sex				
M	19	35	7	
F	37	26	6	0.0347
Age				
≤ 67 yr	27	34	5	
> 67 yr	29	27	7	0.565
Site				
Upper	16	33	7	
Medium	10	11	3	
Lower	25	16	1	
Extensive	5	1	2	0.0183
Circumferential spread				
One-third	25	28	8	
Middle	17	17	1	
Complete	14	16	4	0.582
Craniocaudal extension				
< 3 cm	10	14	5	
≥ 3 cm	46	47	8	0.27
Distance from the anal verge				
≥ 1 cm	1	4	1	
< 1 cm	55	57	12	0.402
Histotype				
NOS	49	59	13	
Mucinous	7	2	0	0.0851
cT				
2	4	8	0	
3	38	42	12	
4	14	11	1	0.265
Histological grade				
1	3	2	0	
2	39	50	11	
3	14	9	2	0.512
Dworak grade				
0	14	6	3	
1	25	23	8	
2	16	13	2	
3	0	11	0	
4	1	8	0	0.0013
Death due to disease				
No	30	50	9	
Yes	26	11	4	0.0043
Recurrence				
No	25	47	9	
Yes	31	14	4	0.0013

cases scored as Dworak 4. Hence, although acellular mucin pools are not mentioned in Dworak TRG[11], we may presume that due to the absence of neoplastic cells, they should be considered as complete tumor regression (Dworak 4). Our results are in line with those reported by other authors[15,17-19]. However, some of the previous analyses could be flawed by the short follow-up of the patients, which was limited to 3 or 5 years[16-18,30], or by sampling restricted to the macroscopically abnormal areas in the rectum[16]. In our cases with acellular mucin pools, the median follow-up time was 71 mo. Additionally, we overcame the possibility of the incorrect assessment of tumor response by embedding the entire surgical specimen and by cutting three additional leveled sections from each paraffin block in cases with questionable residual tumor. In our opinion, cutting step levels is mandatory when seeing acellular mucin pools after CRT. Indeed, this histological feature might represent either a treatment effect or residual tumor. Only careful histological examination of the entire surgical specimen with leveled sections allows for the exclusion of residual tumor. Moreover, a pre-treatment biopsy might not be representative of the entire tumor and may not show mucinous histology. Additionally, mucinous adenocarcinoma may show only rare foci of neoplastic cells that can be hard to identify.

Our results also showed that yN status is an independent predictor of shorter DFS and CSS in patients with rectal cancer submitted to neo-adjuvant CRT. Interestingly, 13% of patients with cN0 status had yN+ after therapy. This result highlights the importance of pathological examination after CRT to ensure appropriate staging and therapy of patients, as was already underscored[31]. Since N remaining unchanged/upstaging was significantly more frequent in tumors showing extensive involvement of the rectum or localization in the lower rectum, surgical resection and follow-up histological examination might have particular relevance in rectal carcinomas showing those features.

Tumor craniocaudal extension higher than 3 cm was an additional significant and independent prognostic parameter associated with worse DFS and CSS in our analyses. Since this feature was not associated with TNM variation or with tumor regression after therapy, we may speculate that cancers with higher craniocaudal extension might have had occult metastases undetectable at the moment of yTNM staging. However, our data suggest that particular attention should be given in the treatment and follow up of tumors existing at a higher extent in the rectum.

Finally, T and N remaining unchanged/upstaging was significantly more frequent in female patients. Although we are not able to explain this phenomenon, the role of hormones in the biological aggressiveness of rectal cancer should be better investigated in the future.

In conclusion, we confirmed the reproducibility

pre-treatment sample, and this finding suggests that acellular mucin deposits in surgical specimens after CRT may represent a radiation effect, as was already hypothesized[28]. Of note, all 7 patients with acellular mucin pools had an unremarkable clinical course, with no recurrence or death from rectal cancer. This outcome supports the College of American Pathologists' consensus recommendation that acellular mucin pools should not be regarded as residual disease in rectal cancer treated with neo-adjuvant CRT[29]. Accordingly, we did not observe any significant difference in CSS and DFS between cases with acellular mucin pools and

Table 6 Statistical correlations between Tumor Node Metastasis stage variation and clinico-pathological variables

Variable	pTNM stage variation			*P* value
	None	Downstaging	Upstaging	
Sex				
M	53	81	7	
F	27	41	6	0.655
Age				
≤ 67 yr	36	65	5	
> 67 yr	44	57	8	0.372
Site				
Upper	26	60	7	
Medium	11	18	3	
Lower	35	41	1	
Extensive	8	3	2	0.0194
Circumferential spread				
One-third	31	55	8	
Middle	25	36	1	
Complete	24	31	4	0.396
Craniocaudal extension				
< 3 cm	17	29	5	
≥ 3 cm	63	93	8	0.4
Distance from the anal verge				
≥ 1 cm	3	7	1	
< 1 cm	77	115	12	0.747
Histotype				
NOS	70	117	13	
Mucinous	10	5	0	0.043
Histological grade				
1	4	2	0	
2	60	108	11	
3	16	12	2	0.135
cT				
2	4	8	0	
3	58	100	12	
4	18	14	1	0.185
cN				
cN0	27	58	13	
cN+	53	64	0	< 0.0001
Dworak grade				
0	20	11	3	
1	35	33	8	
2	22	26	3	
3	2	19	0	
4	1	33	0	< 0.0001
Death due to disease				
No	47	101	9	
Yes	33	11	4	< 0.0001
Recurrence				
No	42	91	9	
Yes	38	31	4	0.0051

and prognostic value of Dworak TRG in rectal cancer treated with neo-adjuvant CRT; however, our data suggested that a simplified two-tiered system may be used with better results. Acellular mucin pools in the surgical specimen may be considered as complete tumor regression, but step levels are warranted in those cases to exclude residual mucinous carcinoma. Finally, particular attention should be paid to rectal carcinomas showing the mucinous histotype, poor

differentiation and extensive/lower involvement of the rectum due to the higher probability of absent/partial regression and TNM remaining unchanged/upstaging in those cases.

COMMENTS

Background
Patients with locally advanced rectal cancer are currently submitted to neo-adjuvant chemo-radiotherapy (CRT) to improve resectability and sphincter preservation and to decrease the probability of local recurrence. Post-treatment Tumor Node Metastasis stage (yTNM) and Tumor Regression Grade (TRG) are used to measure tumor response in surgical specimens obtained after CRT. Several systems, such as the Dworak system, are used to score tumor regression. However, all these types of systems suffer from low interobserver reproducibility.

Research frontiers
A major issue in the interpretation of tumor histological response to CRT is related to the presence of acellular mucin pools. It is not clear whether these pools should be interpreted as complete regression. Finally, the value of clinico-pathological factors in predicting tumor regression after neo-adjuvant CRT remains to be determined.

Innovations and breakthroughs
This article shows that, compared to the currently used four-tiered system a simplified two-tiered, Dworak TRG is preferable for determining score response to CRT because of its higher reproducibility and prognostic significance. Mucinous histotype and poor differentiation are significantly associated with lower response to neo-adjuvant CRT, as highlighted by higher frequency of absent/limited histological regression and TNM remaining unchanged/upstaging in those tumors.

Applications
A simplified two-tiered, Dworak TRG could be used in routine practice to increase reproducibility and prognostic relevance of TRG. A watch and wait approach should be used with caution in rectal carcinomas showing mucinous histotype and poor differentiation in endoscopic pre-treatment biopsy due to their significant association with low response to pre-operative CRT.

Terminology
TRG: measure of the histological response to neo-adjuvant chemoradiotherapy, and it can be assessed by using different systems, such as that proposed by Dworak and colleagues in 1997, which is based on the presence of tumor cells and fibrosis in a surgical specimen. Acellular mucin pools: the presence of mucin deposits devoid of neoplastic cells in rectal surgical specimen after neo-adjuvant CRT that need to be differentiated from residual mucinous carcinoma.

Peer-review
A major finding reported in this study is the possible use of a two-tiered system to score TRG after neo-adjuvant chemoradiotherapy. Strength of this study is the high number (215 cases) of rectal carcinomas analyzed.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic significance of preoperative and postoperative CK19 and CEA mRNA levels in peripheral blood of patients with gastric cardia cancer

Yu-Feng Qiao, Chuan-Gui Chen, Jie Yue, Ming-Quan Ma, Zhao Ma, Zhen-Tao Yu

Yu-Feng Qiao, Chuan-Gui Chen, Jie Yue, Ming-Quan Ma, Zhao Ma, Zhen-Tao Yu, Department of Esophageal Cancer, Tianjin Medical University Cancer Institute and Hospital, Key Laboratory of Cancer Prevention and Therapy of Tianjin City, Tianjin 300060, China

Author contributions: Qiao YF designed the study, analyzed the data, and wrote the paper; Chen CG and Yue J collected the data; Ma MQ and Ma Z performed the data analysis; Yu ZT designed the study and revised the paper; all authors have read and approved the final version to be published.

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Correspondence to: Zhen-Tao Yu, MD, PhD, Department of Esophageal Cancer, Tianjin Medical University Cancer Institute and Hospital, Key Laboratory of Cancer Prevention and Therapy of Tianjin City, Huanhuxi Road, Tiyuanbei, HeXi District, Tianjin 300060, China. qyf800117@sina.com
Telephone: +86-22-23340123
Fax: +86-22-23359984

Abstract

AIM

To evaluate the clinical and prognostic significance of preoperative and postoperative cytokeratin 19 (CK19) and carcinoembryonic antigen (CEA) mRNA levels in peripheral blood of patients with gastric cardia cancer (GCC).

METHODS

We detected the preoperative and postoperative mRNA levels of CK19 and CEA in peripheral blood of 129 GCC patients by using reverse transcription-polymerase chain reaction and evaluated their clinical and prognostic significance by univariate Kaplan-Meier survival analysis and multivariate Cox proportional hazard analysis. A new prognostic model which stratified patients into three different risk groups was established based on the independent prognostic factors.

RESULTS

Elevated preoperative and postoperative CK19 and CEA mRNA levels in peripheral blood of GCC patients were associated with lymph node metastasis. Univariate analysis showed that tumor size, histological grade, depth of tumor invasion, lymph node metastasis, preoperative CK19 mRNA, and preoperative and postoperative CEA mRNA levels were correlated with the prognosis of GCC patients. The multivariate analysis showed that lymph node status (P = 0.018), preoperative CK19 (P = 0.035) and CEA (P = 0.011) mRNA levels were independent prognostic factors for overall survival (OS). The 5-year OS rates for the low-, intermediate-, and high-risk groups were 48.3%, 22.6%, and 4.6%, respectively (P < 0.001).

CONCLUSION

Elevated preoperative CK19 and CEA mRNA levels may be regarded as promising biomarkers for predicting lymph node metastasis and poor prognosis in patients with GCC. This new prognostic model may help us identify the subpopulations of GCC patients with the highest risk.

Key words: Gastric cardia cancer; Cytokeratin 19; Carcinoembryonic antigen; Clinicopathological factor; Prognosis

Core tip: This is a retrospective study that evaluated the clinical and prognostic significance of the preoperative (pre-) and postoperative (post-) mRNA levels of cytokeratin 19 (CK19) and carcinoembryonic antigen (CEA) in peripheral blood of patients with gastric cardia cancer. Increased pre- and post-CK19 and CEA mRNA levels were associated with positive lymph node metastasis. Elevated pre- but not post-CK19 and CEA mRNA levels were independent prognostic factors for overall survival (OS). A new prognostic model was established based on independent prognostic factors (lymph node status, pre-CK19 and pre-CEA mRNA levels), and there was a significant difference in OS among the three different risk groups.

INTRODUCTION

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide[1]. Recently, the incidence of gastric cardia cancer (GCC) has increased around the world[2,3]. The prognosis of patients with GCC was worse than that of patients with non-cardiac GC[4]. Although the important advances in surgical techniques and adjuvant chemotherapies have improved patient outcomes, the 5-year survival rate remains low (10%-30%)[5]. Metastasis and recurrence after surgery are the main causes of treatment failure and poor prognosis[6]. Therefore, it is of great significance to establish molecular biomarkers for GCC to detect early metastasis and improve survival.

Carcinoembryonic antigen (CEA), one of the most commonly used serum tumor markers in GC, plays an important role in tumor metastasis and may be partially associated with prognosis in GC[7]. Cytokeratin 19 (CK19), which originates from epithelial cells, is a reliable tissue-specific marker for epithelial tumor micrometastasis and is highly expressed in the gastrointestinal tract but not in normal lymph node tissue or blood. The detection of CEA and CK19 expression has been used to examine tumor micrometastasis and predict the prognosis in GC and esophageal squamous cell carcinoma[8,9]. Nevertheless, there is no valid marker to predict the lymph node metastasis and prognosis in patients with GCC. Recently, quantitative real-time polymerase chain reaction (qRT-PCR) technology, a sensitive, specific and rapid method, has been widely used to detect the presence of circulating cancer cells in peripheral blood, the expression of tumor markers, and micrometastases, as well as predict prognoses[10]. However, there are few reports on the detection and clinical significance of CEA and CK19 mRNAs in the peripheral blood of GCC patients. With the decrease in tumor burden after surgery, the levels of tumor marker expression will change. Some studies have supported the use of preoperative (pre-) CEA levels as prognostic markers in GC[11,12], whereas other studies have reported the prognostic value of postoperative (post-) CEA levels[13]. There are limited data regarding the prognostic significance of pre- and post-CK19 levels in GCC.

In the present study, we detected the pre- and post-CEA and CK19 mRNA levels in peripheral blood of patients with GCC by using qRT-PCR and estimated the clinical and prognostic value of these biomarkers in patients with GCC.

MATERIALS AND METHODS

Patients and peripheral blood sample collection

A total of 129 patients who were diagnosed with GCC and underwent curative surgery between January 2009 and December 2011 at Tianjin Medical University Cancer Institute and Hospital were recruited for the study. The inclusion criteria of the study were as follows: (1) complete clinicopathological and follow-up data; (2) no neoadjuvant chemotherapy, radiotherapy or chemoradiotherapy; (3) radical gastrectomy for the

primary tumor and D2 lymph node dissection following the Japanese Research Society for Gastric Cancer guidelines[14]; (4) no gross or microscopic residual or recurrent gastric tumor; (5) no distant metastases prior to surgery; and (6) no other synchronous malignancies. The histological diagnosis and tumor-node-metastasis (TNM) staging were based on 7th edition of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) TNM staging system[15]. The study and acquisition of blood specimens were approved by the Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. In addition, all of the patients provided written informed consent.

Total RNA isolation and cDNA synthesis

Three to five millilambert peripheral blood samples were obtained through a catheter inserted into a peripheral vessel and collected into EDTA tubes from each GCC patient before and after surgical resection. Sample processing was performed within 2 h after blood collection. Karyocytes were isolated from the blood samples using a lymphocyte separation medium according to the manufacturer's instructions (Solarbio, Beijing, China). Briefly, blood samples were subjected to Ficoll-sodium diatrizoate density gradient centrifugation. Then, after discarding the plasma layer, the samples were mixed with a wash buffer and centrifuged at 200 *g* for 10 min. The pelleted cells were resuspended in red blood cell lysis buffer (Solarbio) and centrifuged another two times. The remaining cells, which are karyocytes in PB, were washed with PBS, and the total cellular RNA isolation and cDNA synthesis were performed as previously described[9]. Total RNA was extracted from the cell lysate using TRIzol reagent (Invitrogen, Gaithersburg, MD, United States) according to the manufacturer's instructions. The concentration and purity of RNA were determined by using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, United States). A260/A280 ratios in the range of 1.8 to 2.0 were considered satisfactory for purity standards in this study. First-strand cDNA was synthesized using the SuperScript First-Strand cDNA Synthesis kit (Invitrogen) according to the manufacturer's instructions and then stored at -20 ℃ for subsequent quantitative polymerase chain reaction experiments.

Real-time PCR

The mRNA expression levels of CK19 and CEA were detected by real-time PCR using the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, United States). Glyceraldehyde-3-phosphatedehydrogenase (GADPH) was amplified as an internal control to correct for differences in the amount of total RNA per sample by normalizing the mRNA levels of CK19 and CEA to the corresponding GAPDH levels. The relative levels of the normalized gene expression were calculated with the equation $2^{-\Delta CT}$, in which ΔCT = CT gene- CT control. All of the reactions were run in triplicate. The primers targeting CK19, CEA and GAPDH were as follows: 5'-ATGAAAGCTGCCTTGGAAGA-3' (CK19, forward) and 5'-TGATTCTGC- CGCTCACTATCAG-3' (CK19, reverse); 5'-AACTGGTGT- CCCGGATATCA-3' (CEA, forward) and 5'-ATATTCTTTGCTCCTTGCCA-3' (CEA, reverse); 5'-AGAAGGCTGGGGCTCATTTG-3' (GAPDH, forward) and 5'-AGGGGCCATCCACAGTCTTC-3' (GAPDH, reverse). The following thermocycling conditions were used under the standard mode according to the manufacturer's recommendations: 30 s at 95 ℃ followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 34 s.

Follow-up

After curative resection, all patients were followed regularly every 3 mo during the first two years, every 6 mo during the third to fifth years, and every 1 year thereafter until death or the last follow-up. Postoperative follow-up observations involved physical examinations, laboratory blood tests, tumor markers, chest radiography, abdominal computed tomography scan, and endoscopy examinations. The final follow-up date was December 2015. Overall survival (OS) was calculated from the date of surgery to death or the final follow-up date.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 statistical software (SPSS Inc, Chicago, IL, United States). The receiver operating characteristic (ROC) curves were plotted to determine the optimal cutoff points for pre- and post-CK19 and CEA mRNA levels in predicting 5-year survival. Interdependence between the CK19 mRNA levels, CEA mRNA levels, and clinical data were calculated using the χ^2 test and are displayed in cross-tables. Survival analysis was performed using the Kaplan-Meier method and compared by the log-rank test. The variables significantly affecting OS were investigated by multivariate analysis according to the Cox proportional hazard model. *P* values < 0.05 were considered statistically significant.

RESULTS

Patients and characteristics

A total of 129 patients with GCC were included in our study. There were 27 (20.9%) females and 102 (79.1%) males, and the median age was 61 years (range, 38-84 years). All patients were histologically confirmed with adenocarcinoma after surgery. According to the histopathological grading, well and moderately differentiated tumors were observed in 99 (76.7%) patients, and 30 (23.3%) patients presented poorly differentiated or undifferentiated tumors. Based on the criteria of the 7th edition of the UICC/AJCC TNM staging system, 34 (26.4%) and 95 (73.6%) patients

Figure 1 Receiver operating characteristic curves for preoperative and postoperative cytokeratin 19 and carcinoembryonic antigen mRNA levels in patients with gastric cardia cancer according to overall survival. A: Preoperative CK19 mRNA level; B: Postoperative CK19 mRNA level; C: Preoperative CEA mRNA level; D: Postoperative CEA mRNA level. AUC: Area under the curve; CK19: Cytokeratin 19; CEA: Carcinoembryonic antigen; ROC: Receiver operating characteristic.

had T1-T2 and T3-4 disease, respectively. Postoperative histological examinations confirmed that lymph node metastasis was present in 51 (39.5%) cases. With regard to the TNM stage, 11 (8.5%) cases were subsequently diagnosed with stage I, 75 (58.1%) with stage II, and 43 (33.3%) with stage III.

Correlation between the pre- and post-CK19 mRNA levels and clinicopathological factors

According to the ROC curve shown in Figure 1, the cutoff values of the pre- and post-CK19 mRNA levels were set at 55.0 and 53.6, respectively. Based on these cutoff values in predicting 5-year survival, the sensitivity and specificity were 69.7% and 69.8%, respectively, for pre-CK19 mRNA, and 57.9% and 58.5%, respectively, for post-CK19 mRNA. The corresponding areas under the curve (AUCs) were 0.712 and 0.610, respectively. Thus, we divided the patients into "low pre-CK19" ($<$ 55.0, n = 63) and "high pre-CK19" ($>$ 55.0, n = 66) groups as well as "low post-CK19" ($<$ 53.6, n = 67) and "high post-CK19" ($>$ 53.6, n = 62) groups.

The correlations between the pre- and post-CK19 mRNA levels and the clinicopathological characteristics are shown in Table 1. The pre-CK19 mRNA levels were significantly correlated with tumor size (P = 0.008), lymph node status (P = 0.033), and TNM stage (P = 0.012), but showed no significant correlations with

other measured clinicopathological characteristics (P > 0.05 for all). Furthermore, the post-CK19 mRNA levels were only correlated with lymph node status (P = 0.048) and not with any other clinicopathological characteristics measured (P > 0.05 for all).

Correlation between the pre- and post-CEA mRNA levels and clinicopathological factors

According to the plotted ROC curves (Figure 1C and D), the optimal cutoff values of the pre- and post-CEA mRNA levels were set at 9.3 and 8.8, respectively. Based on these cutoff values in predicting 5-year survival, the sensitivity and specificity were 72.4% and 59.7%, respectively, for pre-CEA mRNA, and 67.1% and 54.7%, respectively, for post-CEA mRNA. The AUCs were 0.753 and 0.666, respectively. Thus, we divided the patients into "low pre-CEA" ($<$ 9.3, n = 52) and "high pre-CEA" ($>$ 9.3, n = 77) groups as well as "low post-CEA" ($<$ 8.8, n = 69) and "high post-CEA" ($>$ 8.8, n = 70) groups.

The associations between the pre- and post CEA mRNA levels and the clinicopathological characteristics are shown in Table 2. Pre-CEA mRNA levels were closely correlated with tumor size (P = 0.031), lymph node status (P = 0.002), and TNM stage (P = 0.048). However, no statistical significance was observed in the correlation between pre- CEA mRNA levels and the other measured clinicopathological features (P > 0.05

Table 1 Correlation between the preoperative and postoperative cytokeratin 19 mRNA levels and the clinicopathological features of 129 patients with gastric cardia cancer *n* (%)

Clinicopathological feature	Cases	CK19 mRNA level (pre-)		χ^2 test		CK19 mRNA level (post-)		χ^2 test	
		Low	High	χ^2	*P* value	Low	High	χ^2	*P* value
Gender				1.484	0.223			1.663	0.197
Male	102	47 (46.1)	55 (53.9)			50 (49.0)	52 (51.0)		
Female	27	16 (59.3)	11 (40.7)			17 (63.0)	10 (37.0)		
Age (yr)				0.411	0.521			0.052	0.820
≤ 60	59	27 (45.8)	32 (54.2)			30 (50.8)	29 (49.2)		
> 60	70	36 (51.4)	34 (48.6)			37 (52.9)	33 (47.1)		
Tumor size (cm)				6.928	0.008			3.250	0.071
≤ 4	75	44 (58.7)	31 (41.3)			44 (58.7)	31 (41.3)		
> 4	54	19 (35.2)	35 (64.8)			23 (42.6)	31 (57.4)		
Histological grade				0.474	0.491			0.030	0.861
Well/moderately differentiated	99	50 (50.5)	49 (49.5)			51 (51.5)	48 (48.5)		
Poorly differentiated/ undifferentiated	30	13 (43.3)	17 (6.7)			16 (53.3)	14 (46.7)		
Depth of tumor invasion				3.088	0.079			3.015	0.082
T1-T2	34	21 (61.8)	13 (38.2)			22 (64.7)	12 (35.3)		
T3-T4	95	42 (44.2)	53 (55.8)			45 (47.4)	50 (52.6)		
Lymph node status				4.528	0.033			3.913	0.048
Negative	78	44 (56.4)	34 (43.6)			46 (59.0)	32 (41.0)		
Positive	51	19 (37.3)	32 (62.7)			21 (41.2)	30 (58.8)		
TNM stage				8.861	0.012			2.848	0.241
I	11	9 (81.8)	2 (18.2)			7 (63.6)	4 (36.4)		
II	75	39 (52.0)	36 (48.0)			42 (56.0)	33 (44.0)		
III	43	15 (34.9)	28 (65.1)			18 (41.9)	25 (58.1)		

CK19: Cytokeratin 19.

for all). Furthermore, there was a significant correlation between post-CEA mRNA levels and tumor size (*P* = 0.016), lymph node status (*P* = 0.008), and TNM stage (*P* = 0.025), but no statistical significance was observed in the correlation between post-CEA mRNA levels and the other measured clinicopathological features (*P* > 0.05 for all).

Univariate and multivariate survival analyses of clinicopathological characteristics for OS of patients with GCC

The median follow-up period for the entire cohort was 42 mo (range, 6-84 mo). The 5-year OS rate for all the patients was 31.7% with a median survival time of 39 mo.

The 5-year OS curves of patients based on the pre- and post-CK19 mRNA and CEA mRNA levels are shown in Figure 2. The 5-year OS rates for the low pre-CK19 and high pre-CK19 groups were 42.1% and 21.5%, respectively (χ^2 = 9.183, *P* = 0.002; Figure 2A). The 5-year OS rates for low post-CK19 and high post-CK19 groups were 36.5% and 27.4%, respectively (χ^2 = 2.773, *P* = 0.096; Figure 2B). The 5-year OS rates for low pre-CEA and high pre-CEA groups were 52.2% and 19.5%, respectively (χ^2 = 12.890, *P* = 0.000, Figure 2C). The 5-year OS rates for low post-CEA and high post-CEA groups were 39.5% and 25.4%, respectively (χ^2 = 4.721, *P* = 0.030; Figure 2D).

Univariate Kaplan-Meier analysis showed that tumor size (*P* = 0.015), histological grade (*P* = 0.039), depth of tumor invasion (*P* = 0.010), lymph node status (*P* < 0.001), pre-CK19 mRNA level (*P* = 0.002), pre-CEA mRNA level (*P* < 0.001), and post-CEA mRNA level (*P* = 0.030) significantly affected the prognosis of patients with GCC (Table 3).

The seven factors with prognostic potential for OS were subsequently subjected to multivariate analysis using the Cox proportional hazards model. As shown in the Table 3, the multivariate survival analysis indicated that lymph node status (*P* = 0.018), pre-CK19 mRNA levels (*P* = 0.035), and pre-CEA mRNA levels (*P* = 0.011) were independent prognostic factors for patients with GCC.

Prognostic model and risk groups

To better identify GCC patients at high risk for lymph node metastasis and poor OS, we proposed a new prognostic model by combining the three identified independent prognostic factors and stratified patients into three groups as follows: the low-risk group, comprising patients with 0 or 1 risk factor; the intermediate-risk group, comprising patients with 2 factors; and the high-risk group, comprising patients with all 3 factors. Finally, there were 57, 34, and 26 patients in the low-, intermediate-, and high-risk groups, respectively. The 5-year OS rates for the low-, intermediate-, and high-risk groups were 48.3%, 22.6%, and 4.6%, respectively, and a statistically

Table 2 Correlation between the preoperative and postoperative carcinoembryonic antigen mRNA levels and clinicopathological features in 129 patients with gastric cardia cancer *n* (%)

Clinicopathological feature	Cases	CEA mRNA level (pre-)		χ^2 test		CEA mRNA level (post-)		χ^2 test	
		Low	High	χ^2	*P* value	Low	High	χ^2	*P* value
Gender				1.891	0.169			1.327	0.249
Male	102	38 (37.3)	64 (62.7)			44 (43.1)	58 (56.9)		
Female	27	14 (51.9)	13 (48.1)			15 (55.6)	12 (44.4)		
Age (yr)				0.413	0.521			1.121	0.290
≤ 60	59	22 (37.3)	37 (62.7)			24 (40.7)	35 (59.3)		
> 60	70	30 (42.9)	40 (57.1)			35 (50.0)	35 (50.0)		
Tumor size (cm)				0.078	0.780			5.757	0.016
≤ 4	75	31 (41.3)	44 (58.7)			41 (54.7)	34 (45.3)		
> 4	54	21 (38.9)	33 (61.1)			18 (33.3)	36 (66.7)		
Histological grade				3.024	0.082			2.423	0.12
Well/moderately differentiated	99	44 (44.4)	55 (55.6)			49 (49.5)	50 (50.5)		
Poorly differentiated/ undifferentiated	30	8 (26.7)	22 (73.3)			10 (33.3)	20 (66.7)		
Depth of tumor invasion				4.653	0.031			0.966	0.326
T1-T2	34	19 (55.9)	15 (44.1)			18 (52.9)	16 (47.1)		
T3-T4	95	33 (34.7)	62 (65.3)			41 (43.2)	54 (56.8)		
Lymph node status				9.871	0.002			7.012	0.008
Negative	78	40 (51.3)	38 (48.7)			43 (55.1)	35 (44.9)		
Positive	51	12 (23.5)	39 (76.5)			16 (31.4)	35 (68.6)		
TNM stage				6.063	0.048			7.379	0.025
I	11	6 (54.5)	5 (45.5)			9 (81.8)	2 (18.2)		
II	75	35 (46.7)	40 (53.3)			34 (45.3)	41 (54.7)		
III	43	11 (25.6)	32 (74.4)			16 (37.2)	27 (62.8)		

CK19: Cytokeratin 19; CEA: Carcinoembryonic antigen; TNM: Tumor-node-metastasis.

Table 3 Univariate and multivariate analyses of the clinicopathological variables for overall survival in 129 patients with gastric cardia cancer

Clinicopathological feature	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gender	1.377 (0.797-2.377)	0.245		
Age (yr)	0.812 (0.529-1.247)	0.336		
Tumor size(cm)	1.688 (1.097-2.596)	0.015	1.305 (0.816-2.089)	0.267
Histological grade	1.277 (1.008-1.619)	0.039	1.104 (0.860-1.418)	0.436
Depth of tumor invasion	1.985 (1.162-3.393)	0.010	1.191 (0.660-2.151)	0.562
Lymph node status	2.894 (1.864-4.492)	0.000	1.848 (1.109-3.079)	0.018
Pre-CK19 mRNA	1.932 (1.247-2.992)	0.002	1.625 (1.035-2.553)	0.035
Post-CK19 mRNA	1.431 (0.932-2.197)	0.096		
Pre-CEA mRNA	2.337 (1.443-3.784)	0.000	1.918 (1.162-3.166)	0.011
Post-CEA mRNA	1.609 (1.039-2.492)	0.030	1.213 (0.753-1.955)	0.427

CK19: Cytokeratin 19; CEA: Carcinoembryonic antigen; HR: Hazard ratio.

significant difference was observed (χ^2 = 28.319, *P* < 0.001; Figure 3).

DISCUSSION

Invasion and distant metastasis are the leading factors influencing the clinical outcome of patients with GC[16]. Many GC patients with resectable tumors died of postoperative distant metastasis and had a poor prognosis. GCC has been reported to be a distinct clinical entity based on its pathogenesis and risk factors and has a higher incidence of lymph node metastasis and a poorer prognosis than non-cardiac GC[17,18]. Therefore, identifying one method or marker to determine the potential of cancer cell spreading and disease progression in patients with GCC will certainly help to tailor postoperative adjuvant therapies and improve clinical outcomes. However, there are no markers available that can predict lymph node metastasis and evaluate the prognosis of patients with GCC.

Numerous factors are associated with patient prognosis, including the expression of growth factors and their receptors, cell cycle regulators, cell adhesion

Figure 2 Kaplan-Meier survival curves according to preoperative and postoperative cytokeratin 19 and carcinoembryonic antigen mRNA levels in patients with gastric cardia cancer. A: Preoperative CK19 mRNA level; B: Postoperative CK19 mRNA level; C: Preoperative CEA mRNA level; D: Postoperative CEA mRNA level. *P* values were calculated by the log-rank test and *P* < 0.05 denoted significance. OS: Overall survival; CK19: Cytokeratin 19; CEA: Carcinoembryonic antigen.

Figure 3 Cumulative survival curves for 129 patients with gastric cardia cancer according to risk groups by Kaplan-Meier survival analysis. The 5-year OS rates for the low-, intermediate-, and high-risk groups were 48.3%, 22.6% and 4.6%, respectively (*P* < 0.001). OS: Overall survival.

molecules and matrix-degrading enzymes, all of which play important roles in tumor cell proliferation, invasion, and metastasis[19]. For GC, several tumor-specific markers, including CEA and CK19 in either serum or tumor tissue (as evaluated by either enzyme immunoassay kit or immunohistochemistry, respectively), have been used to detect metastasis and predict prognosis[8,11]. Recently, qRT-PCR has been shown to have a sensitivity 10- to 100-fold higher than the routine immunological methods and to be a

reliable method for detecting circulating tumor cells, measuring the mRNA expression of tumor markers, and predicting patient prognosis[10]. In addition, compared with the acquisition of bone marrow, lymph nodes or tumor tissues, peripheral blood collection is a minimally invasive procedure and can be collected throughout the entire disease process[6]. All these data have garnered increasing attention in this field of research to analyze the clinical and prognostic value of CEA and CK19 mRNAs in the peripheral blood of GCC patients by using qRT-PCR.

In the present study, we detected the CK19 and CEA mRNA levels in peripheral blood of GCC patients by using qRT-PCR and analyzed the correlation between CK19 and CEA mRNA levels with clinicopathological variables. Our results showed significant correlations between the pre-CK19 mRNA levels and tumor size, lymph node status, and TNM stage as well as between the pre-CEA mRNA levels and T status, lymph node status, and TNM stage. Furthermore, a significant association could be found between post-CEA mRNA levels and tumor size, lymph node status, and TNM stage. However, the post-CK19 mRNA levels were only correlated with lymph node status. These results indicated that pre- and post-CEA mRNA and CK19 mRNA levels had different clinical values in GCC, and it seems that pre-CEA and CK19 mRNA levels may

have a stronger prognostic role than post-CEA and CK19 mRNA levels. During disease progression, tumor cells lose their intercellular adhesion molecules, and unpredictable lymphatic spread can occur. Lymph node metastasis is one of the most important prognostic factors for GCC. However, it is difficult to detect lymph node micrometastasis by routine pathological examination[8]. In the present study, our results showed that both pre- and post-CEA mRNA levels as well as pre- and post-CK19 mRNA levels were associated with positive lymph node metastasis. Our results was similar to the reports by Yanagita *et al*[20] and Kochi *et al*[11], who demonstrated that CK19 and CEA mRNA levels in peripheral blood were correlated with lymph node metastasis in GC. CK19, a cytoskeletal protein, is exclusively expressed in epithelial tissues and any tumor tissues derived from the epithelium. If the CK19 transcript is detected in lymph nodes of patients with epithelial tumors, the presence of disseminated cancer cells could be considered[21]. Therefore, our results indicated that both CEA mRNA and CK19 mRNA levels may serve as potential markers to detect lymph node metastasis in GCC.

Previous studies have suggested the potential role of CEA in monitoring disease recurrence and treatment response as well as in predicting the prognosis of many malignancies[22-24]. Cytokeratins are components of epithelial cells and the intermediate filaments of epithelial cancer cells; these proteins are involved in the dynamic remodeling of tumor cells during invasion and metastasis. CK19, a tissue-specific marker for epithelial tumor micrometastasis, has been reported to be a prognostic marker in esophageal cancer[9], hepatocellular carcinoma[25], and lung cancer[21]. Based on the previously published data of CEA and CK19 in other malignancies, we further explored the relationships between pre- and post-CEA and CK19 mRNA levels with OS in patients with GCC. To analyze the prognostic value of pre- and post-CEA and CK19 mRNA levels in GCC, Kaplan-Meier survival analysis and multivariate analysis were performed in the following analysis. The Kaplan-Meier survival analysis showed that both pre- and post-CEA mRNA levels as well as elevated pre-CK19 mRNA levels (but not post-CK19 mRNA levels) were correlated with a poor prognosis in GCC. In addition, the current study showed that the rate of elevated pre-CEA mRNA expression was 59.6%, which was higher than the rate of increased pre-CK19 mRNA expression (51.2%). These results indicated that CEA mRNA levels in peripheral blood of GCC patients may have a stronger prognostic prediction power than CK19 mRNA and that CEA mRNA levels seem to be a potential marker with a high sensitivity for prognostic prediction. Our observation was similar to that from a study by Ikeguchi *et al*[26], who proposed that CEA was more reliable than cytokeratins in detecting disseminated tumor cells. Multivariate analysis further showed that

lymph node status, pre-CK19 mRNA levels and pre-CEA mRNA levels negatively affected the prognosis of GCC patients. These data indicate that pre-CK19 and CEA mRNA levels in peripheral blood of GCC patients who underwent a radical gastrectomy are more accurate indicators of prognosis than post-CK19 and CEA mRNA levels. One reason may be because the levels of CK19 and CEA mRNAs are proportional to the tumor load and TNM stage, and the CK19 and CEA mRNA levels will inevitably decrease after tumor removal. Thus, the pre-CK19 and CEA mRNA levels may have a stronger power to reflect the entire status of disease and predict prognosis compared with post-CK19 and CEA mRNA levels.

In clinical practice, we noticed that GCC patients with three independent risk factors (positive lymph node metastasis, elevated pre-CK19 and CEA mRNA levels) tended to have a poorer prognosis. Therefore, we proposed a prognostic model based on these three risk factors and classified GCC patients into low-, intermediate- and high-risk groups. We further compared the survival curves of the three groups and found that there was a significant difference in OS among the three different risk groups. This prognostic model can be easily constructed and may potentially help clinicians make a more accurate judgment for prognosis and individual therapeutic treatment to improve survival and quality of life based on the risk stratification. GCC patients with a high-risk score may benefit from closer monitoring or more aggressive postoperative adjuvant therapy.

In conclusion, to the best of our knowledge, this is the first report showing that both elevated CK19 and CEA mRNA levels are correlated with positive lymph node metastasis in GCC. Elevated pre-CK19 and CEA mRNA levels were independently associated with poor prognosis in GCC patients undergoing curative surgery. Additionally, the new proposed prognostic model may help clinicians provide better individual therapeutic approaches and improve the outcome of patients with GCC based on the TNM stage.

ACKNOWLEDGMENTS

COMMENTS

Background

Gastric cardia cancer (GCC) is a distinct clinical entity, with a higher incidence of lymph node metastasis and poor prognosis. Meanwhile, there is no valid marker to predict the lymph node metastasis and prognosis in GCC. Carcinoembryonic antigen (CEA) and cytokeratin 19 (CK19) are related to lymph node metastasis and prognosis in gastrointestinal tumors. The level of tumor marker expression will change after surgery. Real-time polymerase chain reaction (qRT-PCR) technology, a sensitive, specific and rapid method,

has been widely used to detect the expression of tumor markers. However, the clinical and prognostic significance of CEA and CK19 mRNAs in GCC patients has been few reported.

Research frontiers

Recent studies have reported that pre- or post-CEA and CK19 mRNA levels are correlated with lymph node metastasis and prognosis in gastric cancer. However, few studies have reported and compared the clinical and prognostic significance of pre- and post-CEA and CK19 mRNA levels in GCC.

Innovations and breakthroughs

qRT-PCR, a sensitive, specific and rapid method, was used to detect the expression of CEA and CK19 mRNAs in GCC. In addition, this study is the first to report and compare the clinical and prognostic significance of pre- and post-CEA and CK19 mRNA levels in GCC.

Applications

These results provide evidence that either pre- and post-CEA or pre- and post-CK19 mRNA levels could help predict lymph node metastasis in patients with GCC, and the prognostic value of pre-CEA and pre-CK19 mRNA levels supersedes that of post-CEA and post-CK19 mRNA levels for clinical applications.

Terminology

CK19, a cytoskeletal protein, is exclusively expressed in epithelial tissues and any tumor tissues derived from the epithelium. CEA is a glycoprotein found in epithelial cells of colon cancer and colonic mucosa.

Peer-review

This is a strong study that evaluated the clinical and prognostic significance of pre- and post-CK19 and CEA mRNA levels in GCC.

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ORIGINAL ARTICLE

Retrospective Study

Proteomic profiling of fetal esophageal epithelium, esophageal cancer, and tumor-adjacent esophageal epithelium and immunohistochemical characterization of a representative differential protein, PRX6

Jun-Hui Guo, Guo-Lan Xing, Xin-Hui Fang, Hui-Fang Wu, Bo Zhang, Jin-Zhong Yu, Zong-Min Fan, Li-Dong Wang

Jun-Hui Guo, Henan Province Hospital of TCM, Zhengzhou 450000, Henan Province, China

Jun-Hui Guo, The Second Affiliated Hospital of Henan University of Chinese Medicine, Zhengzhou 450000, Henan Province, China

Jun-Hui Guo, Bo Zhang, Jin-Zhong Yu, The Second Affiliated Hospital of Henan University of Chinese Medicine, Zhengzhou 450000, Henan Province, China

Guo-Lan Xing, Zong-Min Fan, Li-Dong Wang, Henan Key Lab For Esophageal Cancer Res, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Xin-Hui Fang, Henan Province People's Hospital, Zhengzhou 450003, Henan Province, China

Hui-Fang Wu, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou 450000, Henan Province, China

Abstract

AIM
To understand the molecular mechanism of esophageal cancer development and provide molecular markers for screening high-risk populations and early diagnosis.

METHODS
Two-dimensional electrophoresis combined with mass spectrometry were adopted to screen differentially expressed proteins in nine cases of fetal esophageal epithelium, eight cases of esophageal cancer, and eight cases of tumor-adjacent normal esophageal epithelium collected from fetuses of different gestational age, or esophageal cancer patients from a high-risk area of esophageal cancer in China. Immunohistochemistry (avidin-biotin-horseradish peroxidase complex method) was used to detect the expression of peroxiredoxin (PRX)6 in 91 cases of esophageal cancer, tumor-adjacent normal esophageal tissue, basal cell hyperplasia, dysplasia, and carcinoma *in situ*, as well as 65 cases of esophageal epithelium from fetuses at a gestational age of 3-9 mo.

RESULTS
After peptide mass fingerprint analysis and search of protein databases, 21 differential proteins were identified; some of which represent a protein isoform. Varying degrees of expression of PRX6 protein, which was localized mainly in the cytoplasm, were detected in adult and fetal normal esophageal tissues, precancerous lesions, and esophageal cancer. With the progression of esophageal lesions, PRX6 protein expression showed a declining trend ($P < 0.05$). In fetal epithelium from fetuses at gestational age 3-6 mo, PRX6 protein expression showed a declining trend with age ($P < 0.05$). PRX6 protein expression was significantly higher in well-differentiated esophageal cancer tissues than in poorly differentiated esophageal cancer tissues ($P < 0.05$).

CONCLUSION
Development and progression of esophageal cancer result from interactions of genetic changes (accumulation or superposition). PRX6 protein is associated with fetal esophageal development and cancer differentiation.

Key words: Fetal esophageal epithelium; Esophageal squamous cell carcinoma; Tumor-adjacent esophageal epithelium; Proteomics

Core tip: This was a retrospective study to identify 21 significantly differentially expressed proteins that may be related to the development and growth of fetal esophageal epithelium or the development and progression of esophageal cancer. Peroxiredoxin (PRX)6 protein was localized mainly in the cytoplasm, and detected in adult and fetal normal esophageal tissues, precancerous lesions, and esophageal cancer. With the progression of esophageal lesions, PRX6 protein expression showed a declining trend. In epithelium from fetuses at gestational age 3-6 mo, PRX6 expression showed a declining trend with age. PRX6 protein expression was significantly higher in well-differentiated than poorly differentiated esophageal cancer tissues. PRX6 protein is associated with fetal esophageal development and esophageal cancer differentiation.

INTRODUCTION

Esophageal cancer is one of the most common thoracic malignancies that pose a serious threat to human health. There are about 400000 newly diagnosed cases of esophageal cancer and about 300000 related deaths worldwide each year, with the majority of patients diagnosed in developing countries. China is one of the countries or regions with a high incidence and mortality rates of esophageal cancer. Since the 1990s, the overall incidence and mortality rates of esophageal cancer have declined in China; however, esophageal cancer is still the leading cause of cancer-related death in Northern Henan Province and Chaoshan Region of Guangdong Province; both of which are high-risk areas for esophageal cancer. Squamous cell carcinoma (SCC) is the most important histological subtype of esophageal cancer in China. Esophageal cancer, especially esophageal SCC, is still a key research field for thoracic surgical research in China. Although China has a high incidence of esophageal cancer, > 90% of patients are diagnosed at a middle or late stage. As a result, the 5-year survival rate for Chinese esophageal cancer patients has not obviously declined over recent decades. In patients with early esophageal cancer who have undergone surgical or minimally invasive endoscopic treatment, the 5-year survival rate can reach > 90%. Therefore, early diagnosis is important for its prognosis. Clarifying the molecular mechanisms underlying the development and progression of esophageal cancer and identifying biological markers for screening high-risk populations are therefore of great clinical importance for early diagnosis. In the present study, we collected fetal esophageal tissues, esophageal cancer tissues, different degrees

Figure 1 Flow chart of proteomic analysis.

of precancerous lesions, and tumor-adjacent normal esophageal epithelial tissues from fetuses of different gestational age, or esophageal cancer patients in a high-risk area for esophageal cancer in China. We utilized proteomic technology to analyze the differential protein expression profiles in the development and growth of fetal esophageal epithelium and the development and progression of esophageal cancer, with the aim of clarifying the molecular mechanism of esophageal cancer development.

MATERIALS AND METHODS

Specimens
Esophageal cancer specimens were collected from 91 patients who underwent surgery at the Yaocun Esophageal Cancer Hospital (Linzhou, Henan Province, China) from 2000 to 2005. No patients underwent chemotherapy or radiotherapy. There were 51 men and 40 women, with a median age of 58 years (range, 35-78 years).

Tumor-adjacent normal esophageal mucosal epithelial tissues were collected under a microscope. A solitary lesion was considered only when the lesion was separated from other lesions by ≥ 1 cm of morphologically normal esophageal epithelium. Tumor-adjacent esophageal epithelium was regarded as normal regardless of whether it was completely or only partially normal.

Fetal esophageal tissue specimens were collected from 65 fetuses (30 male and 35 female), and they had a gestational age of 3 (*n* = 7), 4 (*n* = 13), 5 (*n* = 9), 6 (*n* = 10), 7 (*n* = 10), 8 (*n* = 10), or 9 (*n* = 6) mo. All fetuses were legally aborted fetuses or fetuses induced with misoprostol in the Family Planning Service Center of Hui County (Henan Province, China). Their parents had no prior history of usage of special drugs.

The age of fetuses was determined based on the date of the last menstrual period, the crown-heal length, and morphology of the hand and foot[1,2].

Equipment and reagents
WK600 nitric oxide laser gas analyzer (Weck, United States), Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems, United States), and GS-800 Calibrated Densitometer (Bio-Rad, United States) were used. Mouse anti-peroxiredoxin (PRX)6 monoclonal antibody was purchased from Chemicon (United States); ABC kit was purchased from Vector Laboratories (United States); DAB substrate kit was purchased from Beijing Zhong Shan-Golden Bridge Biological Technology Co. Ltd. (China); and lyophilized bovine serum albumin was purchased from Sigma (United States).

Proteomic analysis
Proteomic analysis was performed based on the study flow chart presented in Figure 1.

Solid-phase isoelectric focusing electrophoresis: Following removal of cover film, dried immobilized pH gradient (IPG, pH 3-10) strips (17 cm long) were plated in a fixed-length strip holder with the gel side down. After dispelling the air, 2 mL cover fluid (mineral oil) was applied to minimize evaporation. The holder was covered and placed on the IPG electrode platform, and isoelectric focusing electrophoresis was performed according to the protocol described in Table III-1. After electrophoresis was completed, Milli-Q water-wetted filter paper was used to wipe the mineral oil, and the strips were placed in a rehydration tray with the gel side up. Eight milliliters of equilibrium buffer Ⅰ consisting of 6 mol/L urea, 2% SDS, 0.375 mol/L Tris-HCl (pH 8.8), 20% glycerol and 2%

dithiothreitol was added. After shaking on a horizontal rotator at 15 rpm for 15 min, the strips were taken out and put into equilibrium buffer II consisting of 6 mol/L urea, 2% SDS, 0.375 mol/L Tris-HCl (pH 8.8), 20% glycerol and 2.5% iodoacetamide for 15 min. The strips were finally dried and used for the second dimension electrophoresis.

SDS-PAGE: SDS-PAGE was performed using Tris-glycine-SDS buffer (5 × : Tris 15.1 g, glycine 94 g, and SDS 5 g, dissolved in 1000 mL Milli-Q water). SDS-PAGE gels were prepared according to Table III-2. The gel was poured and allowed to polymerize. Pre-stained protein markers (10-15 μL) were spotted on 0.25 cm^2 Whatman filter paper (3 mm), which was subsequently placed on the "+" end of the IPG strip. IPG strips were imbedded in the second dimension and sealed using 0.5% agarose sealing solution (low melting point agarose 0.5 g and 10 μL 1% bromophenol blue, dissolved in 100 mL 1 × electrophoresis buffer). Electrophoresis was carried out at a constant current of 10 mA per gel for 30 min, which was then raised to 40 mA. After electrophoresis was completed, gels were removed and stained.

Silver nitrate staining and coomassie blue staining: Silver nitrate staining was performed as described previously[3]. For Coomassie blue staining, SDS-PAGE gels were placed in Coomassie blue solution (Coomassie blue R-250 1.16 g, absolute ethanol 500 mL, and glacial acetic acid 100 mL, dissolved in 1000 mL Milli-Q water) and incubated at room temperature for 2 h. The gels were then put into a destaing solution (30% absolute ethanol and 10% glacial acetic acid) until the background staining was removed.

Image scanning and analysis: Stained SDS-PAGE gels were scanned using a GS-800 calibrated densitometer at an optical resolution of 300 dpi and pixel of 8 bits. Images were analyzed with PDQuest 7.1.1 software. Image analysis included detecting spots on gels, editing spot and editing match, and correction of molecular weight (Mr) and isoelectric point (pI) of protein spots.

Tryptic digestion of in-gel proteins: For 2-D gels used for mass spectrometry (MS) analysis, the loading amount of protein was raised to 1 mg. 2-D gel electrophoresis was performed as described above. To ensure the integrity and purity of protein spots, they were cut and subjected to tryptic digestion at 2 wk after staining[4].

MALDI-TOF-MS analysis: MALDI-TOF-MS analysis of tryptic-digested fragments was performed by the Mass Spectrometry Laboratory of Shanghai Jikang Biochemical Technology Co. Ltd. (China). α-Cyano-4-hydroxycinnamic acid was dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid to prepare a saturated solution, and 1 μL of the solution was mixed with 1 μL tryptic-digested fragments. The mixture (1 μL) was loaded onto the mass spectrometer. Positive-ion mass spectra were measured. Two peaks of self-digested trypsin (842.51 and 2211.1046) were used as internal standards.

Immunohistochemistry
The avidin-biotin-horseradish peroxidase complex (ABC) method was used. Paraffin embedded sections were dewaxed, rehydrated through graded ethanol solutions, and washed three times with PBS. After endogenous peroxidase activity was blocked with 0.3% H_2O_2, sections were incubated with normal horse/goat serum (1:50) for 20 min, followed by incubation with a primary antibody diluted with 2% BSA (sPLA2, 1:200; PRX6, 1:500) at 4 ℃ overnight. Sections were then incubated with a secondary antibody diluted with 2% BSA (1:200) for 45 min and ABC (1:1:50) for 30 min at room temperature. Following diaminobenzidine coloration, sections were counterstained with hematoxylin for 15-30 s and mounted. A negative control was run by replacing the primary antibody with PBS, and p53-positive esophageal cancer tissue was used as a positive control[5]. Staining intensity was scored as follows: 0 (no staining); 1 (light yellow granules on the membrane or in the cytoplasm); 2 (yellow granules); and 3 (brown granules). Five hundred cells were counted in five microscopic fields per slide to calculate the percentage of stained cells, which was scored as follows: 0 (< 5%); 1 (5%-25%); 2 (25%-75%); and 3 (> 75%). Negative, positive and strongly positive staining was considered when the sum of the score of staining intensity score and the score of the percentage of stained cells was 0, 1-4, and > 4, respectively.

Quality control
Differential protein spots that were present on 2-D gels for esophageal cancer and fetal esophageal tissues but not on gels for normal esophageal epithelial tissues were chosen for further analysis. To ensure its reproducibility, 2-D gel electrophresis for each sample was repeated at least twice.

Statistical analysis
Statistical analyses were performed using SPSS version 10.0 software. Kruskal-Wallis and Mann-Whitney tests were used for comparing intergroup differences, with statistical significance set at $\alpha = 0.05$.

RESULTS

Selection of landmark protein spots on 2-D gel images
By comparing different 2-D gel images for different samples, four landmark protein spots (A-D) that were present on all gel images were selected (Figures 2 and 3). Comparison of these spots with protein markers showed that their Mr was 40.2, 14.2, 16.8

Figure 2 2-D gel images for tumor-adjacent normal esophageal epithelial tissue, esophageal squamous cell carcinoma tissue, and fetal esophageal tissue.

Figure 3 A typical differential protein spot in tumor-adjacent normal esophageal epithelial tissue, esophageal squamous cell carcinoma tissue, and fetal esophageal tissue. The spot was rarely detected in normal esophageal epithelial tissue, but was overexpressed in esophageal squamous cell carcinoma and fetal esophageal tissues.

and 28 kD, respectively, and PDQuest version 7.1.1 software determined their PI as 5.9, 7.1, 3.4 and 4.2, respectively.

Peptide mass fingerprint analysis and search of protein databases

Protein spots that were significantly differentially expressed on 2-D gel images were cut and then subjected to in-gel tryptic digestion and MALDI-TOF analysis to obtain the peptide mass fingerprint (PMF). PMF analysis and search of protein databases were then performed to identify differentially expressed protein spots. Table 1 lists the names of proteins, their theoretical and observed pI and Mr, number of matched peptide fragments, sequence coverage and MOWSE score. When searching protein databases, we restricted the above parameters to improve the accuracy of the search. After PMF analysis and search of protein databases, 21 differential proteins were identified; some of which represent a protein isoform.

Immunohistochemical staining for PRX6

PRX6 protein was stained brown and mainly localized on the cell membrane, and occasionally in the cytoplasm and nucleus (Figures 4 and 5).

In morphologically normal adult esophageal epithelial tissues, moderate staining of PRX6 protein was

observed. With the progression of esophageal lesions, the positive rate of PRX6 protein expression showed a declining trend, especially prominent in dysplasia (DYS) and carcinoma *in situ* (CIS) (Figure 4), in which weak staining of PRX6 protein predominated. In tumor-adjacent esophageal epithelial tissues, the rate of strongly positive staining ($\geq$ 4 points) decreased from 43% in normal epithelium (NOR) to 35% in basal cell hyperplasia. In DYS, moderately or weakly positive staining of PRX6 protein (< 4 points) predominated. The majority of CIS lesions (79%) showed no staining for PRX6 protein, with weak staining predominating. Interestingly, cancer cells in SCC showed varying degrees of PRX6 staining, with strongly positive staining ($\geq$ 4 points) predominating. In seven cases of well-differentiated SCC, varying degrees of PRX6 staining were observed. With the decrease in the differentiation degree of SCC, PRX6 protein expression gradually declined ($P < 0.05$) (Table 2).

In fetal esophageal epithelial tissues, the positive rate of PRX6 protein was 87%. In esophageal epithelial tissues from fetuses at a gestational age of 4-6 mo, strongly positive staining of PRX6 protein ($\geq$ 4 points) predominated. In esophageal epithelial tissues from fetuses at a gestational age of 3, 7 or 8 mo, moderately or weakly positive staining (< 4 points) predominated. In esophageal epithelial tissues from

Figure 4 Peroxiredoxin 6 expression in different types of esophageal lesions (DAB, magnification × 200). NOR: Normal epithelium; BCH: Basal cell hyperplasia; DYS: Dysplasia; CIS: Carcinoma *in situ*.

Table 1   Identification of differentially expressed proteins in tumor-adjacent normal esophageal epithelial tissue, esophageal squamous cell carcinoma tissue, and fetal esophageal tissue

Spot	Protein name and theoretical pI and Mr	Observed pI and Mr	No. of matched peptide fragments	Sequence coverage	MOWSE score
2	USP30 6.87/45546	9.3/32	2	4	21
4	Thyroid hormone receptor interactor 4.56/11 8489	7.8/31	3	56	51
5	Cardiac muscle 5.34/22563	6.25/29	5	24	66
6	Chain A, crystal structure of a recombinant glutathione transferase 5.09/23202	6.2/24	5	43	85
7	Dermatopontin precursor 4.7/23989	5.3/21.5	5	29	82
10	Heat shock protein 27 7.83/22313	7.1/20.5	5	29	79
11	S100 calcium-binding protein A9 5.71/13234	6.3/15	5	40	74
12	G-gamma-hemoglobin 6.17/11033	7.5/16	3	45	65
13	MB protein 9.24/10863	8.4/15.5	3	32	65
14	Immunoglobulin heavy chain variable region 9.78/13026	8.6/14	3	34	70
15	PREDICTED: hypothetical protein 5.27/6706	8.1/12	2	44	52
16	Immunoglobulin heavy chain variable region 9.04/13053	7.1/11	2	26	49
17	Heat shock 27kDa protein 1 5.98/22768	9.2/16	6	33	80
18	hCG1659706 10.28/5287	9.5/21	2	64	37
19	Smooth muscle protein 8.56/22461	9.4/19	6	26	86
21	hCG1997574 9.07/29453	7.3/32	7	31	93
22	Heat shock 27 kDa protein 1 5.98/22768	6.8/32	6	35	84
23	Disabled homolog 1 (Drosophila) 9.40/22903	7.15/31	6	26	66
24	Phosphoglycerate mutase 1 (brain), 6.40/24669	7.3/31	8	40	117
25	Peroxiredoxin 6 6.00/25019	7.1/27.8	8	41	116
26	Chain A, human triosephosphate isomerase of new crystal form 6.50/26666	7.3/30	10	38	160

fetuses at a gestational age of 8 mo, both strongly positive staining and negative staining were observed (Figure 5). Statistical analysis showed that PRX6 protein expression in esophageal epithelial tissues from fetuses at a gestational age of 4 or 7 mo differed significantly compared with that from fetuses of other gestation age groups ($P < 0.05$, Table 3).

DISCUSSION

The development and progression of esophageal cancer is a multistage process involving many

Figure 5 Peroxiredoxin 6 expression in esophageal tissues from fetuses at a gestational age of 3-8 mo (DAB, magnification × 200).

Table 2 Expression of PRX6 protein in different types of esophageal lesions *n* (%)

Tissue	PRX6		
	No. of cases	Negative	Positive
Tumor-adjacent			
NOR	37	3 (8)	34 (92)
BCH	37	3 (8)	34 (92)
DYS	29	18 (62)	11 (38)
CIS	28	22 (79)	6 (21)
Esophageal cancer			
W-SCC	7	0	7 (100)
M-SCC	16	3 (19)	13 (81)
P-SCC	6	5 (83)	1 (17)

P < 0.05, NOR *vs* DYS, CIS, M-SCC or P-SCC; BCH *vs* CIS, DYS, M-SCC or P-SCC; DYS *vs* W-SCC or M-SCC; CIS *vs* W-SCC or M-SCC; P-SCC *vs* W-SCC or M-SCC. NOR: Normal; BCH: Basal cell hyperplasia; DYS: Dysplasia; CIS: Carcinoma *in situ*; W-SCC: Well differentiated squamous cell carcinoma; M-SCC: Moderately differentiated; P-SCC: Poorly differentiated.

Table 3 Expression of PRX6 expression in esophageal tissues from fetuses of different gestational age *n* (%)

Age (mo)	PRX6		
	No. of cases	Negative	Positive
3	6	0	6 (100)
4	4	0	4 (100)
5	6	1 (17)	5 (83)
6	6	0	6 (100)
7	5	1 (20)	4 (80)
8	6	1 (17)	5 (83)
9	6	2 (33)	4 (67)

P < 0.05, 3 mo *vs* 4 mo, 4 mo *vs* 7 mo, or 6 mo *vs* 7 mo.

genes[6-9]. Proteomics is the study of the entire set of proteins expressed by a single cell or tissue. With the aid of proteomics, changes in protein molecules directly related to tumor development and progression can be identified, thus allowing to find markers for early diagnosis of tumors and monitoring of curative effects. This study utilized the 2-DE-based proteomic technology to identify differentially expressed proteins in fetal esophageal tissues, esophageal cancer tissues, and tumor-adjacent normal esophageal epithelial tissues from fetuses of different gestational age, or esophageal cancer patients from a high-risk area for esophageal cancer in China. Twenty-one significantly differentially expressed proteins were identified, which may be related to the development and growth of fetal esophageal epithelium or the development and progression of esophageal cancer.

PRX6 protein belongs to the antioxidant PRX family, which is ubiquitously expressed in prokaryotes and eukaryotes[10] and has six mammalian members[11]. Chevallet *et al*[12] found that PRX6 expression continued to rise within 1-24 h after oxidative stress. Besides antioxidant properties, PRXs can also be radiation protective, stimulate cell proliferation, and participate in cell signal transduction. This study found that PRX6 expression was elevated in esophageal cancer tissues where cell metabolism and proliferation are active, suggesting that increased PRX6 may inhibit the excessive production of reactive oxygen species and maintain cell homeostasis to protect cells from damage. Similar to our results, PRX protein expression was also found to be elevated in malignant

mesothelioma, lung cancer, and oral cancer[13-15]. In our previous proteomic study of esophageal SCC, PRX1 protein expression was upregulated, but PRX2 expression was downregulated[16], indicating that the development and progression of esophageal SCC involves changes in a variety of antioxidant proteins, and different antioxidant proteins or protein isoforms may have different functions in the development and progression of esophageal cancer. Further studies should be performed to clarify the exact mechanisms of these antioxidant proteins.

The most important finding of this study is that expression of PRX6 protein decreased with progression of esophageal lesions. In tumor-adjacent normal esophageal epithelial tissues, PRX6 protein was highly expressed; however, PRX6 protein expression was significantly reduced in CIS. PRX6 protein expression was positively correlated with the degree of tumor differentiation in esophageal SCC. With the decrease in tumor differentiation, PRX6 protein expression decreased. In the fetal esophageal epithelium, PRX6 protein expression showed a fluctuating pattern with the increase of gestational age. The intensity of PRX6 protein expression was positively correlated with the degree of differentiation of epithelial cells. PRX6 protein expression was highly expressed in well-differentiated epithelial cells, but lowly expressed or not expressed in lowly differentiated epithelial cells.

Both fetal esophageal epithelium and carcinoma tissues have active cell proliferation and metabolism, which may result in increased peroxide generation. PRX6 protein in fetal esophageal epithelium and carcinoma tissues may have antioxidant effects by eliminating peroxides produced by the cell metabolism, thus protecting cells from damage. Loss of expression of PRX6 protein in CIS may promote cell proliferation and facilitate the progression of precancerous lesions. However, the dynamic expression pattern of PRX6 protein in esophageal precancerous lesions and fetal esophageal epithelium suggests that PRX6 protein is associated with the multistage evolution of esophageal cancer and may inhibit cell proliferation and promote the differentiation of esophageal epithelial cells. Loss of expression of PRX6 protein in CIS is likely to promote cell proliferation and facilitate the progression of precancerous lesions. In poorly differentiated carcinoma tissue, loss of PRX6 protein may lead to rapid cell proliferation. Under certain conditions, PRX6 protein may also be involved in the induction of differentiation of cancer cells, thus resulting in the formation of highly differentiated carcinoma tissue. In fetal esophageal epithelium, PRX6 protein expression showed no significant correlation with gestational age, but was, to some extent, associated with the morphology of epithelial cells. In the base layer where cells are arranged neatly, the level of PRX6 protein expression was high. In contrast, when the arrangement of epithelial cells was disorderly, PRX6 protein exhibited low expression. These findings

suggest that PRX6 protein plays an important role in the process of differentiation of fetal esophageal epithelial cells. We speculate that PRX6 protein may promote the trans-differentiation from precancerous lesions to normal esophageal epithelium. However, the precise role of PRX6 protein in cell differentiation requires further research.

This study utilized the 2-DE based proteomic technology to analyze the differential protein expression profiles in fetal esophageal tissues, esophageal cancer tissues, and tumor-adjacent normal esophageal epithelial tissues from fetuses of different gestational age, or esophageal cancer patients from a high-risk area for esophageal cancer in China. We identified 21 significantly differentially expressed proteins that may be related to the development and growth of fetal esophageal epithelium or the development and progression of esophageal cancer, such as dermatopontin, S100A9, HSP27, and PRX6. Our results suggest that the development and progression of esophageal cancer are a result of interactions of many genetic changes (accumulation or superposition). These proteins are related not only to esophageal cancer, but also to other tumors. The identification of proteins associated with esophageal cancer and fetal esophageal development can improve the understanding of the molecular mechanism of esophageal cancer develop-ment, and may help provide a fetal esophageal model for esophageal cancer. PRX6 protein plays an important role in the induction of differentiation of esophageal epithelial cells, and PRX6 protein expression level may be used as an important marker for evaluating cell differentiation. In this regard, further research of molecular mechanism of PRX6 is of great significance.

COMMENTS

Background
The fetal esophageal epithelium is developed from unmatured monolayer columnar epithelium gradually into a matured stratified squamous epithelium, differentiated from immature gradually to mature, epithelial cells differentiated from the active division and proliferation epithelial stem cells gradually matured into stratified squamous epithelium; Adult esophageal epithelial carcinogenesis is the differentiation of mature epithelial tissue in the role of carcinogenic factors, the emergence of hyperplasia and abnormal differentiation eventually developed into cancer. Obviously, the two have the opposite differentiation process. The aims of this study were to: (1) find the protein molecules that are differentially or identically expressed in the process of maturation and differentiation of fetal esophageal epithelium and carcinogenesis of adult esophageal epithelium; and (2) to strengthen the understanding of the molecular mechanism of esophageal carcinogenesis, so as to provide an important theoretical basis for the screening and detection of early molecular markers in high-risk population.

Research frontiers
Clarifying the molecular mechanisms underlying the development and progression of esophageal cancer and identifying biological markers for screening high-risk populations are of clinical importance for early diagnosis. Utilize the proteomic technology to analyze the differential protein expression profiles in the development and growth of fetal esophageal epithelium and the development and progression of esophageal cancer, with an aim to clarify the molecular mechanism of esophageal cancer development.

Innovations and breakthroughs

The authors identified 21 differentially expressed proteins by peptide mass fingerprinting and database analysis in the study, and some proteins contained different isoforms.

Applications

Peroxiredoxin (PRX)6 protein expression level may be used as an important marker for evaluating cell differentiation. In this regard, further research of molecular mechanism of PRX6 is of great significance.

Terminology

MALDI-TOF-MS: matrix-assisted laser desorption/ionization time of flight mass spectrometry.

Peer-review

This is an excellent study. In this study, the authors analyzed the differential protein expression profiles in fetal.

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ORIGINAL ARTICLE

Retrospective Study

Helicobacter pylori infection with intestinal metaplasia: An independent risk factor for colorectal adenomas

Ye Yan, Yi-Na Chen, Qian Zhao, Chao Chen, Chun-Jing Lin, Yin Jin, Shuang Pan, Jian-Sheng Wu

Ye Yan, Yi-Na Chen, Qian Zhao, Chao Chen, Yin Jin, Shuang Pan, Department of Gastroenterology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang Province, China

Chun-Jing Lin, Jian-Sheng Wu, Medical and Health Care Center, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang Province, China

Correspondence to: Dr. Jian-Sheng Wu, Professor, Medical and Health Care Center, The First Affiliated Hospital of Wenzhou Medical University, No. 1 Nan Bai Xiang street, Wenzhou 325000, Zhejiang Province, China. wzwujs@163.com
Telephone: +86-577-55578033
Fax: +86-577-55578033

Abstract

AIM
To explore the association between *Helicobacter pylori* (*H. pylori*) infection status, intestinal metaplasia (IM), and colorectal adenomas.

METHODS
We retrospectively reviewed 1641 individuals aged $\geq$ 40 years who underwent physical examination, laboratory testing, ^{13}C-urea breath testing, gastroscopy, colonoscopy, and an interview to ascertain baseline characteristics and general state of health. Histopathological results were obtained by gastric and colorectal biopsies.

RESULTS
The prevalence of *H. pylori* infection and adenomas was 51.5% (845/1641) and 18.1% (297/1641), respectively. *H. pylori* infection was significantly correlated with an increased risk of colorectal adenomas (crude OR = 1.535, 95%CI: 1.044-1.753, *P* = 0.022; adjusted OR = 1.359, 95%CI: 1.035-1.785, *P* = 0.028). Individuals with IM had an elevated risk of colorectal adenomas (crude OR = 1.664, 95%CI: 1.216-2.277, *P* = 0.001; adjusted OR = 1.381, 95%CI: 0.998-1.929, *P* = 0.059). Stratification based on *H. pylori* infection stage and IM revealed that IM accompanied by *H. pylori* infection was significantly associated with an increased risk of adenomas (crude OR = 2.109, 95%CI: 1.383-3.216, *P*

= 0.001; adjusted OR = 1.765, 95%CI: 1.130-2.757, *P* = 0.012).

CONCLUSION
H. pylori-related IM is associated with a high risk of colorectal adenomas in Chinese individuals.

Key words: *Helicobacter pylori*; Chinese population; Colorectal neoplasms; Intestinal metaplasia; Chronic gastritis

Core tip: This retrospective study revealed *Helicobacter pylori* (*H. pylori*)-related intestinal metaplasia (IM) to be an independent risk factor for colorectal adenomas in Chinese individuals aged $\geq$ 40 years. Clinically, it may be useful for patients with *H. pylori* infection and IM to undergo colonoscopy screening and surveillance.

INTRODUCTION
Colorectal cancer (CRC) is the fifth most common cancer and the fifth most common cause of cancer death in China[1]. CRC mostly arises from colorectal adenomas through the adenoma-to-carcinoma sequence[2]. Common risk factors, such as age, family history, smoking, alcohol consumption, diet, and lifestyle, contribute to colorectal neoplasm development[3]. It is well known that *Helicobacter pylori* (*H. pylori*) is classified as a class 1 carcinogen, as it infects the gastric mucosa and causes inflammation that drives the progression of the gastritis-atrophy-metaplasia-dysplasia-cancer sequence[4]. *H. pylori* infection was first recognized as a risk factor for colorectal neoplasm in the 1990s[5]. Some reports have indicated a positive association between *H. pylori* infection and colorectal neoplasm[5-13], but this has been disputed by others[14-18]. The pathophysiological mechanism of how *H. pylori* induces colorectal neoplasm is still unclear. A recent study associated the presence of *H. pylori* infection and intestinal metaplasia (IM) with a significantly elevated risk of colorectal adenomas[9]. Therefore, we aimed to conduct a further analysis to evaluate the relationship between *H. pylori*-related IM and colorectal adenomas.

MATERIALS AND METHODS
Patient selection
From September 2014 to January 2016, 15622 indivi-

duals from an asymptomatic healthy population underwent health check-ups at the Medical and Health Care Center of The First Affiliated Hospital of Wenzhou Medical University. All individuals underwent physical examination, laboratory testing, and an interview to ascertain baseline characteristics and general state of health. Among this large study group, 1720 individuals aged $\geq$ 40 years underwent the ^{13}C-urea breath test, gastroscopy, and colonoscopy. Individuals with a previous history of *H. pylori* eradication therapy or polyp resection were excluded from the study. In addition, individuals were excluded if they had inflammatory bowel disease, gastric dysplasia, or malignancies, including gastrointestinal cancer. Ultimately, the data of 1641 individuals were included in our analysis.

Diagnostic criteria
The following baseline characteristics were obtained from self-report questionnaires for analysis: age, body mass index (BMI), family history, personal medical history, smoking, and alcohol consumption. Among the 1641 individuals included in the study group, 1550 (94%) had antrum biopsies, 498 (30%) had corpus biopsies, 120 (7%) had cardia biopsies, and 337 (21%) had biopsies at multiple sites. According to the histopathological results of the gastric mucosa, individuals were divided into two groups: IM (+) group and IM (-) group (including normal mucosa, chronic non-atrophic gastritis, and chronic atrophic gastritis). According to results of colorectal biopsies, individuals were divided into three groups: non-polyp group, non-adenomatous polyp group (including hyperplastic polyps and inflammatory polyps), and adenoma group. Polyps located in the cecum, ascending, and transverse colon were classified as "proximal lesions", those located in the descending colon, sigmoid, and rectum were classified as "distal lesions", and those located on both sides were classified as "bilateral lesions". Polyps were grouped based on number: one, two or more. Polyps were also grouped based on size: 0-9 mm and $\geq$ 10 mm. Gastroscopy and colonoscopy were performed with a GIF-H260 gastroscope and a CF-H260AI colonoscope (OLYMPUS, Tokyo, Japan), respectively. The ^{13}C-urea breath test was used to identify *H. pylori* infection and was performed with an infrared spectrometer with a sensitivity of 97.8%, specificity of 96.8%, and accuracy of 97.5%[19]. All examinations were performed on the same day.

Statistical analysis
Statistical analyses were performed using SPSS version 19 (Armonk, NY). Data for continuous variables are expressed as the mean ± SD, and between-group differences were evaluated using the *t* test. Categorical variables were evaluated using a χ^2 test. Odds ratios (ORs) and 95%CIs were obtained by logistic regression analysis. Statistical significance was established for two-sided *P* values < 0.05.

Parameter	Non-polyp 1058	Adenoma 297	Non-adenomaous polyp 286	[1]P value	[2]P value
Age	49.72 (7.974)	53.17 (8.450)	51.91 (8.456)	< 0.001	< 0.001
Male/female	625/433	241/56	221/65	< 0.001	< 0.001
BMI	23.95 (2.963)	24.50 (2.978)	24.9 (3.017)	0.05	< 0.001
Smoker (+/-)	211/847	118/179	120/166	< 0.001	< 0.001
Alcohol (+/-)	140/918	78/219	67/219	< 0.001	< 0.001
TC	5.457 (1.130)	5.481 (1.340)	5.427 (1.021)	0.753	0.682
TG	1.879 (1.763)	2.025 (2.438)	2.111 (1.189)	0.25	0.052
HDL	1.325 (0.342)	1.285 (0.316)	1.246 (0.389)	0.538	0.001
LDL	3.269 (0.814)	3.259 (0.815)	3.271 (0.822)	0.857	0.967
FBG	5.060 (1.230)	5.118 (1.220)	5.316 (1.610)	0.478	0.004

Table 1 Baseline characteristics of subjects *n* (%)

[1]Two-sided *P* values for the difference between the adenoma and non-polyp groups were based on the χ^2 test and *t* test; [2]Two-sided *P* values for the difference between the non-adenomatous polyp and non-polyp groups were based on the χ^2 test and *t* test. BMI: Body mass index; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; FBG: Fasting blood glucose.

RESULTS

The prevalence of *H. pylori* infection was 51.5% (845/1641), and the prevalence rates of IM, non-adenomatous polyps, and adenomas were 18.3% (300/1641), 17.4% (286/1641) and 18.1% (297/1641), respectively. Baseline characteristics of patients with colorectal adenomas and non-adenomatous polyps and those without polyps are summarized in Table 1. No significant differences were observed in mean serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), or fasting blood glucose (FBG) levels between the adenoma and non-polyp groups. Additionally, there were no significant differences in TG, TC or LDL between the non-adenomatous polyp and non-polyp groups. The patients' mean age was 53.17 (8.450) years for the colorectal adenoma group, 51.91 (8.456) years for the non-adenomatous polyp group, and 49.72 (7.974) years for the non-polyp group, with patients in the non-polyp group being significantly younger than for patients in the colorectal adenoma group (*P* < 0.001) and the non-adenomatous polyp group (*P* < 0.001). The mean BMI was higher in the colorectal adenoma group (*P* = 0.05) and non-adenomatous polyp group (*P* < 0.001), compared to the non-polyp group. The frequency of male sex in the colorectal adenoma group, non-adenomatous polyp group, and non-polyp group was 81.11% (241/297), 73.78% (221/286), and 59.07% (625/1058), respectively. Smoking (*P* < 0.001) and alcohol consumption (*P* < 0.001) rates were both higher in the adenoma and non-adenomatous polyp groups than in the non-polyp group. Therefore, age, sex, BMI, smoking, and alcohol consumption were identified as risk factors in the adenoma group, and used to control for confounding effects in the following analyses. For the non-adenomatous polyp group, age, sex, BMI, smoking, alcohol consumption, HDL level, and FBG level were identified as risk factors, and used to control for confounding effects in the following analyses.

Based on *H. pylori* infection status, we divided individuals into two groups. As reported in Table 2, there were no significant differences in mean age or sex between the *H. pylori* positive and *H. pylori* negative groups. In addition, the incidence of adenomas was higher in the *H. pylori* positive group than in the *H. pylori* negative group, with a crude OR of 1.535 (95%CI: 1.044-1.753, *P* = 0.022) and an adjusted OR of 1.359 (95%CI: 1.035-1.785, *P* = 0.028, Table 3). Moreover, there was no significant association between non-adenomatous polyps and *H. pylori* infection. The association of polyps with *H. pylori* infection was highest for single polyp (OR = 1.328, 95%CI: 1.032-1.708, *P* = 0.027), polyp size of 0-9 mm (OR = 1.352, 95%CI: 1.098-1.666, *P* = 0.005), and proximally located polyps (OR = 1.457, 95%CI: 1.062-1.998, *P* = 0.020).

Compared to the IM (-) group, individuals in the IM (+) group were older (*P* < 0.001), with a higher proportion of men (*P* = 0.009, Table 4). The frequency of adenoma was more prevalent in the IM (+) group than in the IM (+) group, with a crude OR of 1.664 (95%CI: 1.216-2.277, *P* = 0.001) and an adjusted OR of 1.381 (95%CI: 0.998-1.929, *P* = 0.059; Table 3). The frequency of non-adenomatous polyps in the IM (+) group and IM (-) group was 20.3% and 16.8%, respectively, with a crude OR of 1.436 (95%CI: 1.035-1.993, *P* = 0.030) and an adjusted OR of 1.225 (95%CI: 0.930-1.612, *P* = 0.148, Table 3). The association of polyps with IM (+) was highest for patients with more than one polyp (OR: 1.766, 95%CI: 1.278-2.441, *P* = 0.001), a polyp size of 0-9 mm (OR: 1.526, 95%CI: 1.176-1.981, *P* = 0.001), and proximally located polyps (OR: 1.703, 95%CI: 1.171-2.475, *P* = 0.005).

The risk for adenoma was significantly higher in the presence of both *H. pylori* infection and IM. Next, we further classified all individuals into four groups (Table 5): Group A: *H. pylori* (-) and IM (-); Group B: *H. pylori* (+) and IM (-); Group C: *H. pylori* (+) and IM (+); and Group D: *H. pylori* (-) and IM (+). The risk of adenomas among the four groups of *H. pylori*-

Table 2 Correlation between *Helicobacter pylori* infection and colorectal neoplasms

Parameter	H. pylori (+) 845	H. pylori (-) 796	OR (95%CI)	P value
Age	50.91 (8.315)	50.54 (8.208)	1.006 (0.994-1.017)	0.355
Female	292	262	1	
Male	553	534	0.929 (0.757-1.140)	0.482
Non-polyp	519	539	1	
Non-adenomaous polyp	158	128	1.282 (0.986-1.667)	0.064
Adenoma	168	129	1.535 (1.044-1.753)	0.022
Polyp number				
One	179	140	1.328 (1.032-1.708)	0.027
Two or more	147	117	1.305 (0.995-1.711)	0.054
Polyp size				
0-9 mm	306	235	1.352 (1.098-1.666)	0.005
≥ 10 mm	20	22	0.944 (0.509-1.751)	0.855
Polyp location				
Proximal	108	77	1.457 (1.062-1.998)	0.020
Bilateral	64	57	1.166 (0.800-1.700)	0.424
Distal	154	123	1.300 (0.997-1.696)	0.053

Correlation between *Helicobacter pylori* (*H. pylori*) (+) and *H. pylori* (-) by logistic regression analysis.

Table 3 Logistic regression model of the association between *Helicobacter pylori* infection, intestinal metaplasia, and colorectal neoplasm after adjustments for confounding factors

	Non-adenomaous polyp		Adenomas	
	Adjusted [a]OR 95%CI	[1]P value	Adjusted [b]OR (95%CI)	[2]P value
H. pylori (+)	1.225 (0.930-1.612)	0.148	1.359 (1.035-1.785)	0.028
IM	1.265 (0.896-1.787)	0.173	1.381 (0.988-1.929)	0.059

[1]Adjusted for age, gender, body mass index (BMI), smoking habit, alcohol consumption, high-density lipoprotein level, and fasting blood glucose level by logistic regression analysis; [2]Adjusted for age, gender, BMI, smoking habit, and alcohol consumption by logistic regression analysis. *H. pylori*: *Helicobacter pylori*.

Table 4 Correlation between gastric lesions and colorectal neoplasm

Parameter	IM (+) 300	IM (-) 1341	OR (95%CI)	[1]P value
Age	53.12 (8.490)	50.19 (8.118)	1.041 (1.026-1.056)	< 0.001
Female	80	474	1	
Male	220	867	1.503 (1.137-1.988)	0.004
Non-polyp	168	890	1	
Adenomas	71	226	1.664 (1.216-2.277)	0.001
Non-adenomaous polyps	61	225	1.436 (1.035-1.993)	0.030
Polyp number				
One	66	253	1.382 (1.006-1.898)	0.046
Two or more	66	198	1.766 (1.278-2.441)	0.001
Polyp size				
0-9 mm	121	420	1.526 (1.176-1.981)	0.001
≥ 10 mm	11	31	1.880 (0.927-3.813)	0.080
Polyp location				
Proximal	45	135	1.703 (1.171-2.475)	0.005
Bilateral	28	85	1.595 (1.013-2.510)	0.044
Distal	59	204	1.434 (1.029-1.997)	0.033

[1]Correlation between IM (+) and IM (-) by logistic regression analysis. IM: Intestinal metaplasia.

related gastric lesions is reported in Table 5. No significant differences were noted between Group A and Group B (crude OR: 1.214, 95%CI: 0.961-1.761, *P* = 0.198). However, the presence of *H. pylori*-related IM was significantly associated with an increased risk for colorectal adenomas, with a crude OR of 2.109 (95%CI: 1.383-3.216, *P* = 0.001) and an adjusted OR of 1.765 (95%CI: 1.130-2.757, *P* = 0.012). The progression of non-*H. pylori*-related IM did not increase the risk of adenomas, with a crude OR of 1.527 (95%CI: 0.954-2.444, *P* = 0.078) and an adjusted OR of 1.222 (95%CI: 0.741-2.012, *P* = 0.432).

Table 5 Correlation between stage of *H. pylori*-related gastric lesions and adenoma

	H. pylori	IM	Adenoma 297	Non-polyp 1018		Crude OR	*P* value	Adjusted [1]OR	[1]*P* value
Group A	(-)	(-)	113	488	B:A	1.214 (0.906-1.626)	0.194	1.190 (0.876-1.617)	0.262
Group B	(+)	(-)	113	402	C:A	2.109 (1.383-3.216)	0.001	1.765 (1.130-2.757)	0.012
Group C	(+)	(+)	42	86	D:A	1.527 (0.954-2.444)	0.078	1.222 (0.741-2.015)	0.432
Group D	(-)	(+)	29	82					

[1]Adjusted for age, gender, body mass index, smoking habit, and alcohol consumption by logistic regression analysis. IM: Intestinal metaplasia.

DISCUSSION

Our study, which included asymptomatic individuals who underwent the ^{13}C-urea breath test, gastroscopy, and colonoscopy, identified *H. pylori*-related IM as an independent risk factor for colorectal adenomas in Chinese individuals aged ≥ 40 years. Age, sex, BMI, smoking, and alcohol consumption were included as confounders to adjust the correlation between *H. pylori*-related IM and colorectal adenomas. *H. pylori* infection was significantly associated with an increased risk of colorectal adenomas. These results are consistent with previous studies that reported a positive correlation between *H. pylori* infection and colorectal adenomas[5-10]. Additionally, individuals with IM had an elevated risk of colorectal adenomas. A large population based case-control study that enrolled 156000 individuals showed a positive association between IM and colorectal adenomas (adjusted OR = 1.24, 95%CI: 1.17-1.32), but without including an analysis of the relationship between *H. pylori*-related IM and colorectal adenomas[9]. Furthermore, a recent study showed that individuals with IM were more likely to have adenomas with high-grade intraepithelial lesions (OR = 3.218, 95%CI: 0.767-13.509)[20]. To our knowledge, no study has analyzed the relationship between *H. pylori*-related IM and colorectal neoplasm. Thus, we conducted an analysis that stratified individuals based on *H. pylori* infection stage and IM. Based on this stratification, we drew the following conclusion: *H. pylori* infection without IM did not increase the risk of colorectal adenomas, whereas IM accompanied by *H. pylori* infection did increase the risk of colorectal adenomas. Therefore, longstanding *H. pylori* infection may be crucial to the development of colorectal adenomas because IM is usually a chronic sequela of *H. pylori* infection. Our analysis may also explain the inconsistencies in previous studies, with some of studies having reported a positive correlation between *H. pylori* infection and colorectal adenomas, while other studies reported either a null or inverse association[14-17]. This may be due to racial differences or discrepancies in the prevalence of *H. pylori* infection and IM in different regions. Differences among studies could also be associated with: the dominant use of hospital-based data, which may result in a patient selection bias; small sample sizes; different diagnostic tests used for *H. pylori* identification; differences

in prior history of *H. pylori* eradication therapy or previous colorectal polyp removal among patients; as well as other uncontrolled confounding factors. In addition, our results revealed that the presence of *H. pylori* infection was significantly associated with an elevated risk of proximal polyps, as previously reported by Hong *et al*[21] for proximal neoplasms. Conversely, other studies have reported an association between *H. pylori* and an elevated risk of distal neoplasms[15,22].

Various interpretations have been proposed to explain the mechanisms by which *H. pylori* infection increases the risk for colorectal adenomas. According to the most commonly described pathogenesis, persistent *H. pylori* infection elicits hypergastrinemia, which has a trophic effect on epithelial cell growth and proliferation, contributing to colorectal carcinogenesis[23]. Indeed, gastrin and the cholecystokinin type B/gastrin receptor are expressed in human colonic polyps, with activation occurring early in the adenoma-carcinoma sequence[24]. Several epidemiological reports have confirmed a positive relationship between hypergastrinemia and an increased risk for colorectal neoplasm[15,25,26], although these findings have been disputed[27-29]. *H. pylori* infection, aging, alcohol consumption, smoking, excessive salt intake, and bile reflux are deemed as risk factors correlated with IM[30,31]. Foci of IM tend to appear first at the antrum-corpus junction, extending to both the antrum and the corpus and replacing the normal gastric parietal cells[32]. Reduced gastric acid secretion triggered by IM might cause hypergastrinemia. In addition, hypochlorhydria hampers protein assimilation, which may increase some metabolites and unabsorbed nutrients, resulting in bacterial overgrowth and colonic disorders and contributing to colorectal carcinogenesis[33,34]. Therefore, *H. pylori*-related IM might aggravate colorectal carcinogenesis.

Our study had several limitations that need to be acknowledged. First, we did not measure the serum gastrin level, which is the key mechanism accounting for the contribution of *H. pylori* to colorectal carcinogenesis. Second, biopsies were taken from multiple (*i.e.*, three or more) sites in only 21% of patients, lowering the rate of gastric disease detection. Third, we used the ^{13}C-urea breath test to determine the presence of an infection. However, the ^{13}C-urea breath test is less reliable than histological staining, such as Giemsa staining, in evaluating *H. pylori* colonization in biopsy tissue. Fourth, this was a single

center study with a small sample size. A multicenter study with a large sample size should be conducted.

In conclusion, our research demonstrated that Chinese people who have *H. pylori*-related IM do have a high risk of colorectal adenomas. Given a high prevalence of colorectal adenocarcinoma in China, it is necessary for patients with *H. pylori* infection and IM to undergo colonoscopy screening and surveillance.

COMMENTS

Background

Previous studies demonstrated a positive correlation between *Helicobacter pylori* (*H. pylori*) infection and colorectal neoplasm. A recent study showed that *H. pylori* infection and intestinal metaplasia (IM) both significantly elevated the risk of colorectal adenomas However, no study has analyzed the relationship between *H. pylori*-related IM and colorectal neoplasm.

Research frontiers

Colorectal cancer mostly arises from colorectal adenomas through the adenoma-to-carcinoma sequence. Early diagnosis of adenoma is very important to lower the mortality. It is necessary for individuals with *H. pylori* infection and IM to have colonoscopy screening and surveillance.

Innovations and breakthroughs

This study identified *H. pylori*-related IM as an independent risk factor for colorectal adenomas in Chinese individuals aged ≥ 40 years.

Applications

The presented research demonstrated that Chinese people who have *H. pylori*-related IM do have a high risk of colorectal adenomas. Given a high prevalence of colorectal adenocarcinoma in China, it is necessary for patients with *H. pylori* infection and IM to undergo colonoscopy screening and surveillance.

Peer-review

In this manuscript, the authors aimed to explore the association between *H. pylori* infection status, IM, and colorectal adenoma, and concluded that *H. pylori*-related IM was associated with a high risk of colorectal adenomas in Chinese individuals. The study was well designed and the results were very interesting. Therefore, the reviewer considers that it can be accepted after some English corrections.

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ORIGINAL ARTICLE

Clinical Trials Study

Clinicopathological and prognostic significance of aberrant Arpin expression in gastric cancer

Tao Li, Hong-Mei Zheng, Nai-Mei Deng, Ying-Jian Jiang, Jiang Wang, Dian-Liang Zhang

Tao Li, Hong-Mei Zheng, Ying-Jian Jiang, Jiang Wang, Dian-Liang Zhang, Center of Colon and Rectum, Qingdao Municipal Hospital, Qingdao University, Qingdao 266011, Shandong Province, China

Nai-Mei Deng, Infection Department, Qingdao Municipal Hospital, Qingdao 266011, Shandong Province, China

Correspondence to: Dian-Liang Zhang, MD, PhD, Center of Colon and Rectum, Qingdao Municipal Hospital, Qingdao University, No. 1 Jiaozhou Road, Qingdao 266011, Shangdong Province, China. phdzdl@yahoo.com
Telephone: +86-532-82852057
Fax: +86-532-82852058

Abstract

AIM

To detect the expression of Arpin, and determine its correlation with clinicopathological characteristics and the prognosis of gastric cancer (GC) patients.

METHODS

A total of 176 GC patients were enrolled as study subjects and classified into groups according to different clinicopathological variables. GC mucosal tissues were obtained *via* surgery. Another 43 paraffin-embedded tissue blocks of normal gastric epithelium (> 5 cm away from the edge of the tumor) were included in the control group. Immunohistochemistry (IHC) for the Arpin and Arp3 proteins was performed on the formalin-fixed, paraffin-embedded GC tissues. Additionally, expression of the Arpin protein in 43 normal gastric tissues was also determined using IHC.

RESULTS

Expression of the Arpin protein in GC was lower than that in normal gastric mucosa (30.68% *vs* 60.47%, $P < 0.001$). A χ^2 test of the 176 GC samples used for IHC showed that decreased Arpin expression was associated with advanced TNM stage ($P < 0.01$) and the presence or absence of lymph node metastasis (80.92% *vs* 35.56%, $P < 0.001$). Additionally, a significant correlation was observed between the expression of Arpin and the presence of the Arp2/3 complex in GC tissues ($\chi^2 = 30.535$, $P < 0.001$). Moreover, a multivariate Cox

regression analysis revealed that Arpin expression [hazard ratio (HR) = 0.551, *P* = 0.029] and TNM stage (HR = 5.344, *P* = 0.001) were independent prognostic markers for overall survival of GC patients. Regarding the 3-year disease-free survival (DFS), the recurrence rate of GC patients with low Arpin expression levels (median DFS 19 mo) was higher than that in the high-Arpin-expression group (median DFS 34 mo, *P* = 0.022).

CONCLUSION
Low Arpin levels are associated with clinicopathological variables and a poor prognosis in GC patients. Arpin may be regarded as a potential prognostic indicator in GC.

Key words: Clinicopathological characteristics; Gastric cancer; Arpin; Arp2/3 complex; Prognosis

Core tip: Arpin, a newly found Arp2/3 complex inhibitor reported in Nature, in 2013, was shown to restrict the rate of actin polymerization and control cell migration. However, little is known about whether the expression of Arpin is altered in gastric cancer (GC) tissues, and the detailed mechanisms for invasion and metastasis of GC remain unknown. Our research shows that expression level of Arpin is low in GC, and decreased Arpin expression is associated with the characteristics of clinical pathology and poor prognosis of GC patients. It may be regarded as a potential prognosis indicator for clinical outcomes in GC.

INTRODUCTION

Gastric cancer (GC) remains the fourth most common malignancy and the third leading cause of cancer-related death worldwide, despite its steadily decreasing incidence and mortality since 1930s[1,2]. Although the early detection rate of GC has increased, many patients still suffer from distant metastasis, resulting in a median survival of only 3-5 mo[3-6]. At present, understanding of the multidisciplinary treatment of cancer is a concern of doctors and researchers. However, surgery is still the treatment of choice for most early solid tumors and even some advanced malignant tumors. With the progress made in molecular biology research, accumulating evidence has shown that the carcinogenesis of the gastric mucosa is

a multi-factor, multi-step, and multi-stage development process that involves a variety of related genes. Moreover, stages of GC development are affected by different genes. Therefore, it is necessary to identify novel markers that can accurately reflect the biological characteristics of GC tumors, provide new therapeutic strategies, and predict clinical outcomes.

Invasion and metastasis are the two main characteristics of malignant tumors. In general, invasion and metastasis accompany the movement of cancer cells from the primary focus in cancer tissues to other normal tissues or organs, and actin polymerization is necessary for this movement. Actin, a structural protein composed of actin filaments, exists in two forms, monomers and polymers. The actin polymerization process can be divided into two distinct steps: actin monomer polymerization in the nucleus, followed by the addition of actin monomers to the formed nuclei or fibers. Cell movement results from the mutual cooperation of polymerization and depolymerization of the cytoskeleton itself and adhesion and desorption between different parts of the cell and extracellular matrix. Moreover, the formation of lamellipodia is closely associated with actin polymerization. Lamellipodia play an important role in the process of exploration of the external environment by cancer cells and the formation of new adhesion contacts with the extracellular matrix, which allow motility and spreading. The process of lamellipodiaformation is often driven by spatially and temporally regulated actin polymerization at the leading edge[7]. The molecular mechanisms of cancer cell motility and migration are more complicated than that expected, the movement and migration of cancer cells is a result of multi-step process initiated by the formation of membrane protrusions in response to migratory and chemotactic stimuli. It is generally believed that lamellipodia in driving cancer cell migration plays a main role, and it is caused by adhesion force to pull the cell body forward in the basement membrane. However, many cytokines can form new actin fibers, and each factor can form a specific network. The actin-related protein 2/3 (Arp2/3) complex, the most investigated molecule by far, is the sole machine that generates branched actin networks, and is also considered to be a key regulator of cell motility[8]. It has been reported to be involved in the development and migration of some cancers, such as pancreatic, gastric, colorectal, and breast cancer[9-12]. By binding actin filaments to the side of an existing filament and initiating branch formation, the Arp2/3 complex accomplishes its role of nucleating actin filaments[13]. Therefore, the Arp2/3 complex is thought to be involved in cancer cell invasion and metastasis and is controlled by the tumor-stromal interaction, but the specific guiding mechanism of cell invasion in tumors has not been extensively explored. Relevant research results show that the Arp2/3 complex and its activators, such as the WAVE complex, are deregulated

in diverse cancers. The Arp2/3 complex plays an important role in the invasion and metastasis of tumor cells. However, it is worth noting that the Arp2/3 complex is also vital for the maintenance of normal cell function.

In 2013, Dang *et al*[14] reported a new protein, Arpin, which contains 220 amino acid residues that are localized to the cell membrane. It is encoded by the *C15ORF38* gene, which is present in multicellular animals and unicellular amoebas. Interestingly, this protein contains a carboxyl structure region but no C-terminal end, helix, or single actin binding domain. However, it does inhibit the Arp2/3 complex, resulting in the inhibition of actin polymerization. Arpin contains the putative binding site of the Arp2/3 complex[14], which has been reported to be closely related to the development and migration of cancer cells due to its key role in filopodia initiation[15,16]. Arpin can guide the direction of cell movement, and it is also known as "the steering factor". However, this process is not dependent on the formation of lamellipodia and is not based on actin dynamics. Arpin acts in a dose-dependent manner and induces the cell to move. Arpin and its acidic motif can compete with nucleation promoting factors for Arp2/3 binding, thereby inhibiting the activation of the Arp2/3 complex[14,17], resulting in the inhibition of actin filament polymerization. Related research has shown the significant relationship between decreased Arpin, the clinical features of breast cancer, and the poor prognosis of breast cancer patients with low Arpin expression relative to the 5-year replase-free survival[18]. However, little is known about the expression of Arpin in GC tissue and its relationship with the clinicopathological characteristics and prognosis of GC patients. In this study, we investigated the issues mentioned above, preliminarily determined the correlation between Arpin and GC as well as between Arpin and the Arp2/3 complex, and provided a theoretical and experimental basis for further research of the specific signaling transduction mechanism of Arpin and the Arp2/3 complex in advanced gastric carcinoma.

MATERIALS AND METHODS

Patients and specimens
The study cohort was composed of samples from 176 patients with gastric adenocarcinoma, including 110 men and 66 women (mean age 56.3 years) who had undergone a gastrectomy at Qingdao Municipal Hospital and the Affiliated Hospital of Qingdao University between March 2013 and August 2013. Following surgery, routine chemotherapy was administered to patients with advanced disease, but no radiation treatment was administered to any of the patients included in the present study. The eligibility criteria for this study included the following: (1) histologically proven adenocarcinoma; (2) no history of gastrectomy

or other malignancy; (3) no other gastric tumors such as gastric stromal tumors; (4) availability of complete clinicopathological and survival data; (5) patients had not received neoadjuvant chemotherapy; and (6) no distant metastasis. This study was approved by the Human Subjects Institutional Committee of the Affiliated Hospital of Qingdao University. All study participants or their legal guardian provided informed written consent prior to study enrollment.

Clinicopathological data
The clinicopathological data were recorded prospectively for the retrospective analysis. The clinicopathological data of the 176 GC patients included age, gender, size of the primary tumor, depth of invasion, lymph node metastasis, TNM stage, degree of differentiation, and location of the primary tumor. The TNM stages of the specimens subjected to immunohistochemistry (IHC) assays were as follows: 70 (39.78%) in TNM stage Ⅰ, 50 (28.41%) in stage Ⅱ, and 56 (31.81%) in stage Ⅲ. Forty-five patients did not have lymph node metastasis, while 131 patients exhibited metastasis. Another 43 paraffin-embedded tissue blocks of normal gastric epithelium (> 5 cm away from the edge of the tumor) were included in the control group. The clinicopathological factors of GC patients are shown in Table 1.

IHC
IHC staining for Arpin was performed in the obtained specimens, which consisted of serial 4-μm-thick sections of 10% formalin-fixed, paraffin-embedded tissue. After blocking endogenous peroxidase activity to reduce nonspecific binding by boiling in a pressure cooker for 3 min, the samples were placed in 3% hydrogen peroxide (H_2O_2) for 10 min. Then the samples washed with buffer and incubated with a goat polyclonal antibody specific to Arpin (sc-242049; Santa Cruz Biotechnology Inc., Bergheimer, Heidelberg, Germany, final dilution 1:25) at 4 ℃ for 24 h, followed by three washes in buffer. The secondary antibody consisted of biotinylated anti-goat immunoglobulin. Then, the slides were incubated with avidin-biotin-conjugated peroxidase. After washing and staining with 3,3-diaminobenzidine tetrahydrochloride and H_2O_2, a brown pigment was obtained after counterstaining with hematoxylin.

Because Arp2 exhibits the same distribution as Arp3 in tumorous tissue, we concluded that identical expression of Arp2 and Arp3 indicates the formation of Arp2/3 complex. Therefore, Arp3 IHC staining (Figure 1) represented the distribution of the Arp2/3 complex[11]. The sections were incubated with an anti-Arp3 polyclonal antibody (diluted to 1:5000). Sections of GC tissue, in which Arp3 was confirmed to be expressed by immunoblot analysis, were used as the positive control for Arp3 immunostaining. For the negative control, normal rabbit serum was substituted

Table 1 Association analysis of immunohistochemistry staining for Arpin *vs* clinicopathological factors of gastric cancer, *n* (%)

Clinicopathological features	Number of patients (*n* = 176)	Arpin expression		χ^2	*P* value
		High (*n* = 54)	Low (*n* = 122)		
Age (yr)					
> 50	97 (55.11)	29 (29.90)	68 (70.10)	0.063	0.802
≤ 50	79 (44.89)	25 (31.65)	54 (68.35)		
Gender					
Male	110 (62.50)	31 (28.18)	79 (71.82)	0.865	0.353
Female	66 (37.50)	23 (34.85)	43 (65.15)		
Tumor size (cm)					
> 5	120 (68.18)	37 (30.83)	83 (69.17)	0.004	0.949
≤ 5	56 (31.82)	17 (30.36)	39 (69.64)		
Depth of invasion					
T1 + T2	44 (25.00)	24 (54.55)	20 (45.45)	15.79	0.000
T3 + T4	132 (75.00)	30 (22.73)	102 (77.27)		
Lymph node metastasis					
Yes	131 (74.43)	25 (19.08)	106 (80.92)	32.404	0.000
No	45 (25.57)	29 (64.44)	16 (35.56)		
TNM stage					
I	70 (39.77)	30 (42.86)	40 (57.14)	9.379	0.009
II	50 (28.41)	14 (28.00)	36 (72.00)		
III	56 (31.82)	10 (17.86)	16 (82.14)		
Histology					
Well + moderate	80 (45.45)	21 (26.25)	59 (73.75)	1.354	0.245
Poor	96 (54.55)	33 (34.38)	63 (65.62)		
Tumor site					
Upper	66 (37.50)	24 (36.36)	42 (63.64)	1.648	0.439
Middle	16 (9.09)	4 (25.00)	12 (75.00)		
Low	94 (53.41)	26 (27.66)	68 (72.34)		

Figure 1 Expression of Arp3 by tumor cells. Arp3 is expressed in the invasive cancer cells. Immunohistochemistry of Arp3; original magnification × 400.

for the primary antibody. Smooth muscle cells of small blood vessels were used as endogenous-negative controls in each section. The secondary antibody consisted of biotinylated anti-goat immunoglobulin.

Immunohistochemical analysis and evaluation

Tumor cells in which the cytoplasm was stained dark brown under light microscopy were considered positive for Arpin IHC staining. Both the staining intensity and the percentage of stained cells were evaluated for the quantification of Arpin expression. Cells with no staining were scored as 0 points, 1 point represented weak staining intensity, 2 points represented moderate staining intensity, and 3 points represented strong staining intensity. Additionally, we assessed the percentage of stained tumor cells: 0% corresponded to 0 points, less than 25% corresponded to 1 point, 25%-50% corresponded to 2 points, and more than 50% corresponded to 3 points. The final score for Arpin expression was equal to the sum of the two types of scores. A staining score ranging from 0 to 3 points represented low expression, and a score more than 3 points was considered a high expression level[19].

If more than 10% of the tumor cells expressed Arp3, expression by the tumor cells was considered positive. Otherwise, Arp3 expression was considered negative.

Statistical analysis

SPSS 16.0 software (SPSS, Chicago Statistical IL, United States) was used for the statistical analysis. The differences in clinicopathological variables were analyzed by the χ^2 test. The McNemar test was applied to determine the correlation between expression of Arpin and the Arp2/3 complex. Survival curves for 3-year disease-free survival (DFS) were constructed using the Kaplan-Meier method and compared by a log-rank test. The significance of survival variables was evaluated using a multivariate Cox proportional hazard regression analysis, which further showed the independent effect of Arpin expression on DFS. A value of *P* < 0.05 represented a significant difference.

Figure 2 Representative photomicrographs of Arpin immunohistochemical staining. A: Indicates the high expression in normal gastric tissue; B: Indicates low expression in gastric carcinoma. Original magnification × 200.

Table 2 Immunohistochemistry staining for Arpin in gastric cancer and normal gastric tissues, n (%)

Group	No. of patients (n = 219)	Arpin expression		χ^2	P value
		High (n = 80)	Low (n = 139)		
Normal	43	26 (60.47)	17 (39.53)	13.221	< 0.001
Cancer	176	54 (30.68)	122 (69.32)		

Table 3 Association analysis of expression of Arpin protein vs TNM stage of gastric cancer, n (%)

TNM stage	No. of patients	Arpin expression		P value
		High (n = 54)	Low (n = 122)	
I	70 (39.77)	30 (42.86)	40 (57.14)	0.096[1]
II	50 (28.41)	14 (28.00)	36 (72.00)	0.213[2]
III	56 (31.82)	10 (17.86)	46 (82.14)	0.003[3]

[1]Stage I vs II; [2]Stage II vs III; [3]Stage I vs III.

Table 4 Correlation of Arpin and Arp2/3 complex expressions in gastric cancer patients

Arp2/3 complex	Arpin		χ^2	P value
	High (n = 54)	Low (n = 122)		
Positive (n = 114)	27	87	30.535	0.000
Negative (n = 62)	27	35		

McNemar χ^2 = 30.535, P = 0.000.

RESULTS

Expression of Arpin protein is decreased in GC tissues

The expression of Arpin in GC tissues was lower than that in normal gastric mucosa (30.68% vs 60.47%, P < 0.001, Figure 2 and Table 2).

Relationship between Arpin expression and clinicopathological parameters

Correlations between Arpin expression and clinicopathological characteristics are shown in Table 1. Analysis of the IHC results from 176 tumor samples showed that Arpin protein expression was lower in stage III (82.14%) than in stage I (57.14%) or II (72.00%). Low Arpin protein expression was significantly associated with advanced TNM stage (stage III vs I, P < 0.01, Table 3). The protein expression level of Arpin in tumor tissues was significantly correlated with the presence or absence of lymph node metastasis (80.92% vs 35.56%, P < 0.001). In addition, we found no significant correlation between the expression of Arpin protein and other clinical parameters, such as tumor location and size, patient age, gender, and histological type.

Correlation between the expression of Arpin and the Arp2/3 complex in GC tissues

The expression levels of Arpin and the Arp2/3 complex in gastric carcinoma were significantly correlated (McNemar χ^2 = 30.535, P < 0.001, Table 4).

Low Arpin expression is associated with a poor prognosis in GC patients

The effects of Arpin expression and clinicopathological characteristics on DFS were evaluated by Kaplan-Meier analysis and log-rank tests. The results showed that GC patients in the low Arpin expression group had a higher recurrence rate (median DFS 19 mo) than those in the high Arpin expression group (median DFS 34 mo, P = 0.022, Figure 3). We found that the 3-year DFS was 31.00% in the low Arpin expression group and 46.00% in the high Arpin expression group. Univariate analyses of clinical variables considered as potential predictors of survival are shown in Table 5. Further analysis using a multivariate Cox proportional hazards model showed that Arpin expression, together with TNM stage, was strongly associated with DFS. Arpin expression [hazard ratio (HR) = 0.551, P = 0.029] and TNM stage (HR = 5.344, P = 0.001) were independent prognostic indicators of DFS in GC

Figure 3 Kaplan-Meier analysis of disease-free survival based on Arpin expression in all 176 patients.

Table 5 Univariate and multivariate survival analysis of 3-year DFS in 176 patients with gastric cancer

Variable	Univariate analysis		Multivariate analysis	
	HR	*P* value	HR	*P* value
Age	1.14	0.488		
Gender	1.373	0.098		
tumor size	1.411	0.08		
Depth of invasion	1.818	0.017	1.117	0.732
Lymph node metastasis	2.498	0.035	1.413	0.225
tumor site	1.336	0.612		
TNM stage	4.985	< 0.001	5.344	0.001
Arpin expression	0.494	0.005	0.551	0.029

patients (Table 5).

DISCUSSION

To our knowledge, the present study is the first to report the clinical significance of Arpin expression in GC patients, although reduced expression of Arpin and its influence on poor prognosis need to be confirmed in further follow-up studies with larger samples.

In this study, all participants with GC were treated surgically. The sections were obtained from postoperative gastric specimens. In addition, the Arpin and Arp3 IHC analysis was conducted using postoperative gastric slides. The results showed that the Arpin protein was significantly decreased in tumor samples relative to the normal tissues in the control group. Therefore, we speculate that due to the decreased expression of Arpin, tumor cells acquire the ability to invade and metastasize in GC, and the degree of malignancy of tumors is closely related to the clinical stage. We then assessed the correlation between Arpin expression and tumor staging. We found that low Arpin expression was significantly associated with advanced TNM stage, and we concluded that Arpin could play a significant role in tumor biology. In the later stage, lower Arpin expression levels indicate greater malignancy. Whether patients in TNM stage IV, with liver metastasis and peritoneal metastasis are correlated with the expression of Arpin deserves intensive study. Further research may accompany with the expression of Arpin with sensitivity of anti-cancer drug and the correlation of the expression of Arpin with cadherin. Because tumor invasion depth, lymph node metastasis, and distant metastasis are the main pathological basis of malignant tumor staging, we further analyzed the correlation between Arpin expression and local invasion of regional lymph nodes. The IHC results showed that low Arpin expression was significantly associated with the depth of invasion and local lymph node metastases. The results of this research may provide new approaches for determining

whether loss of Arpin function is associated with increased cell motility in the epithelial-mesenchymal transition during the progression of cancer. Moreover, the inhibitory effect of Arpin on cell migration could potentially be used to control metastasis. However, the tumor size had no significant effect on the test results. Due to the characteristics of the samples and other factors, our results may be different from previous experimental results.

The expression of the Arp2/3 complex was detected in gastric carcinoma tissue in our experiments. Some observations suggest that stromal cells that express Arp2/3 move and grow in advance of the cancer cells to prepare an environment that facilitates cancer cell invasion. Myofibroblasts, which are considered cancer-induced stromal cells, have been shown to affect the adhesion and movement of cancer cells[19]. Additionally, Arpin inhibits the Arp2/3 complex at the lamellipodium tip. Our research results showed that the expression of Arpin and Arp2/3 complex expression in gastric carcinoma were significantly correlated. Determination of whether a negative correlation exists between these two proteins and the specific mechanism concerning how these proteins affect cell migration will require further study with larger samples. Our experiments may have also indirectly shown a decrease in Arpin expression in GC tissues.

More significantly, in order to investigate the effect of Arpin on the prognosis of GC patients, we compared the 3-year DFS in the Arpin low expression group and the high expression group. Consistently, our study demonstrated that Arpin expression and the TNM stage were independent prognostic indicators of DFS. We propose that Arpin likely plays a role in GC metastasis and the prognosis. However, the exact molecular events leading to cancer metastasis and a poor prognosis have not yet been well elucidated, and further research is required.

The present study had some limitations. We could not visualize the specific signal transduction and regulation mechanism of Arpin and the Arp2/3 complex in GC tissue due to technical difficulties. Arpin is a newly discovered protein, its impact on GC patients and the specific mechanism that it guides cell migration may

help us develop new therapeutic target. The study of mechanism about signal path is the direction of our future research. Thus, more work is required in the future.

In summary, the present study showed that Arpin was decreased in GC tissues. Additionally, this low expression pattern was found to be significantly correlated with aggressive clinicopathological features. In addition, the specific regulatory mechanisms of Rac, WAVE, Arpin and the Arp2/3 complex in the development of GC are still unclear. In recent years, targeted therapeutics for key molecular drivers of cancer progression have been developed[20]. Arpin may be used as a potential biomarker that could provide important information about tumor progress and could even be a possible target for GC therapy.

COMMENTS

Background
Invasion and metastasis are the two main characteristics of malignant tumors. Gastric cancer (GC), the fourth most common malignancy, and is the third leading cause of cancer-related death worldwide. Arpin, a newly found Arp2/3 complex inhibitor reported in Nature, 2013, which was involved in the development and migration of cancer cells for its key role in the filopodia initiation. Thus, further investigations have been carried out about the role of the Arpin protein in GC.

Research frontiers
Arpin, a newly found Arp2/3 complex inhibitor, simultaneously acts on cell speed and directional persistence, which are strongly coupled parameters. Loss of the Arp2/3 inhibitory protein Arpin produces a similar poor outcome in breast cancer as high expression of the NCKAP1 subunit of the Arp2/3 activatory WAVE complex. Moreover, Arpin downregulation may contribute to the initiation and development of breast cancer metastasis. Therefore, as a potential predictive marker, Arpin deserves future studies.

Innovations and breakthroughs
In this study, the authors for the first time investigated the expression of Arpin in GC tissue and its relationship with clinical pathological characteristics and prognosis of GC patients, preliminarily determined the correlations between Arpin and GC, Arpin and Arp2/3 complex, and provided a theoretical and experimental basis for further research of the specific signal transduction mechanism of Arpin and Arp2/3 complex in advanced gastric carcinoma. The authors could not visualize the specific signal transduction and regulation mechanism of Arpin and Arp2/3 complex in GC due to a lack of technical assistance.

Applications
Arpin protein expression was significantly decreased in tumor samples. Low Arpin associates with clinicopathological variables and poor prognosis in GC patients. Arpin may be regarded as potential prognosis indicator of GC. This provides us a more powerful tool and technical guidance to evaluate the clinicopathologic features and prognosis of patients with GC. Further studies are needed to clarify the detailed mechanisms involved.

Peer-review
The authors detected the expression of Arpin, and evaluated its correlation with clinicopathologic characteristics and prognosis of GC patients. They concluded that low Arpin occurs and associates with clinicopathological characteristics and poor prognosis in GC patients. This is a well written interesting study, which is the first report conducted to determine the value of Arpin in patients with GC.

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ORIGINAL ARTICLE

Observational Study

Clinical features and natural history of cryptogenic cirrhosis compared to hepatitis C virus-related cirrhosis

Luca Rinaldi, Fabio Nascimbeni, Mauro Giordano, Chiara Masetti, Barbara Guerrera, Annalisa Amelia, Maria Chiara Fascione, Stefano Ballestri, Dante Romagnoli, Rosa Zampino, Riccardo Nevola, Enrica Baldelli, Natalina Iuliano, Valerio Rosato, Amedeo Lonardo, Luigi Elio Adinolfi

Luca Rinaldi, Mauro Giordano, Barbara Guerrera, Annalisa Amelia, Maria Chiara Fascione, Rosa Zampino, Riccardo Nevola, Natalina Iuliano, Valerio Rosato, Luigi Elio Adinolfi, Internal Medicine, Clinical Hospital of Marcianise, Second University of Naples, 80100 Naples, Italy

Luca Rinaldi, Mauro Giordano, Barbara Guerrera, Annalisa Amelia, Maria Chiara Fascione, Rosa Zampino, Riccardo Nevola, Natalina Iuliano, Valerio Rosato, Luigi Elio Adinolfi, Department of Medical, Surgical, Neurological, Metabolic, and Geriatric Sciences, Second University of Naples, 80100 Naples, Italy

Fabio Nascimbeni, Chiara Masetti, Dante Romagnoli, Enrica Baldelli, Amedeo Lonardo, Outpatient Liver Clinic and Internal Medicine, NOCSAE, Baggiovara, Azienda USL, Modena; University of Modena and Reggio Emilia, 41121 Modena, Italy

Stefano Ballestri, Internal Medicine, Pavullo Hospital, Azienda Sanitaria Locale, Pavullo, 41121 Modena, Italy

Author contributions: Rinaldi L, Nascimbeni F, Lonardo A and Adinolfi LE contributed to conception and designed the study, analysis and interpretation of data, and wrote the manuscript; Giordano M, Masetti C, Guerrera B, Amelia A, Fascione MC, Ballestri S, Romagnoli D, Zampino R, Nevola R, Baldelli E, Iuliano N and Rosato V maintained the database, followed the patients and reviewed the literature; all the authors made critical revision and approved the final version of the manuscript.

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Abstract

AIM
To characterize natural history of cryptogenic cirrhosis (CC) and compare its clinical features and outcomes to those of hepatitis C virus (HCV)-related cirrhosis.

METHODS
A prospective cohort of 102 consecutive patients at their first diagnosis of CC were enrolled in this study. The clinical data and outcomes were compared to an age-

and Child-Pugh class-matched cohort of 110 patients with HCV-related cirrhosis. Diagnosis of cirrhosis was based on compatible clinical and laboratory parameters, ultrasound/endoscopic parameters and, whenever possible, on histological grounds and transient elastography. All cases of cirrhosis without a definite etiology were enrolled in the CC group. The parameters assessed were: (1) severity of liver disease at the time of first diagnosis; (2) liver decompensation during follow-up; (3) hepatocellular carcinoma (HCC); (4) orthotopic liver transplantation; and (5) death. The independent associated factors were evaluated by multiple logistic regression analysis, and survival and its determinants by the Kaplan-Meier model, log-rank test and Cox regression.

RESULTS
At the first observation, median age was 66 and 65 years and male gender was 36% and 58% for CC and HCV cirrhosis, respectively. CC showed Child-Pugh class A/B/C of 47%/31%/22%, respectively. Compared to HCV cirrhosis, CC exhibited a significantly higher prevalence of metabolic syndrome (12% *vs* 54%, respectively), overweight/obesity, high BMI, impaired glucose tolerance, high blood pressure, dyslipidemia, hyperuricemia, cardiovascular diseases, extrahepatic cancer, and gallstones. Over a median period of 42 mo of follow-up, liver decompensation, HCC development and death for CC and HCV-related cirrhosis were 60.8%, and 54.4%, 16.7% and 17.2%, 39.2% and 30%, respectively. The median survival was 60 mo for CC. Independent predictors of death were age and Child-Pugh class at diagnosis. CC showed an approximately twofold higher incidence of HCC in Child-Pugh class A.

CONCLUSION
Undiagnosed nonalcoholic fatty liver disease has an etiologic role in CC that is associated with a poor prognosis, early HCC development, high risk of cardiovascular disease and extrahepatic cancer.

Key words: Liver cirrhosis; Hepatocellular carcinoma; Metabolic syndrome; Nonalcoholic fatty liver disease; Cardiovascular diseases

Core tip: We evaluated the features and outcomes of cryptogenic cirrhosis (CC) compared to age- and Child-Pugh class-matched hepatitis C virus-related cirrhosis at baseline and over a 42-mo follow-up. At diagnosis, the median age of CC was 66 years and Child-Pugh classes A/B/C were 47%/31%/22%, respectively. Among CC cases higher prevalences of metabolic syndrome, overweight/obesity, high BMI, impaired glucose metabolism, high blood pressure, dyslipidemia, hyperuricemia, cardiovascular diseases, extrahepatic cancer, and gallstones were observed. Although in the two groups we detected a similar incidence of liver decompensation, hepatocellular carcinoma (HCC) and

death, an earlier development of HCC was observed in CC. Age and Child-Pugh were predictors of death. Most CC cases are the consequence of undiagnosed nonalcoholic fatty liver disease.

INTRODUCTION
Liver cirrhosis is a major health burden worldwide ranking among the top ten causes of years of life lost in high-income countries[1]. More than 1000000 of deaths worldwide were reported due to cirrhosis in 2010[2]. The number of cirrhotic patients and the related complications and mortality rates are rising[3]. Cirrhosis is the shared, end-stage result of etiologically diverse chronic liver diseases, with a different geographic distribution, which can follow an indolent course and remains asymptomatic until complications or be discovered incidentally at necropsy[4]. In other cases, asymptomatic cirrhosis is usually detected incidentally with laboratory tests or imaging studies performed for unrelated reasons[4]. Although the clinical manifestations of cirrhosis are stereotypic irrespective of its etiology, the identification of the causes is important to define specific therapeutic and surveillance strategies. [3]A substantial number of cases of cirrhosis remain of unknown origin and are therefore designated as "cryptogenic cirrhosis" (CC), which accounts for up to 30% of cases of cirrhosis and about 10% of liver transplants[5,6].

There are no standardized diagnostic criteria for CC and it is best defined by exclusion. Nevertheless, a proportion of CC cases are deemed to result from the progression of previously unrecognized nonalcoholic steatohepatitis (NASH)[7-9]. Models of the natural history of cirrhosis are based on findings in individuals with viral or alcoholic cirrhosis[10,11] and the outcome of CC is far less characterized than cirrhosis with a definite etiology. This is of importance given that the natural history of NASH-cirrhosis offers clues to preventing cardiometabolic risk and hepatocellular carcinoma (HCC)[12,13].

On these grounds and given the paucity of published data, we aimed at characterizing the clinical features and long-term course of CC in a case series of Italian patients consecutively observed at two Italian tertiary liver units. To do so, the features and outcome of CC were compared with those of matched hepatitis C virus (HCV)-related cirrhosis cases.

MATERIALS AND METHODS

In this prospective cohort study we enrolled consecutive patients receiving for the first time a diagnosis of cirrhosis of unknown origin, defined as CC, at two Italian tertiary hepatology centers, the University of Naples and the University of Modena and Reggio Emilia, from January 2008 to June 2015. During the same period, we also enrolled, as control group, an age- and child-Pugh class matched cohort of patients with a diagnosis of HCV-related cirrhosis.

The diagnosis of cirrhosis was based on compatible clinic and laboratory parameters (hepatic encephalopathy, jaundice, ascites, platelet < 90000 mm^3, albumin < 2.5 g/dL), ultrasound/endoscopic parameters (coarse-pattern, irregular liver surface, clear-cut evidence of portal hypertension such as splenomegaly and esophago-gastric varices)[5] and, whenever possible, on histological ground. In addition, in a subset of patients we performed transient elastography by Fibroscan® as a further diagnostic support, and cut-off values of liver stiffness > 12.5 kPa were considered consistent with liver cirrhosis[14].

The criteria for the exclusion of diagnosis of CC were: cases with histological clear-cut features of fatty changes and cirrhosis with definite etiology such as, excessive alcohol consumption (defined as ≥ 30 g of alcohol per day for men and 20 g per day for women); chronic use of hepatotoxic drugs (based on medical history); HCV antibody positivity; hepatitis B surface antigen positivity; autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis or genetic liver diseases such as hemochromatosis, alpha1-antitrypsin deficiency or Wilson's disease (based on clinical grounds, appropriate serum bio-markers or imaging findings)[15].

For HCV-related cirrhosis the diagnosis was based on the presence of serum HCV-Ab and HCV RNA.

The following outcomes were assessed: (1) severity of liver disease at the time of first diagnosis; (2) liver decompensation (*i.e.*, at least 1 episode of ascites, encephalopathy, or variceal bleeding either at presentation or during follow-up; (3) HCC, diagnosed according to current guidelines[16]; (4) orthotopic liver transplantation (OLT); and (5) death. At time of first diagnosis, the cirrhosis stage was assessed by Child-Pugh score. Death was considered to be liver-related if resulting from liver decompensation/hepatorenal syndrome and/or spontaneous bacterial peritonitis and/or variceal bleeding and/or HCC and/or OLT complications. Survival was calculated starting from the first diagnosis of cirrhosis. Extra-hepatic malignancies and cardiovascular events, particularly the presence of coronary artery disease, were recorded. In order to address a potential source of bias, we compared the prevalence of coronary artery disease in the CC cohorts with the HCV-related cirrhosis cohort and an age-matched (1:3) pathological control [652 patients hospitalized in the same period for non-liver conditions such as chronic obstructive pulmonary disease, acute pneumonia, type 2 diabetes (T2D), renal diseases]. Past coronary artery disease was diagnosed based on clinical charts (laboratory analysis, ECG, cardiac ultrasonography, coronary angiography).

All data were collected on the basis of "*a priori*" codification of parameters on a computerized database which was regularly updated during follow-up. The Ethics Boards of the two participating hospitals approved the study protocol.

The main metabolic features were defined as follows: T2D from a previous diagnosis, use of anti-diabetic medications, fasting glucose ≥ 126 mg/dL or HbA1c ≥ 6.5%, impaired fasting glucose (IFG) with blood glucose ≥ 100 mg/dL, central obesity with BMI ≥ 30 kg/m^2 and/or waist circumference ≥ 94 cm in men and ≥ 80 cm in women; overweight with BMI ≥ 25 kg/m^2 and < 30 kg/m^2, high blood pressure by a previous diagnosis or blood pressure ≥ 130/85 mmHg or anti-hypertensive medications. Dyslipidemia was based on a previous diagnosis of dyslipidemia, use of lipid-lowering drugs, triglycerides ≥ 150 mg/dL, and/or serum total cholesterol ≥ 200 mg/dL, and/or HDL < 50 mg/dL in women and < 40 mg/dL in men. According to modified IDF criteria of the AHA/NHBLI[17], metabolic syndrome was diagnosed in the presence of at least 3 of these metabolic risk factors: T2D/IFG, central obesity/overweight, hypertension and dyslipidemia. Hyperuricemia was defined by serum uric acid ≥ 6 mg/dL in women and ≥ 7 mg/dL in men.

Statistical analysis

Quantitative variables were expressed as mean ± SD or median (range), as appropriate, and categorical variables as percentage. Numerical variables were compared using the Mann-Whitney U test. χ^2 and Fisher's exact tests were used for qualitative data when appropriate. Two-sided P values of less than 0.05 were considered statistically significant.

Multiple logistic regression analysis was performed to determine the independent determinants of outcomes variables (liver decompensation and HCC).

Overall survival was evaluated by Kaplan-Meier model. The log-rank test was used to compare overall survival and the Cox regression to identify factors associated with overall survival. Statistical tests were performed using SPSS 17.0 software (SPSS, Inc, Chicago, IL, United States).

RESULTS

Demographic, laboratory and clinical features of cirrhotic patients at time of diagnosis

Based on inclusion and exclusion criteria, 102 patients with diagnosis of CC were enrolled in this study: 70 from Naples and 32 from Modena. They account for approximately 4%-5% of all cases of cirrhosis observed in the same period in our Units (Figure 1).

Figure 1 Selection and enrollment of cirrhotic patients to the study. HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease.

Figure 2 Prevalence of metabolic factors and of cardiovascular complications in cryptogenic cirrhosis and hepatitis C virus-related cirrhosis. All factors are significantly higher in cryptogenic cirrhosis (*P* < 0.01 *vs* HCV-related cirrhosis). HCV: Hepatitis C virus; IFG: Impaired fasting glucose.

Table 1 Demographic, clinical, and laboratory features of patients included in the study with cryptogenic cirrhosis and hepatitis C virus-related cirrhosis

	Cryptogenic cirrhosis	Hepatitis C virus cirrhosis	*P* value
No. of patients	102	110	
Age at diagnosis (yr), median	66 (38-84)	65 (48-86)	NS
Male sex (M:F ratio)	36 (1:1.8)	58 (3:1)	< 0.01
BMI (mean ± SD)	30 ± 5.7	27.1 ± 3.9	< 0.05
Low alcohol intake	11.8%	12.8%	NS
Past and current smokers	29.4%	27.2%	NS
AST (U/L), mean ± SD	47 ± 43	122 ± 88	< 0.01
ALT (U/L), mean ± SD	35 ± 25	102 ± 63	< 0.01
Y-GT (U/L), mean ± SD	126 ± 124	82 ± 61	< 0.05
Alph (U/L), mean ± SD	172 ± 181	198 ± 180	NS
Total bilirubin (mg/dL), mean ± SD	2.8 ± 5.2	2.6 ± 4.2	NS
Albumin (g/dL), mean ± SD	3.4 ± 0.8	3.4 ± 0.9	NS
INR, mean ± SD	1.3 ± 0.6	1.4 ± 0.8	NS
Platelets (10^3/mm^3), mean ± SD	120 ± 80	115 ± 60	NS
Creatininemia (mg/dL), mean ± SD	1.1 ± 0.7	1.1 ± 0.6	NS
Ferritin (ng/mL), mean ± SD	163 ± 153	186 ± 162	NS
α-fetoprotein (ng/mL), median	3.4 (0.6-3300)	36 (2-600)	NS
Child-Pugh class A/B/C (%)	47/31/22	48/29/23	NS
HBsAb/HBcAb positive	27.5%	42.7%	< 0.03
Extrahepatic tumors	12.6%	4.4%	NS

NS: Not significant.

there was a higher prevalence of male (F:M = 1:3). Among CC patients, 14.1% were ex-smokers and 15.3% were active smokers. 11.8% consumed low amount of alcohol (< 30 g of alcohol per day for men and < 20 g per day for women); similar values were observed for HCV cirrhotic patients (data not sowed).

Aminotransferases were normal or slightly elevated in CC; mean ALT values were 1.5-fold higher than cut-off levels for both men and women, whereas, they were significantly higher in HCV-related cirrhosis cases.

The majority of patients were overweight/obese (82.3%) and the mean BMI was 30.0 ± 5.7 kg/m^2; 70% had IFG/T2D, 55.9% had high blood pressure and 50.0% had dyslipidemia 52.9% of patients had full-blown MS. We found hyperuricemia in 29.2%, 14.4% had hypothyroidism and 58.7% had gallstones or had previously undergone cholecystectomy for gallstones. As showed in Figure 2 these metabolic conditions were significantly lesser in HCV patients.

Cholelithiasis or cholecystectomy were present in 58.7% of CC and in 24.2% of HCV cirrhosis (*P* < 0.01). Hypothyroidism was present in CC and HCV cirrhosis in 14.4% and 5.6% (*P* < 0.01), respectively. There was a history of extrahepatic tumors in 12.4% of CC patients and in 5% of HCV patients; 43.3% of patients had a positive history for cardiovascular complications,

Comparison of CC patients according to enrollment center revealed no significant differences as far as demographic, biochemical, and clinical characteristics were concerned (data not shown). Accordingly, data from these 2 cohorts were processed together. As a control group, 110 matched patients with HCV-related cirrhosis were enrolled.

The demographic, clinical, and laboratory characteristics of the studied subjects (CC and HCV cirrhosis) are summarized in Table 1, and the prevalences of metabolic features is shown in Figure 2. The mean age at diagnosis of CC patients was 66 ± 11 years [median (range): 66 (38-84) years]. There was a majority of women (63.7%, F:M = 1.8:1), whereas, in HCV cohort

Table 2 Characteristics and complications of the 102 patients with cryptogenic cirrhosis according to metabolic syndrome components

	With full metabolic syndrome, *n* = 54 (52.9%)	Without full metabolic syndrome, *n* = 36 (35.8%)	Without components of metabolic syndrome, *n* = 12 (11.8%)
Age (yr), median	66 (48-84)	66 (42-84)	70 (38-81)
Female sex	70.4%	58.3%	50%[a]
Obesity	69%	39%	0%[a]
Overweight	27%	55.5%	0%[a]
IFG/T2D	81.8%	61%	33%[a]
Ascites	50%	61.3%	55%
HCC	20%	14%	18%
Cardiovascular diseases	48%	41.6%	25%[a]

[a]*P* < 0.05. HCC: Hepatocellular carcinoma; IFG: Impaired fasting glucose.

Table 3 Outcome observed during follow up of the 102 patients with cryptogenic cirrhosis and 110 patients with hepatitis C virus-related cirrhosis

	Cryptogenic cirrhosis	Hepatitis C virus cirrhosis
Follow up, median, mo (range)	42 (10-96)	40.8 (10-95)
Liver decompensation	60.8%	55.4%
Ascites	54.9%	51.8%
Encephalopathy	25.5%	21.8%
Esophageal variceal bleeding	6.9%	6.3%
Hepatocellular carcinoma	16.7%	17.2%
Orthotopic liver transplantation	1.0%	3.6%
Death	39.2%	30.0%
Liver-related	77.5%	78.8%
Cardiovascular events	10.0%	9.1%
Non-liver-related cancer	7.5%	3.0%
Other causes	5.0%	9.1%
Time to death, median, mo (range)	26 (1-96)	28 (4-86)

in the majority of cases coronary artery disease. Interestingly, the prevalence of coronary artery disease in our CC cohort was significantly higher than that found in two age-matched control groups (*P* = 0.002). Coronary artery disease was present in 33.3% of CC patients *vs* 21.6% in the cohort of HCV cirrhotic patients used as a control for liver disease *vs* 12.0% in a heterogeneous cohort of 652 patients hospitalized for reasons other than liver diseases [median age 66 (47-86), 48% men].

Twenty-eight patients (27.5%) had anti-HBV antibodies: 21 were HBsAb positive and 7 only HBcAb positive. All but one of these patients were serum HBV DNA negative. The HBV DNA positive patient was excluded from the study. A higher prevalence of anti-HBV-Ab was observed in the HCV cohort (42, 7%).

At the first observation, 46.7% of patients had Child-Pugh class A cirrhosis, 31.1% were Child-Pugh B and 22.2% were Child-Pugh C; 67.7% of CC had esophageal varices, a prevalence similar to that observed in HCV-related cirrhosis.

Table 2 shows the metabolic characteristics of patients with CC. More than 88% of patients were overweight/obese and a full metabolic syndrome was observed in 52.9%. In patients with metabolic syndrome, higher prevalences of female, obesity and IFG or diabetes were present. A minority of patients (11.8%) was lean and without component of metabolic syndrome. The lean subgroup showed a significant lower prevalence of cardiovascular diseases than the overweight/obese patients.

Outcome observed during follow-up
The median follow-up period was of 42 mo (range 10-96 mo) for CC and 40.8 mo (range 10-95 mo) for HCV-related cirrhosis (Table 3).

Liver decompensation: During follow up, 60.7% of patients with CC developed liver decompensation: ascites occurred in 54.9%, encephalopathy in 25.5% and esophageal variceal bleeding in 6.9% (Table 3). A similar incidence of ascites, encephalopathy and variceal bleeding was observed in the HCV cohort (Table 3). A similar incidence of liver decompensation for patients with a full metabolic syndrome, those with a component of metabolic syndrome and lean.

Liver transplantation: Only 1 patient with CC and 4 (3.6%) HCV cirrhotic patients, underwent OLT (Table 3). The patient with CC died after liver transplantation.

HCC development: Overall, among CC, 18 patients (17.6%) developed HCC, in 6 of them HCC and cirrhosis were diagnosed at the same time and in 12 during follow up (Table 4). Of the HCV cirrhotic patients, 19 (17.2%) had HCC, 4 of whom at enrollment and 15 developing during follow up (Table 4). The cumulative incidence of HCC developing during follow up was 3.5% per year for CC and 4.5% per year for HCV cirrhosis. The median age of patients with HCC was 73 years for CC and 72 for HCV; the male:female ratio was 1.5:1 and 3:1 for CC and HCV, respectively. The Child-Pugh classification was: A, 39%; B, 39%; C, 22% with a Child A:B/C ratio of 0.50 for CC, and A, 21%; B, 42%; C, 37% with a Child A:B/C ratio of 0.27 for HCV cirrhosis (Figure 3). The prevalence of HBV markers (HBsAb/HBcAb) in CC and HCV was 55.5% and 57,7%, respectively. Of the CC patients, HCC was found in 16 patients (20.5%) with one or more

Figure 3 Occurrence of hepatocellular carcinoma among 102 patients with cryptogenic cirrhosis and 110 patients with hepatitis C virus-related cirrhosis and its distribution among Child-Pugh class. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Table 4 Characteristics, prevalence and incidence of hepatocellular carcinoma in 102 patients with cryptogenic cirrhosis and in 110 patients with hepatitis C virus-related cirrhosis

	Cryptogenic cirrhosis	HCV cirrhosis
HCC at first diagnosis	6 (5.8%)	4 (3.6%)
HCC during follow-up	12 (12.5%)	15 (14.1%)
HCC overall	17.6%	17.2%
Annual rate of incidence	3.5%	4.5%
Age, median, yr (range)	73 (63-82)	72 (60-80)
Male sex (M:F ratio)	52% (1.5:1)	58% (3:1)
Child-Pugh A/B/C (%)	39/39/22	21/42/37
Ratio Child-Pugh A:(B-C)	0.5	0.27
α-fetoprotein (ng/mL), median (range)	48 (6-800)	66 (6-680)

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

metabolic components, and in 2 lean patients (17%) (Table 2).

Survival: Death in CC and HCV cirrhosis occurred in 40 patients (39.2%) and 33 patients (30%), respectively (Table 3). The vast majority of deaths in both CC and HCV cirrhosis were liver-related (Table 3).

The mortality rate for CC patients with full metabolic syndrome, with a component of metabolic syndrome and the lean was 42.5%, 36.1% and 33.3%, respectively.

The median time to death after cirrhosis detection was 26 mo for CC and 28 mo for HCV-related cirrhosis (Table 3).

The median survival in CC was 60 mo (95%CI: 42-78); the calculated probability of overall survival was 86%, 67% and 35% at 12, 36 and 120 mo, respectively.

Predictors of outcome in CC: Table 5 shows the predictors of both cirrhosis decompensation and development of HCC. By univariate analysis, the predictors for liver decompensation were age and Child-Pugh class at the first observation, and baseline serum sodium and creatinine levels. Multivariate analysis showed that Child-Pugh class at presentation was the only independent factors associated with liver decompensation. The only predictors for HCC were baseline serum sodium and creatinine levels; hyponatremia was found to be independently associated with HCC also at multivariate analysis.

Age and Child-Pugh class at presentation, and the baseline serum sodium and creatinine levels were the variables significantly associated with survival. Multivariate Cox regression analysis confirmed that age and Child-Pugh class at presentation were independent predictors of survival. Sex, BMI, T2D, dyslipidemia and cardiovascular complications did not emerge as significant predictors of any outcomes.

Variables significantly associated with liver-related outcomes are summarized in Table 6. Figure 4 shows comparison of survival according to age, Child-Pugh class at presentation and baseline serum sodium and creatinine levels.

DISCUSSION

This study evaluated the features of CC and associated factors in a cohort at their first diagnosis, as well as developments during follow up. Five major characteristic were identified: (1) CC cases had a high prevalence of metabolic syndrome and oncologic/cardiovascular co-morbidities; (2) CC patients without full-blown metabolic syndrome had, in a high proportion, at least one or more metabolic derangements and only a minority of patients did not have any metabolic alterations; (3) CC was associated with a poor prognosis, and with a significant occurrence of liver decompensation and HCC; (4) age, advanced liver damage and kidney impairment were predictors of liver decompensation and death in CC; and (5) a high proportion of HCC was observed in an early stage of CC.

The CC cohort was compared to age- and child-Pugh-matched HCV-related cirrhosis cohort. CC showed a higher prevalence of female sex, of metabolic features, of cardiovascular diseases, extrahepatic malignancy, and lower serum levels of aminotransferases. However, during follow up, there were no differences in the overall incidence of liver decompensation, development of HCC and survival. Of concern, the incidence of HCC in CC child-Pugh class A was 1.8 times higher than that observed in patients with HCV cirrhosis.

Our series recapitulates the demographic and clinical sketch of CC, a disease with a high prevalence of females in their sixties, associated with slightly elevated aminotransferases values and metabolic derangements[7-9,18-20]. The prevalence of body weight excess and IFG/T2D in our cohort, however, ranks among the highest ever reported in a CC case series[8,9,18,19,21]. It is important to highlight that T2D

Figure 4 Cumulative probability of overall survival after cirrhosis detection according to different significant predictors of mortality. A: Cumulative probability of overall survival according to age at diagnosis; P = 0.002 by log-rank test; B: Cumulative probability of overall survival according to Child Class at diagnosis; P < 0.001 by log-rank test; C: Cumulative probability of overall survival according to baseline sodium levels; P = 0.020 by log-rank test; D: Cumulative probability of overall survival according to baseline creatinine levels; P = 0.017 by log-rank test.

Table 5 Predictors of outcomes of the 102 patients with cryptogenic cirrhosis

Outcome	Univariate analysis, OR (95%CI)	P value	Multivariate analysis, OR (95%CI)	P value
Liver decompensation				
Age at diagnosis	1.06 (1.01-1.11)	< 0.01	0.97 (0.90-1.05)	NS
Male sex	1.00 (0.41-2.40)	NS	0.85 (0.18-4.12)	NS
Child B *vs* A	40.0 (8.03-199)	< 0.001	111 (11-1101)	< 0.001
Child C *vs* A	60.8 (7.2-513)	< 0.001	102 (7-1480)	< 0.001
Renal impairment[1]	3.75 (1.14-12.3)	< 0.023	2.90 (0.40-21.1)	NS
Hyponatremia[2]	4.45 (1.50-13.2)	< 0.005	2.89 (0.48-17.4)	NS
Hepatocellular carcinoma				
Age at diagnosis	1.04 (0.99-1.10)	NS	1.03 (0.95-1.12)	NS
Male sex	2.30 (0.79-6.70)	NS	2.38 (0.67-8.44)	NS
Child B *vs* A	1.09 (0.31-3.84)	NS	0.62 (0.13-2.86)	NS
Child C *vs* A	1.67 (0.46-6.10)	NS	0.76 (0.14-4.04)	NS
Renal impairment[1]	3.62 (1.18-11.0)	< 0.019	2.37 (0.52-10.9)	NS
Hyponatremia[2]	4.37 (1.18-13.15)	< 0.006	4.11 (1.14-14.9)	< 0.031

[1]Renal impairment: serum creatinine > 1.20 mg/dL; [2]Hyponatremia: serum Na < 135 mE/L. In addition to the variables reported in the table, analysis included also: ALT/AST, α-fetoprotein, metabolic syndrome, obesity, serum cholesterol, type 2 diabetes mellitus, cardiovascular events. NS: Not significant.

often develops as complication of cirrhosis *per se* and, therefore, it may be argued that it results from, rather than precedes cirrhosis[22]. Nevertheless, the concurrence of either the full-blown metabolic

syndrome (54%) or its individual features (in particular overweight/obesity), which are not classically found in cirrhosis due to other etiologies, and the high rate of cardiovascular co-morbidities, are in agreement

Table 6 Predictors of mortality of the 102 patients with cryptogenic cirrhosis

Outcome	Univariate analysis, HR (95%CI)	*P* value	Multivariate analysis, HR (95%CI)	*P* value
Overall mortality				
Age at diagnosis	1.07 (1.03-1.10)	< 0.001	1.11 (1.04-1.18)	0.001
Male sex	1.10 (0.55-2.20)	NS	1.07 (0.46-2.48)	NS
Child B *vs* A	9.75 (2.85-33.32)	< 0.001	8.35 (2.16-32.29)	< 0.002
Child C *vs* A	23.62 (6.64-84.05)	< 0.005	34.64 (7.5-160.7)	< 0.001
Renal impairment[1]	2.63 (1.31-5.30)	< 0.007	0.65 (0.28-1.54)	NS
Hyponatremia[2]	2.66 (1.34-27)	< 0.005	0.84 (0.36-1.95)	NS

[1]Renal impairment: serum creatinine > 1.20 mg/dL; [2]Hyponatremia: serum Na < 135 mE/L. In addition to the variables reported in the table, analysis included also: ALT/AST, α-fetoprotein, metabolic syndrome, obesity, serum cholesterol, type 2 diabetes mellitus, cardiovascular events. NS: Not significant.

with a primarily metabolic etiology in a substantial number of the cases of cirrhosis observed in our study. Supporting this view, nearly one-third of our patients had hyperuricemia, another cardio-metabolic risk factor associated with a higher risk of cirrhosis[23], and with development and progression of nonalcoholic fatty liver disease (NAFLD)[24,25], which is more common in CC patients compared to cirrhosis due to identifiable etiologies[19]. Further suggesting a pathogenic link of NAFLD with CC, a high proportion of our patients had either hypothyroidism or gallstones/cholecystectomy, which may be additional clues to NAFLD as a predisposing factor to CC[26-29] such as first suggested by Ludwig in 1980[30]. Thus, the overall clinic-pathologic analysis of our cohort of patients at their first diagnosis of CC seems to indicate that the leading cause include previously unrecognized NAFLD.

We also found that only a small number of patients with CC (about 12% of our population) were lean and did not have any components of metabolic syndrome. These patients were characterized by an older age and lower prevalence of T2D and cardiovascular diseases and thus had different features from those of other dysmetabolic CC patients. It is not possible to clearly establish the putative underlying etiology of cirrhosis in this subset of patients, but occult alcohol consumption or viral, autoimmune or genetic factors may account for the development of CC. However, the clinical outcome such as development of ascites, HCC and death was similar for the overweight/obese patients and the HCV-related cirrhosis groups, suggesting that, irrespective of underlying mechanisms, once cirrhosis is established it follows a similar natural course.

The natural history of CC, in term of morbidity and mortality, has been explored only marginally, and with conflicting results[8,21,31,32]. At variance with previous studies that had suggested a mild course of disease[31-35], our cohort of CC patients had a high rate of complications of cirrhosis such as liver failure and HCC that were similar to those observed in the HCV-related cirrhosis cases. In agreement with some of the previous studies[8,18,21], such complications may arise as the inaugural manifestation of liver diseases. Of concern, we confirm that the hepatocarcinogenic

potential of CC is not negligible[21,35,36]. Indeed, the cumulative incidence per year of HCC was 3.5%, slightly higher than those reported in two previous studies, 2.6% and 2.7%, respectively[32,36]; this incidence was slightly lower than that observed (4.5%) in HCV-related cirrhosis cohort. Overall, our results are in agreement with other studies[18,37,38], which reported a substantial risk of HCC developing in CC.

The estimated long-term prognosis of our patients was poor; the overall median survival was of about 5 years and only one-third of patients survived at 10 years. This prognosis is much more severe than that reported for metabolic-cirrhosis[29,34,35], and is in agreement with two previous studies on CC[34,39]. Although mortality was overwhelmingly liver-related, our cohort had a high prevalence of cancer and cardiovascular diseases. The prevalence of coronary artery disease in our cohort was significantly higher than that found in two age-matched groups of patients at high cardiovascular risk owing to either traditional (T2D, kidney and lung diseases) or emerging (HCV cirrhotic patients) risk factors[40]. Finally, in our study, a single patient underwent OLT and died of complications after surgery. Owing to age and co-morbidities, patients with NASH-cirrhosis and CC are less likely to receive OLT[32], have a high risk of NASH or cryptogenic liver disease recurrence after OLT[41], and a higher rate of postoperative complications and mortality than other etiologies[42].

In accordance with a previous study on CC[21], our study showed that age at presentation and severity of liver and kidney tests predicted liver failure and death. No association was found between BMI, T2D and/or other metabolic features with liver-related outcomes. This could be the result of the limited number of events and/or of the high prevalence of metabolic factors.

Of interest, was the observation that no condition predict the development of HCC and that a high proportion was observed in the early phase of cirrhosis. Previous, studies have shown consistently that HCC in NAFLD patients may develop even without cirrhosis, is diagnosed late and, therefore, has a poor prognosis[37,38]. The role of genetic factors in the development of HCC has consistently been suggested by several studies[43-45].

Our study has a number of points of strength in that we have characterized the features of a cohort of cirrhotic patients with a previously unknown liver disease as well as the predictors of the outcomes. Considering the scarcity of published data on such population, we have reason to believe that our findings may help to identify these patients earlier, possibly before the development of cirrhosis, with the aim of preventing and rapidly treating hepatic and extrahepatic complications, thus improving survival and quality of life for these individuals.

As our study was conducted at two tertiary centers, this may have created a selection bias. In addition, it is a cohort study including previously unknown cirrhotic patients with an advanced stage of liver disease and, therefore, ~~so~~ the natural history of CC might not have been faithfully characterized. Moreover, this study specifically identifies risk factors and natural history of CC in the center and south of Italy and our findings may not apply to other ethnicities or geographical area. Finally, the number of patients may be relatively too small to identify all predictors of the outcome. However, we highlight that the size of our cohort was not inferior to those previously published case-series on CC[8,21,31,32].

In conclusion, the data of this study confirm that CC likely results from the progression of unrecognised NASH in a large proportion of cases; hence it may be better defined as "metabolic cirrhosis" in most cases. The subset of lean patients with CC, however, may suggest non-metabolic risk factors, and this group therefore deserves further investigation. The predictors of hepatic complications and death in patients with CC are similar to those of cirrhosis due to HCV. Patients with CC have a high risk of developing severe liver complications and a poor prognosis owing to liver-related death and cardiovascular and oncologic co-morbidities. The risk of HCC developing in CC, even in the early phase, is not negligible, and thus warrants the setting up of surveillance programs for early detection of NAFLD-NASH in patients with dysmetabolic features.

COMMENTS

Background
Cryptogenic cirrhosis (CC) are often incidentally discovered or diagnosed in the late stage, are poor characterized as well as natural history is not well known.

Research frontiers
CC have a high clinical impact considering that account for up to 30% of cases of cirrhosis and about 10% of liver transplantsand thus, there is a need to know their characteristics and outcomes. This study investigated the clinical features at first diagnosis, the outcomes and associated conditions of CC.

Innovations and breakthroughs
The results showed that CC are associated with metabolic features, poor prognosis, hepatocellular carcinoma (HCC) development in early stage, cardiovascular diseases and extrahepatic cancer risk. The data suggest

an etiologic role of undiagnosed nonalcoholic fatty liver disease in a high percentage of cases of CC.

Applications
The data are utilizing in the clinical setting to propose screening strategies for early detection of liver impairment in dysmetabolic subjects and early screening for HCC.

Terminology
CC is cirrhosis of unknown etiology, in particular with no history of alcoholism or previous infective hepatitis. At present, in a large proportion of cases, CC may be best-defined "metabolic cirrhosis".

Peer-review
This is an interesting manuscript by Rinaldi *et al* regarding the natural history of CC compared to hepatitis C virus-related cirrhosis. The manuscript is well-structured, the methodology and the sample size seem appropriate, and overall the topic is relevant for the field.

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ORIGINAL ARTICLE

Observational Study

Competing risk analysis on outcome after hepatic resection of hepatocellular carcinoma in cirrhotic patients

Alessandro Cucchetti, Carlo Sposito, Antonio Daniele Pinna, Davide Citterio, Matteo Cescon, Marco Bongini, Giorgio Ercolani, Christian Cotsoglou, Lorenzo Maroni, Vincenzo Mazzaferro

Alessandro Cucchetti, Antonio Daniele Pinna, Matteo Cescon, Giorgio Ercolani, Lorenzo Maroni, Department of Medical and Surgical Sciences, DIMEC, S.Orsola-Malpighi Hospital, Alma Mater Studiorum, University of Bologna, 40138 Bologna, Italy

Carlo Sposito, Davide Citterio, Marco Bongini, Christian Cotsoglou, Vincenzo Mazzaferro, Gastrointestinal Surgery and Liver Transplantation, Fondazione IRCCS Istituto Nazionale Tumori (National Cancer Institute), 20133 Milan, Italy

Author contributions: Cucchetti A and Sposito C had the original idea and designed the study; Cucchetti A, Pinna AD and Mazzaferro V wrote the manuscript; Cucchetti A performed statistical analysis; Sposito C, Cescon M and Ercolani G interpreted results and added important intellectual content in manuscript preparation; Citterio D, Bongini M, Cotsoglou C and Maroni L collected data and reviewed clinical charts.

Institutional review board statement: The study was reviewed and approved by the Local Ethics Committees.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent according to Italian law.

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Data sharing statement: No additional data are available.

Abstract

AIM
To investigate death for liver failure and for tumor recurrence as competing events after hepatectomy of hepatocellular carcinoma.

METHODS
Data from 864 cirrhotic Child-Pugh class A consecutive patients, submitted to curative hepatectomy (1997-2013) at two tertiary referral hospitals, were used for competing-risk analysis through the Fine and Gray method, aimed at assessing in which circumstances the oncological benefit from tumour removal is greater than the risk of dying from hepatic decompensation. To accomplish this task, the average risk of these two competing events, over 5 years of follow-up, was calculated through the integral of each cumulative incidence function, and represented the main comparison parameter.

RESULTS
Within a median follow-up of 5.6 years, death was

attributable to tumor recurrence in 63.5%, and to liver failure in 21.2% of cases. In the first 16 mo, the risk of dying due to liver failure exceeded that of dying due to tumor relapse. Tumor stage only affects death from recurrence; whereas hepatitis C infection, Model for End-stage Liver Disease score, extent of hepatectomy and portal hypertension influence death from liver failure ($P < 0.05$ in all cases). The combination of these clinical and tumoral features identifies those patients in whom the risk of dying from liver failure did not exceed the tumour-related mortality, representing optimal surgical candidates. It also identifies those clinical circumstances where the oncological benefit would be borderline or even where the surgery would be harmful.

CONCLUSION
Having knowledge of these competing events can be used to weigh the risks and benefits of hepatic resection in each clinical circumstance, separating optimal from non-optimal surgical candidates.

Key words: Hepatocellular carcinoma; Liver failure; Hepatic resection; Survival; Competing risk; Tumour recurrence

Core tip: Optimal candidates for hepatectomy should benefit from the tumour removal that encompasses the risk of dying from post-operative liver function worsening and failure. This means that when evaluating patients for surgery, the competing risks of tumour-related death and of liver failure have to be weighed against each other, and considered from the point of view of available alternative therapies. In the present study, a large cohort of Child-Pugh class A cirrhotic patients submitted to curative (R0) hepatic resection for hepatocellular carcinoma was analysed to provide a competing-risk analysis of these two competing events.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide[1]. It often arises on the background of cirrhosis, making its treatment completely different from other liver malignancies because of the conflicting needs of being oncologically appropriate and of preserving hepatic function. In this regard, a careful preoperative evaluation of both

tumour burden and functional reserve is essential in order to select candidates that will most benefit from surgery[2]. The balance between tumour stage, hepatic curtailment needed to curatively remove the tumour, and the hepatic reserve is critical to obtain a survival benefit, avoiding a pointless oncological outcome, postoperative liver failure, progressive hepatic deterioration and, ultimately, patient premature death. Having these aspects as determinants, hepatic resection is typically indicated in patients able to achieve a significant oncologic benefit from surgery, with low or null probabilities of experiencing liver function worsening[3]. Cirrhotic patients resected for HCC mainly die due to recurrence of the tumour consequences, and/or complications of end-stage liver disease. This means that when evaluating patients for surgery, the competing risks of tumour-related death and of liver failure have to be weighed against each other, and considered from the point of view of available alternative therapies. Having knowledge of these aspects can help in identifying optimal surgical candidates as well as in recognizing sub-optimal and/or non-optimal candidates who will most benefit from other therapies.

Common statistical techniques for time-to-event analysis, including cancer-specific survival, focus on failure-time data with a single type of failure (even if composite such as disease-free survival), are not able to capture competing risks arising when a failure can result from one of several causes and one cause precludes the others[4,5]. Competing risk analysis can more adequately capture the real cause-specific survivals of HCC cirrhotic patients submitted to hepatectomy. However, at present, no data are currently available on the competing risk of these two main end-events. The aim of the present study was to analyze the competing-risk of dying from tumour recurrence or liver failure in a cohort of cirrhotic patients, belonging to Child-Pugh class A, submitted to curative hepatic resection, and to investigate prognostic factors, taking into consideration the competing nature of these two events.

MATERIALS AND METHODS

Study population
Prospectively collected data from two Western centres with similar volumes, expertise and management strategy for HCC (Fondazione IRCCS - Istituto Nazionale Tumori, Milan, Italy and S.Orsola-Malpighi Hospital, Bologna, Italy) were reviewed, and patients submitted to curative resection (R0) of a pathologically proven HCC between 1997 and 2013 were identified (patients with R1 or R2 margin positive resection were excluded). Approval for conducting the study was obtained from the institutional review board at both centres. The study population selection was focused on patients with well-preserved liver function, since they commonly represent typical candidates for hepatic resection. Consequently, only patients belonging to Child-Pugh class A were

retained for the analysis. None of the patients in the study group was treated as an emergency; none had macroscopic tumour portal vein invasion, invasion of adjacent organs or spread to the lymph nodes of the hepatic hilum. The final study population consisted of 864 consecutive Child-Pugh class A cirrhotic patients submitted to curative (R0) hepatectomy. Presence of clinical signs of portal hypertension (PHT) was not considered an absolute contraindication for hepatectomy[6]. Thus, the study cohort also includes patients with total bilirubin > 1 mg/dL and/or with a platelet count < 100000/mL and/or with oesophageal varices at endoscopy, namely, when tumour resection was judged to provide a greater benefit than other available options such as liver transplantation, and loco-regional or systemic therapies.

The following variables were recorded for each patient: age, sex, aetiology of underlying liver disease, presence of oesophageal varices, main serological parameters (total bilirubin, creatinine, international normalized ratio, albumin, platelets count), and main tumour radiological characteristics (number and size of lesions). Presence of PHT was defined as the presence of oesophageal varices or a platelet count < 100000/mL[3]. The extent of the hepatectomy was based on the International Hepato-Pancreato-Biliary Association Classification[7]. Tumours were staged on the basis of preoperative imaging, according to the United Network for Organ Sharing (UNOS)-TNM classification[8].

Following discharge, all patients were observed periodically at follow-up to exclude possible recurrence of HCCs: biochemical liver function tests, serum α-fetoprotein level measurement, and ultrasound were conducted 3 and 6 mo after discharge and then according to an annual or semi-annual surveillance program in the more recent period[9]. Recurrence was diagnosed on the basis of HCC diagnosis guidelines released during the study period. None of the patients in this study group received adjuvant chemotherapy. Patients presenting recurrence were managed with various therapeutic modalities, including re-resection, when possible, and salvage liver transplantation, for selected patients with transplantable recurrence. The patient selection criteria for second hepatic resection were the same as for primary resection. Patients with non-resectable recurrence, and not suitable for liver transplant, were submitted to loco-regional therapies. From the end of 2008, Sorafenib therapy was also adopted, either alone or in combination with loco-regional approaches.

Statistical analysis

Continuous data are reported as median and inter-quartile ranges; categorical data as counts and percentages. Patient survival was measured from the date of hepatic resection until death or the date of the last follow-up. The cause of death was recorded considering that patients would have died because of the tumour in the presence of a disseminated extra- and/or intra-hepatic tumour recurrence, including also those cases where liver function worsened as a consequence of tumour spread (*i.e.*, liver involvement > 50% and/or development of tumour portal vein invasion). On the contrary, patients would have likely died because of liver failure (and/or its complications) in the absence of tumour recurrence and in the presence of clinical signs of end-stage liver disease, or, in cases where recurrence was diagnosed, in all those cases where the burden of tumour relapse did not justify liver failure (*i.e.*, progressive liver worsening in the presence of small recurrences). Controversial cases were discussed between authors Cucchetti A and Sposito C. Cases not fulfilling these criteria were recorded as other causes of death. Follow-up ended at June 2015. Patients submitted to salvage transplantation were censored the day prior to transplant. Survival rates, observed after surgery, were obtained by plotting Kaplan-Meier curves. Cumulative incidences of the competing events of interest were calculated using the Fine and Gray competing risks approach using the STATA syntax stcrreg (StataCorp. *Stata* Statistical Software: Release 12.)[10]. Factors identified having a *P* < 0.10 on simple (univariate) competing risk analysis were entered into a multivariable regression model. A backward stepwise variable-selection process was adopted to identify independent predictors of death for tumour recurrence or for liver failure. A *P* value < 0.05 was considered statistically significant in all the analyses. The cumulative incidence of death for tumour relapse or hepatic failure was thus calculated for each combination of independent variables. The area under the curve of cumulative incidence over time was then calculated using trapezoidal rule and divided by time so it was expressed as average risk within the first 5 years from surgery. Differences between these incidences were compared through standardized differences (d) calculation, a measure of the effect size[11]. In particular, d values < |0.1| indicated very small differences between the means; d values between |0.1| and |0.3| indicated small differences, d values between |0.3| and |0.5| indicated moderate differences, and d values > |0.5| indicated considerable differences[12].

RESULTS

Baseline characteristics of the 864 cirrhotic patients, all belonging to Child - Pugh class A forming the study population, are reported in Table 1. The median age was 66.7 years, ranging between 18 and 85 years, most of the patients were hepatitis C positive (62.3%), had a single tumour (78.2%), were within UNOS-T1/T2 stage (70.6%), and were submitted to the removal of one Couinaud segment or less (sub-segmentectomy) (72.3%).

Table 1 Characteristics of the study population of cirrhotic patients, belonging to Child - Pugh class A, submitted to curative hepatectomy of hepatocellular carcinoma *n* (%)

	n = 864
Age (yr)	67 (61- 72)
≥ 67 yr	432 (50.0)
Gender male	678 (78.5)
Anti-HCV positive	538 (62.3)
HBsAg positive	197 (22.8)
Alcohol/other	120 (13.9)
Creatinine (mg/dL)	0.90 (0.80-1.04)
Albumin (g/L)	4.0 (3.7-4.3)
Bilirubin (mg/dL)	0.85 (0.64-1.19)
INR	1.13 (1.07-1.22)
Platelet count ($\times 10^3$/mL)	142 (102-186)
< 100.000/mL	202 (23.4)
MELD score	9 (8-10)
< 9	428 (49.5)
9-10	298 (34.5)
> 10	138 (16.0)
Oesophageal varices	216 (25.0)
Tumour size (cm)	3.5 (2.3-5.0)
Single nodule	676 (78.2)
UNOS Stage	
T1	84 (9.7)
T2	527 (61.0)
T3	237 (27.4)
T4a	16 (1.9)
Extension of hepatectomy	
Wedge/segmentectomy	625 (72.3)
Bisegmentectomy	146 (16.9)
Three or more segments	93 (10.8)

Continuous variables are reported as medians and interquartile ranges. Tumour features are radiological: 610 patients were within Milan criteria (70.6%). UNOS: United Network for Organ Sharing.

During a median follow-up of the whole cohort of 5.6 years (range: 1 d-13 years), 489 patients experienced tumour recurrence (56.6%) and 334 patients died (38.7%). Death was attributable to tumour recurrence in 212 patients (63.5% of causes of death) and to liver failure in 71 patients (21.2%). The median time between tumor recurrence and death was 1.4 years. In 51 patients, the cause of death was not attributable to either of these two events (15.3%). The 30-d and 90-d post-operative death rates were 1.0% and 2.4%, respectively. The 1-, 3-, 5- and 10-year patient survival probabilities were 90.9%, 70.9%, 54.9% and 29.0%.

Competing risk analysis

Competing risk analysis on cause of death for the entire study population is reported in Figure 1. As can be noted, in the first 16 mo, the predicted risk of dying due to liver failure exceeds that of dying because of tumour recurrence. Afterwards, most deaths were attributable to tumour relapse whereas liver failure mortality remained roughly stable through subsequent years, namely between 4.4% at 1 year and 9.1% at 5 years.

Relationships between clinical variables and the cumulative incidence of death due to tumour recurrence or due to liver failure are reported in Table 2. Tumour stage and extension of hepatectomy were predictors of death due to tumour relapse (*P* = 0.002 and 0.042, respectively). Regarding the cumulative incidence of death attributable to liver failure, hepatitis C infection (*P* = 0.032), Model for End-stage Liver Disease (MELD) score (*P* = 0.001) and PHT (*P* = 0.029) were significantly related to the predicted risk of dying from liver failure.

For completeness of results, the same analysis was conducted for other causes of death but none of the variables analysed was found to be significantly related to death for causes other than tumour recurrence or liver failure; thus, detailed results were omitted.

Prediction of competing events incidences

Results from multivariable competing risk analysis are reported in Table 3. Tumour stage only affects death due to recurrence (*P* = 0.001) whereas hepatitis C positivity (*P* = 0.046), MELD score (*P* = 0.001), extension of hepatectomy (*P* = 0.019) and PHT (*P* = 0.024) influence death as a result of liver failure.

A summary of possible combinations of clinical and tumoral variables is reported in the Figure 2. Results are reported as average risk over 5 years of follow-up, predicted by the multivariable regression model (the integer of the cumulative incidences over time divided by time) and were adjusted for the distribution of hepatitis C positive patients in the present study population (62.3%). The threshold for positive oncologic surgical benefit of liver resection (positive d-values) widens as the T stage progresses, while HCC resectability of HCC in cirrhosis becomes less achievable with the progression of liver dysfunction. Some examples can help to clarify this figure. A patient with a T1 tumour has an average annual risk of dying from cancer of 5.1% and a corresponding risk of dying from liver failure of 1.8%, provided there are no clinical signs of PHT, the patient has a MELD score < 9 and the tumour is removable with a wedge or a segmentectomy (d = 0.182: positive benefit). If the removal of the tumour requires a more extended hepatectomy and/or there are clinical signs of PHT, the risk of dying from liver failure increases to 3.2/3.3% (d < 0.10: borderline benefit), thus nullifying the oncologic surgical benefit. A patient with a T2 tumour has an annual average risk of dying from cancer of 7.9% and, in the presence of a MELD score > 10, clinical signs of PHT and the need for a wedge or a segmentectomy, a corresponding risk of dying of liver failure of 14.6%; thus, the risk of dying from liver failure exceeds the oncologic surgical benefit (d = -0.213: negative effect size).

DISCUSSION

Despite the increasing incidence of HCC, the curative

Table 2 Cumulative incidence of death from tumour recurrence and from liver failure resulting from competing risk analysis

	Death from tumour recurrence			Death from liver failure		
	1-year (%)	3-year (%)	5-year (%)	1-year (%)	3-year (%)	5-year (%)
Age (yr)		P = 0.915			P = 0.267	
< 67	3.8 (0.9)	19.9 (2.2)	28.1 (2.6)	4.5 (1.0)	6.2 (1.2)	7.8 (1.4)
≥ 67	3.2 (0.8)	14.7 (1.9)	28.1 (2.8)	4.3 (1.0)	6.7 (1.3)	10.5 (1.8)
Gender		P = 0.738			P = 0.287	
Male	3.6 (0.7)	17.7 (1.7)	27.7 (2.1)	4.2 (0.8)	6.6 (1.0)	8.5 (1.2)
Female	3.4 (1.4)	15.4 (2.9)	28.2 (4.1)	5.0 (1.6)	7.2 (1.9)	11.1 (2.8)
Hepatitis C infection		P = 0.838			P = 0.032	
Positive	3.7 (0.8)	15.9 (1.8)	27.4 (2.4)	5.3 (1.0)	7.5 (1.2)	10.9 (1.6)
Negative	3.2 (1.0)	19.3 (2.5)	28.3 (3.1)	2.8 (0.9)	4.7 (1.2)	5.9 (1.5)
Portal hypertension[1]		P = 0.515			P = 0.029	
Absent	3.6 (0.8)	17.6 (1.8)	27.2 (2.3)	3.3 (0.8)	5.1 (1.0)	6.9 (1.2)
Present	3.3 (1.0)	16.5 (2.3)	28.8 (3.2)	6.2 (1.4)	8.9 (1.7)	12.9 (2.2)
MELD score		P = 0.760			P = 0.001	
< 9	4.2 (1.0)	16.7 (2.0)	27.7 (2.8)	1.9 (0.7)	3.3 (0.9)	5.2 (1.3)
9-10	2.5 (0.9)	17.7 (2.5)	27.8 (3.2)	4.7 (1.2)	6.0 (1.4)	10.2 (2.1)
> 10	3.6 (1.6)	17.3 (3.6)	27.7 (4.7)	11.0 (2.7)	16.6 (3.2)	17.9 (3.4)
UNOS T-stage		P = 0.002			P = 0.597	
T1	1.0 (0.0)	7.6 (0.2)	14.5 (0.7)	1.2 (1.0)	3.2 (2.3)	10.6 (4.6)
T2	3.0 (0.7)	15.4 (1.8)	25.0 (2.4)	4.1 (0.9)	6.7 (1.2)	8.7 (1.4)
T3-T4a	5.8 (1.5)	26.3 (3.0)	34.6 (3.6)	6.0 (1.5)	7.8 (1.7)	9.3 (2.0)
Hepatectomy extension		P = 0.042			P = 0.052	
Wedge/segmentectomy	3.2 (0.7)	15.2 (1.6)	26.7 (2.3)	3.0 (0.7)	5.5 (1.0)	8.7 (1.4)
Two or more segments	4.4 (1.4)	22.3 (2.9)	30.7 (3.5)	8.1 (1.8)	9.0 (1.9)	10.3 (2.1)

Standard errors are reported in parentheses. [1]Defined as presence of oesophageal varices and/or platelet count < 100000/mL. MELD: Model for End-stage Liver Disease; UNOS: United Network for Organ Sharing.

Table 3 Results from multivariable competing risk regression models

	Sub-Hazard ratio	95%CI	P value
Death for tumour recurrence			
UNOS T-stage (T1 *vs* T2 *vs* T3-4a)	1.59	1.21-2.09	0.001
Removal of more than one segment	1.08	0.76-1.51	0.667
Death for liver failure			
Hepatitis C (positive *vs* negative)	1.79	1.01-3.17	0.046
Portal hypertension (present *vs* absent)	1.84	1.08-3.12	0.024
MELD class (< 9 *vs* 9-10 *vs* > 10)	2.21	1.59-3.07	0.001
Removal of more than one segment	1.89	1.11-3.21	0.019

The multivariable model included variables with a $P < 0.10$ of Table 2. A backward stepwise variable-selection process was selected to obtain estimates of non-significant variables (Removal of more than one segment for death from tumour recurrence; $P = 0.667$). Sub-hazard ratios were used together with the baseline cumulative sub-hazard function (data not reported) to predict individual risks of death for liver failure and for tumour recurrence reported in Figure 2.

approach of hepatic resection remains underused and at times ignored. Recent data from the American College of Surgeons National Cancer Data Base showed that even if hepatic resection was associated with a significant increase in survival among patients with AJCC stage Ⅰ/Ⅱ HCC, only less than 40% of such patients were treated surgically[13]. This proportion increases when patients are managed in academic centres, probably because of a better knowledge of risks and benefits obtainable with hepatic resection[13]. The main concern in offering surgical resection to cirrhotic patients is represented by the need to avoid post-hepatectomy liver failure and persistent function worsening[2,3,6]. Considering that the main causes of death after hepatic resection are tumour relapse and liver failure, a comprehensive knowledge of these

two distinct risks can help in daily clinical practice, especially when comparing surgical and non-surgical therapies[2]. The present results can help in fill this gap of knowledge.

The first result showed that the risk course of the two competing end-events varies with the passage of time (Figure 1). In the mid-term following liver resection, namely in the first 16 mo, the risk of death from tumour relapse was lower than that from liver failure (that does not exceed 5%), confirming the curative value of surgery. The surgical benefit is clinically supported by the fact that diagnosis of recurrence is not associated with dismal prognosis as it used to be, since the improved ability to treat recurrence (even curatively) can significantly prolong survival[9]. These aspects have to be weighed against

Figure 1 Cumulative incidences of death from liver failure, tumour recurrence and other causes after curative hepatic resection for hepatocellular carcinoma. Of note, in the first 16 mo, the risk of dying from liver failure exceeds that of dying because of tumour recurrence, confirming the curative value of surgery.

the risk of liver failure, which, in the absence of a liver transplant, still represents a diagnosis of imminent death. From the present results, it can be said that, starting from the first year onwards, the healing ability of surgery begins to decline and long-term risks and benefits should be evaluated from this time point onwards.

One main clinical indication that can be derived from the present study regards the identification of optimal and non-optimal candidates for liver surgery, even beyond conventional recommendations (Figure 2). It can be suggested that optimal surgical candidates are those patients having a long-term risk of dying due to tumour relapse constantly higher than that of dying from liver failure. In cirrhotic patients with a T1 tumour (single nodule < 2 cm), in the absence of clinical signs of PHT, MELD < 9 and limited extent of liver resection, namely a wedge or a segmentectomy, the average annual risk of dying from cancer is considerably higher (5.1%) than that of dying of liver failure (1.8%). The magnitude of the effect size (d = 0.182) supports the indication for surgery in this kind of patient, who has a tumour that is superficially located and easily removable with limited removal of liver parenchyma. In cases in which the presence of initial signs of liver function worsening (PHT or increased MELD score) or where there is a need for greater parenchymal removal, the risk of dying from liver failure starts to increase, nullifying the oncological benefit (effect size < 0.1) and becoming potentially harmful in more advanced degrees of liver dysfunction (negative effect size). In these cases, patients become non-ideal candidates for surgery, supporting the role of loco-regional treatments.

Patients with a T2 tumour (single nodule 2-3 cm or 2-3 nodules all < 3 cm) represent the majority of surgical candidates[14] and it is worthy of note that in cases of multiple lesions these patients fall beyond conventional recommendations built on the Barcelona Clinic Liver Cancer (BCLC) staging system[3,15,16]. T2 patients are burdened by an average annual risk of dying from tumour recurrence of 7.9%. From the

comparison of the two competing risks, it can be noted that the oncological benefit obtainable from surgery, allows acceptance of a higher risk of liver failure (Figure 2). That is, in the presence of a T2 tumour, clinical signs of PHT should not represent an absolute contraindication to surgery (effect size > 0.1), provided there is a substantial normal liver function (MELD < 9-10)[6,16,17]. In these cases, the risk of tumour-related death was permanently higher than that of death due to liver failure, supporting the concept that the benefit obtainable with surgery is considerably greater than the risk of liver failure. Conversely, a more advanced degree of liver dysfunction and/or the need for more extensive hepatectomies may turn surgery into a harmful treatment that can be justified by the possibility of a subsequent salvage transplantation: a resource-consuming alternative that, on top of poorly predictable outcome, transforms an elective strategy into an emergency procedure.

Tumours still of surgical interest while belonging to UNOS-T3-T4a stages mostly fall within the intermediate stage of BCLC algorithm[3,15,18]. Resectability in these patients should be assessed by experienced surgical groups before offering trans-arterial chemoembolization (TACE)[18] as a (non-curative) alternative treatment option. These tumour stages are burdened by the highest risk of dying from tumour relapse, thus making it possible to prioritise the surgical indication, including also patients with PHT and MELD scores around 10. In fact, surgically-resectable T3-T4a patients are non-ideal candidates for resection but, as recently outlined in a large analysis, a patient who may not be an ideal candidate for resection may still have a better outcome than what is expected when alternative modalities recommended by the current guidelines are applied[2]. Thus, the recommended treatment modality for the intermediate stage represented by TACE[18] can be challenged by liver resection, not in all cases but under the specific circumstances specified above. Notably, in pertinent literature benchmarks, TACE has, in the best-case scenario, a median survival of about 2 years[3,15,18,19], corresponding to an average risk of

	Risk of dying from liver failure			Risk of dying from tumour recurrence		
				T1	T2	T3-T4a
PHT	> 1 segment	MELD	%	5.1	7.9	12.2
No	No	< 9	1.8	0.182	0.287	0.416
No	Yes	< 9	3.2	0.095	0.206	0.343
Yes	No	< 9	3.3	0.090	0.201	0.338
No	No	9-10	3.9	0.058	0.170	O.309
Yes	Yes	< 9	5.7	-0.027	0.087	0.229
Yes	No	9-10	6.8	-0.072	0.042	0.185
No	Yes	9-10	7.1	-0.084	0.030	0.173
No	No	> 10	9.2	-0.160	-0.047	0.097
Yes	Yes	9-10	12.5	-0.263	-0.152	-0.009
Yes	No	> 10	14.6	-0.323	-0.213	-0.070
No	Yes	> 10	15.1	-0.337	-0.227	-0.085
Yes	Yes	> 10	25.4	-0.589	-0.483	-0.343

Positive oncologic benefit of resection

Borderline oncologic benefit of resection

Negative oncologic benefit of resection

Figure 2 Comparison between the predicted average risk of dying for liver failure (rows) and for tumour recurrence (columns) within the first 5 years after surgery, resulting from the competing-risk regression model. The average risks reported derive from: (1) the calculation of the area under the curves (AUC) of the risk of dying from liver failure and from tumour recurrence over time, predicted by the competing-risk model of Table 3; and (2) the division of the obtained AUCs by the time-period considered (5 years). Comparison between these two distinct risks is reported as effect size: values < |0.1| indicated very small differences between the means; values between |0.1| and |0.3| indicated small differences, values between |0.3| and |0.5| indicated moderate differences, and values > |0.5| indicated considerable differences. When the risk of dying of liver failure after resection is greater than that of dying from tumour relapse, effect size returns negative values (dark grey cells).

dying during the first 5 years of about 48%, which is always higher than present figures after resection (Figure 2). In other words, the present results support patients with intermediate stage HCC being offered liver resection when this is judged technically feasible in experienced centres and when the risk of dying from liver cancer exceeds the risk of liver failure. In all other instances, T3-T4a patients should remain with the conventional approach and be considered for TACE.

In determining the risk of dying from liver failure, hepatitis C infection deserves special consideration. The present study population encompasses a time period when direct antiviral agents (DAA) were not available, and only a small proportion of the most recently resected patients are currently receiving DAA. The low probabilities of achieving a cure for hepatitis C infection with standard interferon-based regimens of the past decades, and consequently the low probabilities of slowing down (or stopping) the progression of cirrhosis, are the reasons for its strong impact in the liver failure-related deaths observed in the present study. Although somewhat optimistic, it is reasonable to think that DAA can, in the future, achieve control of the progression of cirrhosis: presuming an improvement in the pro-gression of cirrhosis, the competing role of tumour-

related death will increase the benefit obtainable from hepatic resection.

Some limitations of the present study deserve appropriate discussion. First, it reports the experience from a surgical series and a comparison with non-surgical therapies would be the ultimate goal. Such a comparison is advisable for future studies that consider the competing risks of dying from cancer or liver failure in relationship with other therapeutic modalities. However, it is not strictly necessary in the current analysis to have this control group: these surgical results can be seen as a reference point for other studies of TACE or ablation without all being assessed in the same study. Nevertheless, future comparative studies using a competing risk approach are warranted. Second, the retrospective nature of the present study and the policies adopted in our centres may have determined a surgical population selection not completely representative of all patients suffering from resectable HCC. However, as previously outlined[13], patients managed in academic centres are more frequently offered such a potentially curative treatment, and the present data can be considered representative of a tertiary level hospital experience. Finally, limitations of the multivariable competing risk regression model have also to be taken into account. Even if it comes from a relatively large sample size, the risk of over-fitting the model cannot be excluded and the present results require further external or prospective validations to confirm their validity.

In conclusion, the present study provided a first competing risk analysis of causes of death after hepatic resection of HCC that could be used as a reference for similar analyses conducted on alternative treatments such as ablation or TACE. Ideal candidates for hepatic resection should be represented by those patients having a risk of dying from tumour cancer that is significantly greater than the risk of dying from liver failure.

COMMENTS

Background
The main concern in offering surgical resection to cirrhotic patients with hepatocellular carcinoma (HCC) is represented by the need to avoid post-hepatectomy function worsening. Considering that the main causes of death after surgery are represented by tumour relapse and liver failure, a comprehensive knowledge of these two distinct competing risks can help in clinical practice to distinguish optimal from non-optimal surgical candidates.

Research frontiers
Ideal candidates for hepatic resection should be represented by those patients having a risk of dying from tumour cancer that is significantly greater than the risk of dying from liver failure.

Innovations and breakthroughs
This study is the first one to provide a first competing risk analysis of causes of death after hepatic resection of HCC. The combination of tumour size and number, Model for End-stage Liver Disease score, extension of hepatectomy required to curatively remove the tumour and the absence or presence of

clinical signs of portal hypertension, identifies those patients in whom the risk of dying from liver failure did not exceed the tumour-related mortality, representing optimal surgical candidates.

Applications
Having knowledge of these competing events can be used to weigh risks and benefits of hepatic resection in each clinical circumstance, providing a benchmark to also assess the benefit achievable from non-surgical therapies, such as ablation or embolization.

Terminology
Survival analysis is the analysis of data measured from a specific time of origin until an event of interest or a specified endpoint occurs, where every patient provides two pieces of information: follow-up time and status (*i.e.*, event or censored endpoint). However, a patient may experience an event different from the event of interest. For example, a patient with HCC may die due to causes unrelated to his/her cancer. Such events are termed competing risk events.

Peer-review
A very interesting observation study provided a first competing risk analysis of causes of death after hepatic resection of HCC particular on the patients with Child' A functional class. This manuscript is well written and analyzing. It should benefit to kind in mild that those patients having a risk of dying from cancer resection that significantly overcome the risk of dying from liver failure.

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ORIGINAL ARTICLE

Observational Study

Different phenotypes of monocytes in patients with new-onset mild acute pancreatitis

Man-Li Zhang, Yan-Fang Jiang, Xin-Rui Wang, Li-Li Ding, Hong-Juan Wang, Qing-Qing Meng, Pu-Jun Gao

Man-Li Zhang, Qing-Qing Meng, Department of Hepatology and Gastroenterology, The Second Part of First Hospital of Jilin University, Changchun 130021, Jilin Province, China

Man-Li Zhang, Yan-Fang Jiang, Xin-Rui Wang, Hong-Juan Wang, Department of Central Laboratory, The Second Part of First Hospital of Jilin University, Changchun 130021, Jilin Province, China

Xin-Rui Wang, Hong-Juan Wang, Pu-Jun Gao, Department of Hepatology, The First Hospital of Jilin University, Changchun 130021, Jilin Province, China

Yan-Fang Jiang, Key Laboratory of Zoonosis Research, Ministry of Education, Jilin University, Changchun 130021, Jilin Province, China

Li-Li Ding, Department of Intensive Care Unit, The First Hospital of Jilin University, Changchun 130021, Jilin Province, China

Correspondence to: Pu-Jun Gao, MD, PhD, Department of Hepatology, The First Hospital of Jilin University, 71 Xinmin Street, Changchun 130021, Jilin Province, China. pujun-gao@163.com
Telephone: +86-431-84808239
Fax: +86-431-84808391

Abstract

AIM

To evaluate the numbers of different subsets of monocytes and their associations with the values of clinical measures in mild acute pancreatitis (MAP) patients.

METHODS

The study included one group of 13 healthy controls and another group of 24 patients with new-onset MAP. The numbers of different subsets of monocytes were examined in these two groups of subjects by flow cytometry. The concentrations of plasma interleukin (IL)-10 and IL-12 were determined by cytometric bead array. The acute physiology and chronic health evaluation (APACHE) II scores of individual patients were evaluated, and the levels of plasma C-reactive protein (CRP) as well as the activities of amylase and lipase were measured.

RESULTS

In comparison with that in the controls, significantly increased numbers of CD14+CD163-, CD14+CD163-MAC387+ M1 monocytes, but significantly reduced

numbers of CD14+CD163+IL-10+ M2 monocytes were detected in the MAP patients ($P < 0.01$ or $P < 0.05$). Furthermore, significantly higher levels of plasma IL-10 and IL-12 were observed in the MAP patients ($P < 0.01$ for all). More importantly, the levels of plasma CRP were positively correlated with the numbers of CD14+CD163- ($R = 0.5009$, $P = 0.0127$) and CD14+CD163-MAC387+ ($R = 0.5079$, $P = 0.0113$) M1 monocytes and CD14+CD163+CD115+ M2 monocytes ($R = 0.4565$, $P = 0.0249$) in the patients. The APACHE II scores correlated with the numbers of CD14+CD163+CD115+ ($R = 0.4581$, $P = 0.0244$) monocytes and the levels of plasma IL-10 ($R = 0.4178$, $P = 0.0422$) in the MAP patients. However, there was no significant association among other measures tested in this population.

CONCLUSION
Increased numbers of CD14+CD163- and CD14+CD163-MAC387+ monocytes may contribute to the pathogenesis of MAP, and increased numbers of CD14+CD163+CD115+ monocytes may be a biomarker for evaluating the severity of MAP.

Key words: Mild acute pancreatitis; Monocyte; Cytokine; Acute physiology and chronic health evaluation II score; C-reactive protein

Core tip: This is the first study on the numbers of different phenotypes of peripheral blood monocytes in patients with new-onset mild acute pancreatitis (MAP). Increased numbers of CD14+CD163- and CD14+CD163-MAC387+ M1 monocytes were positively correlated with the levels of plasma C-reactive protein (CRP), which suggest that pro-inflammatory monocytes may participate in the pathogenesis of MAP. The increased numbers of CD14+CD163+CD115+ M2 monocytes were positively correlated with the plasma CRP levels and the acute physiology and chronic health evaluation II scores, suggesting that the numbers of CD14+CD163+CD115+ monocytes may be a valuable biomarker for evaluating the severity of MAP. Our findings may provide new insights into the pathogenic process and immunoregulation of MAP.

INTRODUCTION
Mild acute pancreatitis (MAP), the mild form of acute pancreatitis, accounts for 80% of AP, and is mainly caused by gallstones and alcohol abuse[1]. Once inflammation damages the pancreas, the inactive digestive enzymes, such as trypsinogen, are activated, leading to autodigestion of the pancreas and triggering AP[2]. Immunocompetent cells, including monocytes and macrophages, participate in the development and progression of AP[3-5]. However, how monocytes regulate the pathogenesis of AP has not been clarified.

Monocytes are circulating white blood cells (WBCs), which differentiate into macrophages when they enter the tissue[6]. Macrophages can be classically activated as M1 or alternatively activated as M2 cells[7]. The M1 macrophages can produce many types of pro-inflammatory cytokines, including interleukin (IL)-12, IL-1β and IL-6, and defense against infectious pathogens and tumors. The M2 macrophages are characterized by expressing anti-inflammatory cytokines (*e.g.*, IL-10) and many other factors that regulate immune responses and tissue repair, but may promote tumor growth and metastasis[7,8]. Different types of macrophage responses and their relative IL-10 and IL-12 have been associated with different types of inflammatory diseases and are valuable for evaluating disease severity[9,10]. The M1 and M2 classification is initially proposed for macrophages, and can be extended to human peripheral blood monocytes[11]. The expression of both M1 and M2 markers is detected in circulating peripheral blood mononuclear cells (PBMCs), and the different types of polarized monocytes in peripheral blood are associated with different diseases[11-13]. However, little is known on whether the numbers of peripheral blood different types of polarized monocytes and the levels of plasma IL-10 and IL-12 can be valuable for evaluating the severity of MAP.

Peripheral blood monocytes and macrophages express CD14, which is one of the pattern-recognition receptors and a co-receptor of toll-like receptor[14,15]. MAC387 antibody can recognize calprotectin, a complex of intracellular myeloid-related proteins (MRPs) 8 and MRP14. The blood-derived MAC387+ monocytes/macrophages are recently infiltrating monocytes/macrophages and are important inflammatory components[16-18]. CD163 is a hemoglobin scavenger receptor expressed by peripheral blood monocytes and macrophages, polarizes monocytes/macrophages toward M2-phenotype and is crucial for the resolution of inflammation[19,20]. CD115 is macrophage colony-stimulating factor receptor (CSF-1R) and engagement of CSF-1R by CSF-1 polarizes toward M2-type macrophages and enhances their function[21]. CD115 is expressed by human monocytes. How different subsets of M1 and M2 monocytes regulate the pathogenesis of MAP has not been explored. Notably, anti-CD115 can inhibit inflammatory osteolysis and has potential anti-tumor effect, suggesting that CD115+ M2 monocytes/

Table 1 Demographic and clinical characteristics of participants

Parameter	MAP patients	Healthy controls
No.	24	13
Age (yr)	41 (25-60)	40 (26-59)
Sex, female/male	10/14	6/7
BMI	24.5	23.6
WBC (10^9/L)	9.34 (5.46-16.34)[1]	6.79 (4.87-9.3)
Neutrophils (10^9/L)	6.92 (3.50-14.16)[1]	4.16 (3.01-6.15)
Monocytes (10^9/L)	0.61 (0.23-1.94)	0.51 (0.37-0.61)
AMY (U/L)	302.8 (30.8-1845)[1]	73.05 (24.5-158.3)
LPS (U/L)	1135 (40.08-3200)[1]	72.38 (6-150.4)
CRP (mg/L)	16.62 (1.04-45.87)[1]	3.70 (0.43-7.25)
APACHE II score	6 (4-9)	NA

Data are median (range) or real case number. [1]$P < 0.05$ *vs* the controls. Normal values: WBC: 3.50-9.50 (10^9/L), Monocytes: 0.1-0.6 (10^9/L), AMY: 8-220 (U/L), LPS: 0-190 (U/L), CRP: 0-10 (mg/L). MAP: Mild acute pancreatitis; BMI: Body mass index; WBC: White blood cell counts; AMY: Amylase; LPS: Lipase; CRP: C-reactive protein; APACHE: Acute physiology and chronic health evaluation; NA: Not available.

macrophages are associated with inflammation and tumor growth[21-23]. However, there is no report on the numbers of CD115+ monocytes in MAP patients.

In this study, we characterized the numbers of different subsets of monocytes and the levels of plasma IL-10 and IL-12 in patients with newly diagnosed MAP and health controls. Furthermore, we analyzed the potential association of the numbers of different subsets of monocytes and the levels of plasma IL-10 and IL-12 with the levels of plasma C-reactive protein (CRP) and the acute physiology and chronic health evaluation (APACHE) II scores in MAP patients.

MATERIALS AND METHODS

Patients and controls
A total of 24 patients with new-onset MAP were recruited at the inpatient service of the Department of Gastroenterology, the Second Part of the First Hospital of Jilin University (Changchun, China) from July 2015 to March 2016. All patients met the Atlanta criteria of MAP[24]. Another 13 sex-, age- and ethnicity-matched healthy subjects were recruited from the Physical Examination Center of our hospital during the same period, and served as the controls. These controls had no history of autoimmune diseases, chronic inflammatory diseases, or allergies. The disease severity of individual patients was evaluated by APACHE II score. Patients were excluded if she/he had a history of malignant tumor, autoimmune diseases, any of other chronic inflammatory diseases, or had received immunosuppressive drugs within the past 3 mo. Written informed consent was obtained from individual subjects. The experimental protocol was established according to the guidelines of the Declaration of Helsinki and was approved by the Human Ethics Committee of Jilin University. The

demographic and clinical characteristics of individual participants are shown in Table 1.

Clinical data
The clinical data of each subject were collected from the hospital records. These data included age, sex, height, body weight, body mass index (BMI) and laboratory tests. Individual subjects were subjected to routine laboratory tests for full blood cell counts, the concentrations of plasma CRP, amylase (AMY) and lipase activities. The levels of plasma CRP were determined by scatter turbidimetry using a Siemens special protein analyzer (Siemens Healthcare Diagnostics Products, GmbH, Munich, Germany). The concentrations of plasma AMY and lipase were determined by ADVIA 1650 biochemical analyzer (Bayer, Pittsburg, PA, United States).

Flow cytometry analysis
Heparinized fasting venous blood samples (6 millilitres, mL) were collected from the median cubital vein of individual MAP patients (within 72 h after upper abdominal pain occurred) and control subjects, and PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Little Chalfont, United Kingdom). PBMCs at 1×10^6/tube were stained in duplicate with BV510-anti-CD14, PE-anti-CD115 (BD Biosciences, Franklin Lakes, NJ, United States), PE/Cy7-anti-CD163 and APC/Cy7-anti-206 (Biolegend, San Diego, CA, United States) in the dark at 4 ℃ for 30 min. After being washed, the cells were fixed and permeabilized using a fixation/permeabilization kit (BD Biosciences), followed by intracellular staining with FITC-anti-MAC387 (Abcam, Cambridge, United Kingdom). The fluorescence- and isotype-matched antibodies served as negative controls.

To detect the function, PBMCs (10^6 cells/well) were stimulated in duplicate with 50 ng/mL of lipo-polysaccharide, phorbol myristate acetate and 1.0 μg/mL of ionomycin (Sigma-Aldrich, St. Louis, United States) in 10% fetal bovine serum RPMI 1640 (complete medium) for 2 h at 37 ℃ in 5% CO_2 and exposed to Brefeldin A (GolgiPlug; BD Biosciences) for 4 h, as described previously in a study from our laboratory[15,25]. After being washed, the cells were stained with BV510-anti-CD14 and PE/Cy7-anti-CD163, fixed and permeabilized using the permeabilization solution, followed by intracellular staining with BV421-anti-IL-12 and PE-CF594-anti-IL-10 (BD Biosciences). The real positive and negative cells were distinguished by fluorescence minus one (FMO) and the cells were stained with all of the fluorochromes, except for the one that was being measured. The percentages of different subsets of monocytes were characterized on a FACSAria II (Becton, Dickinson and Company, Franklin Lakes, NJ, United States) and the data were analyzed by FlowJo software (v5.7.2; TreeStar,

Ashland, OR, United States). Finally, the numbers of different subsets of monocytes were calculated, based on the numbers of monocytes in individual subjects and expressed as the numbers of cells per mL.

Cytometric bead array analysis of plasma cytokines

The concentrations of plasma IL-10 and IL-12 were determined by Cytometric bead array (CBA), according to the manufacturer's protocol (BD Biosciences) with minor modification. Briefly, plasma samples (50 μL) from individual subjects were subjected in duplicate to analysis of the levels of plasma IL-10 and IL-12 using the CBA kit on a FACSAria II (Beckton, Dickinson and Company). The concentrations of plasma cytokines were quantified using the CellQuestPro and CBA software (Becton, Dickinson and Company). The limitation of detection for IL-10 and IL-12 is 3.3 pg/mL and 1.9 pg/mL, respectively.

Statistical analysis

Data are expressed as median and range. The difference between two groups was analyzed by the Mann-Whitney *U* nonparametric test. The relationship between variables was evaluated using the Spearman rank correlation test. All the statistical analyses were performed by the SPSS version 19.0 software. A two-sided *P* value of < 0.05 was considered statistically significant.

RESULTS

Increased numbers of M1 monocytes in MAP patients

To understand the importance of monocytes, 24 MAP patients and 13 controls were recruited. Among all the patients, 7 cases were induced by alcohol, 11 cases by hypertriglyceridemia, and 6 by cholelithiasis, and the length of hospital stay was 7-10 d. There was no significant difference in the distribution of age, sex and BMI as well as the number of PBMCs between the MAP patients and controls (Table 1). The numbers of peripheral blood WBCs and neutrophils were significantly higher in the patients than those in the controls (*P* < 0.05; Table 1). The values of AMY, lipase and CRP were significantly higher in the MAP patients than that in the controls. In addition, MAP patients displayed variable values of APACHE II score.

The numbers of peripheral blood CD14+CD163- M1 and CD14+CD163+ M2 monocytes in the MAP patients and controls were characterized by flow cytometry. As shown in Figure 1, the numbers of CD14+CD163- M1 monocytes were significantly higher in the patients than that in the controls (*P* = 0.0049). In contrast, there was no significant difference in the numbers of CD14+CD163+ M2 monocytes between the MAP patients and controls (*P* = 0.8362). Collectively, increased numbers of CD14+CD163- M1 monocytes existed in MAP patients.

Increased numbers of MAC387+ M1 monocytes in the MAP patients

The blood-derived MAC387+ monocytes/macrophages are recently infiltrating monocytes/macrophages and CD14+CD163-MAC387+ monocytes/macrophages are important inflammatory components[16-18]. To further understand the importance of monocytes, the numbers of peripheral blood CD14+CD163-MAC387+ and CD14+CD163+MAC387+ cells in the MAP patients and controls were characterized by flow cytometry (Figure 2). The numbers of CD14+CD163-MAC387+ cells in the MAP patients were significantly higher than that in the controls (*P* = 0.0091). However, there was no statistically significant difference in the numbers of CD14+CD163+MAC387+ cells between the MAP patients and controls (*P* = 0.9619). Thus, increased numbers of MAC387+ M1 monocytes were present in the MAP patients.

Increased numbers of CD115+ M2 monocytes in the MAP patients

Although there was no statistically significant difference in the numbers of CD14+CD163+ M2 monocytes between the MAP patients and controls, there are different subsets of M2 monocytes with different surface markers and varying functions[15,26]. M2 monocytes can express CD206 (the mannose receptor) and/or CD115[15,27]. To understand the importance of different subsets of M2 monocytes, the numbers of peripheral blood CD206+ and/or CD115+ M2 monocytes in the MAP patients and controls were further analyzed by flow cytometry. As shown in Figure 3, the numbers of CD14+CD163+CD115+ and CD14+CD163+CD115+CD206+ monocytes in the MAP patients were significantly higher than those in the controls (*P* = 0.0072 and *P* < 0.0001, respectively). However, there was no statistically significant difference in the numbers of CD14+CD163+CD206+ monocytes between the MAP patients and controls (*P* = 0.9873). Together, increased numbers of CD115+ M2 monocytes were present in the MAP patients.

Increased cytokine production in the MAP patients

M1 monocytes can produce IL-12 and are critical for inflammation. In contrast, M2 monocytes produce IL-10, which controls inflammation and promotes tumor growth. To understand the functions of M1 and M2 monocytes in the pathogenic process of MAP, the numbers of peripheral blood IL-12+ M1 monocytes and IL-10+ M2 monocytes were characterized by flow cytometry. There was no significant difference in the fluorescent intensity of anti-IL-12 and anti-IL-10 signals in monocytes between the MAP patients and controls (data not shown). As shown in Figure 4, the numbers of IL-12+ M1 monocytes were significantly higher in the MAP patients than the controls (*P* = 0.0202). Conversely, the numbers of IL-10+ M2 monocytes were

Figure 1 Flow cytometry analysis of peripheral blood monocytes. Peripheral blood mononuclear cells (PBMCs) were obtained from individual subjects and stained in duplicate with BV510-anti-CD14 and PE/Cy7-anti-CD163 or isotype control antibodies. The cells were gated initially on living mononuclear cells and then on CD14+ monocytes. Subsequently, the frequency of CD14+CD163- M1 and CD14+CD163+ M2 monocytes were determined by flow cytometry and the numbers of each subset of monocytes were calculated, based on total numbers of monocytes. Data are representative charts of flow cytometry and expressed as the mean values of individual subjects (n = 13 for the healthy controls, n = 24 for the patients). A: Flow cytometry analysis; B: Quantitative analysis. The horizontal lines indicate the median values for individual groups. FSC: Forward scatter; SSC: Side scatter; HC: Healthy control; MAP: Mild acute pancreatitis.

significantly lower in the MAP patients than the controls (P = 0.022). As a result, the ratios of the numbers of CD14+CD163-IL-12+ to CD14+CD163+IL-10+ monocytes in the MAP patients were significantly higher than that in the controls. Further CBA analysis indicated that the concentrations of plasma IL-12 and IL-10 were significantly higher in the MAP patients than that in the controls (P = 0.0065 and P = 0.0001, respectively). Hence, increased levels of plasma pro-inflammatory IL-12 and anti-inflammatory IL-10 existed in the MAP patients.

Correlation analysis of clinical parameters with different subsets of monocytes and cytokines in the MAP patients

Finally, the potential relationship between the values of clinical parameters and the numbers of different subsets of monocytes and the levels of cytokines was analyzed in the MAP patients. As shown in Figure 5, the levels of plasma CRP were positively correlated with the numbers of CD14+CD163- (R = 0.5009, P = 0.0127) and

CD14+CD163-MAC387+ (R = 0.5079, P = 0.0113) M1 monocytes, and CD14+CD163+CD115+ (R = 0.4565, P = 0.0249) M2 monocytes, but not with CD14+CD163+ M2 monocytes in this population. Furthermore, the APACHE II scores were positively correlated with the numbers of CD14+CD163+CD115+ (R = 0.4581, P = 0.0244) M2 monocytes, and the levels of plasma IL-10 (R = 0.4178, P = 0.0422) in this population of patients. There was no other significant correlation among these measures in this population. Collectively, increased numbers of CD14+CD163- and CD14+CD163-MAC387+ M1, and CD14+CD163+CD115+ M2 monocytes and elevated levels of plasma IL-10 may contribute to imbalance of pro-inflammatory and anti-inflammatory responses during the process of MAP, and the numbers of CD14+CD163+CD115+ monocytes may serve as a biomarker for evaluating MAP severity.

DISCUSSION

Monocytes and macrophages are important in-

Figure 2 Flow cytometry analysis of peripheral blood MAC387+ monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from individual subjects and stained in duplicate with BV510-anti-CD14 and PE/Cy7-anti-CD163 or isotype controls. After being washed, the cells were fixed and permeabilized using a fixation/permeabilization kit, followed by intracellular staining with FITC-anti-MAC387. The numbers of peripheral blood CD14+CD163+MAC387+ and CD14+CD163-MAC387+ monocytes were analyzed by flow cytometry. Data are representative FACS charts and expressed as the mean numbers of each type of cells in individual subjects. The horizontal lines indicate the median values for each group. FSC: Forward scatter; SSC: Side scatter; HC: Healthy control; MAP: Mild acute pancreatitis.

flammatory cells and their activation is associated with the severity of AP[28]. In this study, the numbers of different subsets of peripheral blood monocytes and their potential association with disease severity were analyzed in 24 MAP patients. In comparison with healthy controls, significantly increased numbers of CD14+CD163-, CD14+CD163-MAC387+ and CD14+CD163-IL-12+ M1 monocytes and reduced numbers of CD14+CD163+IL-10+ monocytes were detected in the MAP patients. Furthermore, significantly higher levels of plasma IL-12 were observed in the MAP patients. More importantly, the levels of plasma CRP were positively correlated with the numbers of CD14+CD163- and CD14+CD163-MAC387+ M1 monocytes in the MAP patients. These data were consistent with a previous report of M1-polarized macrophages at the early stage in rats with AP[29]. Similarly, our previous study had found that peripheral blood polarized M1 monocytes are present

in patients with tuberculous pleural effusion (TPE)[15]. Given that the levels of plasma CRP reflect the degrees of systemic inflammation, these findings support the notion that pro-inflammatory monocytes participate in the pathogenesis of MAP[30,31]. Hence, targeting pro-inflammatory M1 monocytes may be valuable for control of MAP.

MAC387+ macrophages are recently recruiting macrophages, and increased numbers of MAC387+ macrophages are associated with poor prognosis in cancer patients[16-18,32-34]. In this study, we found significantly increased numbers of circulating MAC387+ M1 monocytes in the MAP patients, similar to the findings in patients with encephalitis[16]. Furthermore, the numbers of CD14+CD163-MAC387+ M1 monocytes were positively associated with the levels of plasma CRP in the MAP patients. These data suggest that during the pathogenic process of MAP, continual differentiated M1 monocytes display in MAP patients,

Figure 3 Flow cytometry analysis of peripheral blood CD206+ and CD115+ M2 monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from individual subjects and stained in duplicate with BV510-anti-CD14, PE/Cy7-anti-CD163, PE-anti-CD115, and APC/Cy7-anti-206 or isotype controls. The numbers of peripheral blood CD14+CD163+CD206+, CD14+CD163+CD115+ and CD14+CD163+CD206+CD115+ M2 monocytes were analyzed by flow cytometry. Data shown are representative FACS charts and expressed as the mean numbers of each type of cells per mL of blood in individual subjects. The horizontal lines indicate the median values for each group. FSC: Forward scatter; SSC: Side scatter; HC: Healthy control; MAP: Mild acute pancreatitis.

creating a positive feedback loop to strengthen pro-inflammatory responses. These novel findings may provide new insights into the pathogenesis of MAP.

During the pathogenic process of MAP, inflammatory stimuli damage the pancreatic acinar cells to release many inflammatory cytokines and mediators, which recruit leukocyte infiltrates, including M2 monocytes/macrophages[28,35]. M2 monocytes/macrophages can produce anti-inflammatory molecules that control inflammation and promote tissue repair[36]. In this study, we found significantly increased numbers of CD14+CD163+CD115+ M2 monocytes and significantly elevated levels of IL-10 in MAP patients. More importantly, the numbers of CD14+CD163+CD115+ M2 monocytes were positively correlated with the levels of plasma CRP and the APACHE II scores in MAP patients. Furthermore, the levels of plasma IL-10 were positively correlated with the APACHE II scores

Figure 4 Analysis of peripheral blood IL-12+ M1 and IL-10+ M2 monocytes and the concentrations of plasma IL-10 and IL-12. Peripheral blood mononuclear cells (PBMCs) were isolated from individual subjects and stimulated with lipopolysaccharide /phorbol myristate acetate/ionomycin *in vitro*. The cells were stained in duplicate with BV510-anti-CD14 and PE/Cy7-anti-CD163 or isotype controls. The cells were fixed, and permeabilized, followed by intracellular staining with BV421-anti-IL-12 and PE-CF594-anti-IL-10. The numbers of peripheral blood CD14+CD163-IL-12+ M1 and CD14+CD163+IL-10+ M2 monocytes in individual subjects were determined by flow cytometry. The concentrations of plasma IL-10 and IL-12 in individual subjects were determined by cytometric bead analysis. Data are representative FACS charts or the mean numbers of each type of cells and the mean values of each type of cytokine in individual subjects. The horizontal lines indicate the median values for each group. FSC: Forward scatter; SSC: Side scatter; HC: Healthy control; MAP: Mild acute pancreatitis.

in the MAP patients. However, our previous study had shown a decreased number of peripheral blood CD14+CD163+CD115+ M2 monocytes, which is not significantly associated with the clinical measures in TPE patients[15]. We speculate the different results may stem from the different diseases. Engagement of CD115 by CSF-1 is crucial for the survival, differentiation and possible activation of monocytes/macrophages, and can polarize macrophages toward M2-type[21,23]. Previous studies have shown that anti-CD115 can enhance anti-tumor immunity and ameliorate inflammation in humans and rodents[22,23,37-39]. The increased numbers of CD14+CD163+CD115+ M2 monocytes and elevated levels of plasma IL-10 may reflect a compensatory response during the inflammatory process of MAP[40,41]. Alternatively, CD14+CD163+CD115+ monocytes may through unknown factors promote the pathogenesis of MAP. Given that APACHE II scores and the levels of plasma CRP are two standard measures for the severity of inflammatory diseases, the positive correlations suggest that the numbers of CD14+CD163+CD115+ monocytes and the levels of plasma IL-10 may be valuable for evaluating the disease severity in patients with MAP.

IL-10 is a key anti-inflammatory cytokine and can control inflammation[42]. IL-10 is associated with immunosuppression in the late phase of severe AP[43].

Previous studies have revealed elevated levels of plasma IL-10 and IL-12 in AP patients and that the levels of plasma IL-10 are biomarkers for evaluating the severity of AP[9,10,41,44]. In this study, we also observed significantly higher concentrations of plasma IL-10 and IL-12 in MAP patients. The levels of plasma IL-10 were positively correlated with the APACHE II scores in the MAP patients. However, we found significantly reduced numbers of CD14+CD163+IL-10+ M2 monocytes in the MAP patients. The contradictory data suggest that the elevated levels of plasma IL-10 may stem from other anti-inflammatory cells, such as bone marrow derived mesenchymal stem cells (MSCs), regulatory T and B cells as well as some types of dendritic cells[45]. Indeed, infusion with human MSCs inhibits pancreatitis and ameliorates tissue damage by enhancing regulatory T cell responses in a rat model of MAP[46] and restoration of regulatory B cells inhibits pancreatitis in CD19-/- mice[47]. Accordingly, it is possible that the elevated levels of plasma IL-10 from other anti-inflammatory cells may compensatively limit inflammation during the pathogenic process of MAP. We are interested in further investigating the immunoregulation during the pathogenesis of MAP.

In conclusion, our data indicated significantly increased numbers of pro-inflammatory CD14+CD163-, CD14+CD163-MAC387+ and CD14+CD163-

Figure 5 Correlation analysis. The potential correlations between the numbers of each subset of monocytes and APACHE II scores or CRP levels in those patients were analyzed by the Spearman rank correlation test. Data shown are the values of individual patients (n = 24). CRP: C-reactive protein; APACHE: Acute physiology and chronic health evaluation.

IL-12+ M1 monocytes and reduced numbers of CD14+CD163+IL-10+ monocytes in the MAP patients. Furthermore, the levels of plasma CRP were positively correlated with the numbers of CD14+CD163- and CD14+CD163-MAC387+ M1 monocytes in the MAP

patients. In addition, we detected significantly increased numbers of CD14+CD163+CD115+ monocytes and levels of plasma IL-10 in MAP patients. These data support the notion that pro-inflammatory monocytes participate in the pathogenesis of MAP. Moreover, the

numbers of CD14+CD163+CD115+ monocytes were positively correlated with the levels of plasma CRP and the APACHE II scores in MAP patients, suggesting that the numbers of CD14+CD163+CD115+ monocytes may be a valuable biomarker for evaluating the severity of MAP. To the best of our knowledge, this was the first study on the numbers of peripheral blood different phenotypes of monocytes in patients with new-onset MAP. Our findings may provide new insights into the pathogenic process and immunoregulation of MAP. We recognized that our study had limitations, such as a relatively small sample size and the lack of longitudinally functional study of monocytes during the pathogenic process of MAP. We are interested in further investigating the values of these subsets of monocytes in a bigger population and in the moderate or severe acute pancreatitis to understand their roles in the pathogenesis of different types of AP.

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COMMENTS

Background
Monocytes participate in the inflammatory process of mild acute pancreatitis (MAP) and their function is associated with the severity of MAP.

Research frontiers
To date, it is still unclear how different subsets of monocytes regulate the pathogenesis of MAP.

Innovations and breakthroughs
This study demonstrated that increased numbers of CD14+CD163- and CD14+CD163-MAC387+ M1 monocytes participate in the pathogenesis of MAP. Increased numbers of CD14+CD163+CD115+ M2 monocytes may be a valuable biomarker for evaluating the severity of MAP.

Applications
The present findings may provide new insights into the pathogenic process and immunoregulation of MAP.

Peer-review
The authors investigated the numbers of different subsets of monocytes and their associations with clinical markers of patients with MAP. They found that increased numbers of CD14+CD163- and CD14+CD163-MAC387+ M1 monocytes participated in the pathogenesis of MAP. Increased numbers of CD14+CD163+CD115+ M2 monocytes may be a valuable biomarker for evaluating the severity of MAP. These findings are interesting, and may provide new insights into the pathogenic process and immunoregulation of MAP.

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ORIGINAL ARTICLE

Prospective Study

Clinical correlations of infliximab trough levels and antibodies to infliximab in South Korean patients with Crohn's disease

Eun Hye Oh, Dae-Hyun Ko, Hyungil Seo, Kiju Chang, Gwang-Un Kim, Eun Mi Song, Myeongsook Seo, Ho-Su Lee, Sung Wook Hwang, Dong-Hoon Yang, Byong Duk Ye, Jeong-Sik Byeon, Seung-Jae Myung, Suk-Kyun Yang, Sang Hyoung Park

Eun Hye Oh, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul 05505, South Korea

Dae-Hyun Ko, Department of Laboratory Medicine, Hallym University College of Medicine, Dongtan Sacred Heart Hospital, Hwaseong 18450, South Korea

Hyungil Seo, Kiju Chang, Gwang-Un Kim, Eun Mi Song, Myeongsook Seo, Sung Wook Hwang, Dong-Hoon Yang, Byong Duk Ye, Jeong-Sik Byeon, Seung-Jae Myung, Suk-Kyun Yang, Sang Hyoung Park, Department of Gastroenterology, University of Ulsan College of Medicine, Asan Medical Center, Seoul 05505, South Korea

Ho-Su Lee, Health Screening and Promotion Center, University of Ulsan College of Medicine, Asan Medical Center, Seoul 05505, South Korea

Abstract

AIM
To investigate the clinical implications of infliximab

trough levels (IFX-TLs) and antibodies to infliximab (ATI) levels in Crohn's disease (CD) patients in Asian countries.

METHODS
IFX-TL and ATI level were measured using prospectively collected samples obtained with informed consent from CD patients being treated at Asan Medical Center, South Korea. We analyzed the correlations between IFX-TLs/ATI levels and the clinical activity of CD (quiescent *vs* active disease) based on the CD activity index, C-reactive protein level, and physician's judgment of patients' clinical status at enrollment. The impact of concomitant immunomodulators was also investigated.

RESULTS
This study enrolled 138 patients with CD (84 with quiescent and 54 with active disease). In patients with quiescent and active diseases, the median IFX-TLs were 1.423 μg/mL and 0.163 μg/mL, respectively ($P < 0.001$) and the median ATI levels were 8.064 AU/mL and 11.209 AU/mL, respectively ($P < 0.001$). In the ATI-negative and -positive groups, the median IFX-TLs were 1.415 μg/mL and 0.141 μg/mL, respectively ($P < 0.001$). In patients with and without concomitant immunomodulator use, there were no differences in IFX-TLs (0.632 μg/mL and 1.150 μg/mL, respectively; $P = 0.274$) or ATI levels (8.655 AU/mL and 9.017 AU/mL, respectively; $P = 0.083$).

CONCLUSION
IFX-TL/ATI levels were well correlated with the clinical activity in South Korean CD patients. Our findings support the usefulness of IFX-TLs/ATI levels in treating CD patients receiving IFX in clinical practice.

Key words: Infliximab; Drug effect; Antibody; Crohn's disease; Drug monitoring

Core tip: This study aimed to clarify the clinical implications of infliximab trough levels (IFX-TLs) and antibodies to infliximab (ATI) levels. They were measured using prospectively collected samples in 138 Crohn's disease (CD) patients being treated at Asan Medical Center, South Korea. Correlations between IFX-TLs/ATIs and the clinical activity ($P < 0.001$) were verified in the study. Our findings support the usefulness of IFX-TLs/ATI levels in treating CD patients receiving IFX in clinical practices.

INTRODUCTION

Crohn's disease (CD) is a chronic systemic inflammatory disease that mainly affects the gastrointestinal tract[1]. It is relatively prevalent in developed countries in North America and Europe, affecting up to 0.5% of the general population[2]. However, its prevalence has doubled over the past decade in countries in East Asia[3,4].

The introduction of biologic agents blocking tumor necrosis factor (TNF)-α has greatly modified the treatment strategies for CD and the effects of these agents on remission induction and maintenance has been clearly shown[5]. However, the human body can develop antibodies against infliximab (IFX), the first and most widely used biologic agent for CD treatment. Antibodies to infliximab (ATIs) are thought to be associated with an infusion reaction and reduce the effect of the drug by decreasing its serum level[6,7]. For these reasons, monitoring of the IFX trough levels (IFX-TLs) and ATI levels has been recommended by some experts[8]. However, there are few data clearly defining the relationship among IFX-TLs, ATI levels, and the clinical activity, especially in Asian countries[9]. Besides, the clinical implications and applications of the results in daily clinical practice are still a matter of debate, although the value of the measurement of IFX-TLs/ATI levels for therapy adjustment is undisputable because of practical issues such as cost, the lack of a universally valid assay, and the absence of a cutoff level clearly related to clinical outcomes[10].

In this study, therefore, we analyzed the correlation between IFX-TLs/ATI levels and the clinical activity in South Korean patients with CD using a prospectively collected samples to evaluate the usefulness of therapeutic drug monitoring (TDM) in clinical practice.

MATERIALS AND METHODS

Study population
Between March and May 2015, we enrolled 138 CD patients, aged 17-50 years, receiving IFX as maintenance therapy at the Inflammatory Bowel Disease (IBD) center of Asan Medical Center, a tertiary university hospital in Seoul, South Korea. They gave informed consent prior to being enrolled in the study. All patients unwilling to provide consent were excluded from the study. Patients aged less than 17 years, diagnosed with ulcerative colitis or any other IBD, and on biologic agents other than IFX were also excluded. IFX was administered at an 8-wk interval, mostly at a dose of 5 mg/kg body weight as maintenance therapy[11]. Out of 138 patients, 27 (19.57%) were receiving a double-dose of IFX (10 mg/kg) at the time of enrollment because of a lack of response to the usual maintenance dose. Interval shortening for dose intensification is not reimbursed in South Korea.

Data collection
During the study period, serum samples were

obtained from every patient a few hours before IFX administration. The samples were then stored at -20 ℃ until analysis. IFX-TLs and ATI levels were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits (IDKmonitor® - K9655, K9650; Immundiagnostik AG, Bensheim, Germany).

Measurement of IFX-TLs
Standards, controls, and samples were diluted 200 times and pipetted into wells in duplicate. They were incubated with shaking for 1 h and then for an additional 1 h with conjugate solution at room temperature. After washes, they were mixed with TMB (tetramethylbenzidine) substrate solution and incubated in the dark. Finally, STOP solution was added and the results were checked with an ELISA reader at 450 nm.

Measurement of ATI levels
Controls (negative, positive, and cutoff controls) and samples were mixed with buffers and pretreated with gentle shaking at room temperature for 30 min to dissociate ATIs from IFX. After being washed and pipetted into wells in duplicate, they were incubated overnight at 4 ℃. Then, the samples were incubated for 1 h at room temperature with diluted conjugate solution and for an additional 10-20 min with TMB substrate solution in the dark. Finally, STOP solution was added and the results were checked with an ELISA reader at 450 nm. The result was considered negative if the optical density (OD) of the sample was lower than that of the cutoff control. If the OD was higher than that of the cutoff control, the result was considered positive. The ATI level of the cutoff control was set to 10 AU/mL[9] and the ATI level of the sample was calculated with the formula: ATI level of the sample = mean of the sample ODs/mean of the cutoff control ODs × 10 AU/mL.

Assessment of clinical activity was based on the CD activity index (CDAI), serum C-reactive protein (CRP) level, and physician's judgment of the patients' clinical status at the time of enrollment[12]. If a patient's CDAI score was below 150, the serum CRP was within the normal range (< 0.6 mg/dL), and the physician considered that the effect of IFX had lasted for 8 wk at the time of enrollment, the patient was categorized into the quiescent group. However, if a patient's CDAI score was above 150, the serum CRP was above the upper normal limit ($\geqslant$ 0.6 mg/dL), or the physician considered that the effect of IFX lasted less than 8 wk, the patient was categorized into the active group. In addition, we also investigated if concomitant immunomodulators, such as azathioprine/6-mercaptopurine or methotrexate, were administered at the time of enrollment.

The study protocol was approved by the Institutional Review Board of Asan Medical Center (IRB No. 2015-0173).

Statistical analysis
Demographics and baseline characteristics were summarized using descriptive statistics. Patients were categorized into groups by their ATI status and the clinical activity. Differences between the groups were compared using the Student *t* test. Correlations among IFX-TLs/ATI levels and the clinical activity were analyzed with logistic regression analysis. The diagnostic power of IFX-TLs was investigated using area under the receiver-operator characteristic (ROC) curve analysis to obtain the area under the curve (AUC) and 95%CI. The cutoff value for the IFX-TLs that identified disease activity was determined by identifying the point closest to the 1.0 angle. Data were evaluated with SPSS® statistics version 23.0 for Windows (SPSS Inc., Chicago, IL) and *P* < 0.05 was considered statistically significant.

RESULTS

Patient characteristics
The baseline characteristics of the patients are presented in Table 1. Of the 138 patients with CD, 90 (65.2%) were male; the median age at diagnosis and first infusion of IFX were 21 years [interquartile range (IQR) 19-27 years] and 27 years (IQR 22-33 years), respectively. The average duration from diagnosis to first IFX administration was 52 mo (IQR 13-91 mo) and the median follow-up duration with IFX was 47 mo (IQR 30-73 mo) (Table 1).

Categorization of the patients into groups
Out of the 138 patients, 84 (60.9%) were categorized into the quiescent group and 54 (39.1%) were categorized into the active group based on our assessment of clinical activity (Figure 1). The median IFX-TL of all patients was 0.941 μg/mL (IQR 0.189-2.143 μg/mL). The cutoff value for identifying quiescent disease by ROC analysis was 0.68 μg/mL (AUC 0.90, 95%CI: 0.84-0.95, sensitivity 83%, specificity 84%). The median ATI level of all patients was 8.846 AU/mL (IQR 7.719-16.727 AU/mL). Finally, 91 patients (65.9%; median 7.619 AU/mL; IQR 6.857-8.834) were categorized as ATI negative and 47 patients (34.1%; median 47.381; IQR 15.381-146.630) were categorized as ATI positive (Figure 1).

Among all patients, 65 (47.1%) were taking concomitant immunomodulators (64 patients with azathioprine/6-mercaptopurine and 1 patient with methotrexate) at the time of the study. By clinical activity, 31 out of 84 patients in the quiescent group (36.9%) and 34 out of 54 patients in the active group (63.0%) were taking concomitant immunomodulators. In addition, 44 out of 91 patients in the ATI-negative group (48.4%) and 21 out of 47 patients in the ATI-positive group (44.7%) were taking concomitant immunomodulators.

Figure 1 Scatter diagram of the study patients. Of these 138 subjects, 84 (60.9%) had quiescent disease and 54 (39.1%) had active disease. The overall median infliximab trough level (IFX-TL) value was 0.941 μg/mL. In total, 91 patients were antibodies to infliximab (ATI) negative (65.9%) and 47 patients were ATI positive (34.1%), with an overall median ATI value of 8.846 (IQR 7.719-16.727) AU/mL.

Table 1 Demographics and baseline characteristics of the 138 South Korean patients in this study with Crohn's disease who received infliximab treatment *n* (%)

Variables	Value
Male/female	90/48 (65.2/34.8)
Median age at diagnosis (IQR) (yr)	21 (19-27)
Median age at first infusion (IQR) (yr)	27 (22-33)
Median duration of disease prior to first IFX (IQR) (mo)	52 (13-91)
Indication for IFX treatment	
Luminal	122 (88.4)
Perianal fistulizing	16 (11.6)
Median follow-up of IFX treatment (IQR) (mo)	47 (30-73)
Disease location at diagnosis	
L1 (ileum)	19 (13.8)
L2 (colon)	9 (6.5)
L3 (ileocolon)	108 (78.3)
Not documented	2 (1.4)
Disease behavior at diagnosis	
B1 (non-stricturing non-penetrating)	115 (83.3)
B2 (stricturing)	7 (5.1)
B3 (penetrating)	16 (11.6)
Perianal fistula at diagnosis	
Active	36 (26.1)
Previous	26 (18.8)
Smoking status at diagnosis	
Current smoker	26 (18.8)
Ex-smoker	9 (6.6)
Never smoker	103 (74.6)
Previous major abdominal surgery prior to first IFX	44 (31.9)
Concomitant immunomodulators	
None	73 (52.9)
Azathioprine/6-mercaptopurine	64 (46.4)
Methotrexate	1 (0.7)

IQR: Interquartile range; IFX: Infliximab.

Comparison of differences by group

In patients with quiescent and active disease, the median IFX-TLs were 1.423 μg/mL (IQR 0.877-2.483) and 0.163 μg/mL (IQR 0.002-0.636), respectively (*P* < 0.001), and the difference between the median values

Table 2 Factors affecting the clinical activity of Crohn's disease: Results of logistic regression analysis of each factor

	IFX-TL (μg/mL)	ATI levels (AU/mL)
Overall predictability (%)	82.0	75.9
Quiescent group (%)	82.9	96.3
Active group (%)	80.4	43.1
P value	< 0.001	0.003
Odds ratio (95%CI)	0.103 (0.045-0.236)	1.055 (1.018-1.093)

IFX-TL: Infliximab trough level; ATI: Antibody to infliximab.

was 1.260 μg/mL. The median ATI levels were 8.064 AU/mL (IQR 6.929-9.908) and 11.209 AU/mL (IQR 8.008-118.835) in patients with quiescent and active disease, respectively (*P* < 0.001), and the difference between the median values was 3.145 AU/mL (Figure 2). In the ATI-negative and -positive groups, the median IFX-TLs were 1.415 μg/mL (IQR 0.570-2.495) and 0.141 μg/mL (IQR 0.002-0.869), respectively (*P* < 0.001), and the difference between the median values was 1.274 μg/mL (Figure 3). In patients with and without concomitant immunomodulator use, there were no differences in IFX-TLs (0.632 μg/mL and 1.150 μg/mL, respectively; *P* = 0.274) or ATI levels (8.655 AU/mL and 9.017 AU/mL, respectively; *P* = 0.083) (Figure 4).

Correlation analysis of variables

After excluding 5 patients without the tendencies of the other patients (3 active patients with high IFX-TLs and low ATI levels and 2 quiescent patients with low IFX-TLs and high ATI levels), we used logistic regression analysis to analyze IFX-TLs and ATI levels as independent factors affecting the clinical activity of CD. The analysis found an explanation power of 54% and 37.4% and overall predictability of 82% and 75.9% for IFX-TL and ATI, respectively (Table 2). By analyzing these 2 factors (IFX-TLs and ATI levels) together, 69 out of 82 patients in the quiescent group (84.1%) and 38 out of 51 patients in the active group (74.5%) were correctly predicted, with an overall predictability of 80.5%. The odds ratios of IFX-TLs and ATI levels were 0.150 (95%CI: 0.065-0.349, *P* < 0.001) and 1.028 (95%CI: 1.003-1.053, *P* = 0.014), respectively (Table 3). Therefore, these factors were verified to be significantly associated with the clinical activity of CD.

DISCUSSION

The most important finding of our current study was that the IFX-TL/ATI levels were well correlated with the clinical activity of CD in South Korean patients. Definite inverse correlations between IFX-TLs and the clinical activity (*P* < 0.001) and between IFX-TLs and ATI levels (*P* < 0.001) were verified in this analysis. Additionally, we found a correlation between ATI levels

A

B

Figure 2 Comparisons of the infliximab trough levels and antibody to infliximab levels between patients with quiescent or active disease. The median infliximab trough levels (IFX-TLs) were 1.423 μg/mL (IQR 0.877-2.483) and 0.163 μg/mL (IQR 0.002-0.636), respectively ($P < 0.001$) (A) and the median antibodies to infliximab (ATIs) levels were 8.064 AU/mL (IQR 6.929-9.908) and 11.209 AU/mL (IQR 8.008-118.835), respectively ($P < 0.001$) (B) in patients with quiescent and active disease.

Figure 3 Comparison of the infliximab trough levels between patients with/without antibody to infliximab. In the ATI-negative and -positive groups, the median infliximab trough levels (IFX-TLs) were 1.415 μg/mL (IQR 0.570-2.495) and 0.141 μg/mL (IQR 0.002-0.869), respectively ($P < 0.001$). ATI: Antibodies to infliximab.

Table 3 Factors affecting the clinical activity of Crohn's disease: Results of logistic regression analysis of the 2 factors together

	IFX-TL (μg/mL)	ATI levels (AU/mL)
Overall predictability (%)	80.5	
Quiescent group (%)	84.1	
Active group (%)	74.5	
P value	< 0.001	0.027
Odds ratio (95%CI)	0.150 (0.065-0.349)	1.028 (1.003-1.053)

IFX-TL: Infliximab trough level; ATI: Antibody to infliximab.

and the clinical activity ($P < 0.001$). To the best of our knowledge, this study is the first to evaluate and define the correlations between IFX-TLs/ATI levels and the clinical activity in South Korean CD patients. In addition, the number of study subjects (138 patients) was larger than that of previous Asian studies (less than 100 patients). Thus far, only a few Asian studies have evaluated the usefulness of IFX drug monitoring in IBD patients[9,13,14]. In 2012, a study from Japan evaluated the clinical utility of a novel methodology to measure serum ATI levels in 58 patients with CD. This study found that patients positive for ATIs had significantly lower serum trough levels of infliximab ($P < 0.01$) and significantly higher clinical activity scores ($P < 0.001$) than patients negative for ATI[9]. Another Japanese study revealed a correlation between the clinical efficacy of IFX and serum IFX-TLs in 57 patients with CD ($P < 0.01$)[14].

In recent decades, anti-TNF-α agents have been introduced and become widely used in the management of CD. This change in the treatment paradigm for CD may alter the natural history of CD. Several studies have reported that the prognosis of CD, such as the hospitalization or surgery rate, improved after

the introduction of anti-TNF-α therapy[15]. In Asian countries, the incidence of CD has rapidly increased and anti-TNF-α agents have been used increasingly earlier and more frequently in recent decades[4,16]. However, despite the clinical efficacy of this treatment, up to 40% of patients do not respond to induction therapy with anti-TNF-α agents[17-19]. Additionally, about 20% of initial responders may lose responsiveness to anti-TNF-α therapy each year[5]. Because of this phenomenon, the importance of TDM, such as that of drug and anti-drug antibody levels, has been highlighted recently for the establishment of appropriate strategies in clinical practice. This TDM-based approach has been especially emphasized in maintenance therapy, in cases of a loss of response, persistent elevation of CRP, and persistent mucosal lesions[20-25]. In addition, this kind of strategy provides significant cost savings compared with conventional IFX dose intensification in CD patients with a loss of response[26]. However, in the TAXIT trial, which has taken a more proactive approach than before, with TDM applied to patients still responding to maintenance therapy, a TDM-based dose adjustment was not superior to dose adjustment based on symptoms alone[27]. In other words, we should selectively adjust TDM-based personalized treatment strategies to improve the outcomes of IBD patients in daily clinical practice. Additionally, the major limitation of previous studies regarding ATI was an inability to identify whether or not ATIs were neutralizing the drug, because the presence of ATI in serum does not

A

B

Figure 4 Comparisons of infliximab trough levels and antibody to infliximab levels in patients with/without immunomodulators. There were no differences in the median infliximab trough level (IFX-TL) (0.632 μg/mL and 1.150 μg/mL, respectively; P = 0.274) (A) and median antibodies to infliximab (ATIs) (8.655 AU/mL and 9.017 AU/mL, respectively; P = 0.083) (B) levels between the 2 groups according to the use of concomitant immunomodulators.

necessarily correlate with a loss of response[28].

Our optimal cutoff value for IFX-TLs for identifying disease activity was 0.68 μg/mL, which is consistent with previous studies. A study from Denmark found an optimal cutoff value of IFX ≥ 0.5 μg/mL for the prevention of a loss of response to IFX[29]. A Japanese study reported that more potent effects were achieved at higher serum IFX trough levels and that the threshold of the clinical responses was an IFX trough level of 1.0 μg/mL[14]. Another study from Japan suggested an optimal cutoff value of IFX-TLs of 0.6 μg/mL for CRP[13]. However, previous studies suggested various cutoff values of IFX for predicting the efficacy of IFX treatment from below 1 μg/mL to over 7 μg/mL[10]. This heterogeneity could be due to different methodologies, different study designs and subject characteristics, and different endpoints.

In our current analysis, IFX-TLs/ATI levels were not significantly different between the groups treated with and without concomitant immunomodulators. The addition of immunomodulators to anti-TNF-α treatment can improve the efficacy of anti-TNF-α treatment in IBD[30,31], especially in patients who are naïve to both immunomodulators and IFX, and can decrease ATI formation, even at suboptimal doses[32]. However, we did not observe any ability for immunomodulators such as azathioprine/6-mercaptopurine or methotrexate to increase IFX-TLs or decrease ATI formation, possibly because of the heterogeneity of the study subjects and the limitation of a study design. In general, patients in the active group seemed more frequently to be in a combination regimen to maximize the efficacy of IFX than patients in the quiescent group. In our study, 34 out of the 54 patients in the active group received concomitant immunomodulators and 31 out of the 84 patients in the quiescent group received concomitant immunomodulators at the time of enrollment (63.0% *vs* 36.9%, P = 0.003).
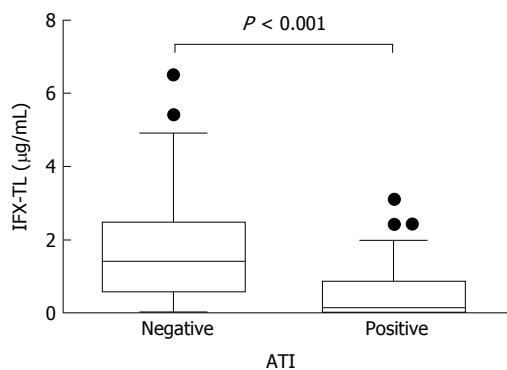
Among our study patients, we observed 5 patients not following the tendencies of the other patients (3 active patients with high IFX-TLs and low ATI levels and 2 quiescent patients with low IFX-TLs and high

ATI levels). For the former 3 patients, there are 2 possible interpretations of this situation: (1) high inflammatory burden of the disease; or (2) factors other than TNF-α that play a major pathologic role in these patients[33]. In this situation, we should consider dose intensification or switching to another class of drugs such as anti-integrins. For the latter 2 patients, their clinical activity is low despite low IFX-TLs and high ATI levels. In other words, their clinical activity is controlled regardless of anti-TNF-α therapy. In this situation, we should consider stopping the anti-TNF-α therapy if long-term deep remission is achieved[33].

The major potential limitation of this study was the use of time-consuming and difficult-to-apply ELISA-based commercial kits to obtain IFX-TL/ATI values. The usual turnaround time of ELISA-based kits for IFX-TLs/ATI levels is at least 8 h, which would delay the target dosage adjustment of the subsequent infusion. However, recent efforts to replace commonly used ELISA-based kits with a rapid, user-friendly, point-of-care IFX assay would make immediate target dosage adjustment possible in the near future[34]. Another limitation of our current study was the lack of analysis of mucosal healing. In our institution, we routinely check CDAI and perform blood tests, including CRP, in CD patients on IFX maintenance therapy during follow-up. Therefore, we used the CDAI, CRP and the physician's judgement of the duration of the IFX effect to assess the clinical activity of CD in this study. Likewise, previous studies used various outcomes to evaluate the usefulness of IFX-TLs/ATI levels, including clinician's assessment, patient-reported outcomes such as CDAI or the Harvey-Bradshaw index, or surrogate markers such as CRP[12]. A recent study from Japan evaluating the relationship between serum IFX-TLs and disease activities in 45 patients with CD showed that endoscopic activity negatively correlated with serum IFX-TLs and that mucosal healing requires a higher IFX trough level than that required to achieve the normalization of other clinical markers such as albumin and CRP[13].

In conclusion, IFX-TL/ATI levels are well correlated

with the clinical activity of South Korean CD patients. Our findings confirm the usefulness of IFX-TLs/ATI levels in treating CD patients receiving IFX in clinical practice. Therefore, we can use IFX-TLs/ATI levels in making decisions in patients with loss of response to IFX therapy. Further larger prospective studies are warranted to establish guidelines and to reveal the ability of concomitant immunomodulators to decrease the formation of ATIs.

COMMENTS

Background

The prevalence of Crohn's disease (CD) is rapidly increasing in Asian countries and the use of infliximab (IFX) is also increasing because the drug is proved to be effective in controlling disease activity. However, about 20% of initial responders to induction therapy with IFX suffer from loss of responsiveness (LOR) to the drug and antibodies to IFX (ATI) are thought to be a cause for the problem. As limited data are available in Asian patients with CD, this study tried to verify the correlations between IFX trough levels (IFX-TLs)/ATI levels and the clinical activity of CD.

Research frontiers

LOR to IFX has become an important issue in treating CD patients and alternative treatment strategies for patients suffering LOR should be raised. Furthermore, there is no clear indication to discontinue IFX maintenance therapy after clinical remission is retained for a long time. Along with clarifying the correlations between IFX-TLs/ATI levels and the clinical activity of CD, this study suggested the ways to making decisions using IFX-TLs and ATI levels in those patients.

Innovations and breakthroughs

This was the first study analyzing relationship between IFX-TLs/ATI levels and the clinical activity of CD in South Korea. Furthermore, our findings support the usefulness of IFX-TLs/ATI levels in treating CD patients receiving IFX in clinical practice.

Applications

Measuring IFX-TLs and ATI levels could be used in making decisions in patients suffering LOR. If high IFX-TL and low ATI level were shown in the patients, we should consider dose intensification or switching to another class of drugs such as anti-integrins. On the other hand, it can be used in to decide discontinuation of IFX maintenance therapy in patients with quiescent disease. If low IFX-TL and high ATI level were shown in the patients, we should consider stopping the anti-tumor necrosis factor (TNF)-α therapy if long-term deep remission is achieved.

Terminology

Infliximab: A drug that works as monoclonal antibody blocking action of TNF-α which is known to be playing a main role in pathogenesis of CD. Trough level: The lowest level of a drug. If a drug is administered periodically, the trough level should be measured just before the administration of the next doses.

Peer-review

This was the first study analyzing and revealing definite correlations among IFX-TLs/ATI levels and clinical activity in CD patients in South Korea. Clinical implications and applications to practices are suggested well.

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Association between *Helicobacter pylori* and end-stage renal disease: A meta-analysis

Karn Wijarnpreecha, Charat Thongprayoon, Pitchaphon Nissaisorakarn, Natasorn Lekuthai, Veeravich Jaruvongvanich, Kiran Nakkala, Ridhmi Rajapakse, Wisit Cheungpasitporn

Karn Wijarnpreecha, Charat Thongprayoon, Ridhmi Rajapakse, Department of Internal Medicine, Bassett Medical Center, Cooperstown, NY 13326, United States

Pitchaphon Nissaisorakarn, Department of Internal Medicine, Jacobi Medical Center, Albert Einstein College of Medicine, Bronx, NY 10461, United States

Natasorn Lekuthai, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Veeravich Jaruvongvanich, Department of Medicine, University of Hawaii, Honolulu, HI 96822, United States

Kiran Nakkala, Cape Fear Center for Digestive Diseases, P.A., Fayetteville, NC 28312, United States

Wisit Cheungpasitporn, Division of Nephrology, Department of Internal Medicine, Mayo Clinic, Rochester, MN 55905, United States

Abstract

AIM
To investigate the prevalence and association of *Helicobacter pylori* (*H. pylori*) with end-stage renal disease (ESRD).

METHODS
SA comprehensive literature search was completed from inception until October 2016. Studies that reported prevalence, relative risks, odd ratios, hazard ratios or standardized incidence ratio of *H. pylori* among ESRD patients were included. Participants without *H. pylori* were used as comparators to assess the association between *H. pylori* infection and ESRD. Pooled risk ratios and 95%CI was calculated using a random-effect model. Adjusted point estimates from each study were combined by the generic inverse variance method of DerSimonian and Laird.

RESULTS

Of 4546 relevant studies, thirty-seven observational studies met all inclusion criteria. Thirty-five cross-sectional studies were included in the analyses to assess the prevalence and association of *H. pylori* with ESRD. The estimated prevalence of *H. pylori* among ESRD patients was 44% (95%CI: 40%-49%). The pooled RR of *H. pylori* in patients with ESRD was 0.77 (95%CI: 0.59-1.00) when compared with the patients without ESRD. Subgroup analysis showed significantly reduced risk of *H. pylori* in adult ESRD patients with pooled RR of 0.71 (95%CI: 0.55-0.94). The data on the risk of ESRD in patients with *H. pylori* were limited. Two cohort studies were included to assess the risk of ESRD in patients with *H. pylori*. The pooled risk RR of ESRD in patients with *H. pylori* was 0.61 (95%CI: 0.03-12.20).

CONCLUSION

The estimated prevalence of *H. pylori* in ESRD patients is 44%. Our meta-analysis demonstrates a decreased risk of *H. pylori* in adult ESRD patients.

Core tip: *Helicobacter pylori* (*H. pylori*) is the most common chronic bacterial infection in gastrointestinal tract of humans. The prevalence and association of *H. pylori* with end-stage renal disease (ESRD), however, are still unclear. To further investigate this potential relationship, we conducted this systematic review and meta-analysis of observational studies reporting the association between *H. pylori* infection and ESRD and prevalence in ESRD patients. We found an estimated prevalence of *H. pylori* in ESRD patients of 44%. In addition, our meta-analysis demonstrates a 0.71-fold decreased risk of *H. pylori* in adult ESRD patients.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is the most common chronic bacterial infection in the gastrointestinal tract of humans[1]. It has been estimated that the prevalence of *H. pylori* infection is up to thirty percent in adult aged 18 to 30 years and to fifty percent in those older than 60 years old[2]. Many studies demonstrated that *H. pylori* infection is associated with a peptic and duodenal ulcer, chronic gastritis, and gastric cancer[3,4]. Recently, epidemiologic studies have demonstrated associations between *H. pylori* infection and extra-gastrointestinal organ involvements including coronary artery disease, dyslipidemia, insulin resistance, and hematologic disorders[5-7].

End-stage renal disease (ESRD) is a common and serious chronic disease worldwide that continues to increase in prevalence by approximately 21000 cases per year in the United States[8]. Although there is no visible evidence demonstrated that *H. pylori* infection is directly associated with renal disease, patients with ESRD usually have gastrointestinal problems such as gastritis, dyspeptic symptoms or ulcers[9-11]. Interestingly, recent investigations have demonstrated an association between *H. pylori* infection and ESRD[12-14]. In addition, an increase in renal resistance index due to systemic inflammation state *H. pylori* infection was also described[15-18]. However, many studies reported the conflict data regarding the association between *H. pylori* infection in ESRD and also the prevalence of *H. pylori* infection in ESRD patients[19-42]. Thus, we conducted the systematic review and meta-analysis that summarized all available evidence to determine the prevalence of *H. pylori* infection among ESRD patients and the association between *H. pylori* infection and ESRD.

MATERIALS AND METHODS

Literature search

Three investigators (Wijarnpreecha K, Thongprayoon C and Cheungpasitporn W) independently reviewed published studies indexed in MEDLINE and EMBASE database from their inception to October 2016 using the search strategy that included the terms for "Helicobacter", "hemodialysis", and "renal disease" as described in Item S1 in online Supplementary Data 1. A search for additional articles utilizing references from included studies was also performed. There was no confinement on language in the literature search. We conducted this systematic review following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis statement.

Selection criteria

The inclusion criteria were: (1) observational studies appraising the association between *H. pylori* and ESRD and prevalence in hemodialysis; (2) prevalence, odds ratios, relative risks, or hazard ratios with 95%CI were presented; and (3) individuals without *H. pylori* were used as comparators in cohort studies while individuals without ESRD were used as comparators in the cross-sectional and case-control studies. Wijarnpreecha K, Thongprayoon C and Cheungpasitporn W individually examined the titles and abstracts of the studies. After

Figure 1 Literature review process.

the first phase, the full text of the included studies was subsequently examined to ascertain if they met the inclusion criteria. Discrepancies were also settled by discussion with all investigators.

Data abstraction

A structured data collection form was utilized to obtain the data from included studies including title of the study, year of publication, country where the study was conducted, name of the first author, demographic of subjects, method used to diagnose *H. pylori*, prevalence of *H. pylori*, effect estimates (hazard ratios, odds ratios, relative risks) with 95%CI, and factors adjusted in the multivariate analysis. To ensure the certainty, this data extraction process was reviewed by all investigators. The quality of each study was individually appraised by each investigator. We utilized the validated Newcastle-Ottawa quality assessment scale for cohort and case-control studies[43] and modified Newcastle-Ottawa scale[44] for the cross-sectional study.

Statistical analysis

MetaXL software (EpiGear International Pty Ltd)[45] was used for meta-analysis of prevalence. Otherwise, data analysis was performed using the Review Manager 5.3 software from the Cochrane Collaboration (London, United Kingdom). Adjusted point estimates from each study were combined by the generic inverse variance method of DerSimonian and Laird, which assigned the weight of each study based on its variance[46]. We used a random-effect model due to the high likelihood of between-study variance from different study designs, populations, and *H. pylori* testing. Cochran's *Q* test and

I^2 statistic were used to ascertain the between-study heterogeneity. A value of I^2 of 0%-25%, 25%-50%, 50%-75%, and > 75% embodied insignificant, low, moderate and high heterogeneity, respectively[47].

RESULTS

Of 4546 potentially relevant articles, 4466 articles were excluded due to the title and abstract not meeting inclusion criteria. Subsequently, 43 articles were excluded (6 articles were not observational studies, and 37 articles did not describe the outcomes of interest). Finally, thirty-seven observational studies (2 cohort[14,48] and 35 cross-sectional studies[12,13,16,19-42,49-56]) met all inclusion criteria. The literature retrieval, review, and selection process are shown in Figure 1. The characteristics and quality assessment of the included cross-sectional studies are presented in Table 1 while the characteristics of the included cohort studies are shown in Table 2.

The prevalence of H. pylori among ESRD patients

Thirty-five cross-sectional studies were included in the analyses to assess the prevalence and association of *H. pylori* with ESRD. The estimated prevalence of *H. pylori* among ESRD patients was 44% (95%CI: 40%-49%, I^2 = 80%), as demonstrated in Figure 2. Subgroup analysis was also performed on thirty-two studies[12,13,16,19-23,25-28,30-42,49-51,53-56] that provided prevalence on adult subjects and three studies[24,29,52] that provided prevalence on pediatric patients and showed estimated prevalences of *H. pylori* among adult ESRD patients of 44% (95%CI: 39%-49%, I^2

Table 1 Main characteristics of the cross-sectional studies included in this meta-analysis

Study	Country	Year	Study sample	*H. pylori* testing	*H. pylori* prevalence (%)	OR	Study quality
Offerhaus *et al*[36]	The Netherland	1989	Dialysis	Antibody	22/50 (44%)	0.96 (0.42-2.22)	S 3 C 0 O 2
Shousha *et al*[55]	United Kingdom	1990	Dialysis	Histology	12/50 (24%)	0.43 (0.20-0.90)	S 3 C 0 O 2
Loffeld *et al*[34]	The Netherland	1991	HD	Antibody	13/30 (43%)	1.24 (0.58-2.64)	S 3 C 1 O 2
Davenport *et al*[22]	United Kingdom	1991	HD	Antibody	27/76 (36%)	1.29 (0.75-2.22)	S 3 C 1 O 2
Ala-Kaila *et al*[16]	Finland	1991	HD	Histology	3/23 (13%)	0.68 (0.17-2.64)	S 3 C 0 O 2
Gladziwa *et al*[27]	Germany	1993	HD	Cumulative evaluation (urease, test, histology, culture and direct examiniation)	12/35 (34%)	0.44 (0.19 -1.00)	S 3 C 0 O 2
Giachino *et al*[25]	Italy	1994	HD	Urease test, histology and culture	13/40 (32%)	0.51 (0.20-1.28)	S 3 C 0 O 2
De Vecchi *et al*[51]	Italy	1995	HD and PD	Antibody	HD and PD 37/67 (55%) HD 17/29 (59%) PD 20/38 (53%)	HD and PD 0.39 (0.18-0.81) HD 0.54 (0.18-1.62) PD 0.30 (0.11-0.81)	S 3 C 1 O 2
Jaspersen *et al*[31]	Germany	1995	HD	Urease test and histology	7/34 (21%)	0.44 (0.18-1.09)	S 3 C 0 O 2
Seyrek *et al*[39]	Turkey	1996	HD	Antibody	13/91 (14%)	0.56 (0.21-1.50)	S 3 C 1 O 2
Krawczyk *et al*[33]	Poland	1996	HD	Urease test and histology	13/21 (62%)	0.93 (0.27-3.20)	S 3 C 1 O 2
Ozgür *et al*[38]	Turkey	1997	HD	Urease test	28/47 (60%)	0.83 (0.41-1.69)	S 3 C 0 O 2
Hruby *et al*[30]	Poland	1997	HD	Antibody, culture	9/26 (35%) by culture 16/26 (62%) by antibody	0.68 (0.19-2.44) by culture 0.53 (0.13-2.12)	S 3 C 0 O 2
Yildiz *et al*[42]	Turkey	1999	HD	Antibody	31/47 (66%)	0.79 (0.34-1.84)	S 3 C 0 O 2
Fabrizi *et al*[23]	United States	1999	HD	Antibody	127/228 (56%)	1.11 (0.74-1.66)	S 3 C 1 O 2
Tamura *et al*[40]	Japan	1999	HD and PD	Urease test, histology, and culture	25/49 (51%)	0.88 (0.40-1.96)	S 3 C 0 O 2
Gür *et al*[28]	Turkey	1999	HD	Urease test and histology	25/45 (56%)	1.04 (0.45-2.40)	S 3 C 0 O 2
Araki *et al*[50]	Japan	1999	HD and PD	Histology and culture	29/63 (46%)	0.45 (0.22-0.91)	S 3 C 1 O 2
Karari *et al*[32]	Kenya	2000	CRF (HD - 36%)	Urease test and histology	41/77 (53%)	0.90 (0.48-1.70)	S 3 C 1 O 2
Nakajima *et al*[53]	Japan	2002	HD	Urease test, histology, and culture	14/51 (28%)	0.30 (0.11-0.81)	S 3 C 0 O 2

Tsukada *et al*[41]	Japan	2003	HD	Histology	9/36 (25%)	0.28 (0.02-3.82)	S 3 C 2 O 2
Olmos *et al*[37]	Argentina	2003	HD	Antibody	44/93 (47%)	0.62 (0.35-1.11)	S 3 C 2 O 2
Nakajima *et al*[54]	Japan	2004	HD	Antibody	51/138 (37%)	0.35 (0.22-0.58)	S 3 C 1 O 2
Nardone *et al*[35]	Italy	2005	HD	Urease test, histology, urea breath test and stool antigen	7/11 (64%)	3.04 (0.82-11.13)	S 3 C 0 O 2
Blusiewicz *et al*[19]	Poland	2005	HD	Urease, histology	19/30 (63%)	0.71 (0.24-2.07)	S 3 C 0 O 2
Khedmat *et al*[13]	Iran	2007	HD	Urease test	46/73 (63%)	3.20 (1.88-5.44)	S 3 C 0 O 2
Khazaei *et al*[52]	Iran	2008	HD - children	Urease test, and histology	16/24 (67%)	8.00 (2.19-29.25)	S 3 C 0 O 2
Gioè *et al*[26]	Italy	2008	HD	Urease test, and histology	75/142 (53%)	1.39 (0.86-2.23)	S 3 C 0 O 2
Abdulrahman *et al*[49]	Saudi Arabia	2008	ESRD	Histology	16/40 (40%)	0.22 (0.09-0.56)	S 3 C 1 O 2
Asl *et al*[12]	Iran	2009	HD	Histology	23/40 (58%)	2.81 (1.13-6.99)	S 3 C 1 O 2
Sugimoto *et al*[56]	Japan	2009	HD	Antibody	262/539 (49%)	0.26 (0.19-0.35)	S 3 C 0 O 2
Chang *et al*[21]	South Korea	2010	HD	Urease test and histology	12/33 (36%)	0.30 (0.12-0.74)	S 3 C 0 O 2
Hooman *et al*[29]	Iran	2011	HD - children	Histology	19/68 (28%)	1.59 (0.65-3.92)	S 3 C 0 O 2
Genç *et al*[24]	Turkey	2013	HD and PD - children	Antibody	17/33 (52%)	0.69 (0.26-1.83)	S 3 C 1 O 2
Chang *et al*[20]	Taiwan	2014	ESRD	Urease test and histology	81/144 (56%)	0.54 (0.38-0.77)	S 4 C 2 O 3

H. pylori: *Helicobacter pylori*; HD: Hemodialysis; PD: Peritoneal dialysis.

= 81%), and 47% (95%CI: 24%-71%, I^2 = 84%) among ESRD children, respectively as demonstrated in Supplementary Figures 1 and 2.

The association between H. pylori and ESRD

We found a marginal but not significantly decreased risk of *H. pylori* infection in overall ESRD subjects compared with non-ESRD subjects[12,13,16,19-42,49-56] with pooled RR of 0.77 (95%CI: 0.59-1.00, I^2 = 79%) (Figure 3). Subgroup analysis based on ageing as described above, we found a significant decreased risk of *H. pylori* infection among adult ESRD patients[12,13,16,19-23,25-28,30-42,49-51,53-56] with pooled RR of 0.71 (95%CI: 0.55-0.94, I^2 = 79%) compared with non-ESRD patients (Supplementary Figure 3). Nevertheless, we did not find a significant association between *H. pylori* infection and ESRD among ESRD children[24,29,52]; pooled RR = 1.93 (95%CI: 0.55-6.82,

I^2 = 77%), (Supplementary Figure 4).

The data on the risk of ESRD in patients with *H. pylori* were limited. Two cohort[14,48] studies were included to assess the risk of ESRD in patients with *H. pylori*. The pooled risk RR of ESRD in patients with *H. pylori* was 0.61 (95%CI: 0.03-12.20).

Evaluation for publication bias

A funnel plot assessing publication bias for the association between *H. pylori* infection in overall ESRD subjects was demonstrated in Figure 4. The funnel plot of the association between *H. pylori* infection in overall ESRD subjects was symmetric and suggested no publication bias.

DISCUSSION

In this meta-analysis summarizing all presently

Figure 2 Forest plot of overall prevalence of *Helicobacter pylori* infection among end-stage renal disease patients.

Study	Prev (95%CI)	% Weight
Offerhaus *et al*	0.44 (0.30, 0.58)	2.9
Shousha *et al*	0.24 (0.13, 0.37)	2.9
Loffeld *et al*	0.43 (0.26, 0.62)	2.5
Davenport *et al*	0.36 (0.25, 0.47)	3.2
Ala-Kalia *et al*	0.13 (0.02, 0.30)	2.2
Gladziwa *et al*	0.34 (0.19, 0.51)	2.6
Giachino *et al*	0.33 (0.19, 0.48)	2.7
De Vecchil	0.55 (0.43, 0.67)	3.1
Jaspersen *et al*	0.21 (0.08, 0.36)	2.6
Seyrek *et al*	0.14 (0.08, 0.22)	3.3
Krawczyk *et al*	0.62 (0.40, 0.82)	2.2
Ozgur *et al*	0.60 (0.45, 0.73)	2.9
Yildiz *et al*	0.66 (0.52, 0.79)	2.9
Tamura *et al*	0.51 (0.37, 0.65)	2.9
Gur *et al*	0.56 (0.41, 0.70)	2.8
Fabrizi *et al*	0.56 (0.49, 0.62)	3.6
Araki *et al*	0.46 (0.34, 0.58)	3.1
Karari *et al*	0.53 (0.42, 0.64)	3.2
Nakajima *et al* (1)	0.27 (0.16, 0.41)	2.9
Tsukada *et al*	0.25 (0.12, 0.41)	2.6
Olmos *et al*	0.47 (0.37, 0.58)	3.3
Nakajima *et al* (2)	0.37 (0.29, 0.45)	3.5
Nardone *et al*	0.64 (0.33, 0.90)	1.6
Blusiewicz *et al*	0.63 (0.45, 0.80)	2.5
Khedmat *et al*	0.63 (0.52, 0.74)	3.2
Khazaei *et al*	0.67 (0.46, 0.84)	2.3
Gioe *et al*	0.53 (0.45, 0.61)	3.5
Hosseini *et al*	0.57 (0.42, 0.73)	2.7
Sugimoto *et al*	0.49 (0.44, 0.53)	3.8
Chang *et al* (1)	0.36 (0.21, 0.54)	2.6
Hooman *et al*	0.28 (0.18, 0.39)	3.1
Genc *et al*	0.52 (0.34, 0.69)	2.6
Chang *et al* (2)	0.56 (0.48, 0.64)	3.5
Hruby *et al*	0.35 (0.17, 0.54)	2.4
Abdulrahman *et al*	0.40 (0.25, 0.56)	2.7
Overall	0.44 (0.40, 0.49)	100.0

$Q = 174.36$, $P = 0.00$, $I^2 = 80\%$

Table 2 Main characteristics of the cohort studies included in this meta-analysis		
Study	**Lo *et al*[48]**	**Lin *et al*[14]**
Country	Hong Kong	Taiwan
Study design	Cohort study	Cohort study
Year	2004	2015
Study sample	Type 2 diabetic patients with clinical proteinuria and renal insufficiency	*H. pylori*-infected and non-infected patients without ESRD
H. pylori testing	Antibody Positive *H. pylori* (Titer > 1.1 U/mL)	Diagnosis of *H. pylori* infection (ICD-9 041.86) was used from inpatient database of The Taiwan National Health Insurance Research Database
ESRD definition	Doubling of baseline serum creatinine concentration or need for dialysis or serum creatinine ≥ 500 μmol/L	ESRD was identified from Registry for Catastrophic Illness Patient Database
Adjusted HR	0.12 (0.03, 0.52)	2.58 (2.33, 2.86)
Confounder adjustment	Sex, *H. pylori* status, serum creatinine, hemoglobin, systolic blood pressure, ACE inhibitors, Hepatitis B surface antigen status	Age, sex, comorbidity
Quality assessment (Newcastle-Ottawa scale)	Selection: 3 Comparability: 2 Outcome: 3	Selection: 4 Comparability: 2 Outcome: 3

H. pylori: *Helicobacter pylori*; HD: Hemodialysis; PD: Peritoneal dialysis; ESRD: End-stage renal disease.

Study or subgroup	log[risk ratio]	SE	Weight	Risk ratio IV, random, 95%CI	Year	Risk ratio IV, random, 95%CI
Offerhaus *et al*	-0.04082	0.424747	2.9%	0.96 (0.42, 2.21)	1989	
Shousha *et al*	-0.84397	0.383693	3.1%	0.43 (0.20, 0.91)	1990	
Ala-Kalia *et al*	-0.38566	0.699677	2.0%	0.68 (0.17, 2.68)	1991	
Davenport *et al*	0.254642	0.276834	3.5%	1.29 (0.75, 2.22)	1991	
Loffeld *et al*	0.215111	0.386609	3.0%	1.24 (0.58, 2.65)	1991	
Gladziwa *et al*	-0.82098	0.423656	2.9%	0.44 (0.19, 1.01)	1993	
Giachino *et al*	-0.67334	0.473545	2.7%	0.51 (0.20, 1.29)	1994	
De Vecchil	-0.94161	0.383693	3.1%	0.39 (0.18, 0.83)	1995	
Jaspersen *et al*	-0.82098	0.459433	2.8%	0.44 (0.18, 1.08)	1995	
Seyrek *et al*	-0.57982	0.501559	2.6%	0.56 (0.21, 1.50)	1996	
Krawczyk *et al*	-0.07257	0.630736	2.2%	0.93 (0.27, 3.20)	1996	
Hruby *et al*	-0.38566	0.651206	2.1%	0.68 (0.19, 2.44)	1997	
Ozgur *et al*	-0.18633	0.361308	3.1%	0.83 (0.41, 1.69)	1997	
Fabrizi *et al*	0.10436	0.206103	3.7%	1.11 (0.74, 1.66)	1999	
Yildiz *et al*	-0.23572	0.430759	2.9%	0.79 (0.34, 1.84)	1999	
Araki *et al*	-0.79851	0.362198	3.1%	0.45 (0.22, 0.92)	1999	
Gur *et al*	0.039221	0.427035	2.9%	1.04 (0.45, 2.40)	1999	
Tamura *et al*	-0.12783	0.405417	3.0%	0.88 (0.40, 1.95)	1999	
Karari *et al*	0.322601	0.322601	3.3%	1.38 (0.73, 2.60)	2000	
Nakajima *et al* (1)	-1.20397	0.509325	2.6%	0.30 (0.11, 0.81)	2002	
Olmos *et al*	-0.47804	0.294434	3.4%	0.62 (0.35, 1.10)	2003	
Tsukada *et al*	-1.27297	1.339866	0.8%	0.28 (0.02, 3.87)	2003	
Nakajima *et al* (2)	-1.04982	0.247296	3.6%	0.35 (0.22, 0.57)	2004	
Nardone *et al*	1.111858	0.66533	2.1%	3.04 (0.83, 11.20)	2005	
Blusiewicz *et al*	-0.34249	0.549659	2.4%	0.71 (0.24, 2.09)	2005	
Khedmat *et al*	1.163151	0.271048	3.5%	3.20 (1.88, 5.44)	2007	
Khazaei *et al*	2.079442	0.661219	2.1%	8.00 (2.19, 29.24)	2008	
Gioe *et al*	0.329304	0.243067	3.6%	1.39 (0.86, 2.24)	2008	
Abdulrahman *et al*	-1.51413	0.466359	2.7%	0.22 (0.09, 0.55)	2008	
Hosseini *et al*	1.033184	0.423291	2.9%	2.81 (1.23, 6.44)	2009	
Sugimoto *et al*	-1.34707	0.155844	3.8%	0.26 (0.19, 0.35)	2009	
Chang *et al* (1)	-1.20397	0.464071	2.7%	0.30 (0.12, 0.74)	2010	
Hooman *et al*	0.463734	0.458386	2.8%	1.59 (0.65, 3.90)	2011	
Genc *et al*	-0.37106	0.497803	2.6%	0.69 (0.26, 1.83)	2012	
Chang *et al* (2)	-0.61619	0.180158	3.8%	0.54 (0.38, 0.77)	2014	
Total (95%CI)			100.0%	0.77 (0.59, 1.00)		

Heterogeneity: Tau^2 = 0.46; χ^2 = 162.07, df = 34 (P < 0.00001); I^2 = 79%
Test for overall effect : Z = 1.95 (P = 0.05)

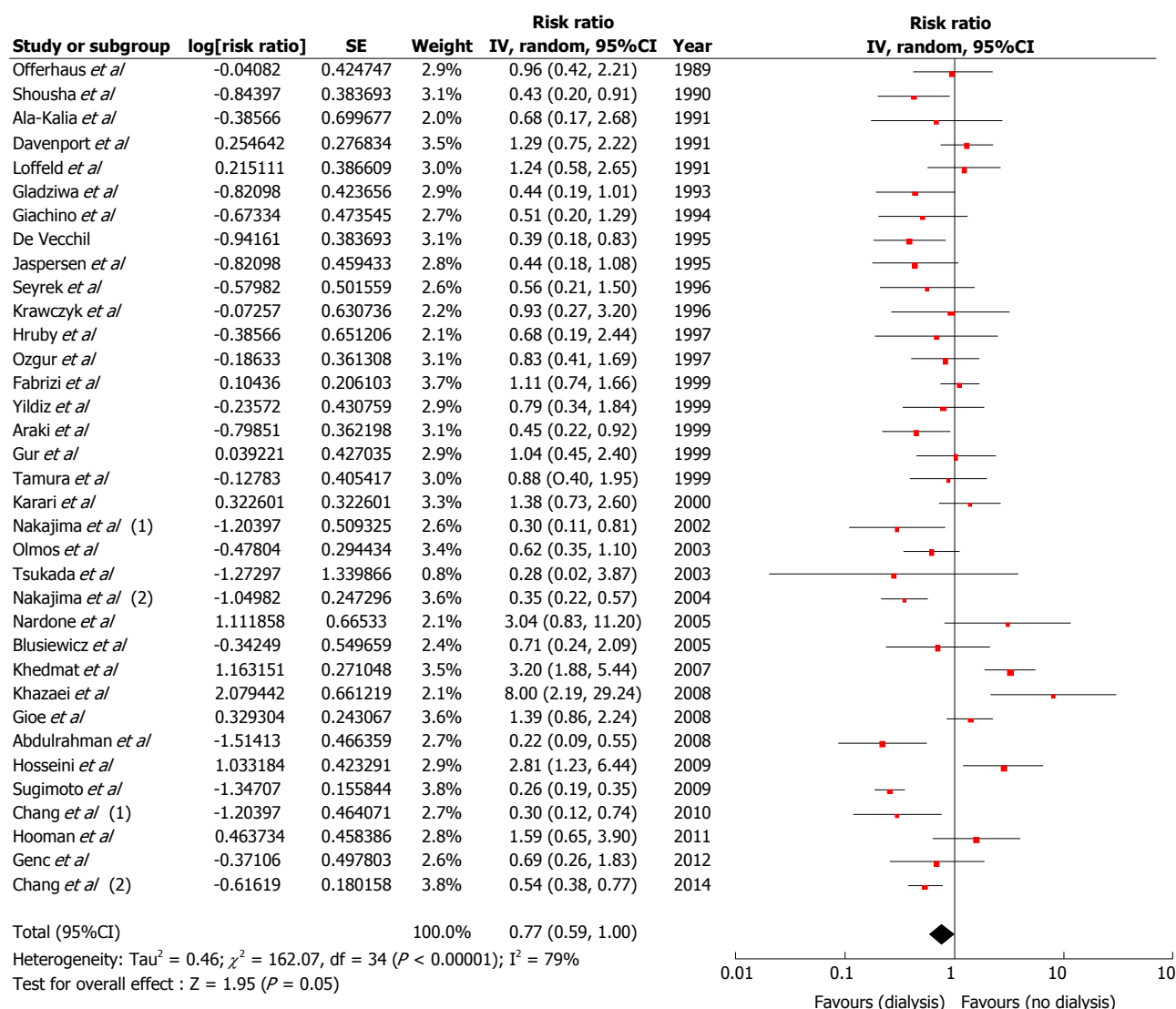Favours (dialysis)   Favours (no dialysis)

Figure 3 Forest plot of the association between *Helicobacter pylori* infection and end-stage renal disease.
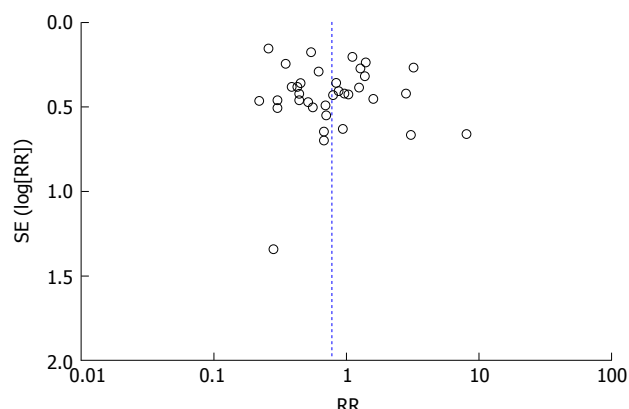
available data on the prevalence of *H. pylori* infection among ESRD patients and the association between *H. pylori* infection and ESRD, we demonstrated an estimated prevalence of *H. pylori* in ESRD patients of 44%. In addition, we found a 0.71-fold decreased risk of *H. pylori* in adult ESRD patients.

Although the precise explanation of reduced risk of *H. pylori* among adult ESRD patients is still unclear, there are several plausible explanations for this association. First, it has been postulated in previous studies that administering antibiotics and antacid more frequently in ESRD patients may contribute to lower the prevalence of *H. pylori* infection[39,53]. Previous study proposed that ESRD patients may have a lower risk of *H. pylori* infection from routinely used of antacids to prevent renal osteodystrophy by reducing intestinal phosphate absorption[16]. Second, patients with ESRD have higher levels of inflammatory cytokines including tumor necrotic factor, interleukin-6 and -8 from infiltrative inflammatory cells in gastric mucosa[57] and chronic circulatory failure[58,59] could

lead to gastric mucosal damage and progress to gastric atrophy or atrophic gastritis, increased in gastric pH mucosa, and eventually eradication of *H. pylori* infection[60-62].

Although the included studies in this meta-analysis are almost of good quality, there are several limitations to this study that need to be addressed. Firstly, there was a statistical heterogeneity in the completed analysis. Possible sources of this heterogeneity include differences in confounder-adjusted methods (*e.g.*, age, gender, ethnicity and socioeconomic status), different test to detect *H. pylori* infection in each study, various grades of uremia. Secondly, our subgroup analysis revealed significantly decreased the risk of *H. pylori* infection among adult subjects with ESRD but not in children likely due to a limitation in some studies. Although the number of study assessing *H. pylori* in children was limited and the insignificant finding in ESRD children could be from the lack of power, further studies are required to determine the role of aging in the underlying pathogenesis of *H. pylori* infection

Figure 4 Funnel plot of the association between *Helicobacter pylori* infection and end-stage renal disease.

among ESRD patients. Lastly, this study is a meta-analysis of observational studies. Thus, our study demonstrated an association, but could not establish causality as unknown confounders could play a role in the association between prevalence of *H. pylori* among hemodialysis and association between *H. pylori* and ESRD.

In conclusion, our meta-analysis demonstrated an estimated prevalence of *H. pylori* in ESRD patients of 44%. In addition, our meta-analysis demonstrates a decreased risk of *H. pylori* in adult ESRD patients. ESRD could be a potential protective factor for *H. pylori* infection.

COMMENTS

Background
Helicobacter pylori (*H. pylori*) is the most common chronic bacterial infection in the gastrointestinal tract of humans. Epidemiologic studies showed the link between *H. pylori* infection and extra-gastrointestinal tract including end-stage renal disease (ESRD). However, many studies reported the conflict data regarding the association between *H. pylori* infection in ESRD and also the prevalence of *H. pylori* infection in ESRD patients.

Research frontiers
The results of those epidemiologic studies were inconsistent. To further investigate this possible association of *H. pylori* infection and ESRD and determine the prevalence of *H. pylori* among ESRD patients, the authors conducted this systematic review and meta-analysis of observational studies reporting the association between *H. pylori* and ESRD and prevalence of *H. pylori* among ESRD patients.

Innovations and breakthroughs
The authors found an estimated prevalence of *H. pylori* in ESRD patients of 44% (95%CI: 40%-49%). Moreover, the authors also found a decreased risk of *H. pylori* infection among adult ESRD patients with pooled RR of 0.71 (95%CI: 0.55-0.94).

Applications
This study demonstrated a significantly decreased risk of *H. pylori* infection among ESRD patients. This finding suggests that ESRD may be an independent potential protective factor for *H. pylori* infection.

Peer-review
This meta-analysis investigated the prevalence and association of *H. pylori*

with end-stage renal diseases and demonstrated a decreased risk of *H. pylori* in adult ESRD patients. The context is well organized and the conclusion is of interest.

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CASE REPORT

Preventing pancreatic fistula after distal pancreatectomy: An invagination method

Nagato Katsura, Yasuhiro Kawai, Takashi Gomi, Kenji Okumura, Takahiko Hoashi, Seijun Fukuda, Katsushi Takebayashi, Kenji Shimizu, Masugi Satoh

Nagato Katsura, Takahiko Hoashi, Seijun Fukuda, Kenji Shimizu, Department of Surgery, Misugikai Otokoyama Hospital, Otokoyama Izumi, Yawata, Kyoto 614-8366, Japan

Yasuhiro Kawai, Takashi Gomi, Kenji Okumura, Katsushi Takebayashi, Masugi Satoh, Department of Surgery, Misugikai Satoh Hospital, Yobuhigashimachi Hirakata, Osaka 573-1124, Japan

Abstract

Following an increase in the use of the GIA stapler for treating a pancreatic stump, more techniques to prevent postoperative pancreatic juice leakage have been required. We describe one successful case using our new technique of invaginating the cut end of the pancreas into the stomach to prevent a pancreatic fistula (PF) from occurring. A 50-year-old woman with pancreatic cancer in the tail of the pancreas underwent distal pancreatectomy, causing a grade A PF. We resected the distal pancreas without additional reinforcement to invaginate the stump into the gastric posterior wall with single layer anastomosis using a 3-0 absorbable suture. The drain tubes were removed on the third postoperative day. Although a grade A PF was noted, the patient was discharged on foot on the eleventh postoperative day. Our technique may be a suitable method for patients with a pancreatic body and tail tumor.

Key words: Amylases; Pancreatectomy; Pancreatic fistula; Pancreatic juice; Neoplasms

Core tip: More techniques for preventing postoperative pancreatic juice leakage have been required since the use of GIA stapler has increased. We describe one successful case wherein our new technique of invaginating the cut end of the pancreas into the stomach was used to prevent a pancreatic fistula (PF) from occurring. A 50-year-old woman with pancreatic

cancer in the tail of the pancreas underwent distal pancreatectomy. Although a grade A PF was noted, the patient was discharged on foot on the eleventh postoperative day. Our technique may be a suitable method for patients with a pancreatic body and tail tumor.

INTRODUCTION

Historically, it has been recognized that post-pancreatectomy complications can be severe[1]. Generally, pancreaticoduodenectomy or distal pancreatectomy will cause a pancreatic fistula (PF) to some extent, and it has been said that complete prevention of PF after pancreatectomy is impossible. A PF can cause an intra-abdominal abscess due to an activated bacterial infection, which can result in sepsis, hemorrhage[2-5], and delayed gastric emptying[6]. To prevent these complications, various methods have been reported; however, to date, none of them has become a standard procedure[7]. Here, we describe our experience invaginating the pancreatic stump into the stomach after distal pancreatectomy with an excellent result.

CASE REPORT

A 50-year-old woman had a hard navel mass. She regularly visited the Department of Internal Medicine at our hospital for the treatment of diabetes. In August 2011, she presented to the Department of Dermatology with a main complaint of an umbilical mass; however, she was sent to the Surgical Department because of the diagnosis of an umbilical lesion located deep in the abdomen. The hard mass, which was the size of a thumb, was palpable at her navel. She did not feel any pain.

An abdominal computed tomography (CT) scan showed the enhanced tumor; it was 2 cm in diameter and located in the pancreatic tail (Figure 1). The contrasting effect was poor compared to normal tissue, which was a finding suggestive of pancreatic cancer. The para-aortic lymph node was not swollen; however, low, enhanced foci were scattered in the spleen, which was a finding suggestive of metastasis.

The fluorodeoxyglucose-positron emission tomography (FDG-PET) scan showed swelling in the pancreatic tail with abnormal accumulation (Figure 2). This contrasting pattern along with the CT findings suggested pancreatic cancer. Accumulation in the spleen was noted, so we were unable to rule out the possibility of invasion. There was a small granular shadow with slight accumulation suggestive of lymph node metastases.

Skin thickening and abnormal accumulation in the umbilical region were also noted. Consecutive accumulation was not observed in the peritoneum, which was suggestive of local inflammation, not dissemination. No other abnormality was found.

Operative findings

The patient was placed in a supine position for the operation. First, we used Kocher mobilization at the front part of the inferior vena cava, moving toward the anterior surface of left renal vein to back side of the superior mesenteric artery. We confirmed no para-aortic lymph node swelling. The greater omentum was resected from the spleen to the pancreas, transverse colon, and splenic flexure. The inferior mesenteric vein was set aside. The adhesion between the stomach and pancreas was opened. In addition, the posterior gastric vein was separated, and the coronary vein was preserved. The spleen was separated from the retroperitoneum. Next, the left adrenal gland was resected from the pancreas and preserved intact. The pancreas was cut at the anterior of the superior mesenteric artery, and the pancreatic tail, including the tumor, was extracted. The stump and tumor were quick frozen for pathological examination; the pancreatic duct stump was ligated with 5-0 prolene sutures. The patient was diagnosed as having pancreatic cancer, and the pancreatic stump was not malignant. The gastric posterior wall was transected to approximately 80% of the stump width. Single layer anastomosis was performed with 3-0 absorbable sutures (Figure 3A-E). One soft drain was placed under the left diaphragm and the hiatus of Winslow after washing with 2000 mL of saline. The left side of the greater omentum was used to cover the stomach-pancreas anastomotic region. The operative time was 211 min, and the blood loss was 162 mL.

Results of the pathological examination

The invasive ductal carcinoma of the pancreatic tail, scirrhous, nodular, Infγ , ly0, v1, ne3, mpd(-), s(+), rp(-), PVsp(+), A(-), pcm(-), mdpm(-), and M1(umbilicus) carcinoma, formed the mass (30 × 25 mm) and showed serosa exposure and progress to the outer membrane of the spleen. This mass was a tub1(> tub2)-based tubular, scirrhous adenocarcinoma. It was accompanied by high neurologic and splenic vein invasion. Each excised stump was negative for malignancy.

Cytology of ascites showed that the umbilical region mass, the invasive ductal carcinoma, was a class V adenocarcinoma.

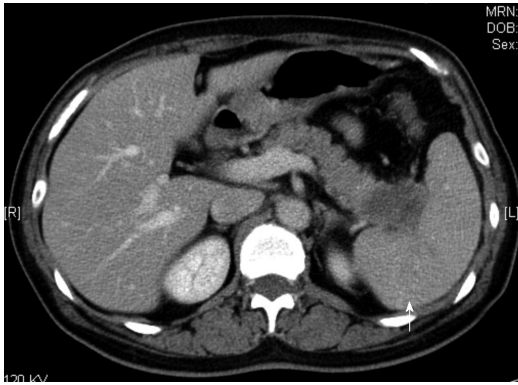The drain was removed after the drain fluid amylase

Figure 1 The low density tumor in the pancreatic tail (white arrow).

Figure 2 Fluorodeoxyglucose-positron emission tomography scan showing the swollen part in the pancreas tail and umbilical region (white arrow) with abnormal accumulation.

level decreased to 190 IU/L on day 3 from 1595 IU/L on day 1. The patient was discharged without any problems on postoperative day 11.

We have followed the patient's pancreatic stump in the stomach postoperatively for 1 year using a gastric fiber scope. After 1 wk, the stump was massive; however, after 3 mo, the gastric mucosa covered almost the entire stump end. After 1 year, we could not detect the stump in the stomach (Figure 4A-C).

DISCUSSION

Ligation of the main pancreatic duct with a fish-mouth-shaped closure of the cut end has long been considered a standard technique for distal pancreatectomy[7]. However, it has generally been said that the probability of PF occurrence is in the range of 32% to 57%[8-12]. This can cause an intra-abdominal abscess with lethal results. When an intra-abdominal hemorrhage occurs, there is a 30% to 50% possibility of death; therefore, it is important to prevent PF after distal pancreatectomy. A recently published systemic review appraised all available surgical alternatives for handling the pancreatic remnant after distal pancreatectomy[1]. However, in many cases, the improved surgical techniques could not significantly reduce the incidence of PF[13].

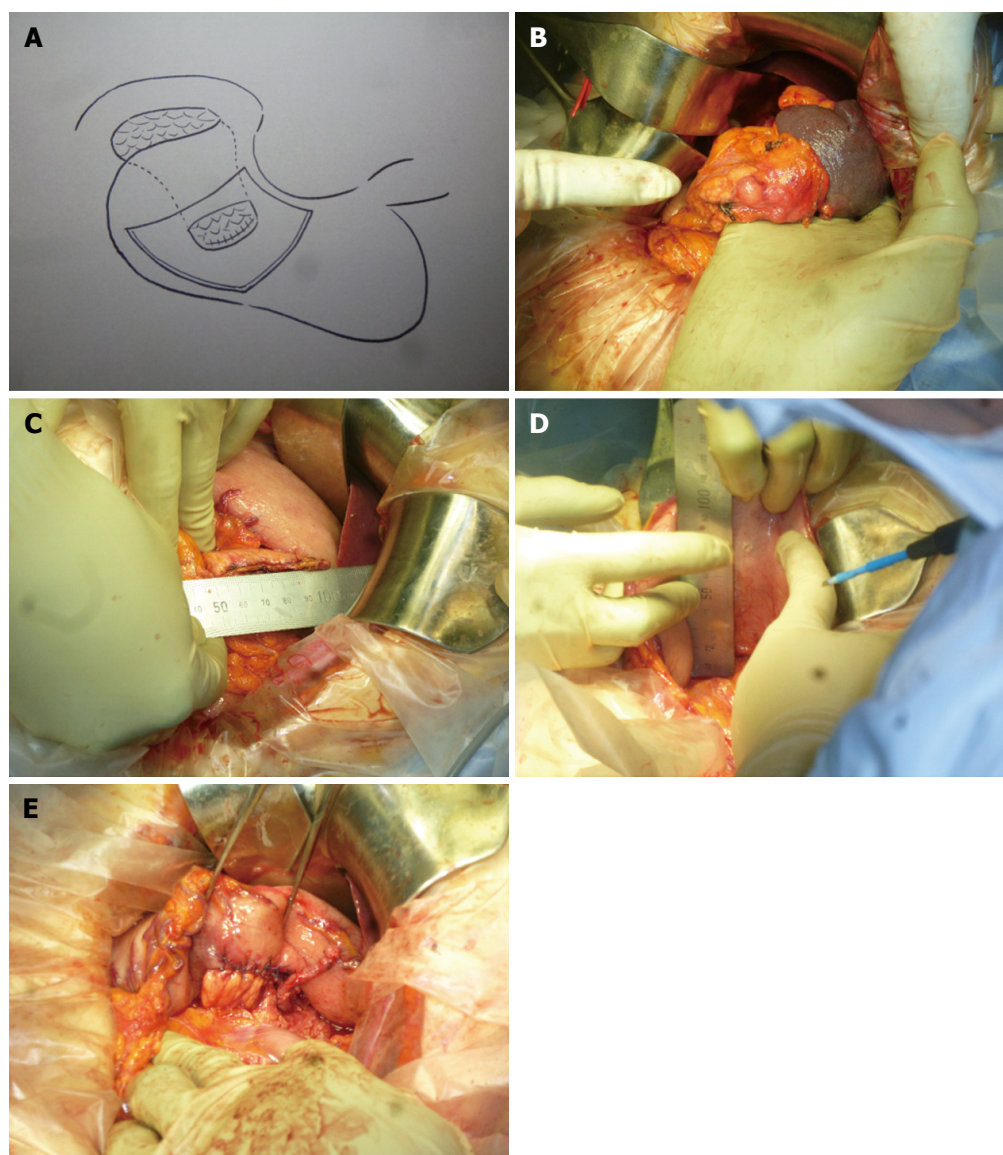Balcom *et al*[14] investigated 190 distal pancre-atectomies over a 10-year period from April 1990 to October 2000 at the Massachusetts General Hospital. They divided the cases into three periods (from September 1998 to October 2000, from July 1995 to August 1998, and from April 1990 to June 1995), and the incidence rates of PF during each period were 12%, 17%, and 14%, respectively. In other words, the incidence of PF did not decrease from 1990 to 2000. As a result, several studies have tried to identify improved methods of preventing PF after distal pancreatectomy. We can classify these methods into the following categories: (1) operation apparatus development; (2) ingenuity in drainage; (3) choice of drugs; and (4) development of operative techniques.

Several apparatuses have been investigated: the supersonic wave surgery aspirator (cavitron ultrasonic surgical aspirator), supersonic wave solidification incision device, and automatic suture device. The frequencies of PF with these aforementioned apparatuses have been reported as 4%, 8%, and 5.5 to 34%, respectively[15,16]. None of the reported apparatuses has completely prevented PF.

There are few studies on the predictive value of amylase in drains[17]. Regarding the contrivance of the drainage method, Molinari *et al*[18] measured serum amylase levels in drainage fluid postoperatively. They reported that it is possible to identify the risk of PF formation and development of complications using an amylase level of > 5000 U/L on postoperative day 1. They also reported that patients may benefit from lengthening the time of intensive postoperative therapy. When a PF was confirmed or suspected, contrast examination of the drain was performed, and they determined the most effective drainage route by studying the flow of contrast media and performing enforced washing of saline (2000 mL/d). Continuous washing was performed for 1-3 wk depending on the clinical situation and amylase level of the drain.

The use of different drugs in an attempt to prevent PF has been reported. Konishi *et al*[19] reported the use of prolamine emulsion, which is regularly used to treat kidney tumors; it was injected into the main pancreatic duct. According to the authors, no patient developed a PF among the 51 cases of distal pancreatectomy. Suzuki *et al*[7] also reported the use of fibrin glue for sealing the pancreatic stump to prevent PF following distal pancreatectomy. They reported that 15.4% of patients in the fibrin glue sealing group and 40.0% of those in the control group were diagnosed as having a PF after distal pancreatectomy. However, they stated the following concerns: the injection of drugs may destroy the lobular structure of the parenchyma and cause atrophic fibrosis of the exocrine glands.

When it comes to the development of operative maneuvers, Kuroki *et al*[20] reported a trial of 20 patients in whom the pancreatic stump was covered with the gastric wall, and they compared these patients to 33 patients in whom the conventional method was performed. Using their new technique, only one case (5.0%) of PF occurred. Conversely, 12 cases (36.4%)

Figure 3 Our procedure. A: We invaginate the pancreatic stump into the posterior wall of the stomach with single layer anastomosis using a 3-0 absorbable suture; B: The pancreatic tail tumor is indicated by the index finger on the left-hand side; C: Measurement of the width of the stump; D: The cutting length is determined at about 80% of the stump width; E: Anastomosis is completed. The pancreatic stump is invaginated into the posterior wall of the stomach.
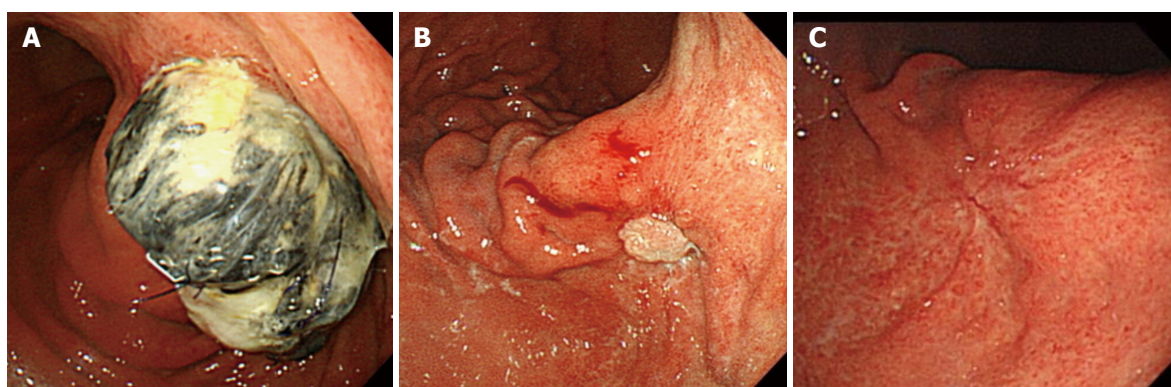
of PF occurred in patients treated with the conventional method. Additionally, they discussed the hardness of the pancreas. Namely, PF can easily occur in a soft pancreas, so the hardness of the pancreas should be considered when comparing the incidence of PF between methods. Lillemoe *et al*[21] reported their method of covering the pancreatic stump with a ligament from the liver, and they asserted that it is easy to use.

Authors of these previous reports declared the superiority of their method; however, we do not believe that there have been enough cases to determine which method is best. These different techniques reflect the clinical heterogeneity in this field[1]. The impact of these techniques is difficult to interpret owing to small sample sizes, non-randomized study designs, and inconsistent study populations and fistula definitions.

Therefore, we developed our new method of in-

vaginating the pancreatic stump into the stomach. Using this method, all leakage from the pancreatic stump flows into the stomach, so the results would not be dependent on the stiffness of the pancreas. This procedure does not require high-level techniques, and it only takes approximately 20 min to complete. The predicted disadvantages of this method include the following: (1) delayed gastric emptying may occur due to deformity of the stomach after fixation of the pancreas; (2) if a major leakage occurred from the gastropancreatic anastomosis, very severe complications would follow; and (3) if a future operation of the stomach is needed, it would be difficult to perform. However, this technique has been used for several years for pancreatoduodenectomy, with no major problems[22,23]. However, another limitation is that this procedure would not be beneficial in all cases.

Figure 4 The change of the invaginated pancreatic stump in the stomach. A: The invaginated pancreatic stump in the stomach after 1 wk; B: The stump after 3 mo; C: The stump after 1 year.

If the pancreatic tumor would have been on the left side of the portal vein and if the pancreatic resection line could not be moved to the left side of the portal vein, then we would not have been able to perform the invagination safely. In our case, if we had diagnosed the umbilical tumor as a metastasis of pancreatic cancer before the operation, pancreatectomy would not have been indicated. However, preoperative FDG-PET findings suggested that an inflammatory change had occurred, and we did not detect other metastases. We aimed to perform curative surgery and hoped to administer chemotherapy early. For these reasons, we needed to prevent PF as much as possible. Our patient was discharged on the eleventh day postoperatively and has not had any problems to date. We believe that our new method can be an effective procedure in some cases with distal pancreatectomy to prevent PF. We will continue to perform our method and collect data to examine its adequacy.

In conclusions, we obtained an excellent result in distal pancreatectomy invaginating the pancreatic stump into the stomach. Our new method may effectively prevent PF.

ACKNOWLEDGMENTS

COMMENTS

Case characteristics
A 50-year-old woman with a palpable umbilical mass at her navel.

Clinical diagnosis
A 2-cm-diameter tumor located in the pancreatic tail, along with low, enhanced foci scattered in the spleen. These findings suggested that the pancreatic cancer had already metastasized.

Imaging diagnosis
The abdominal computed tomography scan showed a 2-cm-diameter tumor located in the pancreatic tail. The para-aortic lymph node was not swollen; however, low, enhanced foci were scattered in the spleen. The fluorodeoxyglucose-positron emission tomography scan showed swelling in the pancreatic tail with abnormal accumulation.

Pathological diagnosis
Cytology of ascites showed that the umbilical region mass, the invasive ductal carcinoma, was a class V adenocarcinoma.

Treatments
The distal pancreas was resected without additional reinforcement to invaginate the stump into the gastric posterior wall with single layer anastomosis using a 3-0 absorbable suture.

Related reports
Ligation of the main pancreatic duct with a fish-mouth-shaped closure of the cut end has long been considered a standard technique for distal pancreatectomy. However, the probability of pancreatic fistula (PF) occurrence ranges from 32% to 57%.

Term explanation
Pancreatectomy is performed to excise pancreatic tumors. This resection commonly results in a PF, which can further result in major complications.

Experiences and lessons
There is a high probability of PF occurrence after pancreatectomy. Here, we describe the technique of invaginating the pancreatic stump into the stomach. This method may effectively prevent PF.

Peer-review
The authors describe a new innovative approach for preventing postoperative PF.

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