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EDITING
Editorial Board of *World Journal of Gastroenterology*,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
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EDITORIAL OFFICE
Jian-Xia Cheng, Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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EDITORIAL

Postoperative ileus: Impact of pharmacological treatment, laparoscopic surgery and enhanced recovery pathways

Knut Magne Augestad, Conor P Delaney

Knut Magne Augestad, Conor P Delaney, Division of Colorectal Surgery, University Hospitals Case Medical Center, Cleveland, OH 44106-5047, United States
Knut Magne Augestad, Department of Digestive Surgery, Telemedicine and Integrated Care, University Hospital North Norway, 9037 Tromsø, Norway
Author contributions: Augestad KM wrote the paper; Delaney CP supervised in the writing process, and critically revised the final version.
Supported by North Norwegian Health Authorities Research Fund
Correspondence to: Conor P Delaney, MD, PhD, Division of Colorectal Surgery, University Hospitals Case Medical Center, 11100 Euclid Avenue, Cleveland, OH 44106-5047, United States. conor.delaney@uhhospitals.org
Telephone: +1-216-8448087 Fax: +1-216-8445957
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Abstract

Almost all patients develop postoperative ileus (POI) after abdominal surgery. POI represents the single largest factor influencing length of stay (LOS) after bowel resection, and has great implications for patients and resource utilization in health care. New methods to treat and decrease the length of POI are therefore of great importance. During the past decade, a substantial amount of research has been performed evaluating POI, and great progress has been made in our understanding and treatment of POI. Laparoscopic procedures, enhanced recovery pathways and pharmacologic treatment have been introduced. Each factor has substantially contributed to decreasing the length of POI and thus LOS after bowel resection. This editorial outlines resource utilization of POI, normal physiology of gut motility and pathogenesis of POI. Pharmacological treatment, fast track protocols and laparoscopic surgery can each have significant impact on pathways causing POI. The optimal integration of these treatment options continues to be assessed in prospective studies.

Key words: Postoperative ileus; Pathophysiology; Cost utilization; Pharmacologic treatment; Laparoscopic surgery; Enhanced recovery pathways

Peer reviewers: Robert V Rege, MD, Department of Surgery, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas, TX 75390-9031, United States; Piers Gatenby, MA, MD, MRCS, Department of Surgery, Royal Free and University College Medical School, London, NW3 2PF, United Kingdom

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INTRODUCTION

All patients with a bowel resection develop postoperative ileus (POI), an interruption of bowel function after surgery[1-4]. POI is characterized by a transient cessation of bowel function with a variable reduction in motility sufficient to prevent effective transit of intestinal contents[5]. POI is the single most important determinant of length of stay (LOS) after abdominal surgery, and thus has significant implications for individual patients and hospital resource utilization.

POI has been discussed by surgeons for more than two centuries[6,7]. In 1906, Finney divided POI into three subgroups according to pathophysiology: mechanical, septic and adynamic[8]. After a century of debate a conse-

nsus conference in 2006[5] proposed a definition of POI as: "transient cessation of coordinated bowel motility after surgical intervention, which prevents effective transit of intestinal contents or tolerance of oral intake". Primary POI was defined as such cessation occurring in the absence of any precipitating complication, whereas secondary POI was defined as that occurring in the presence of a precipitating complication (infection, anastomotic leak, *etc*.). Patients undergoing major abdominal surgery are at highest risk for developing POI, which is related to the degree and length of manipulation of the intestines. Other surgical procedures may also be associated with POI, such as cardiac surgery, orthopedic surgery and trauma[9-12]. In addition to surgery and trauma, postoperative opioid analgesics that are necessary to manage postoperative pain contribute significantly to the incidence of POI.

Clinically POI is characterized by the inability to tolerate a solid diet, delayed passage of flatus and formed stool, pain and abdominal distention, nausea, vomiting, and accumulation of gas or fluids in the bowel[9,13]. Several pharmacological substances have been introduced to treat or prevent POI[14-26]. There has however been little if any proven success in pharmacologic limitation of POI until the reports investigating selective antagonism of opioid receptors.

In addition to pharmacological treatment, fast track or enhanced recovery pathways (ERP) and laparoscopic surgery have introduced new dimensions in treatment of POI. ERP shorten the postoperative recovery period[27] and laparoscopic surgery shortens the average length of POI[28]. The objective of the present paper is to highlight POI and its consequences for patients and society, and the impact pharmacological treatment, laparoscopic surgery and ERP have upon the pathogenesis and duration of POI.

COST IMPLICATIONS OF POI

POI as a complication of major abdominal surgery can have a substantial clinical and economic impact. POI is associated with increased postoperative morbidity, reduced patient satisfaction, and increased length of hospital stay. Moreover, POI-related increases in LOS and use of resources translated into increased costs for the health care system[29]. It has been estimated that POI accounts for a significant amount of perioperative health care costs in the US[5], with estimated total hospital costs attributable to POI $1.28 billion[5].

A recently published study by Iyer *et al*[30] identified 17 000 patients undergoing colectomy in the US Premier Perspective database. The mean hospital LOS was significantly longer in patients with POI compared with patients without POI (13.8 d *vs* 8.9 d). POI in colectomy patients is a significant predictor of increased hospital resource utilization.

Clearly, reducing the LOS associated with POI has become a health care priority for the surgical community, with interventions reducing the LOS having great implications both for the individual patient and for hospital cost utilization.

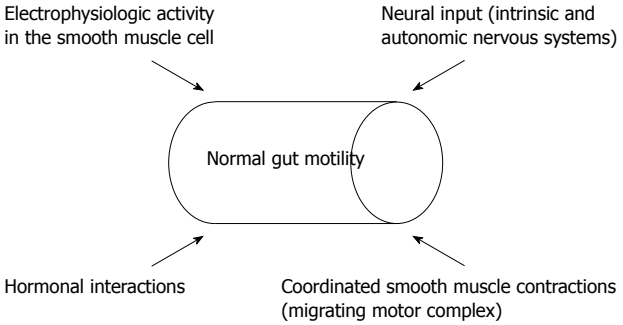

Figure 1 Normal physiology of gut motility.

Bell *et al*[31] recently published a paper, where the economic effect of the use of alvimopan in four randomized controlled trials (RCT) trials was analyzed. This paper used LOS data from the North American alvimopan trials, and calculated cost by extrapolation of cost from the Premier Perspective database, and cost of medication, using mathematical modeling techniques. Compared with placebo, alvimopan was associated with a significantly shorter mean time to gastrointestinal (GI) recovery and a mean hospital LOS of one full day less than placebo. The mean estimated hospital cost was $879-$977 less for patients who received alvimopan compared with placebo. Bell *et al*[31] suggest that use of alvimopan compared with placebo may have a cost-saving effect in the hospital setting. It is however unresolved if alvimopan is more cost effective than optimal use of laparoscopic surgery and ERP protocols, and further research is needed.

PATHOGENESIS OF POI

Normal bowel function is a complex interaction between GI motility, mucosal transport and defecation reflexes. The motility of the intestines is dependent upon the electrophysiological activity in the smooth muscle cells, neural input from the intrinsic and autonomic nervous system, hormonal interactions and coordinated smooth muscle interaction[32] (Figure 1). The migrating motor complex (MMC) has a central position in the normal gut motility[10,33]. The MMC is divided into four phases and regulates the gut motility (contractile pattern) between meals[34] and occurs approximately once every 1-2 h; Phase Ⅰ: Oscillating smooth muscle membrane potentials without actual muscle contractions. Phase Ⅱ: Occurrence of intermittent muscle contractions. Phase Ⅲ: Contractions increase to the maximal contractile frequency (i.e. stomach 3 contractions/min and duodenum 11 contractions/min). Phase Ⅳ: cessation of contractions, and the bowel returns to Phase Ⅰ.

In addition, the nervous system (enteric and central)[35], hormones[36] and smooth muscle activity play roles. Ingestion of food activates these mechanisms and turns off the MMC contractile pattern.

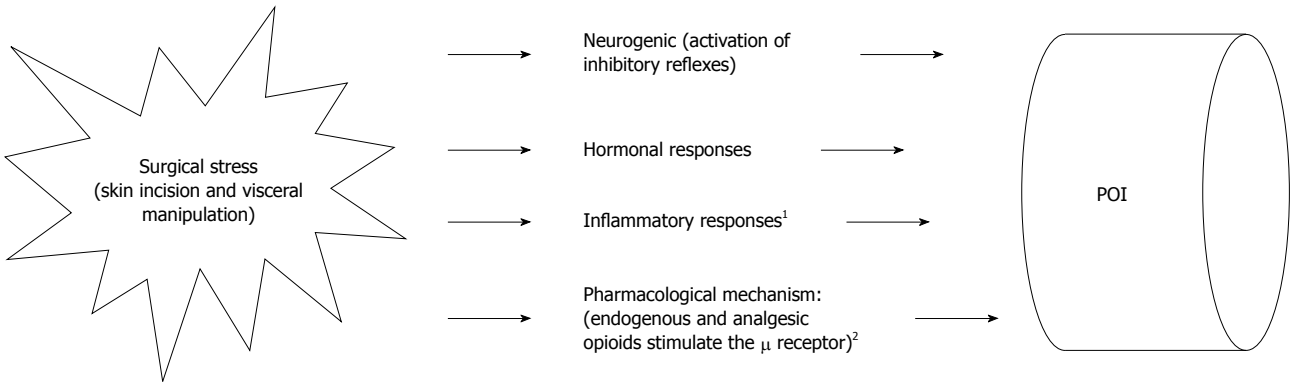

Figure 2 Pathogenesis of postoperative ileus. [1]Laparoscopic surgery decreases the surgical stress and inflammatory response; [2]Primarily exogenous opioids, e.g. morphine, binding to μ-receptors in the GI tract, which results in disorganized and non propulsive motility and, thus, prolongs ileus. In addition, activation of opioid receptors, which occurs following major abdominal surgery, inhibits acetylcholine release, reduces gastrointestinal motility, and has been demonstrated to play a key role in POI regulatory pathways[10,29,36-38]. Alvimopan inhibits this effect by blocking the pheripheral opioid μ receptor.

The interactions between these regulatory components are complex and much is unknown. Contractions in the colon differ from the small intestine, with irregular oscillations and contractile patterns[10,35].

Although multiple factors are thought to contribute to the pathogenesis of POI, four major pathways have been identified (Figure 2): (1) Neurogenic: surgical stress (i.e. skin incision and bowel manipulation) response stimulates inhibitory neural reflexes resulting in decreased bowel motility. This activation of inhibitory reflexes inhibits the normal MMC pattern and is activated as early as at the moment of skin incision (by somatic fibers) and from manipulation of the intestines (by visceral fibers)[37]; (2) Inflammatory: bowel manipulation and resection stimulates normally inactive macrophages and neutrophil recruitment with release of inflammatory mediators that reduce bowel motility; this includes endogenous opioid peptides. Manipulation also causes secretion of proinflammatory cytokines[37]. An increase in the degree of surgical manipulation leads to increased accumulation of neutrophils, macrophages, mast cells, T cells, natural killer cells and dendritic cells which in turn leads to increased inflammation in the intestine and tissue damage[32]. All these factors induce paralysis of the intestine and in turn POI; (3) Hormonal: surgical stress results in elevation of corticotrophin-releasing factor, which stimulates release of inflammatory mediators in the bowel[38]. In addition, a wide variety of local factors, hormones and neurotransmitters may play a role in POI (i.e. Substance P, Nitric Oxide and Calcitonin gene-related peptide CGRP)[10]; and (4) Pharmacologic: primarily exogenous opioids, e.g. morphine, binding to μ-receptors in the GI tract, which results in disorganized and non-propulsive motility and thus prolongs ileus. In addition, activation of opioid receptors, which occurs following major abdominal surgery, inhibits acetylcholine release, reduces GI motility, and has been demonstrated to play a key role in POI regulatory pathways[10,32,39-41].

Opioid-based regimens are the most common treatments to effectively manage post-surgical pain. However, morphine and other μ-opioid receptor agonists can prolong the duration of POI through delayed gastric emptying, reduced GI motility, and disrupted colonic myoelectric activity[42,43]. In addition to exogenous opioids, studies have suggested a role for endogenous opioid peptides in various postoperative responses, including ileus[43-46].

PHARMACOLOGICAL TREATMENT AND POI

Up to the year 2000, several pharmacological agents had been studied in order to prevent POI, however none of these agents was effective enough to become part of routine established practice[14-26].

Chewing sugarless gum following elective intestinal resection is associated with improved outcomes. Gum chewing is thought to promote physiological simulation of the cephalic-vagal axis, thereby increasing bowel motility and GI stimulation[47-49]. There exist two meta-analyses analyzing the effects of chewing gum[50,51], however, all of the included trials in these meta-analyses were small, and only two trials had an adequate sample size calculation.

The meta analysis showed an improvement in first time to bowel movement of 23 h and a reduction in LOS of 1.1 d, and is similar to the effect shown by alvimopan. An adequately powered, methodologically rigorous trial of gum chewing is however required to confirm if there are any benefits[52].

After five RCT, alvimopan represented a novel breakthrough in pharmacological treatment of POI. Alvimopan is a selective peripherally acting μ-opioid antagonist administered orally, blocking the μ-opioid receptor and minimizing the paralytic effect opiates have upon the intestines. The medication blocks the peripheral μ-receptor with high affinity, therefore minimizing the paralytic effect opiates have upon the intestines (Figure 2). This molecule is large and polar and therefore does not cross the blood brain barrier and therefore does not impair analgesia. During the last 5 years five RCTs have been conducted upon alvimopan and its effect upon POI[53-58]. In all these trials

Table 1 Overview of RCT trials 2004-2008 of alvimopan

Author	Number of patients	Primary end point	GI[2] improvement (h)	GI[3] improvement (h)	Hazard ratio	DCO improvement (h)
Wolff *et al*[57]	510	GI[3]	20.0[1]	15.0[1]	1.28[1]	13.0[1]
			28.0[2]	22.0[2]	1.54[2]	20.0[2]
Viscusi *et al*[56]	666	GI[3]	16.4[1]	7.5[1]	1.20[1]	14.2[1]
			13.7[2]	9.9[2]	1.24[2]	15.2[2]
Delaney *et al*[55]	451	GI[3]	15.2[1]	14.1[1]	1.45[1]	14.0[1]
			10.5[2]	7.5[2]	1.28[2]	7.2[2]
Ludwig *et al*[53]	654	GI[2]	20.0[2]	16.0[2]	1.50[2]	17.0[2]
Buchler *et al*[58]	911	GI[3]	14.3[1]	8.5[1]	1.18[1]	8.1[1]
			10.7[2]	4.8[2]	1.37[2]	5.9[2]

[1]6 mg alvimopan; [2]12 mg alvimopan. Hazard ratio: The ratio of two hazard rates. Hazard rate is defined as the probability to failure given survival to date. DCO: Discharge order; GI[2]: The time to recovery of GI function, a composite end points that represent full upper and lower recovery. i.e. time that the patient first tolerated solid food and time that the patient first passed a bowel movement; GI[3]: The time to recovery of GI function, a composite end points that represents full upper and lower recovery. i.e. time that the patient first tolerates solid food, or time that the patient first passes flatus or a bowel movement.

recovery of bowel function was enhanced, except one from Europe when lower opioid doses were used. The primary endpoints in these trials were time to recovery of GI function, measured as either GI[2] or GI[3], composite measures that were developed to track recovery of upper and lower GI function. In these trials GI[2] was defined as "the time that the patient first tolerated solid food and had passed a bowel movement". GI[3] was defined as "the time that the patient first tolerates solid food, and the patient first passes flatus or a bowel movement".

The results from these trials lead to Food and Drug Administration approval in May 2008. A summary of the trial results is shown in Table 1.

The definition of lower GI recovery has been controversial and debated, and up to date there exists no consensus upon this definition. All of the RCTs chose GI[3] as their primary endpoint for GI recovery, except the study by Ludwig *et al*[53], as GI[2] had been noted to be a more robust endpoint by the collaborative study group. GI[3] uses documentation of passage of flatus which can be subject to considerable variability[59], since the patient has to be conscious and willing to report it. In this way it was advocated that GI[2] is a more objective endpoint[53], i.e. a composite end point that was represented by the time the patient first tolerated solid food and time that the patient first passed a bowel movement. In the trial by Ludwig *et al*[53], GI[2] recovery was primarily driven by time to first bowel movement, as this was the later occurring of the two components of GI[2] recovery. In the RCTs there are no obvious dose response curves for alvimopan, and a dose of 12 mg does not give an increased GI recovery, although data were slightly more consistent through the trials (Table 1). This phenomenon is common for new drugs, particularly biologic agents, where effects on a receptor are not directly related to the concentration of the agent. These discrepancies may also be attributed to differing patient populations. However, a pooled analysis has shown that a 12 mg dose provided more consistent benefits across both sexes and all ages[54].

Alvimopan has had fewer side effects than placebo. Treatment adverse events reported in the published RCTs were most commonly nausea and vomiting, but these were less common in the alvimopan treated groups[60]. All studies on alvimopan have been conducted for open abdominal surgery.

Alvimopan will increase pharmacy expenditures; the cost of acquiring gum is substantially lower but more research is needed upon the effect of gum. Whether there is an additive effect upon GI recovery of pharmacological treatment (i.e. alvimopan and/or chewing gum) in combination with laparoscopic surgery and ERP protocols is unresolved.

LAPAROSCOPIC SURGERY AND POI

The greatest advance in limiting POI to date has probably resulted from the expanded use of laparoscopic surgery and the advantage of limiting tissue trauma. Recent studies suggest that laparoscopic surgery causes a lesser degree of mast cell activation and inflammation, and thus prolonged POI[61] (Figure 2). Laparoscopic surgery has many potential advantages over conventional open surgery, including smaller incisions, earlier GI recovery, shorter hospital stay and less pain[62].

Studies of large national databases suggest a higher rate for all commonly identified complications for open compared to laparoscopic colectomy. In a recent published study by Senagore *et al*[63], 4419 cases of open laparotomy were compared to 2728 cases of laparoscopy. All perioperative complications were more frequent in the open laparotomy group. Other database studies, identifying more than 30 000 cases of colectomies, shows superior results of laparoscopic colectomies compared to open cases[64]. Similarly, most other trials have demonstrated significant reductions in time to recovery of GI function after laparoscopic colectomy compared with open techniques, which translate into decreased hospital LOS. In a recent published study[28], mean GI recovery and LOS after laparoscopic colectomy were accelerated compared with those for patients in open laparotomy bowel resection.

Table 2 Effect of interventions to improve gastrointestinal recovery and reduce LOS

Trials	Type of study	Intervention	Improvement passage flatus	Improvement first bowel movement	Decrease in LOS
Noble *et al*[52]	Meta analysis	Chewing gum	14 h	23 h	1.1 d
Delaney *et al*[54]	Pooled analysis	Alvimopan	12 h[1] (6 mg) 15 h[1] (12 mg)	15 h[2] (6 mg) 18 h[2] (12 mg)	18.4 h[3]
Delaney *et al*[28]	Observational multicenter study	Laparoscopic surgery	NA	0.7 d[2]	1.7 d[3]
Walter *et al*[69]	Meta analysis	ERP	NA	NA	3.64 d

[1]Defined as GI[3]; [2]Defined as GI[2]; [3]Discharge order written. Standard elective open colorectal resection is usually associated with LOS of 8-12 d. Pharmacological treatment, laparoscopic surgery and ERP have substantially decreased LOS after surgery. LOS: Length of stay; ERP: Enhanced recovery pathways; NA: Not available.

The primary end points were time to upper and lower GI recovery (GI[2]: toleration of solid food and bowel movement) and postoperative LOS.

Overall POI-related morbidity (postoperative nasogastric tube insertion or investigator-assessed POI resulting in prolonged hospital stay or readmission) was similar between the open bowel resection and laparoscopic colectomy populations, suggesting that POI continues to cause significant morbidity regardless of the surgical approach[28].

Overall, there is substantial evidence that laparoscopic surgery helps accelerate GI recovery after surgery. Although the results in a study by Basse *et al*[65] did not show a benefit with laparoscopy, in our opinion the burden of evidence supports laparoscopic surgery as the standard approach when appropriate.

ERP AND POI

ERP have become part of the standardized postoperative recovery pathway at most hospitals. These protocols include many different elements and interventions, up to 20 elements in a recent published consensus review[66]. The main ERP elements are: avoidance of bowel preparation, preoperative fasting and carbohydrate loading, opioid sparing analgesia and mid thoracic epidural, antibiotic prophylaxis, laparoscopic surgery, small surgical incisions, no nasogastric tubes, normothermia, operative and postoperative fluid restrictions, no abdominal drains, suprapubic urinary drainage, oral diet at will, and early mobilization.

The benefits of fast track protocols (improved recovery, shortening of hospital stay, and earlier recovery of GI function) have been established in several RCTs[67]. Two systematic reviews of controlled and RCT supports the use of fast-track colorectal surgery[68,69]. ERP appear to be safe and shorten hospital stay 1-4 d after elective open colorectal surgery.

During the recent years, old dogmas have been challenged, such as keeping patients fasting after surgery. Allowing patients to eat normal food at will from the first day after major GI surgery does not increase morbidity, including the frequency of POI, when compared with traditional care with nil-by-mouth and enteral feeding[70]. LOS was reduced by approximately 3 d in the group allowed normal food at will. Similarly the use of a nasogastric tube after surgery has been debated. In fact routine nasogastric decompression does not accomplish any of its intended goals (including increased GI recovery) and so should be abandoned in favor of selective use of the nasogastric tube[71].

The best postoperative analgesic regime after surgery has been addressed in several trials, and is still debated. A recent published study advocates the use of thoracic epidural analgesia[72], however this conclusion is contested in a systematic review by Levy *et al*[73]. According to this review there is a paucity of data assessing the benefits of postoperative analgesic regimes following laparoscopic colorectal surgery and none of the protocols were shown to be clearly superior. Low *et al*[74] outlines the concerns of hypotension (increased risk of cardiovascular complications) and splanchnic hypoperfusion (increased risk of anastomotic leak) that are observed in connection with epidural analgesia. The best postoperative analgesic regime is therefore still unresolved; however epidural analgesic regime is included in most published ERP protocols, including a recent published consensus review[66]. Interestingly, while several randomized studies have suggested earlier recovery of GI function with epidurals, this has only been shown for opioid free epidurals, and has never translated into shorter hospital stay.

Over the last decade it has been increasingly realized that the pathophysiology of POI is multifactorial. Thus ideally ERP, and the care provided to colectomy patients should beneficially influence all four pathophysiologic pathways in the pathogenesis of POI (neurogenic, pharmacologic, hormonal, inflammatory, Figure 2)[75]. Although it is not yet clear which pathway is ideal, there is good consensus that fast track, ERP should be used at all modern hospitals, and included in all postoperative care.

FUTURE PERSPECTIVES

Standard elective colorectal resection is usually associated with a complication rate of 20%-30% and a postoperative stay of 8-12 d[67]. The introduction of pharmacological treatment, fast track protocols and laparoscopic surgery has changed this perspective (Table 2).

The introduction of laparoscopy in colorectal surgery improves early postoperative outcome, and randomized

trials have shown promising short term benefits with reduction of LOS by 3-4 d.

Despite rigorous research and several RCT trials during the past decade, the use of alvimopan is still debated, and for unclear reasons its incorporation into practice has been less than might be expected[76,77]. Several important questions remains unresolved, particularly the effect of alvimopan after laparoscopic surgery. After laparoscopic surgery, patients have less pain, lower opioid requirements and a shorter recovery time. It is unclear if alvimopan will have an additive effect upon the duration of POI and consequently LOS for this patient group.

Similarly, all of the alvimopan RCT studies were performed upon patients using a simple and standardized ERP protocol in the treatment and placebo groups. While ERP protocols may include up to 20 different elements, it is unclear which of these elements have the greatest impact upon POI and LOS. Similarly it is unclear what additive effect alvimopan may have upon POI and LOS for patients using ERP protocols. Recent studies[28,78,79] have however included fast track protocols in combination with laparoscopic surgery with promising results upon POI. Future POI research should address fast track protocols, laparoscopic surgery and pharmacological treatment, and the optimal combination of these interventions.

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EDITORIAL

Management of gallstones and gallbladder disease in patients undergoing gastric bypass

Bernabé M Quesada, Gustavo Kohan, Hernán E Roff, Carlos M Canullán, Luis T Chiappetta Porras

Bernabé M Quesada, Gustavo Kohan, Hernán E Roff, Carlos M Canullán, Luis T Chiappetta Porras, Department of Surgery, Cosme Argerich Hospital, Alte. Brown 240, ZC 1155 Buenos Aires, Argentina
Author contributions: All authors contributed equally.
Correspondence to: Bernabé M Quesada, MD, Department of Surgery, Cosme Argerich Hospital, Alte. Brown 240, ZC 1155 Buenos Aires, Argentina. bmquesada@hotmail.com
Telephone: +54-114-1212925 Fax: +54-114-1212925
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Abstract

The appropriate management of gallstones and gallbladder disease in patients undergoing gastric bypass remains unknown. Several therapeutic modalities are used and include performing cholecystectomy on all patients at the time of gastric bypass, performing concomitant cholecystectomy only when patients have gallstones and performing cholecystectomy only in the presence of both symptoms and gallstones. Some groups administer ursodeoxycholic acid for gallstone prevention in the postoperative period. All treatment modalities are analyzed and their results and rationality are discussed.

Key words: Gallbladder; Gastric bypass; Morbid obesity; Gallstones; Cholecystectomy

Peer reviewers: Florencia Georgina Que, MD, Department of Surgery, Mayo Clinic, 200 First Street Southwest, Rochester, MN 55905, United States; Masayuki Sho, MD, PhD, Professor, Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, 634-8522, Japan; Jai Dev Wig, MS, FRCS, Former Professor and Head, Department of General Surgery, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

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INTRODUCTION

Rapid weight loss after bariatric surgery is one of many known risk factors for gallstone development, along with age, female gender, parity, race, obesity, genetics, very-low-calorie diets, short bowel syndrome, gallbladder motor dysfunction, diabetes, drugs and gastrointestinal surgery, among many others[1-25].

Traditionally cholecystectomy was indicated only in the presence of both gallstones and symptoms, but recently some have advocated elective cholecystectomy in selected cases in the absence of symptoms and even in the absence of gallstones[26-29].

Sustained weight loss after gastric bypass is achieved by a combination of gastric restriction and a variable degree of malabsorption[30-32] and has therefore a greater risk for gallstone development than purely restrictive procedures like adjustable gastric banding[33-35]. The appropriate management of gallstones and gallbladder disease in these patients is still under debate and several therapeutic modalities are used[36], including simultaneous cholecystectomy to all patients at the time of gastric bypass, regardless of the presence or absence of gallstones and/or symptoms (prophylactic approach)[37], simultaneous cholecystectomy only to patients with gallstones (elective or selective approach)[38] and expectant management with or without the prophylactic administration of ursodeoxycholic acid until symptoms develop (conventional approach)[39].

The objective of the paper is to discuss the rationale and the results obtained with these therapeutic modalities.

PROPHYLACTIC APPROACH

This consists of performing simultaneous cholecystectomy on all patients at the time of gastric bypass, regardless of the presence or absence of gallstones and/or symptoms.

The rationale behind this approach is based on the elevated incidence of gallstone development after gastric bypass compared to the normal population and the low sensitivity and specificity of ultrasonography for detecting gallstones in patients with morbid obesity[37,40-42]. A minimal morbidity rate with the addition of cholecystectomy is required.

In the series of Fobi *et al*[37] abnormal findings in the gallbladder were found in 75% of surgical specimens despite a negative preoperative ultrasound. Most of these patients had gallstones but other findings include cholesterolosis and cholecystitis. The addition of cholecystectomy to open gastric bypass added only an average of 15 min and the authors report no specific morbidity related to it.

Nougou *et al*[43] found some pathology in nearly 82% of specimens after simultaneous cholecystectomy with laparoscopic gastric bypass. 8.3% of patients did not undergo simultaneous cholecystectomy because it was judged to be dangerous. In the remaining patients, cholecystectomy added only 19 min on average to the procedure, with no extra ports addition. The authors do not report specific morbidity related to cholecystectomy.

Guadalajara *et al*[41] performed simultaneous cholecystectomy on 89 patients undergoing open gastric bypass and found a postoperative incidence of gallstones of 24% while the preoperative ultrasound incidence was only 16%.

Liem *et al*[44] performed simultaneous cholecystectomy on all patients undergoing open gastric bypass and found an incidence of gallbladder pathology of 80%.

A summary of these results is presented in Table 1.

ELECTIVE/SELECTIVE APPROACH

This consists of performing simultaneous cholecystectomy only on patients with asymptomatic gallstones diagnosed pre or intraoperatively.

The rationale behind this approach is based on an assumed higher incidence of symptomatic gallbladder disease in patients with gallstones in comparison to those without them. Some groups administer prophylactic ursodeoxycholic acid to patients without gallstones and therefore not submitted to concomitant cholecystectomy. A low morbidity rate is also required to support this approach.

Hamad *et al*[38] performed simultaneous cholecystectomy on 16.9% of patients at the time of gastric bypass. Operative times were significantly longer for patients undergoing simultaneous cholecystectomy and total hospital stay was almost doubled in comparison to gastric bypass without concomitant cholecystectomy. A significantly higher major morbidity rate was observed for patients undergoing simultaneous cholecystectomy but no specific morbidity was directly related to it. The most common pathological finding in the specimens was cholecystitis (99% of the cases). All patients without simultaneous cholecystectomy received 300 mg of ursodeoxycholic acid twice a day orally for a 6 mo period. During follow up 2.3% of these patients developed symptomatic gallstone disease and required cholecystectomy after an average of 12.4 mo.

Villegas *et al*[45] performed simultaneous cholecystectomy on 14% of patients after intraoperative diagnosis of gallstones or sludge with the aid of laparoscopic ultrasound. The global need for a subsequent cholecystectomy was 7%. Patients completing prophylactic ursodeoxycholic acid treatment had a significantly lower need of subsequent cholecystectomy.

In the series of open gastric bypass of Caruana *et al*[46] the diagnosis of gallstones was made by intraoperative palpation of the gallbladder. The authors did not report significant morbidity related to the addition of cholecystectomy. A subgroup of 125 patients that did not undergo simultaneous cholecystectomy was followed for at least 16 mo, requiring 8% of them to have a subsequent cholecystectomy for symptomatic gallstone disease.

Ahmed *et al*[47] retrospectively analyzed a series of 400 consecutive patients and found only significant differences in terms of operative times, which were 29 min longer for patients undergoing simultaneous elective cholecystectomy. No information about the incidence of symptomatic gallstone disease in the population without simultaneous elective cholecystectomy is given.

The group of the Universidad Católica de Chile reports a rate of simultaneous elective cholecystectomy of 10.9%[48]. The only significant differences were found for operative times, which were higher for the population undergoing simultaneous elective cholecystectomy. No information regarding the incidence of symptomatic gallstones in the population without simultaneous elective cholecystectomy was given.

Taylor *et al*[49] performed simultaneous cholecystectomy on 15% of patients. They reported the lowest need for subsequent cholecystectomy without the administration of prophylactic ursodeoxycholic acid, with only 3% of patients requiring it.

Tucker *et al*[50] performed simultaneous cholecystectomy on 7.2% of patients. A subgroup of patients was not submitted to this approach although they had gallstones present at the time of gastric bypass. The need for subsequent cholecystectomy in these patients was 17.6% whereas for patients without gallstones at the time of gastric bypass it was 6%.

A summary of these results is presented in Table 1.

CONVENTIONAL APPROACH

This consists of performing cholecystectomy only in the presence of both gallstones and symptoms, following the present guidelines for gallstone disease management[51,52]. The rationale behind this approach is to

Table 1 Results for cholecystectomy

Author	Yr	Indication for cholecystectomy	Increased morbidity	Ursodesoxicolic acid administration	Need for subsequent cholecystectomy
Fobi *et al*[37]	2002	Prophylactic open	No	NA	NA
Nougou *et al*[43]	2008	Prophylactic laparoscopic	No	NA	NA
Guadalajara *et al*[41]	2006	Prophylactic open	No	NA	NA
Liem *et al*[44]	2004	Prophylactic open	No	NA	NA
Hamad *et al*[38]	2003	Selective laparoscopic	Yes	Yes	2.30
Villegas *et al*[45]	2004	Selective laparoscopic	NR	Yes	7.00
Caruana *et al*[46]	2005	Selective open	No	No	8.00
Ahmed *et al*[47]	2007	Selective laparoscopic	No	No	NR
Escalona *et al*[48]	2008	Selective laparoscopic	No	No	NR
Taylor *et al*[49]	2006	Selective open	NR	No	3.00
Tucker *et al*[50]	2008	Selective laparoscopic	No	NR	11.80
Swartz *et al*[53]	2005	Conventional	NA	Yes	14.70
Fuller *et al*[54]	2007	Conventional	NA	Yes	7.69
Ellner *et al*[55]	2007	Conventional	NA	No	9.00
Portenier *et al*[56]	2007	Conventional	NA	No	8.10
Papasavas *et al*[39]	2006	Conventional	NA	No	7.83
Patel *et al*[57]	2006	Conventional	NA	No	6.00
Patel *et al*[58]	2009	Conventional	NA	No	4.90
Cosme Argerich Hospital	2010	Conventional	NA	No	9.84

NA: Not applicable; NR: Not reported.

indicate cholecystectomy only for the patients requiring it and since most of the subsequent cholecystectomies are performed when a significant weight loss is achieved, the operation is done in a leaner and healthier patient.

Swartz *et al*[53] found an incidence of subsequent cholecystectomy of 14.7%, with a significant lower incidence for patients completing prophylactic ursodeoxycholic acid treatment. In Fuller and coworker's experience, the need for subsequent cholecystectomy in patients completing prophylactic ursodeoxycholic acid treatment was 7.69%[54].

Ellner *et al*[55] and Portenier *et al*[56] did not administer prophylactic ursodeoxycholic acid treatment and found an incidence of subsequent cholecystectomy of 9% and 8.1%, respectively.

Papasavas *et al*[39] did an interesting study in which they retrospectively reviewed the records of 644 patients undergoing gastric bypass without simultaneous cholecystectomy. All of them received postoperative prophylactic ursodeoxycholic acid treatment. The need for a subsequent cholecystectomy in patients with gallstones present at the time of gastric bypass was 8.3%, which is similar to the incidence observed for patients without gallstones (6.9%) and to the incidence for patients without preoperative ultrasound screening (8.3%). After obtaining these results, this group no longer screens for gallstones preoperatively. Similarly, Patel *et al*[57] at the UCLA School of Medicine found an incidence of subsequent cholecystectomy without preoperative screening and without postoperative ursodeoxycholic acid administration of 6%. A subsequent analysis by the same group with a longer follow up showed an even lower rate of subsequent cholecystectomy of 4.9%[58].

In our experience at Cosme Argerich Hospital from Buenos Aires, the global need for cholecystectomy after gastric bypass, without prophylactic ursodeoxycholic acid treatment, was 9.84% (unpublished data). For patients with gallstones present at the time of surgery a subsequent cholecystectomy was needed in 5% and for patients without gallstones in 10.71% (*P*: not significant). Based on our own data, the natural history of patients with asymptomatic gallstones undergoing gastric bypass is very much like the natural history of asymptomatic gallstones in the general population[59-61].

A summary of these results is presented in Table 1.

URSODEOXYCHOLIC ACID TREATMENT

The preventive administration of ursodeoxycholic acid proved to be significantly better than placebo in preventing gallstone formation in a double blind, prospective and randomized study conducted by Sugerman *et al*[62]. A daily dose of 600 mg was associated with the lowest rate of gallstone formation and the lowest incidence of adverse events. Patients that developed gallstones showed a lower compliance rate. The effect of the 6 mo treatment seems to last for at least 1 year, since at that moment patients were reevaluated with ultrasonography and the incidence of gallstones was significantly less compared to the placebo arm. Unfortunately, there is no mention in the study regarding how many of those patients that developed gallstones were actually symptomatic and therefore required cholecystectomy, since the actual standard of care for non-obese patients indicates a cholecystectomy only when both symptoms and gallstones are present (SSAT NIH). A true benefit for ursodeoxycholic acid would be a lower rate of delayed cholecystectomy over the placebo group.

Wudel *et al*[63] compared, in a randomized double-blind fashion, a cohort of 60 patients without gallstones at the time of open gastric bypass and prescribed them

a 6-mo course of ursodeoxycholic acid, ibuprofen or placebo. 71% of the patients subsequently developed gallstones and no benefit of the two therapies investigated could be demonstrated because of an extremely low compliance rate of 28%.

A recently published meta-analysis by Uy *et al*[64] concluded that ursodeoxycholic acid administration prevents gallstone formation after bariatric surgery, but no meta-analysis of symptomatic gallstones could be done, since only one paper addressed this topic and unfortunately did not include patients undergoing gastric bypass[65].

CONCLUSION

Prophylactic and selective management can be safely performed and the only significant difference with patients not submitted to concomitant cholecystectomy is mostly observed in operative times that are higher in those who do undergo cholecystectomy. Data obtained from conventional management studies, with or without ursodeoxycholic acid administration, show that most of the patients remain asymptomatic even when they develop gallstones and therefore do not require a subsequent cholecystectomy, so that the risks of performing a concomitant cholecystectomy might be unwarranted. Treatment with ursodeoxycholic acid prevents gallstone formation after gastric bypass but most of the studies conducted show a low compliance rate and do not mention the true benefit of the treatment, which would be a lower cholecystectomy rate.

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TOPIC HIGHLIGHT

Ki-Baik Hahm, MD, PhD, Professor, *Series Editor*

Connection between inflammation and carcinogenesis in gastrointestinal tract: Focus on TGF-β signaling

Suntaek Hong, Ho-Jae Lee, Seong Jin Kim, Ki-Baik Hahm

Suntaek Hong, Laboratory of Cancer Cell Biology, Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, Incheon 406-840, South Korea
Ho-Jae Lee, Laboratory of Chemoprevention, Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, Incheon 406-840, South Korea
Seong Jin Kim, Laboratory of Cell Regulation and Carcinogenesis, Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, Incheon 406-840, South Korea
Ki-Baik Hahm, Laboratory of Translational Medicine, Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, Incheon 406-840, South Korea; Department of Gastroenterology, Gachon Graduate School of Medicine, Incheon 406-840, South Korea
Author contributions: Hong S wrote the manuscript; Lee HJ contributed to article processing; Kim SJ mentored all the research regarding TGF-β; Hahm KB organized, revised and orchestrated the manuscript.
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Correspondence to: Ki-Baik Hahm, MD, PhD, Laboratory of Translational Medicine, Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, Incheon 406-840, South Korea. hahmkb@gachon.ac.kr
Telephone: +82-32-8996055 Fax: +82-32-8996054
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Abstract

Inflammation is a primary defense process against various extracellular stimuli, such as viruses, pathogens, foods, and environmental pollutants. When cells respond to stimuli for short periods of time, it results in acute or physiological inflammation. However, if the stimulation is sustained for longer time or a pathological state occurs, it is known as chronic or pathological inflammation. Several studies have shown that tumorigenesis in the gastrointestinal (GI) tract is closely associated with chronic inflammation, for which abnormal cellular alterations that accompany chronic inflammation such as oxidative stresses, gene mutations, epigenetic changes, and inflammatory cytokines, are shared with carcinogenic processes, which forms a critical cross-link between chronic inflammation and carcinogenesis. Transforming growth factor (TGF)-β is a multi-potent cytokine that plays an important role in regulation of cell growth, apoptosis and differentiation. Most importantly, TGF-β is a strong anti-inflammatory cytokine that regulates the development of effector cells. TGF-β has a suppressive effect on carcinogenesis under normal conditions by inhibiting abnormal cell growth, but on the other hand, many GI cancers originate from uncontrolled cell growth and differentiation by genetic loss of TGF-β signaling molecules or perturbation of TGF-β adaptors. Once a tumor has developed, TGF-β exerts a promoting effect on the tumor itself and stromal cells to enhance cell growth, alter the responsiveness of tumor cells to stimulate invasion and metastasis, and inhibited immune surveillance. Therefore, novel development of therapeutic agents to inhibit TGF-β-induced progression of tumor and to retain its growth inhibitory activities, in addition to anti-inflammatory actions, could be useful in oncology. In this review, we discuss the role of TGF-β in inflammation and carcinogenesis of the GI tract related to abnormal TGF-β signaling.

Key words: Inflammation; Carcinogenesis; Transforming growth factor-β; Gastrointestinal tract

Peer reviewer: Kazuaki Takabe, MD, PhD, Assistant Professor of Surgery & Assistant Professor of Biochemistry and Molecular Biology, Surgical Oncology, VCU Massey Cancer Center, Virginia Commonwealth University/Medical College of Virginia, PO Box 980011, Richmond, VA 23298-0011, United States

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TRANSFORMING GROWTH FACTOR-β IN HOMEOSTASIS AND INFLAMMATION OF GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract is composed of the esophagus, stomach, small intestine and large intestine; it is the longest and largest surface among human organs, and is the first contact place for various pathogens and food antigens. Therefore, the GI tract should have many lymphoid organs to control the untoward or harmful immune reactions. The immune system of the GI tract can induce a series of host defensive reactions such as recruitment of immune cells, activation of anti-inflammatory enzymes, and secretion of anti-inflammatory cytokines[1]. To prevent pathological inflammation against various antigens exposed to the GI tract, the human body has developed various regulatory mechanisms: the development of regulatory T (Treg) cells, the maintenance of the gut barrier, and immune reactions. Since the inflammatory process in the GI tract is started by food antigens, extracellular pollutants and pathogenic infections, disruption of the intestinal barrier results in intestinal inflammation by inducing the pro-inflammatory reactions of immune cells[2].

Therefore, in order to maintain homeostasis of the GI tract, tolerance is a prerequisite to maintain the balance between pro-inflammation and anti-inflammation. Although many types of regulatory immune cells perform their suppressive functions in the GI tract to acquire tolerance, this activity is mediated by a relatively small number of materials including interleukin (IL)-10 and transforming growth factor (TGF)-β, which are well known regulators of immune reactions in the GI tract[3,4].

TGF-β is a multifunctional cytokine with critical roles in many cellular pathways including cell growth, apoptosis, differentiation and immune reactions[5,6]. TGF-β is secreted as a latent inactive protein and needs to be activated *via* conformational change or protein cleavage by protease or thrombospondins. Since TGF-β1 knockout mice show a dramatic phenotype and develop severe autoimmunity that leads to death within 2 wk after birth[7,8], and T-cell specific disruption of TGF-β signaling results in serious inflammatory changes through constitutively activated T cells in the gut and lung, as a similar phenotype to whole TGF-β knockout mice[9], TGF-β is thought to be a strong anti-inflammatory cytokine. In the regulation of intestinal inflammation, TGF-β inhibits T-cell proliferation as well as blocking differentiation of $CD4^+$ and $CD8^+$ naïve T cells into helper T cells by inhibiting expression of the transcription factors such as T-bet, STAT4 and GATA-3[10]. Furthermore, TGF-β suppresses immune reactions through the induction of Treg cells. In fact, TGF-β has been shown to be essential for the induction and maintenance of peripheral $CD4^+CD25^+$ Treg cells by activation of Foxp3 expression[11].

However, TGF-β also has pro-inflammatory effects through the differentiation of Th17 cells by induction of retinoic-acid-receptor-related orphan nuclear receptor γt, a Th17-specific transcription factor[12]. Conclusively, cross-talk with surrounding tissues may be important for activity of TGF-β in GI tract inflammation. TGF-β has a critical role in regulation of inflammatory processes, therefore, it should be tightly regulated by various mechanisms. Dysregulated or attenuated TGF-β signaling has been suspected in the pathogenesis of various inflammation-related diseases including chronic inflammatory disorders and cancer. T-cell specific deficiency of furin, which activates latent TGF-β, leads to spontaneous autoimmune disease such as colitis and intestinal inflammation in murine models[13]. Similarily, overexpression of mutant TGF-β, which has a defect in binding activity with integrins, shows similar phenotypes to TGF-β null mice, such as vascular defects, multi-organ inflammation, and lack of Langerhans cells[14].

Disruption of Smad3 in mice also shows defects in mucosal immunity and results in early death after birth. Smad3 mutant mice exhibit large amounts of infiltration of T cells and bacterial abscess formation in the GI tract[15]. Activation of Smad3 in patients with inflammatory bowel disease (IBD) is diminished compared to unaffected persons. Isolated cells from IBD patients do not respond to treatment with TGF-β, and do not activate phosphorylation of Smad3, even in the presence of high concentrations of TGF-β[16,17]. In addition, interaction between Smad3 and Smad4 is markedly decreased in lamina propria mononuclear cells of IBD patients. All of these pathogenic phenotypes seem likely to originate from overexpression of Smad7, which is inhibitory for TGF-β signaling. The anti-inflammatory activity of TGF-β comes from inhibition of nuclear factor (NF)-κB activation, but it is lost in the intestine of IBD patients due to high levels of Smad7[18]. An *in vitro* mouse model of colitis treated with trinitrobenzene sulfonic acid or oxazolone shows similar results to those in humans[19]. When anti-sense oligonucleotide against Smad7 is given to mice to reduce the level of Smad7, TGF-β-induced phosphorylation of Smad3 is markedly increased. Conversely, the inflammatory phenotypes of the colon, such as weight loss and microscopic changes, are recovered to normal states after treatment with Smad7 antisense nucleotides. These data indicate that precise regulation of TGF-β signaling, including activation of Smad3 and level of Smad7, is important for homeostasis during inflammation of the GI tract. In contrast to these data, Smad7 also has anti-inflammatory activity against tumor necrosis factor (TNF) signaling and mediates the suppressive activity of TGF-β in primary macrophages and mouse skin[20]. This discrepancy might originate from

differences in cell lines, mouse strains, and experimental systems.

TGF-β LIGANDS AND THEIR SIGNALS

TGF-β sends signals through the heterodimeric complex formation of type I and type Ⅱ serine/threonine kinase receptors. When the ligands interact with receptor Ⅱ, type I receptor is activated by phosphorylation of serine residues. Activated receptor complexes recruit the adaptor proteins, receptor-activated Smad proteins (R-Smad). For example, Smad2 and Smad3 are R-Smad proteins for TGF-β and Smad1, Smad5 and Smad8 are R-Smad proteins for bone morphogenic protein signaling. R-Smad proteins have two conserved domains that are separated by a middle linker domain. The MH1 domain of the N-terminal region functions as a DNA-binding domain and the C-terminal of the MH2 region regulates the nuclear localization and transcriptional activity of R-Smad proteins. Receptor-mediated phosphorylation of C-terminal serine residues induces activation and nuclear translocation of R-Smad proteins. Activated R-Smad proteins form a complex with common Smad (co-Smad) protein, Smad4. The interaction between R-Smad and co-Smad proteins is mediated through the MH2 domain and induces the translocation into the nucleus of Smad complexes. In the nucleus, Smad complexes interact with Smad binding elements (SBEs) of target genes and regulate the transcriptional activity. TGF-β upregulates or downregulates the expression of target genes, which depend on Smad-interacting partners, coactivators or corepressors[6].

CROSS-LINKING BETWEEN INFLAMMATION AND GI CARCINOGENESIS

Relationship between chronic inflammation and carcinogenesis was originally suggested by Virchow, who first hypothesized in 1893 that the lymphoreticular infiltrate may be the origin of tumor at sites of chronic inflammation. Over 100 years later, his hypothesis was cited as evidenced that chronic inflammatory conditions definitely promote progression of malignant tumors. Inflammatory cells and cytokines act as a tumor promoter that affects cell survival, proliferation, invasion, angiogenesis and chemo-resistance[21-23]. Almost 15% of cancers are reported to occur through chronic inflammation-related processes (Table 1). We have established animal models of GI tumors, all of which were based on the concepts that inflammation leads to tumorigenesis, Barrett's esophagus originates through repeated reflux-induced inflammation in the esophagus, colitic tumors arise from repeated bouts of colitis, and gastric cancer is associated with *Helicobacter pylori* (*H. pylori*) infection.

Reflux esophagitis and esophageal cancer

More than 500 000 patients are diagnosed with esophageal cancer annually, which has the second highest mortality after pancreatic cancer among GI cancers worldwide. The most typical type of esophageal cancer is, in histological terms, squamous epithelial carcinoma, and Asians have a greater prevalence of this histological type of carcinoma than adenocarcinoma. On the other hand, Caucasians have just as many cases of adenocarcinoma as squamous carcinoma[24,25]. Many reasons to explain such differences can be put forward, including lifestyle and genetic factors, but in particular, Barrett's esophagus results in a great discrepancy in incidence between Western and Asian populations. Since the numbers of reflux esophagitis patients are rapidly increasing in Korea, as lifestyle as well as living environment have changed to more western styles, in the near future, it is expected that esophageal adenocarcinoma will be more prevalent among Koreans. Esophageal adenocarcinoma is closely related to Barrett's esophagus, therefore, carcinogenesis has the order of chronic reflux esophagitis followed by Barrett's esophagus and esophageal adenocarcinoma. Reflux esophagitis leads to chronic esophagitis and an increase in Barrett's esophagus, which directly creates a precancerous lesion, for which we have demonstrated that chronic exposure to gastroduodenal contents through duodenoesophageal anastomosis leads to metaplastic changes in the squamous epithelium (Figure 1). Molecular factors related to reflux esophagitis are as follows: increase in cyclooxygenase-2 (COX-2), IL-8, IL-1β, IL-10 and TNF-α; loss of TGF-β signaling; and activation of NF-κB. All of these contribute to development of Barrett's esophagus. This mediates chronic inflammation that increases expression of CDX-like homeobox family genes and the caudal gene family, which results in intestinal metaplasia of squamous epithelial cells. Intestinal metaplasia is the direct cause of esophageal adenocarcinoma and because it shares similar molecular mediators with chronic esophagitis, it is thought to be involved in carcinogenesis[26,27]. Barrett's esophagus is thought to be a precancerous lesion because of the appearance of the following changes: augmentation of the cell cycle and proliferation, increased angiogenesis and aneuploidy, decreased antiproliferative signaling and apoptosis, accompanied by the histological changes involved in the progression from chronic esophagus to Barrett's esophagus.

Table 1 GI tumorigenesis associated with chronic inflammation

	Tumor	Causes
Infection-related		
Chronic gastritis	Gastric cancer	*H. pylori*
Chronic gastritis	Gastric cancer	Cytomegalovirus
Prostate inflammatory atrophy	Prostate cancer	Pathogens
Chronic hepatitis	Hepatocellular cancer	HBV and HCV
Infection-unrelated		
Esophagitis	Esophageal cancer	Gastric acid, alcohol, tobacco
Pancreatitis	Pancreatic cancer	Alcohol, tobacco
IBD	Colorectal cancer	Crohn's disease, UC
Cholecystitis	Gall bladder cancer	Gall bladder stone

GI: Gastrointestinal; *H. pylori*: *Helicobacter pylori*; HBV: Hepatitis B virus; HCV: Hepatitis C virus; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

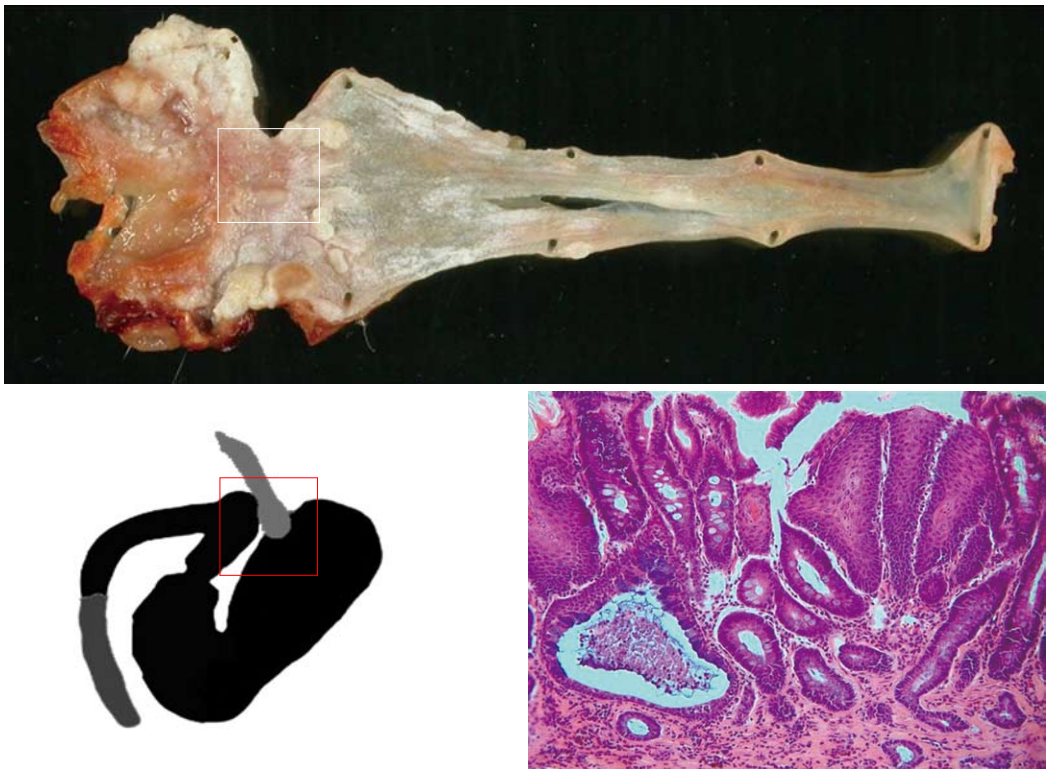

Figure 1 Connection between inflammation and carcinogenesis as demonstrated in a rat model of Barrett's esophagus. With the application of surgical bypass, esophagoduodenostomy (red boxed), overt discolored lesions (white boxed) were noted at the esophagogastric junction, which showed the existence of intestinal metaplasia against a background of squamous epithelium. These animal models provided evidence of the cross-linking between inflammation and premalignant lesions.

Chronic pancreatitis and pancreatic cancer

Pancreatic cancer has the poorest prognosis among GI cancers because patients are diagnosed at a fully progressed stage, and the tumor cells are very aggressive, so that metastasis is very frequent and they do not respond to anti-cancer therapy. Chronic pancreatitis patients have a 3.8-18.5 times higher chance of developing pancreatic cancer[28]. However, severe chronic pancreatitis cannot be discriminated from pancreatic cancer, especially in cases with a history of smoking and alcohol consumption, and a family history. Continuation of chronic pancreatitis for long periods can create a cancer-like molecular environment, such as activation of COX-2, NF-κB and inducible nitric oxide synthetase, production of cytokines such as IL-1, IL-6, IL-8 and TNF-α, and free radical oxygen formation[29,30]. TGF-β plays such an important role in regulating onset of chronic pancreatitis, and we have shown that lack of TGF-β in pancreatic acinar cells leads to increased sensitivity to acute pancreatitis, as well as a favorable environment for chronic pancreatitis.

Chronic atrophic gastritis and gastric cancer

Although there has been a tendency towards a decrease in gastric cancer for the past 80 years, it is still the second highest cause of death following lung cancer. In Korea, about 25-30 people out of every 100 000 develop gastric cancer regardless of their sex, and its mortality rate is ranked as the highest. However, the discovery of a causative pathogen of gastric cancer, *H. pylori*, has raised hopes for preventing gastric cancer through eradication or regulation of the bacterium. The International Agency for Research on Cancer in 1994 defined *H. pylori* as an imminent carcinogenic pathogen, based on epidemiological research and animal studies. This definition is based on epidemiological evidence, and several animal experiments have shown that infection with *H. pylori* causes infiltration of inflammatory cells, oxidative damage and gene mutations. In resected specimens from gastric cancer, the intestinal metaplastic lesions are easily identified around the cancer lesions, with which *H. pylori* infection is closely associated. Although *H. pylori* is a class I carcinogen, gastric cancer is not prevented by *H. pylori* eradication in all patients. This can be explained by the relationship between gastritis, metaplasia and gastric cancer, whereby prevention of *H. pylori*-associated carcinogenesis only benefits those in whom the malignant process has not begun[31]. That is, even though *H. pylori* is completely eradicated, gastric inflammation remains, thus it seems that amelioration of gastric inflammation itself seems to be far more essential in achieving cancer prevention than eradication of the pathogen. This emphasizes that chronic gastritis has a greater chance of causing onset of gastric cancer than the presence of *H. pylori* itself. Furthermore, genetic factors, toxicity of the pathogen, and environmental factors are intertwined in the development of *H. pylori*-induced gastric cancer from inflamma-

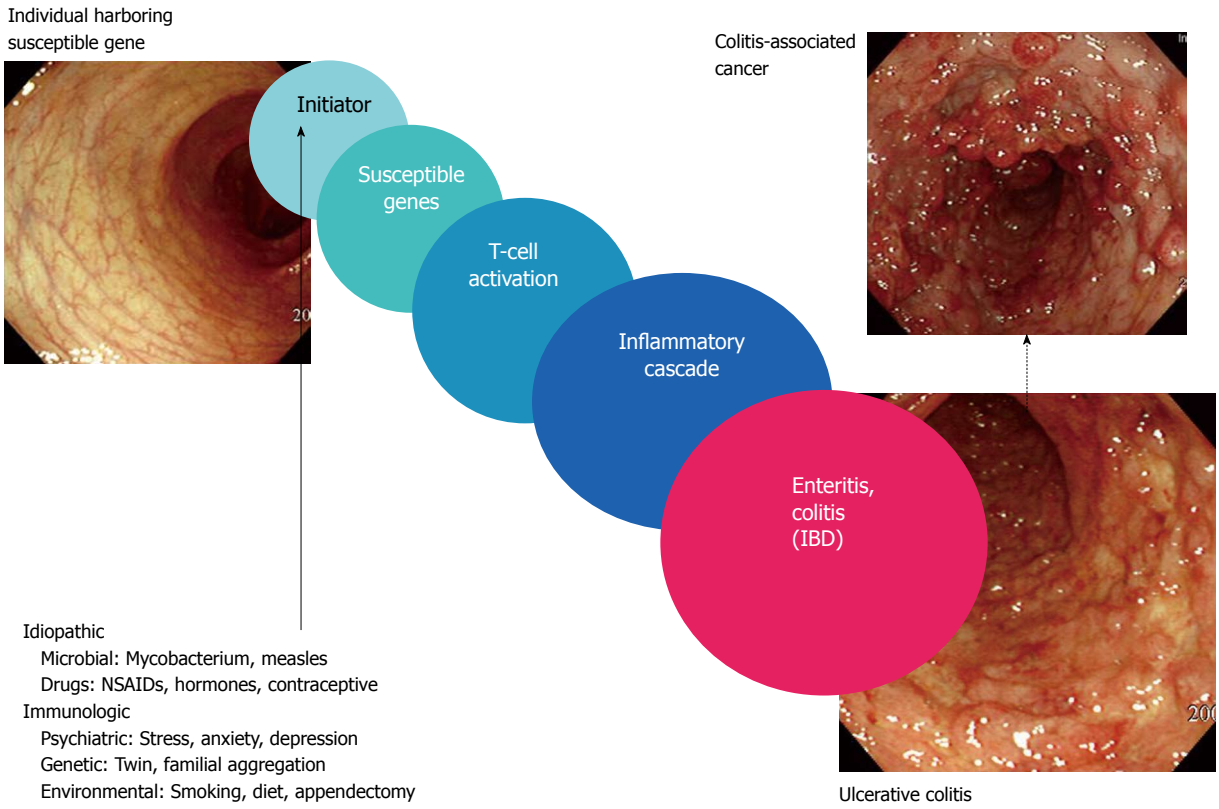

Figure 2 Inflammatory bowel disease (IBD) and colitis-associated cancer. The cause of IBD is idiopathic because several etiologies have been suggested including microorganisms, drugs, psychiatric illness, genetic abnormality, and environmental factors. However, when individuals that harbor IBD susceptibility genes are exposed to the above-mentioned etiological factors, they experience T cell activation, after which perpetual inflammatory cascades lead to enteritis or colitis. Repeated bouts of chronic colitis alone provoke colitis-associated cancer, which emphasizes the cross-linking between inflammation and carcinogenesis. NSAIDs: Nonsteroidal antiinflammatory drugs.

tion, thus alleviation or treatment of inflammation could form the basis of prevention of gastric cancer[32,33]. The contribution of TGF-β to *H. pylori*-associated gastric carcinogenesis has been investigated using pS2-dnR Ⅱ mice, which are defective in TGF-β signaling due to expression of the dominant negative R Ⅱ receptor (TGF-β-dnR Ⅱ) in the stomach, using gastric specific promoter pS2 (gastric trefoil peptide). pS2-dnR Ⅱ mice developed gastric adenocarcinoma with *H. pylori* alone compared to their wild-type littermates, which suggests that the development of gastric cancer after *H. pylori* infection might be influenced by host genetic conditions[34].

IBD and colitis-associated cancer

The most common cause of colorectal cancer is sporadic. However, 10%-15% of these patients continually suffer from chronic IBD for 10 years, which later develops into colitis-associated cancer, namely, colitic cancer. Ulcerative colitis (UC) is a form of chronic IBD that usually has a clinical course of repeated exacerbation and remission, and less commonly presents with an unremitting, fulminating course. Another feature of the clinical course of UC is an increase risk for the development of colitic cancer (Figure 2). Although sporadic colorectal cancer and colitic cancer arise from dysplastic precursor lesions and share several molecular alterations, the nature of the dysplasia and the frequency and timing of several of the key molecular changes differ sufficiently to support our hypothesis that colitic cancer can be prevented by early intervention with strong anti-inflammatory agents or cancer surveillance with strict biomarkers. Etiologically, in the case of sporadic colorectal cancer, most cases are preceded by adenomatous polyps, namely adenoma, followed by the so-called "adenoma-carcinoma" sequence[35]. On the other hand, colitic cancer is contracted at a relatively young age and the crucial factor is the range, extent and period of inflammation, which implies the "inflammation-dysplasia-carcinoma" sequence. Considering the fact that the number of IBD patients is increasing, in the forthcoming 10 years, inflammation-related cancer is expected to increase, thus we should be focusing on cancer prevention through control of inflammation. We have studied colorectal cancer prevention based on an animal model of inflammation-related cancer, and are working on how the control of inflammation itself prevents colorectal cancer. We have performed another similar animal model with repeated bouts of colitic cancer and have shown that potent anti-inflammatory drugs inhibit cancer formation. This suggests that colitis is involved at the beginning as well as the progression of cancer, and control of inflammation is one of the methods for preventing inflammation-related colorectal cancer.

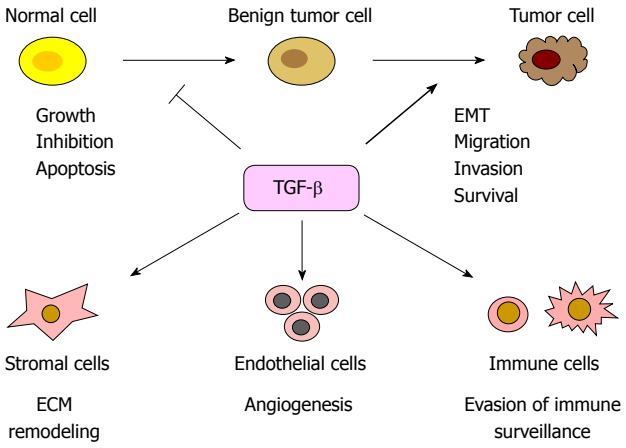

Figure 3 Role of transforming growth factor (TGF)-β in cancer progression. TGF-β is a secreted polypeptide that signals *via* receptor serine/threonine kinases and intracellular Smad effectors. TGF-β inhibits proliferation and induces apoptosis in various cell types. Accumulation of loss-of-function mutations in the TGF-β receptor or Smad genes classifies the pathway as a tumor suppressor in humans. In addition, various oncogenic pathways directly inactivate the TGF-β receptor-Smad pathway, thus favoring tumor growth. On the other hand, all human tumors overproduce TGF-β whose autocrine and paracrine actions promote tumor cell invasiveness and metastasis. Accordingly, TGF-β induces epithelial to mesenchymal transition (EMT).

TGF-β IN GI CARCINOGENESIS

TGF-β signaling is a crucial regulator of intestinal homeostasis and inflammation, therefore, dysregulation of this pathway could be associated with inflammation-related carcinogenesis of the GI tract (Figure 3). Altered signaling pathways can disturb the regulatory mechanism of TGF-β to block chronic inflammation followed by carcinogenesis. As a tumor suppressor gene, TGF-β can inhibit initiation of cell transformation, through growth inhibition and apoptosis in normal epithelium. During changes in the genetic and epigenetic context of transforming cells, the responsiveness of cells to TGF-β is decreased, but the expression and activation of TGF-β are markedly increased[36].

Mouse models have been used to investigate the role of TGF-β in GI inflammation and tumorigenesis. Heterozygotic TGF-β null mice express only 10%-30% of wild-type TGF-β1 protein level, and show increased cell turnover with chemical carcinogens, which results in enhanced tumorigenesis compared with wild-type mice[37]. Furthermore, TGF-β1/Rag2 double knockout mice can live for a long time and develop colon cancer after developing inflammatory lesions[38]. Mutations of TGF-β receptor Ⅱ (TGF-β-RⅡ) are frequently found in colon and gastric cancer with microsatellite instability[39]. Conditional transgenic mice using TGF-β-dnRⅡ with pS2/TFF1 promoter were created and infected with *H. pylori*, and they developed dysplasia as well as adenocarcinoma, which suggests that disruption of TGF-β signaling is related to gastric cancer formation[34]. The findings that TGF-β-dnRⅡ mice have higher levels of gastritis than their wild-type littermates, suggests that TGF-β1 plays an important role in prevention of gastric inflammation as well as cancer. An experiment for inducing IBD was performed on mice that overexpressed TGF-β-dnRⅡ in the small/large intestine using the promoter of ITF (intestinal trefoil factor)/TFF3. TGF-β-dnRⅡ mice showed much more severe colitis than their wild-type littermates when induced with the same concentration of dextran sulfate sodium[40].

Some pancreatic and biliary adenocarcinomas originate from mutation of TGF-β receptor Ⅰ (TGF-β-RⅠ) with very low frequency[41]. In a mouse model, mutation or deletion of bone morphogenetic protein receptor, which is another member of the TGF-β superfamily, also induces chronic inflammatory phenotypes and then precancerous lesions[42].

Deletion of Smad proteins also leads to loss of the regulatory role of immune suppression and induces malignant cell proliferation. Deficiency of Smad4 in mutant mice causes gastric polyps and progression to gastric tumor in old age[43]. In humans, deletion of Smad4 is found in juvenile polyposis syndrome and is a strong risk factor for GI cancer[44]. In addition, T-cell specific deletion of Smad4 results in spontaneous epithelial cancer of the oral cavity, stomach, colon, rectum and duodenum in mice[45]. Smad3 knockout mice also develop metastatic colorectal cancer through dysregulation of cell proliferation. The expression of Smad3 shows low to undetectable levels in 40% of human gastric cancer tissues[46]. Reintroduction of Smad3 into gastric cancer cells restored their responsiveness to TGF-β and blocks tumor formation in nude mice. These data support the suggestion that Smad-mediated TGF-β signaling regulates homeostasis and inflammation in the GI tract.

Mutations in fine tuning of regulatory circuits cause the loss of normal communication within target organs, and defects in regulating stromal and epithelial interactions. In addition to Smad proteins, cofactors of TGF-β signaling can also affect the biological roles of TGF-β and the progress of GI carcinogenesis. Based on mouse model studies, mutations of these cofactors, such as embryonic liver fodrin (ELF), promyelocytic leukemia tumor suppressor, Smad anchor for receptor activation, and filamin lead to malformation of intestinal structures and induce the formation of liver, gastric and colon cancers. In case of ELF, 40%-70% of heterozygotic null mice develop hepatocellular cancer within 15 mo through abnormal angiogenesis or cyclin D1 activation[47,48].

In contrast to its tumor suppressive activity, expression of TGF-β1 is markedly increased in colon, esophageal, gastric, hepatocellular and pancreatic cancer[49]. High levels of TGF-β expression are correlated with tumor progression, metastasis and angiogenesis, which results in poor prognostic outcome. In addition to its oncogenic nature related to TGF-β mutation, TGF-β, which is secreted from tumor cells, can control tumor progression by suppressing cytotoxic immune reactions, stimulating expression of cell survival factors, or regulating autonomous signaling of tumor cells, depending on cell type and context. At the late stage of tumor develop-

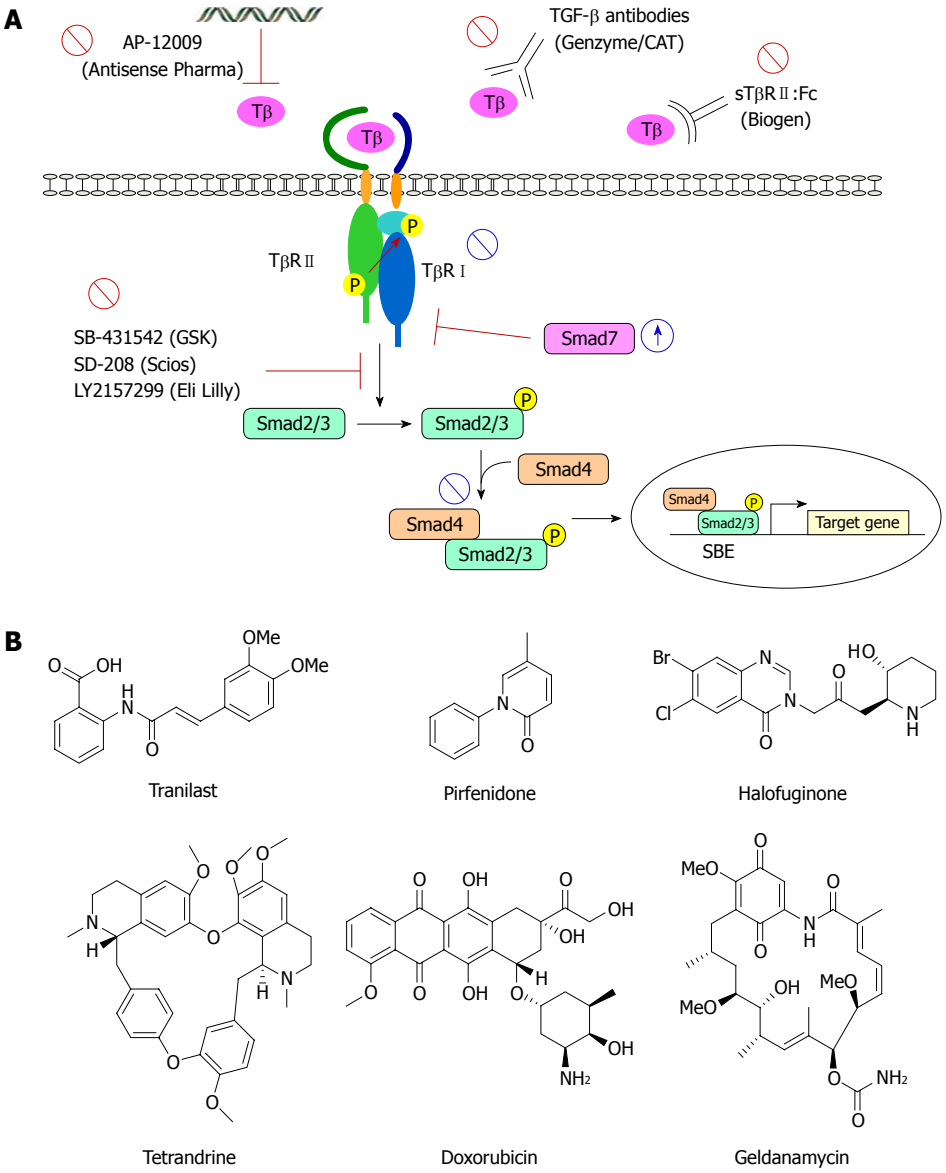

Figure 4 Therapeutic target of TGF-β signaling. A: TGF-β signaling and points of therapeutic intervention; B: Structures of multi-action TGF-β signal inhibitors. SBE: Smad binding element.

ment, invasive and metastatic potentials are important properties, and TGF-β signaling is a crucial pathway for tumor cell invasion and metastasis by inducing epithelial to mesenchymal transition (EMT)[50,51]. EMT progresses by decreasing the expression of E-cadherin to inhibit cell-cell adhesion and by increasing the expression of laminin-5, vimentin, integrins, and fibulin-5 that are involved in cell-extracellular matrix associations[52,53].

THERAPEUTIC TARGET OF TGF-β SIGNALING

Until now, the most successful target-aimed anti-cancer treatment has focused on the suppression of oncogenes that are abnormally activated in cancer cells. As examples, Gleevec® (imatinib), which is a *Bcr-abl* kinase inhibitors, and Herceptin (trastuzumab), which is a monoclonal antibody that interferes with the *Erb*B2/*Neu* receptor, are good examples of target-aimed molecular cancer treatments[54]. However, regarding TGF-β, the development of TGF-β inhibitors for the purpose of cancer treatment has shown no apparent progress so far in spite of TGF-β being known as a potent growth inhibitory factor against epithelial and blood cells. Instead, TGF-β signal transduction has been recognized as one of the representative targets of cancer treatment, aided by recent clinical results and cancer biology with regard to TGF-β. TGF-β ligands, as well as various downstream signal transducers can be a target in the therapeutic regulation of TGF-β signaling (Figure 4A). For instance, a potential target for the therapeutic intervention can be unveiled by inhibiting TGF-β synthesis, suppressing TGF-β activity at the cell surface, removing or neutralizing synthesized TGF-β, or regulating receptors and Smads involved in intracellular signaling pathways. By using EMT6 murine mammary tumor cell line that stably expresses antisense TGF-β transduced with a retroviral vector, it has been

demonstrated that the decreased production of TGF-β inhibited tumor growth[55]. In particular, the decreased secretion level of TGF-β by cancer cells induced tumor regression by increasing the activity of anti-tumor cytotoxic T cells. Currently, the inhibition of TGF-β production by using anti-sense oligonucleotides is one of the most potent methods in clinical cancer treatment. For example, AP-12009, which is synthesized by Antisense Pharma and is especially selective for TGF-β2, has prominent clinical effects on malignant neuroglial tumor patients[56,57]. It is now in phase Ⅱ clinical trials for the treatment of neuroglioma and pancreatic and skin cancer. The suppression of latent TGF-β activation can also be useful for regulating TGF-β activity. It has been reported that the furin protease inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (*dec-RVKR-cmk*), participates in TGF-β activity and inhibits the release of mature bioactive TGF-β molecules *in vitro*[58]. However, furin is not considered as a good selective inhibitor for TGF-β signaling because it is known to be involved in the activity of other growth factors, growth factor receptors, integrin, and matrix metalloproteinase.

Agents that promote TGF-β signaling

Restoration or augmentation of TGF-β signal transduction can be a good strategy for cancer prevention and treatment. For instance, chemical compounds such as retinoid and deltanoid (vitamin D), which are related to the expression or regulation of TGF-β signaling, have been used for chemoprevention of cancer[59,60]. Based on tumor suppressor activity of TGF-β, various attempts have been made to screen compounds that may activate TGF-β signaling. The first attempt, which was made by Seto's group, was to screen small molecular chemical compounds that have similar activities to TGF-β[61,62]. By searching for agents that increased the transcriptional activity of plasminogen activator inhibitor-1 (PAI-1), which is one of target genes of TGF-β, they finally isolated diheteropeptin and spiruchostatin A and B from microbial metabolites. These compounds turned out to increase the expression of PAI-1 and p21 and suppress the growth of lung epithelial cells in nanomolar concentrations. In addition, they had an inhibitory activity against histone deacetylase (HDAC). Lee *et al*[63] have isolated onnamide A and theopederin B from marine sponge, which promotes the transcriptional activity of TGF-β.

Onnamide A and theopederin B induce expression of PAI-1 gene and apoptosis through activation of mitogen-activated protein kinases (MAPKs) such as p38 and JNK, which suggests that they have strong anti-tumor activity. Moses's group[64] identified one TGF-β mimicking agent, A-161906, by performing high-throughput screening using mink lung epithelial cells stably transfected with PAI-1 promoter reporter. As an HDAC inhibitor, A-161906 demonstrated cellular effects similar to those of TGF-β. A-161906 not only inhibited cell proliferation, but also induced growth arrest at the G1-S checkpoint, and expression of the cyclin-dependent kinase inhibitor p21. In addition, based on the fact that Smad4 (DPC4) mutation, which is frequently found in pancreatic cancer, plays a crucial role in the loss of TGF-β-mediated growth inhibition, Sohn's group[65] performed high-throughput screening. Using a stably transfected reporter gene that has six copies of SBE (p6SBE-Luc) in a pancreatic cancer cell line, PANC-1, they screened a library of approximately 16000 compounds from ChemBridge DIVERSet, and finally identified scriptaid compound, which increased Smad transcriptional activity.

It is well known that expression of TGF-β receptors plays an important role in TGF-β-mediated cell growth inhibition. Therefore, increasing expression levels of TGF-β receptors in cancer cell lines, in which TGF-β receptors are mutated or their expression levels are attenuated, may be a good therapeutic target in cancer treatment. In fact, it has been reported that HDAC inhibitors, such as MS-275 and suberoylanilide hydroxamic acid (SAHA), increase response to TGF-β by inducing the expression of TGF-β-RⅡ and TGF-β-RⅠ, respectively, in breast cancer cell lines[66,67]. Besides, it has been found that the expression level of TGF-β-RⅡ is increased by captopril (angiotensin-converting enzyme inhibitor), FTI-27742 (farnesyl transferase inhibitor), and 5-aza-2'-deoxycytidine (DNA methyltransferase inhibitor)[68-70]. Thus, these compounds may be used as anti-cancer drugs or for combination treatments when treating colon or small cell lung cancer that commonly displays decreased expression levels of TGF-β-RⅡ.

Intriguingly, the screened chemical compounds including A-161906 and scriptaid that were identified by high-throughput screening, diheteropeptin, and spiruchostatins are strong HDAC inhibitors. This indicates that the activity of HDAC may play a key role in the regulation of TGF-β signaling. Recently, the FDA has approved SAHA (Vorinostat® Zoniza) as a treatment for cutaneous T-cell lymphoma (CTCL). It has been reported that SAHA activates p21 and induces apoptosis of T cells in CTCL patients[71].

Many transport molecules of TGF-β signaling as well as TGF-β receptors are regulated by epigenetic mechanisms. Therefore, even though HDAC inhibitors do not induce selective activation during TGF-β signal transduction, it is considered that HDAC inhibitors and various chemical compounds similar to or increasing TGF-β activity can be good targets in the prevention and treatment of cancer. Since the most frequent genetic alteration found in cancer is the loss-of-function in tumor suppressor genes, this alteration is very specific to cancer cells and it may be a good therapeutic target in the development of anti-cancer drugs. In order to identify the agents selectively targeting cancer cells with loss-of-function mutations, Wang *et al*[72] have demonstrated a novel screening technique, named pharmacologic synthetic lethal screening, and have identified UA62001, which shows selectivity against DPC4-deficient cell line BxPC-3, and by investigation of

downstream target genes of UA62001, they have found that UA62001 arrests cells in G2/M phase by inhibiting cyclin B/CDC2 expression. In addition, Park *et al*[73] have screened the Maybridge Diversity Set chemical library using a cell-based assay to measure nuclear translocation of Smad proteins. Through high-throughput screening, DAM-1976, which enhances TGF-β signaling, has been identified. DAM-1976 increases sensitivity to TGF-β by prolonging Smad2/3 activation in the nucleus in a TGF-β-independent manner.

Professor Sporn MB, who discovered TGF-β and has been in the vanguard of cancer chemoprevention research, and his colleagues[74] have made many modifications of triterpenoids that possess anti-inflammatory and anti-tumor activity. They have screened derivatives of two known triterpenoids, oleanolic acid, and ursolic acid, and synthesized 2-cyano-3,12-dioxooleana-1, 9-dien-29-oic acid (CDDO), which has the most potent anti-inflammatory activity and low toxicity. It has been reported also that CDDO not only promotes Smad signal transduction, but also displays various biological activities similar to that of TGF-β[75]. Moreover, CDDO is currently in phase Ⅱ clinical trials for the treatment of leukemia and solid tumors, based on its anti-inflammatory and anti-tumor activities, cellular protection, and suppression of tumorigenesis. Therefore, CDDO is considered to be a promising chemopreventive and chemotherapeutic agent in cancer treatment.

TGF-β antibody and soluble TGF-β receptors

The monoclonal antibody that neutralizes TGF-β ligand is one of most developed inhibitors for the clinical application of suppressing TGF-β activity. The human monoclonal antibodies CAT-152 (lerdelimumab), CAT-192 (metelimumab) and GC1008, and the mouse monoclonal antibodies 1D11 and 2G7 against TGF-β have been generated to date. CAT-152 and CAT-192 are the human IgG4 monoclonal antibodies that neutralize TGF-β2 and TGF-β1, respectively. CAT-152 and CAT-192 (Cambridge Antibody Technology) have entered phase Ⅲ and Ⅱ clinical trials for the treatment of wounds after glaucoma filtration surgery and pachyderma, respectively. However, the trials were discontinued in 2004 due to problems concerning their effectiveness[76]. GC1008, developed by Genzyme Corporation, is a pan-TGF-β selective monoclonal antibody that works against all TGF-β isomers. Clinical trials for GC1008 were conducted in idiopathic pulmonary fibrosis patients. Also, it is currently in a clinical trial for the treatment of metastatic skin cancer and renal cancer. The anti-tumor effectiveness of 1D11 and 2G7 has been studied in various animal tumor models. According to the report of Arteaga *et al*[77], intraperitoneal injection of 2G7 suppresses not only tumor growth of MDA-MB-231 breast cancer cell lines, but also lung metastasis. Moreover, 2G7 partially inhibits angiogenesis by reducing the levels of vascular endothelial growth factor in blood plasma. On the other hand, suppression of TGF-β activity can be achieved by using macromolecular proteins such as soluble TGF-β receptors that are able to inhibit competitively interactions between the ligand and the receptor. In particular, soluble TGF-β-RⅡ has been extensively studied in preclinical tumor models. A soluble chimeric protein, composed of the extracellular domains of the TGF-β-RⅡ and the Fc portion of IgG1, has been shown to block TGF-β activity[78]. When tested in a transgenic mouse model that expressed a soluble TGF-β-RⅡ receptor: Fc fusion protein (Fc:TGF-β-RⅡ), metastatic capacity was suppressed without any increase of tumor growth, which indicated that this fusion protein selectively inhibited metastasis-associated TGF-β activity[79]. In addition to soluble TGF-β-RⅡ, soluble TGF-β-RⅢ also suppressed tumor growth and lung metastasis in an MDA-MB-231 xenograft tumor model by antagonizing the tumor-promoting activity of TGF-β[80].

TGF-β receptor kinase inhibitors

TGF-β receptor kinases are not only key molecules in intracellular TGF-β signal transduction, but potential therapeutic targets that can be easily regulated by small molecular compounds. Owing to the success of Gleevec®, many projects, focused on TGF-β receptor kinases, have been carried out along with large pharmaceutical companies. In particular, numerous studies concerning TGF-β-R Ⅰ have been performed to elucidate the domain structures, biochemical activity, and biological functions of TGF-β-R Ⅰ [81]. Thus, TGF-β-R Ⅰ serves as an attractive target for drug development. The development of protein kinase inhibitors can be achieved in several ways, such as target hopping, novel screening and virtual screening.

TGF-β-R Ⅰ kinase inhibitors with various structures have been developed by drug companies. Their various effects including anti-tumor activity confirmed in animal models, relations with TGF-β-induced Smad phosphorylation, activation of reporter genes, cell cycle arrest, and EMT, have been reported. SB-431542, SB-505124 (Glaxo Smith Kline; GSK), LY364947, LY2157299 (Eli Lilly), SD-208 (Scios), and IN-1130 (In2gen) are well known TGF-β-R Ⅰ kinase inhibitors. SB-431542 and SB-505124 compounds from GSK were developed by target hopping based on the structures of the kinases. Phosphorylation of TGF-β-R Ⅰ is inhibited by SB203580, a p38 kinase inhibitor, because TGF-β-R Ⅰ has a gatekeeper residue equivalent to that of p38 kinase, which results in structural similarity to p38 kinase[82]. SB-431542 was synthesized having SB-203580 as a lead compound. It has shown greater selectivity for TGF-β-R Ⅰ compared with other serine/threonine kinases (protein kinase C, extracellular signal-regulated kinase, JNK, p38 MAPK). However, it also has displayed strong inhibitory activity against activin receptor (ALK4) and nodal receptor (ALK7)[83]. In addition, SB-431542 inhibits proliferation of glioma, TGF-β-mediated morphological changes, and cell motility, which suggests that small molecule inhibitors of TGF-β receptor kinases have potential to suppress tumor progression[84].

Based on the growth inhibitory characteristics of TGF-β in epithelial cells, researchers at Eli Lilly (Indianapolis, MN, USA) have identified the heteroarylpyrazole structure of LY364947 through high-throughput screening[85]. After they confirmed that TGF-β-R I and TGF-β-R Ⅱ kinase were the molecular targets of LY364947, the latter became a lead compound in the development of inducers. LY364947 also turned out to be identical to HTS-466274, which was identified by Biogen Inc. through computer modeling of a compound database that contained over 200 000 compounds. The result suggested shape-based virtual screening as a powerful tool to discover and develop new TGF-β-R I kinase antagonists. Furthermore, studies on the relationship between structure and activation, as well as optimization of lead compounds have led to the development of more advanced compounds, such as LY2157299. In particular, LY2157299 entered a phase I clinical trial for the first time as a TGF-β-R I kinase antagonist. Also, studies on its pharmacotoxicity, pharmacokinetics, and pharmacodynamics are currently in progress[86].

Oral bioavailability of SD-208, another type of TGF-β-R I kinase inhibitor under development by Scios Inc., has been verified by demonstrating its ability to inhibit infiltration of glioma cells and metastasis of a highly metastatic breast cancer cell line[87]. Besides, A-83-01 has been found to be more potent in the inhibition of TGF-β-R I than SB-431542, and GW788388 can be administered orally, with improved pharmacokinetics[88,89]. IN-1130, recently developed in Korea as a small molecular inhibitor of TGF-β-R I , has shown kinase selectivity similar to that of SB-431542. IN-1130 also inhibits tumor growth and promotes activation of cytotoxic T cells in xenograft prostate tumor models, which suggests that it might restore TGF-β-induced immunosuppression in cancer cells[90].

Non-selective agents that suppress TGF-β signaling

Besides the selective inhibitory substances, including antibodies against the ligand and receptor kinase inhibitors, non-selective chemical compounds may provide strategies for the regulation of TGF-β signaling. Such agents include compounds that suppress the expression level of TGF-β, increase the expression level of Smad7, an endogenous inhibitory protein, or regulate the stability of TGF-β receptors (Figure 4B). Natural or synthesized agents that suppress TGF-β signaling through these mechanisms generally demonstrate anti-inflammatory, anti-fibrosis, and various biological activities. Among these, tranilast (Rizaben®) has been used as an anti-allergic drug for the treatment of asthma and atopy. Also, it has been known that tranilast has anti-fibrosis and anti-tumor effects. According to animal experiments recently carried out with the highly metastatic murine 4T1 breast cancer cell line, tranilast effectively suppressed metastasis by activating Smad proteins, inhibiting EMT, and strongly suppressing the production of TGF-β and IL-17 that were secreted by immune cells[91]. The suppressive effect of tranilast on metastasis turned out to be greater than that of TGF-β antibody 1D11 or paclitaxel.

In addition, pirfenidone, which is in a clinical trial for idiopathic pulmonary fibrosis, due to its anti-fibrosis effects, suppresses proliferation of neuroglioma by inhibiting expression and activation of TGF-β[92]. Halofuginone, a plant alkaloid and inducer of febrifugine, is a representative small molecular weight molecule in the suppression of collagen synthesis. It was first isolated from *Dichroa febrifuga*, approved by the FDA, and has been used for the treatment of scleroderma. Recently, clinical trials have been conducted on Kaposi's sarcoma to examine its inflammatory activity. Halofuginone has been reported to inhibit TGF-β signaling by decreasing TGF-β-R Ⅱ expression and elevating inhibitory Smad7 expression[93].

Moreover, tetrandrine, another plant alkaloid, and doxorubicin have demonstrated increased expression of Smad7[94,95]. While screening for agents that suppress TGF-β signal transduction, geldanamycin, which is also known as a heat shock protein 90 (Hsp90) inhibitor, was identified. Geldanamycin inhibits TGF-β-induced target gene expression and Smad phosphorylation even at the subnanomolar concentrations. Analysis of its mechanism of action revealed that geldanamycin suppresses TGF-β signaling *via* complex processes. For instance, geldanamycin regulates Hsp90 chaperone-associated stability of TGF-β receptors, suppresses receptor complex formation between TGF-β-R Ⅱ and TGF-β-R I , and induces interaction between Hsp70 and its receptors[96]. In general, Hsp90 inhibitors are widely recognized as a promising therapeutic target for anti-cancer drug development because of their ability to suppress tumor growth by inactivating various proteins that are related to tumorigenesis, such as Bcr-abl, ErbB2 and Raf-1. Various Hsp90 inhibitors, including geldanamycin inducer and 17-(allylamino)-17-demethoxygeldanamycin (17-AGG) are currently undergoing clinical trials. In fact, 17-AGG has completed a phase Ⅲ clinical trial for the treatment of multiple myeloma[97].

CONCLUSION AND FUTURE DIRECTIONS

Although TGF-β signaling has been shown to have some paradoxical actions in tumorigenesis, many factors activate TGF-β signaling for tumor suppression, such as HDAC inhibitor, SAHA, synthetic terpenoid, CDDO, in addition to inhibitors of TGF-β signaling. It is a good strategy to block the initiation of inflammation-associated tumorigenesis through the development of TGF-β mimics in order to achieve chemoprevention. Activators of TGF-β signaling inhibit initiation of tumorigenesis and invasion and metastasis of advanced-stage tumors. This sort of approach will be very helpful for TGF-β-responsive tumor therapy, so-called tumor vaccination. In terms of chemoprevention, the difficulty in applying TGF-β activators to normal people is due to immunosuppressive action and difficulty in detecting the tumor

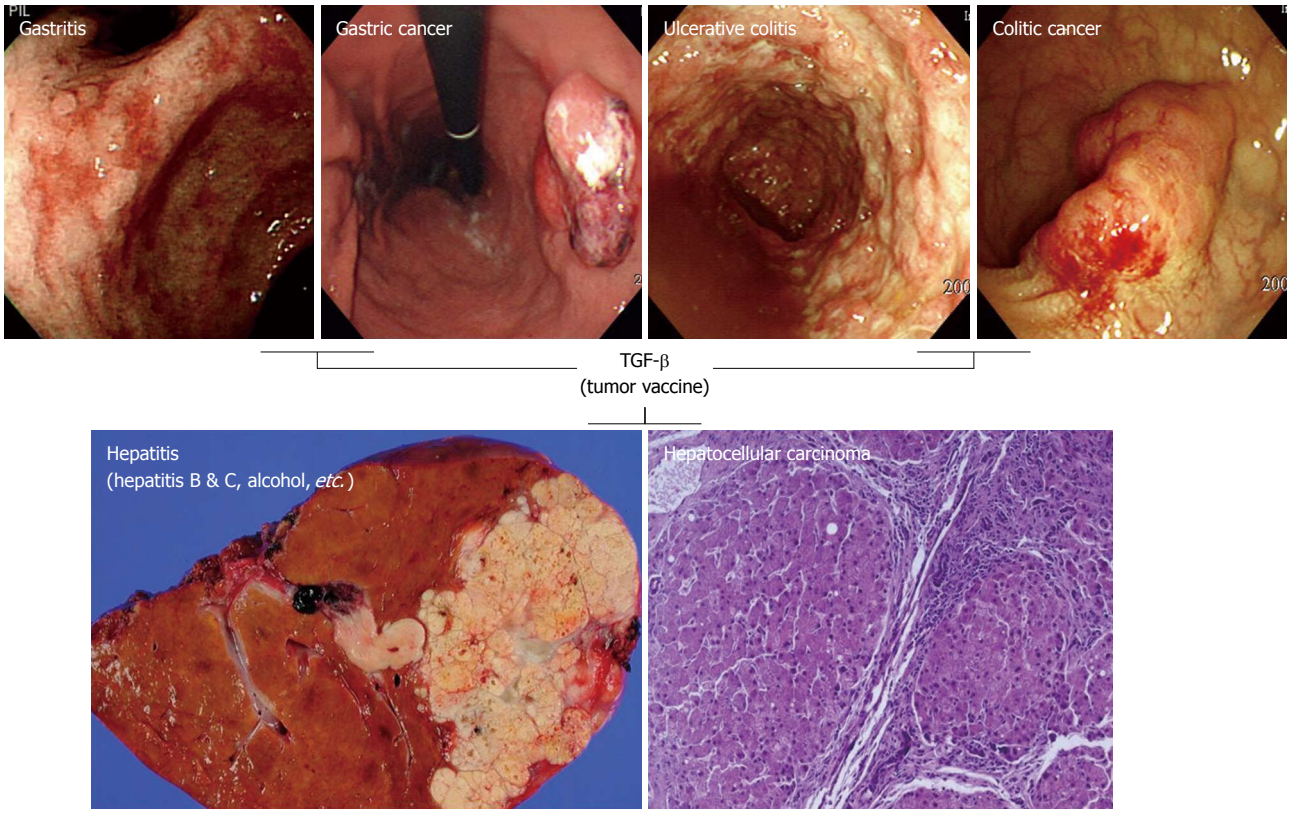

Figure 5 TGF-β1 as pivotal tumor vaccine based on anti-inflammatory actions that block progression from inflammatory lesions to precancerous lesions/cancer. Chronic inflammation induced by biological, chemical, microbial and physical factors is associated with increased risk of human cancer at various sites. Inflammation facilitates initiation of normal cell transformation, growth, and progression to malignancy through production of pro-inflammatory cytokines and diverse reactive oxygen species. In this pathway, TGF-β plays a key role in chemoprevention through anti-inflammatory and anti-mutagenic actions.

at an early stage. Blocking inflammation-associated carcinogenesis with the anti-inflammatory activity of TGF-β signaling can be used as a chemopreventive strategy in patients at high risk of cancer (Figure 5). Even though TGF-β may regulate the inflammatory process by modulating the differentiation of many kinds of T cells, we still do not know the exact role of TGF-β in T-cell biology. Further study of the role of TGF-β in development of GI tract immune cells can lead to future therapeutic approaches for inflammation and tumorigenesis. To date, tumor chemotherapy by suppression of TGF-β signaling has been tried, to enhance the tumor-specific immune reaction, inhibit angiogenesis of the tumor microenvironment, and block invasion and metastasis of tumor cells. However, TGF-β has different effects depending on the type or context of the tumor, and has an anti-inflammatory effect on various immune cells. For these reasons, it is important to select the right patients, time of intervention, therapeutic reagents to combine, and exact targets, to minimize the side effects and maximize successful treatment. Recently, many low molecular size inhibitors of TGF-β receptor kinase have been tested in clinical trials of tumor chemotherapy. Also, cross-talk between TGF-β signaling and other signaling pathways has been studied in detail, and new candidates have been screened and developed to regulate these signaling pathways. Based on results of cooperative activity of TGF-β with oncogenes, combination therapy with conventional anti-tumor reagents and TGF-β signaling inhibitors can enhance the therapeutic effects. Since inflammation-associated carcinogenesis is caused by complex cross-talk between many different cytokine signaling pathways, by collaboration with systems biologists, we can understand in more detail the mechanisms and networks of signaling pathways for inflammation and tumorigenesis. Through this approach, we can design and develop more efficient therapeutic drugs for treatment of GI cancer based on the biological action of TGF-β.

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Acute lung injury and ARDS in acute pancreatitis: Mechanisms and potential intervention

Meng-Tao Zhou, Cheng-Shui Chen, Bi-Cheng Chen, Qi-Yu Zhang, Roland Andersson

Meng-Tao Zhou, Bi-Cheng Chen, Qi-Yu Zhang, Department of Surgery, First Affiliated Hospital, Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China
Cheng-Shui Chen, Department of Respiratory Medicine, First Affiliated Hospital, Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China
Bi-Cheng Chen, Zhejiang Provincial Top Key Discipline in Surgery, Wenzhou Key Laboratory of Surgery, Wenzhou 325000, Zhejiang Province, China
Roland Andersson, Department of Surgery, Clinical Sciences Lund, Lund University and Lund University Hospital, SE-221 85 Lund, Sweden
Author contributions: All authors equally contributed.
Correspondence to: Roland Andersson, MD, PhD, Department of Surgery, Clinical Sciences Lund, Lund University and Lund University Hospital, SE-221 85 Lund, Sweden. roland.andersson@med.lu.se
Telephone: +46-46-172359 Fax: +46-46-147298
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Abstract

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) in acute pancreatitis still represents a substantial problem, with a mortality rate in the range of 30%-40%. The present review evaluates underlying pathophysiological mechanisms in both ALI and ARDS and potential clinical implications. Several mediators and pathophysiological pathways are involved during the different phases of ALI and ARDS. The initial exudative phase is characterized by diffuse alveolar damage, microvascular injury and influx of inflammatory cells. This phase is followed by a fibro-proliferative phase with lung repair, type Ⅱ pneumocyte hypoplasia and proliferation of fibroblasts. Proteases derived from polymorphonuclear neutrophils, various pro-inflammatory mediators, and phospholipases are all involved, among others. Contributing factors that promote pancreatitis-associated ALI may be found in the gut and mesenteric lymphatics. There is a lack of complete understanding of the underlying mechanisms, and by improving our knowledge, novel tools for prevention and intervention may be developed, thus contributing to improved outcome.

Key words: Acute lung injury; Acute respiratory distress syndrome; Acute pancreatitis; Etiology; Pathophysiology

Peer reviewers: Udayakumar Navaneethan, MD, Department of Internal Medicine, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267, United States; Naoaki Sakata, MD, PhD, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

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INTRODUCTION

The incidence of acute pancreatitis is in the range of 300 or more patients per million annually[1,2]. Using the Atlanta classification on severity, about 10% of acute pancreatitis patients are classified as severe[3]. About one-third of all deaths from acute pancreatitis has been reported to occur prior to admission to hospital, and in most cases, is associated with acute lung injury (ALI)[4]. Hospital deaths occur within the first week after admission in 35%-50%[1,5,6] and the cause of death is related to single or multiple organ failure in a majority of cases[7]. In elderly patients, up to 60% of all deaths within the first week are considered to be caused by pancreatitis-

associated ALI and acute respiratory distress syndrome (ARDS)[8]. Independently, ALI is a consequence of a pronounced systemic inflammatory response with increased endothelial and epithelial barrier permeability, with leakage of a protein-rich exudate into the alveolar space and interstitial tissues, thus compromising oxygenation and gas exchange[9]. The magnitude of the systemic inflammatory response determines the concomitant clinical course and outcome[10,11] and this also is true for the severity of the acute-pancreatitis-associated ALI[12] (Figure 1). Respiratory complications are frequent in acute pancreatitis, and respiratory dysfunction, presenting as ALI or ARDS, is a major component of multiple organ dysfunction syndrome (MODS), with a frequent need for ventilatory support[8,13], which contributes to early death in severe acute pancreatitis[14] (Figure 2).

The mortality in ALI has been reported as 30%-60%, and is higher in elderly patients[15,16]. In those who survive, the quality of life is impaired[17]. Overall, ALI and ARDS represent the most common and earliest organ dysfunction in the development of MODS, in which mortality is related to the number of involved organs[18]. This type of secondary ALI, a dominant part of MODS, is also found in severe acute pancreatitis, in which lung injury has been reported to account for a high percentage of deaths.

Acute respiratory failure, including ALI and the more severe form, ARDS, has radiological findings with bilateral pulmonary infiltrates and physiological changes, normal cardiac filling pressures, and a ratio of arterial oxygen pressure and inspiratory oxygen concentration (PaO_2/FiO_2 < 300 mmHg for ALI and < 200 mmHg for ARDS, which reflects pronounced morphological changes)[19]. ALI and ARDS frequently occur in critically ill patients, although the exact incidence in acute pancreatitis has not been stated. If we extrapolate Scandinavian data on ALI and ARDS patients, mortality in the United States is about 36 000 patients per year[20]. More recent mortality rates have also been reported to be 30%-40% and higher in elderly patients[21].

MECHANISMS

Two different phases in ALI and ARDS have been described. Initially, an exudative phase during the first days with diffuse alveolar damage, microvascular injury, type I pneumocyte necrosis, and influx of inflammatory cells and fluid to the pulmonary interstitium has been seen, followed by a fibro-proliferative phase during days 3-7, during which type II pneumocyte hyperplasia, proliferation of fibroblasts and lung repair occur[22]. As a consequence of a pronounced and complex systemic net pro-inflammatory response, both endothelial and epithelial injury is involved in ALI and ARDS (Figure 3). Mediators are several, including cytokines and chemokines, pro-inflammatory mediators and a variety of cells, which regulate the migration and pulmonary infiltration of neutrophils into the interstitial tissue, where they cause injury and breakdown of the pulmonary parenchyma[23]. In order to give a sense of the significance of acute pancreatitis as an etiological factor for ARDS in ICU patients,

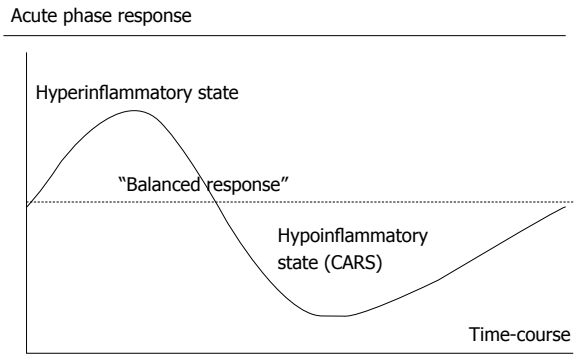

Figure 1 The acute phase response as seen in critical illness, e.g. severe acute pancreatitis.

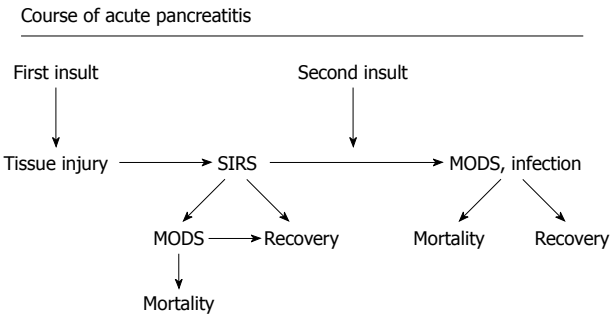

Figure 2 Course of acute pancreatitis. A potential development in severe acute pancreatitis with the first "insult" resulting in a pronounce systemic inflammatory response and potential development of organ dysfunction, and in the worst scenario early mortality. Later during the course, combination of organ dysfunction and infection, potentially pronounced after the second "insult" (translocation from the gut, burst of proinflammatory cytokines, surgery, *etc.*) may result in late mortality. MODS: Multiple organ dysfunction syndrome.

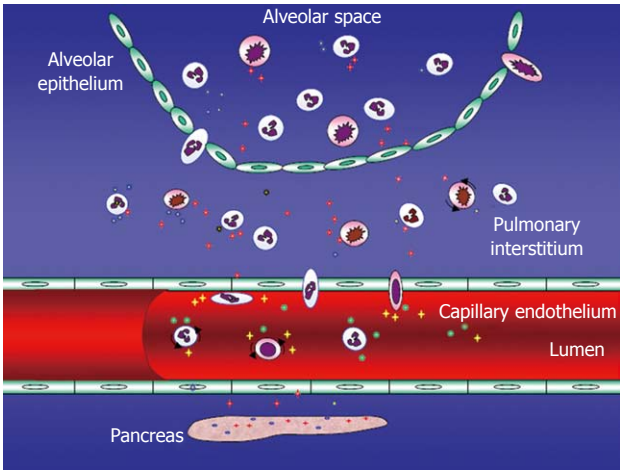

Figure 3 Acute pancreatitis-associated acute lung injury (ALI) - potential mechanisms including endothelial barrier dysfunction. Several adhesion molecules [selectins, intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1) among others] involved in the extravasation of not at least polymorphonuclear neutrophils (PMNs). Tissue injury by not at least these PMNs.

almost one out of seven patients have acute pancreatitis as a primary cause[24].

PATHOPHYSIOLOGICAL MECHANISMS IN SECONDARY ALI

ALI and ARDS may occur secondary to acute pancreatitis, with similar appearances. In general, controlling the source of what is actually fuelling the ALI is important. The features of secondary ALI/ARDS thus involve an initial exudative phase with diffuse alveolar damage, microvascular injury, type- I pneumocyte necrosis and influx of inflammatory cells, followed by a fibro-proliferative phase with lung repair and type- Ⅱ pneumocyte hyperplasia and proliferation of fibroblasts[13]. Both endothelial and epithelial injury is involved. These changes in ALI, which involve endothelial barrier dysfunction, neutrophil and monocyte/macrophage activation, adhesion molecule expression and intracellular signaling, can to a great extent be executed by proteases derived from polymorphonuclear neutrophils (PMNs), and the process seems driven by tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1, with involvement of mast cells, at least during the initiation of leukocyte activation[25-27]. These complex mechanisms that underlie the ALI associated with acute pancreatitis, and the variety of cells involved, which contribute to neutrophil recruitment, adhesion and activation, as well as signal transduction pathways such as tyrosine kinase activation, local transcription of nuclear factor-κB, and expression of multiple inflammatory genes, have been described in a number of experimental studies and reviews[9,13,28,29]. It thus seems well established that inflammatory mediators play a key role in the pathogenesis of ALI and ARDS. These mediators include TNF-α, interleukins-1β, -6, and -10, transforming growth factor-β, granulocyte-macrophage colony-stimulating factor, platelet-activating factor (PAF), selectin and adhesion molecules, complement component C5a, neuropeptide substance P, and chemokines such as MCP-1, and macrophage inflammatory protein-1α. Moreover, one of the results seems to be the production of reactive oxygen and nitrogen species with potential deleterious effects on pulmonary endothelial and epithelial functions[12,29,30]. The neuropeptide substance P possesses pro-inflammatory action that increases vascular permeability, evidently acting through neurokinin-1 receptors. The complement component C5a is a pro-inflammatory chemoattractant that, at least in the experimental setting, seems to increase lung injury, as does the CD40 receptor found on lymphocytes, monocytes and dendritic cells[30]. All of these represent potential targets for future intervention. It is thus the net magnitude of the pro-inflammatory response and the ratio between pro- and anti-inflammatory activity during the process that ultimately determine the outcome of pancreatitis-associated ALI/ARDS. The means of activating leukocytes may be complex and different, and also involve mast cells during the initiation of leukocyte activation. Pulmonary macrophages are likely to be involved in pancreatitis-induced endothelial barrier dysfunction, compromise of type Ⅱ pneumocytes, and tissue injury, and furthermore, the release of matrix metalloproteinases seems essential, not at least, that derived from mast cells[31,32].

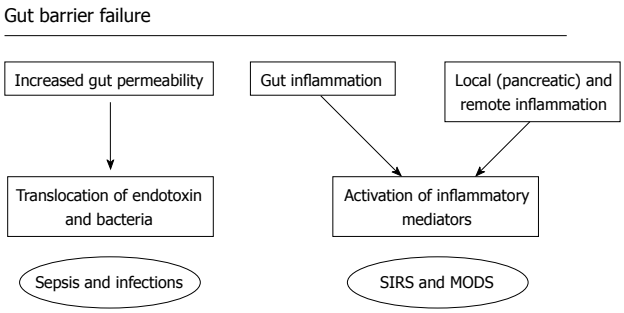

Figure 4 Gut barrier failure. The increase in permeability of the gut barrier from the intestinal lumen may allow translocation of endotoxin and bacteria and there is also activation of immunocompetent cells in the gut wall and gut associated lymphoid tissue, contributing to the inflammatory response, infection, and potentially the development of organ dysfunction.

Pancreatitis-associated ALI has been reported to be related to the effects of pancreatic enzymes, and in particular, phospholipase A_2 is thought to play a role in ALI by damaging pulmonary surfactant, which is a substrate for phospholipase A_2[33]. Furthermore, patients with severe acute pancreatitis have been found to have elevated serum concentrations of phospholipase A_2, which are correlated with the extent of pulmonary complications[34], and a correlation between lung injury score and serum concentration of phospholipase A_2 has been identified[35]. It should be mentioned that the increased systemic levels of phospolipase A_2 that have been reported in this study may be derived not only from the pancreas, but also be of non-pancreatic origin[35]. With all these mechanisms involved, including a variety of different mediators and cells, there have been high expectations on the use of various types of pancreatic protease inhibitors.

DOES THE GUT PLAY A ROLE IN ALI?

The gut barrier not only represents a mechanical line of defense, i.e. a border between the gut lumen and its contents and the rest of the body, but also includes a variety of immunocompetent cells both within the intestinal wall and in associated lymph nodes, which are represented as mucosa-associated lymphoid tissue and gut-associated lymphoid tissue. Preserving an intact gut barrier is thus a complex and meticulous process that is impaired in critical illness such as severe acute pancreatitis, in which the compromised barrier allows for increased intestinal permeability and translocation of bacteria derived from the intestinal lumen and toxins, as well as a gut inflammatory state[10,36-39] (Figure 4).

Mesenteric lymphatics have been found to carry gut-derived factors that contribute to ALI in various

experimental models[40-42]. Interruption of the flow of mesenteric lymph leads to amelioration of ALI, but still we have not identified the exact factors derived from the gastrointestinal tract that are responsible for the pulmonary and systemic effects. This observation represents one potential tool to achieve control of the systemic response and decrease pulmonary injury by modulating factors derived from the gut[43,44].

OUTCOME OF EXPERIMENTAL STUDIES AND POTENTIAL CLINICAL IMPLICATIONS

Our improved understanding of the underlying pathophysiological mechanisms involved in ALI in critical illness has led to a corresponding expectation about potential clinical interventions. This concerns the role of the inflammatory response and signaling mechanisms, such as the protein kinase C pathway[30-32]. Pretreatment and early treatment in experimental acute pancreatitis with, for example, a PAF antagonist and monoclonal antibodies against adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) have been successful[26,27,45]. When evaluating clinical trials with a variety of non-antibiotic interventions in acute pancreatitis, outcome has been less favorable with contradictory results for octreotide and its analogs, as well as the use of the intracellular protease inhibitor gabexate[46]. High expectations have been raised for the use of the highly specific PAF antagonist lexipafant, which has been shown to reduce organ failure and the inflammatory response in patients with predicted severe acute pancreatitis, when administered early[47,48]. A concomitant major study was less convincing, although it did report decreased organ failure inflammatory mediators[49].

FUTURE ASPECTS

Cross-talk between coagulation and inflammation evidently seems to exist, as exemplified by treatment with recombinant human activated protein C in patients with severe acute pancreatitis, in whom a reduction in mortality has been reported[50]. Other components of the coagulation cascade seem to possess inflammatory properties to various degrees. For example, blockers of tissue factor or factor VIIa in experimental severe acute pancreatitis have been shown to ameliorate the associated ALI and decrease neutrophil influx, both when administered as pretreatment and as early treatment[51]. The role of anticoagulants as anti-inflammatory agents in ALI may represent a novel therapeutic option and should be further investigated[52].

The epithelium is involved early in the development of ALI, and produces pro-inflammatory chemokines and triggers neutrophil migration. Furthermore, the epithelium interacts with pulmonary macrophages, which may exacerbate production of pro-inflammatory mediators, thereby increasing recruitment of PMNs from the circulation to the pulmonary interstitial tissue and alveolar lumen. The blocking of chemokines, for example, MCP-1, may thus represent an interesting mode of intervention[53].

Gram-negative infections may be an important predisposing factor for ARDS in acute pancreatitis and endotoxin might potentiate ALI[54]. This emphasizes translocation from the gastrointestinal tract to the systemic circulation and remote organs, as well as the role of the gut-lymph-lung axis. Toll-like receptor 4 (TLR4) compromises the innate immune response and initiates complex signaling pathways when interacting with lipopolysaccharide, which ultimately results in a pro-inflammatory response. Amelioration of the severity of acute pancreatitis and reduced lung injury has been noted in mice that lack TLR4[55], and the lung injury decreases in severity in experimental severe acute pancreatitis treated with nitric oxide, which affects TLR4 gene expression[56]. Therefore, TLR4 has been emphasized as a potential future therapeutic target against inflammatory processes[57].

Heparan sulfate derived from the extracellular matrix or the surface of epithelial cells induces a pro-inflammatory response and has, in its soluble form, been suggested to activate TLR4, thus triggering an endogenous pathway to initiate cytokine production[58]. In experimental acute pancreatitis, heparan sulfate aggravates pancreatic inflammation, and promotes the progression from a local to a systemic inflammatory response, which implies that blocking of heparan sulfate is a potential route of intervention[59]. It has also been suggested that heparan sulfate is important during the initiation of acute pancreatitis, by aggravating the local inflammatory response within the pancreas and thereby potentially promoting the development of a more pronounced systemic inflammatory response, in which heparan sulfate is cleaved off from the epithelial lining of the pancreatic ducts[60]. The role of heparan sulfate and its inhibition thus represents an interesting future line of research, both for the initiation of acute pancreatitis, as well as the development of a systemic inflammatory response.

CONCLUSION

ALI and ARDS in acute pancreatitis remains a major challenge that requires substantial resources. Presently, there is a lack of interventions directed at the underlying pathophysiological mechanisms, although improved understanding may provide us with novel tools for prevention and intervention. Ongoing research on acute pancreatitis and acute respiratory injury gives hope of improvement in the management of this severe and resource-demanding complication, that is still associated with substantial mortality.

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ORIGINAL ARTICLE

Inhibition of hepatitis C virus replication by single-stranded RNA structural mimics

Robert Smolic, Martina Smolic, John H Andorfer, Catherine H Wu, Robert M Smith, George Y Wu

Robert Smolic, Martina Smolic, John H Andorfer, Catherine H Wu, Robert M Smith, George Y Wu, Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-1845, United States

Author contributions: Smolic R, Smolic M, Andorfer JH, Wu CH, Smith RM and Wu GY contributed equally to this work; Smolic R, Smith RM and Wu GY designed the research; Smolic R, Smolic M, Smith RM and Andorfer JH performed the research; Wu CH contributed new reagents/analytic tools; Smolic R, Smolic M and Wu GY wrote the paper.

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Correspondence to: George Y Wu, MD, PhD, Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-1845, United States. wu@nso.uchc.edu
Telephone: +1-860-6792509 Fax: +1-860-6793159

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Abstract

AIM: To examine the effect of hepatitis C virus (HCV) structural mimics of regulatory regions of the genome on HCV replication.

METHODS: HCV RNA structural mimics were constructed and tested in a HCV genotype 1b aBB7 replicon, and a Japanese fulminant hepatitis-1 (JFH-1) HCV genotype 2a infection model. All sequences were computer-predicted to adopt stem-loop structures identical to the corresponding elements in full-length viral RNA. Huh7.5 cells bearing the BB7 replicon or infected with JFH-1 virus were transfected with expression vectors generating HCV mimics and controls. Cellular HCV RNA and protein levels were quantified by real-time polymerase chain reaction and Western blotting, respectively. To evaluate possible antisense effects, complementary RNAs spanning a mimic were prepared.

RESULTS: In the BB7 genotype 1b replicon system, mimics of the polymerase (NS-5B), X and BA regions inhibited replication by more than 90%, 50%, and 60%, respectively. In the JFH-1 genotype 2 infection system, mimics that were only 74% and 46% identical in sequence relative to the corresponding region in JFH-1 inhibited HCV replication by 91.5% and 91.2%, respectively, as effectively as a mimic with complete identity to HCV genotype 2a. The inhibitory effects were confirmed by NS3 protein levels. Antisense RNA molecules spanning the 74% identical mimic had no significant effects.

CONCLUSION: HCV RNA structural mimics can inhibit HCV RNA replication in replicon and infectious HCV systems and do so independent of close sequence identity with the target.

Key words: Hepatitis C virus; Japanese fulminant hepatitis virus; Complementarity; RNA sequence; Hybridization

Peer reviewer: Luc JW van der Laan, PhD, Associate Professor, Departments of Surgery and Gastroenterology & Hepatology, Erasmus MC-University Medical Centre, 's-Gravendijkwal 230, Room L458, Rotterdam, 3015 CE, The Netherlands

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular

carcinoma[1-3]. Current therapeutic options are limited and associated with significant adverse effects[4,5]. Accordingly, there is a strong impetus to develop novel therapeutic options. Because HCV replicates through the interaction of its RNA-dependent RNA polymerase and its RNA template to generate progeny RNA, physical contact between the enzyme and genomic RNA is required[6-8]. We hypothesized that mimics could be created with sufficient resemblance to the natural genomic structure to compete with the HCV genome, resulting in inhibition of replication. HCV RNA has a number of cis-acting replication elements (CREs) whose function could potentially be inhibited by structural RNA mimics[9]. For example, structural mimics based on the HCV internal ribosome entry site (IRES) were recently shown to inhibit HCV translation *in vitro* and in replicon cell culture[10]. Synthesis of (+)-strand RNA molecules was inhibited by RNA aptamers specific for the (-)-strand IRES domain corresponding to the 3'-terminal end of the negative strand of HCV RNA[11,12]. In addition to the IRES[13-15], HCV RNA bears CREs in the positive-strand NS5B[6,8,16,17] and X region[18,19] as well as in the negative strand 3'-terminal region[13,20]. The function of each CRE is assumed to depend on the ability of these structures to bind host factors[21-24] and viral non-structural proteins[8,14,15,25,26]. The objective of the current study was to determine to what extent computer-predicted single-stranded HCV RNA structures could inhibit HCV replication.

MATERIALS AND METHODS

Cloning of HCV RNA structural mimics

For replicon studies, structural mimics were constructed based on RNA sequences of the HCV 1b subgenomic replicon, BB7[27,28] (Table 1). Table 1 shows the regions and the nucleotides from which the mimic sequences were derived. Mimics 5B and X were designed to be identical to the (+) strand of the NS5B and X regions (Figure 1A and D, respectively). Mimics BA (Figure 1B) and EC (Figure 1C) were designed to be identical to the (-) strand of the 3'-terminus. The HCV RNA structural mimic sequences were predicted to adopt stem-loop structures identical to the corresponding CRE in full-length viral RNA, as determined by the Mfold ver 3.2 web server[29,30]. For Japanese fulminant hepatitis virus (JFH-1) infection studies, mimic sequence identities corresponding to HCV JFH-1 genotype 2a were designed to vary from 100% (5B-100), 74% (5B-74) and 46% (5B-46), the latter achieved by changes in the stem regions. The 5B-74 mimic was identical to the mimic (NS5B) used in genotype 1b replicon studies. To construct 5B-46, base-pair exchanges were made at positions 2-15, 18, 30-43, 49-53, 57-60, 75-78, 89-94 in 5B-74 (Figure 2). Regions in the stems or loops in which changes were predicted by Mfold ver 3.2 to alter the secondary structure were left intact. DNA fragments containing each mimic sequence flanked by *Bam*H I and *Hin*dⅢ sites were generated by polymerase chain reaction (PCR) amplification of sequences from pHCV rep1bBB7 (Apath, LLC, St. Louis, MO, USA). As a negative control, an unrelated [hepatitis B virus (HBV) encapsidation signal] was amplified by PCR from plasmid adwR9 (from Dr. T Jake Liang, NIH, Bethesda, MD, USA). The region cloned included HBV sequences flanking a 60 nucleotide (nt) element, so that the total insert length approximated that of the HCV RNA structural mimics. Each insert was cloned into a T7 transcription vector, pENT7 by *Bam*H I /*Hin*dⅢ digestion and ligation. pENT7 is a derivative of pENTR4 (Invitrogen Co., Carlsbad, CA), in which a T7 expression cassette (*Bam*H I and *Hin*dⅢ sites downstream of the consensus T7 RNA polymerase promoter) had been inserted in the multiple cloning site.

For expression of HCV RNA structural mimics as polymerase Ⅱ transcripts in mammalian cells, each insert was subcloned into pSilencer 4.1-CMV puro (Ambion) by *Bam*H I /*Hin*dⅢ digestion and ligation. To confirm fidelity, clones containing pSilencer 4.1-CMV puro with HCV RNA structural mimics were sequenced with primers as recommended by the manufacturer: 5'-AGGCGATTAAGTTGGGTA-3', 5'-CGGTAGGCGTGTACGGTG-3'.

Cloning of a 5B-100 structural mimic

As a positive control for HCV JFH-1 genotype 2a inhibition, a 5B-100 mimic was constructed to be 100% identical to the HCV JFH-1 genotype 2a NS5B coding region (Table 1). This mimic was also predicted using web server Mfold ver. 3.2 to adopt stem-loop structures identical to the corresponding CRE in full-length viral RNA.

Cells

Huh7.5 cells, a human hepatoma cell line, bearing the BB7 HCV genotype 1b replicon were obtained from Apath (St. Louis, MO). Huh7.5 cells, which support JFH-1 replication, were obtained from Dr. Charles M. Rice, Rockefeller University, NY, USA[16,31,32]. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X antibiotic/antimycotic solution (Invitrogen). Cells were passaged every 3-4 d to maintain 75% confluence.

Transfection of plasmids for in situ generation of HCV structural mimics

Huh-7.5 cells containing the BB7 replicon, or cells carrying persistent infection with JFH-1 HCV RNA were seeded at a density of 10^5 cells in 2 mL of growth medium per well in 6-well plates, 6 d before transfection. For transfection, 25 μg of each plasmid encoding a structural mimic were transfected into Huh7.5 cells, 95% confluent, with 15 μL Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was diluted in 250 μL of Opti-MEM I Reduced Serum Medium (Invitrogen) without serum. Lipofectamine 2000 was diluted to the appropriate amount in 250 μL of Opti-MEM I medium. After 5 min incubation, diluted

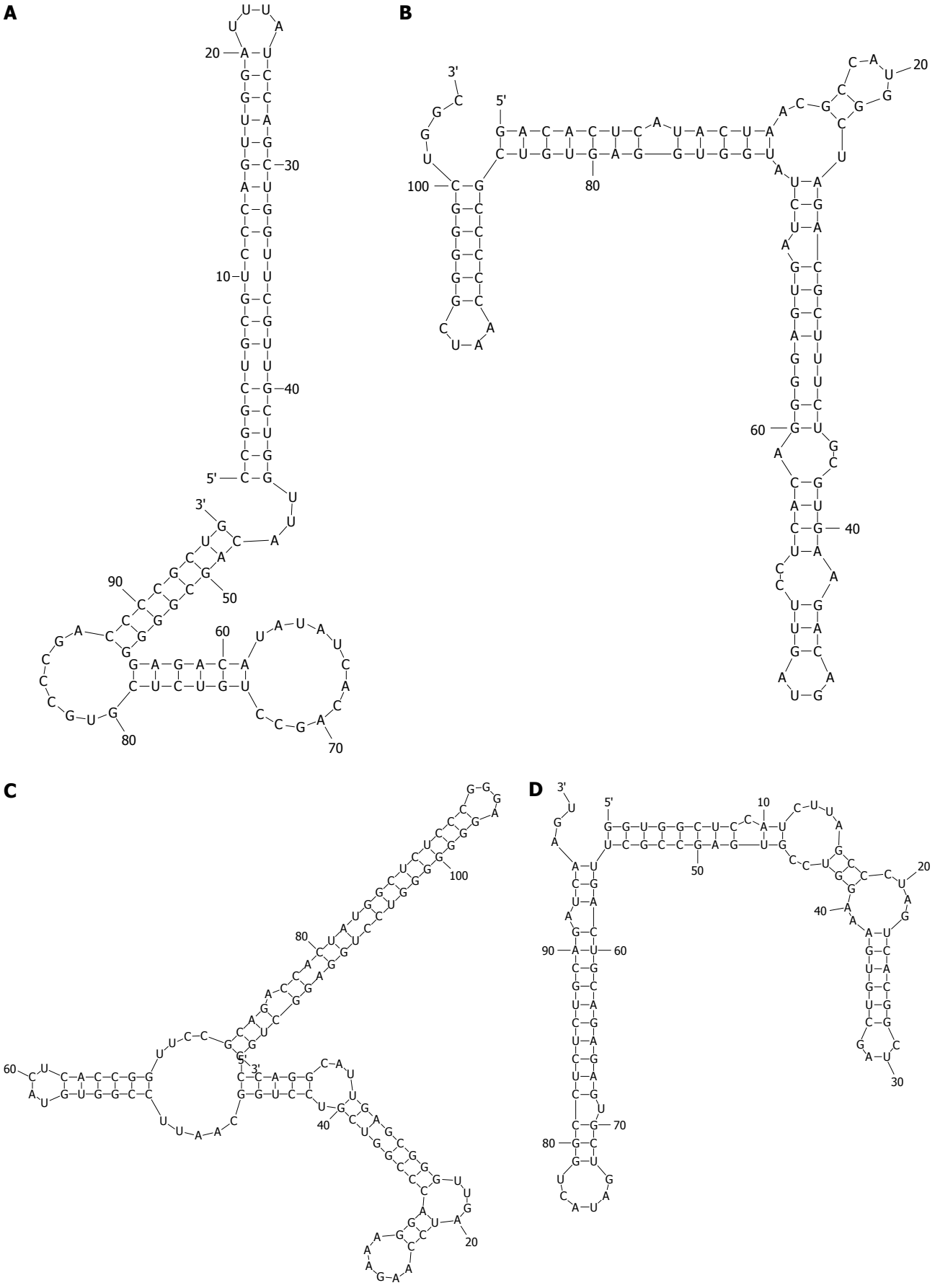

Figure 1 Predicted folding of RNA sequences designed as mimics of hepatitis C virus (HCV) genotype 1b regions. HCV RNA sequences were predicted by Mfold ver 3.2 to adopt stem-loop structures identical to the corresponding sequences in full-length viral RNA. A: 5B-74 corresponding to NS-5B (+) strand; B: BA region 3'-terminus of (-) strand; C: EC region 3'-terminus of (-) strand; D: X region of (+) strand.

Table 1 HCV RNA structural mimics

Structural analogs	Source BB7 sequence (nt)	Source JFH sequence (nt)	Replicon strand	JFH strand	Stem-loop domains
NS-5B (5B-74)	9216-9310		(+) strand		NS5B coding region
X	9508-9605		(+) strand		X region
EC	107-222		(-) strand		3'-terminus
BA	1-104		(-) strand		3'-terminus
5B-100		9284-9378		(+) strand	NS5B coding region

HCV: Hepatitis C virus; JFH: Japanese fulminant hepatitis.

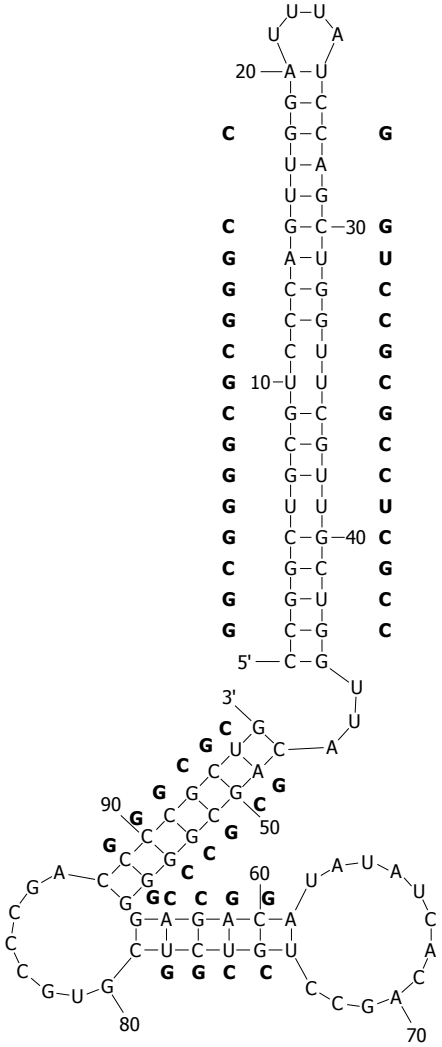

Figure 2 Diagram showing the nucleotide replacements (in bold) in 5B-74 made to create the 5B-46 structural mimic and predicted stem-loop structures identical to the corresponding sequences in 5B-74.

DNA was combined with diluted Lipofectamine 2000, mixed and incubated for 20 min at 25℃. Complexes in 500 μL medium were added. After 6 h of incubation at 37℃ under 50 mL/L CO_2, 1.5 mL of DMEM with 15% FBS were added to each well, and cells were incubated at 37℃ under CO_2 for 48 and 72 h. Cells were harvested and lysates assayed for NS3 protein levels by Western blotting. Total RNA was isolated and HCV RNA levels were determined by real-time reverse transcriptase PCR (RT-PCR).

HCV infection system

A cDNA from hepatitis C genotype 2a virus, JFH-1 strain (from Dr. Takaji Wakita, National Institute of Infectious Diseases, Tokyo, Japan)[33] has been shown to replicate autonomously without drug selection or adaptive mutations[34-37]. This cDNA was used to transfect the full-length JFH-1 genome into Huh7.5 cells to produce infectious HCV[38].

Real-time RT-PCR

RNA was isolated from BB7 replicon cells with Trizol (Invitrogen), and treated with RNase-free DNase (Promega). One microgram of DNase-treated total RNA was reverse transcribed using an iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA). After incubation at 25℃ for 5 min, at 42℃ for 30 min and at 85℃ for 5 min, the resulting cDNA was quantified by real-time RT-PCR with SYBR GREEN according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA) using HCV genotype 1b specific primers: forward primer: 5'-CTGTCTTCACGCAGAAAGCG-3' and reverse primer: 5'-CACTCGCAAGCACCCTATCA-3'. *Homo sapiens* lactate dehydrogenase A (LDHA) mRNA levels in each sample were simultaneously quantified to normalize values of HCV RNA. The primer sequence for LDHA forward primer was: 5'-TAATGAAGGACTTGGCAGATGAACT-3' and reverse primer: 5'-ACGGCTTTCTCCCTCTTGCT-3'. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication as a percent of unrelated control.

For the JFH infection system, RNA replication in transfected cells was quantified by RT-PCR using HCV genotype 2a specific forward primer: 5'-TAGGAGGGCCCATGTTCAAC-3', reverse primer 5'-CCCCTGGCTTTCTGAGATGAC-3'. The PCR conditions were: 2 min at 50℃, 10 min at 95℃ and 15 s at 95℃. After 40 cycles, final extension was performed at 60℃ for 1 min. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication as a percent of unrelated control.

Western blotting analyses

Total protein lysates of Huh7.5 cells stably infected with JFH-1 were evaluated by Western blotting. Cells were harvested in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA) supplemented with protease inhibitors

(Roche). Protein concentrations were determined with Bio-Rad assay as described by the manufacturer. Forty micrograms of protein was resolved by SDS-PAGE, and transferred to Hybond nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ, USA). Membranes were blocked with 5% nonfat milk in PBS, incubated with a 1:1000 dilution of the monoclonal mouse HCV NS3 antibody (Biodesign International, Saco, ME, USA), washed with PBS/0.05% Tween 20 and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Pierce, Rockford, IL, USA) at 1:20 000 dilution. Bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce) and quantified by Gel Imaging software GeneTools (SynGene, Frederick, MD, USA). To ensure comparable loading of samples, blots were incubated with a 1:1000 dilution of a polyclonal rabbit α-tubulin antibody (Abcam Inc., Cambridge, MA, USA), and detected by HRP-conjugated goat anti-rabbit secondary antibody using procedures as described above.

Evaluation of possible antisense effects

Because the 5B-74 structural mimic was single-stranded, and retained significant (74%) sequence identity to JFH-1 genotype 2a sequence, it was possible that the observed inhibition of replication was a result of antisense effects. To assess this possibility, 4 complementary RNAs 21-28 nt long, spanning the 5B-74 mimic were synthesized (IDT-Integrated DNA Technologies, Inc., Coralville, IA, USA). The antisense sequences were: 5B-C1 (nt 5-21), 5'-CCGGCUGCGUCCCAGUUGGAU-3', 5B-C2 (nt 22-43), 5'-UUAUCCAGCUGGUUCGUUGCUG-3', 5B-C3 (nt 44-71), 5'-GUUACAGCGGGGGAGACAUAUAUCACAG-3' and 5B-C4 (nt 72-95), 5'-CCUGUCUCGUGCCCGACCCCGCUG-3'.

One day before transfection, JFH-1-infected Huh7.5 cells were plated in growth medium without antibiotics, to be 95% confluent at transfection. Oligomer-Lipofectamine 2000 complexes were prepared by dilution of 50 pmol or 500 pmol RNA oligomer in 250 μL of Opti-MEM I without serum. Five microliters of Lipofectamine 2000 was diluted in 250 μL Opti-MEM mixed gently and incubated for 5 min at 25℃. The diluted oligomer was combined with diluted Lipofectamine 2000, mixed and incubated for 20 min at 25℃. Complexes were added to each well containing cells and medium. After 6 h, 1.5 mL of 15% FBS diluted in DMEM was added and the cells were incubated at 37℃ in a CO_2 incubator for 48 h. HCV RNA replication was determined by real-time RT-PCR using HCV specific primers. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication as a percent of unrelated control.

RESULTS

For replicon studies, structural RNA mimics were constructed based on sequences of the HCV 1b subgenomic replicon, BB7. Because HCV RNA replication involves generation of both positive and negative strands, structural mimics were designed for both strands. These targets were selected based on previous reports that HCV RNA bears CREs in the positive-strand NS5B coding region[6,8,16,17] and X region[18,19] as well as in the negative strand 3'-terminal region[13,39].

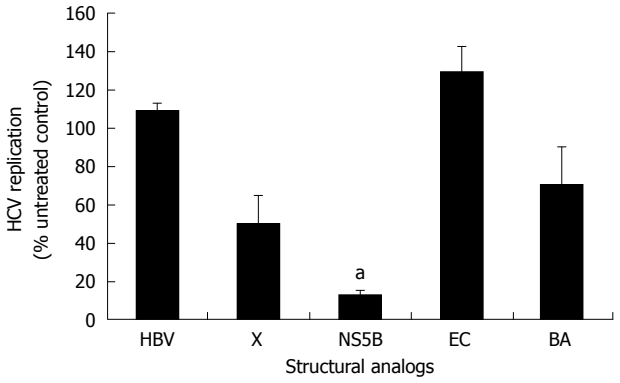

Figure 3 HCV RNA levels in the BB7 replicon cell lysates at 48 h post-transfection with structural mimics. BB7 replicon cells were transfected with plasmids generating structural mimics, and HCV RNA levels were assayed by real-time reverse transcriptase polymerase chain reaction (RT-PCR). The columns and bars represent the mean ± SD, respectively of 3 independent triplicate transfections. Statistically significant differences relative to controls are indicated by ($^aP < 0.05$).

The results of transfection of RNA mimics into BB7 replicon cells quantified by real time RT-PCR are shown in Figure 3. The 5B mimic inhibited HCV replication by more than 90%. Mimics to X and BA regions decreased replication by 50%, and 60%, respectively. The EC mimic actually had no inhibitory effect, and in fact appeared to increase replication, but the effect was not statistically significant. The fact that this sequence, also identical to the natural HCV sequence, failed to inhibit replication indicates that the observed inhibition by the NS5B mimic did not result from a nonspecific effect of HCV RNA. The inhibition by the NS5B plasmid was found to be dose-dependent up to 25 μg (data not shown).

Because the NS5B structural mimic appeared to be the most effective, further experiments were performed to determine whether this mimic might be effective against a different genotype in JFH-1-infected Huh-7.5 cells. It was found that the NS5B, now named 5B-74, mimic which was only 74% identical to the genotype 2a sequences of NS5B was as effective in suppression of HCV replication of JFH-1 genotype 2a as 5B-100 which was 100% identical to the genotype 2a (Figure 4).

The intent of the design of the structural mimics was to create single-stranded versions of the natural structure to compete for protein interactions. However, it is possible that the observed inhibitory effects could have been simply the result of non-specific effects. To explore this possibility, the mimic 5B-46 was prepared in which bases were changed at positions 2-15, 18, 30-43, 49-54, 57-60, 75-78, and 89-94 (Figure 2), with a sequence identity of the stems of only 27.5%, and overall

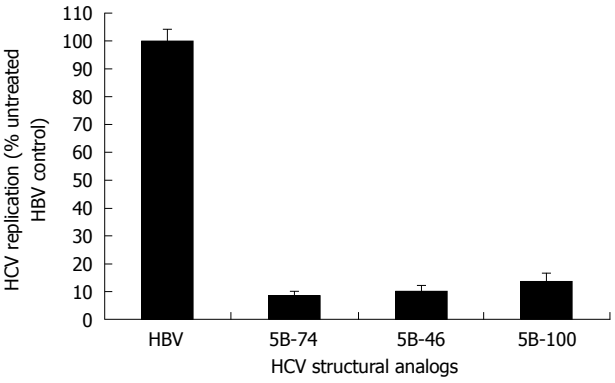

Figure 4 Effects of transfection of NS5B mimics 5B-74, 5B-46 and 5B-100 on Japanese fulminant hepatitis-1 (JFH-1) genotype 2a viral infection in Huh7.5 cells. Cells stably infected with JFH-1 genotype 2a virus were transfected with plasmids generating structural mimics, and HCV RNA levels were assayed by real-time RT-PCR. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication percents of unrelated hepatitis B virus (HBV) control.

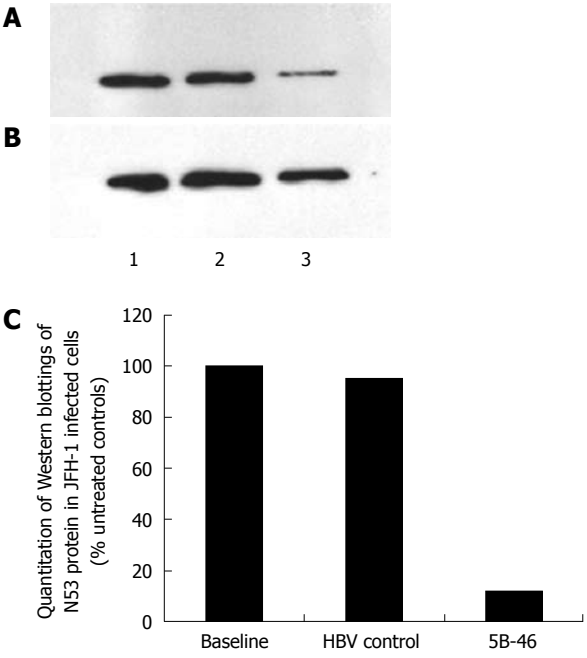

Figure 5 Western blotting analysis of NS3 protease gene expression in JFH-1 infected Huh7.5 cells after transfection of 5B-74 mimic and controls. A: HCV NS3; B: Tubulin, 72 h after transfection. Lane 1: Levels prior to addition of mimic; Lane 2: HBV (unrelated) control; Lane 3: 5B-46 mimic; C: Quantification of Western blottings of NS3 protein in JFH-1-infected cells at baseline, after 72 h exposure to 5B-46, and to HBV unrelated control. Bound NS3 antibodies were detected with SuperSignal chemiluminescent substrate and quantitated by Gel Imaging software GeneTools.

identity of 46%, relative to JFH-1 (genotype 2a) NS5B. The 5B-74 mimic decreased HCV replication by 91%. Furthermore, in spite of a sequence identity of only 46%, the novel mimic 5B-46, decreased HCV replication in JFH-1-infected cells by 90%, not significantly different from the mimic 5B-74 with 74% identity, or the mimic 5B-100 with complete identity to the HCV genotype 2a (Figure 4).

To assess viral protein levels, Western blottings of

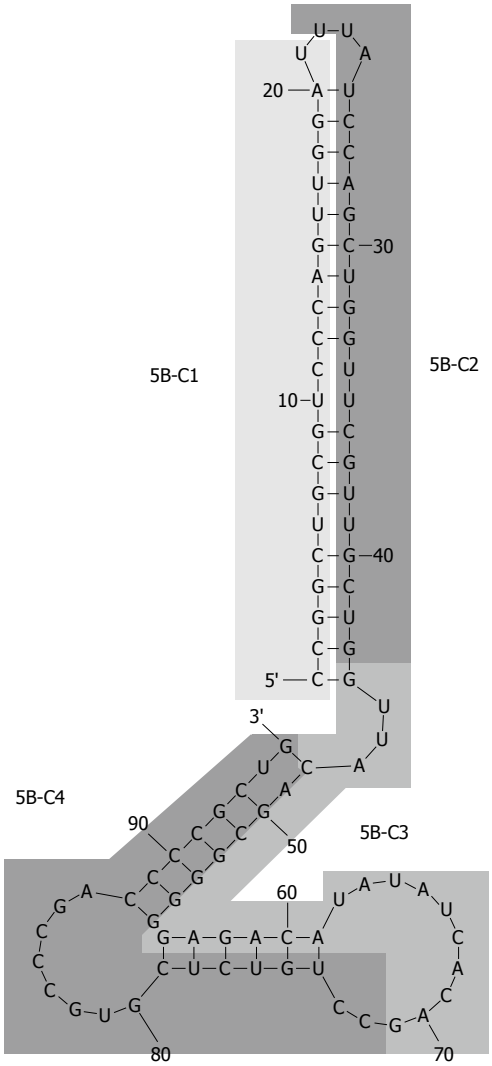

Figure 6 Diagram of single-stranded fragments spanning the entire length of the 5B-74 mimic used as antisense controls.

the NS3 protease were performed. Levels of neither the housekeeping protein tubulin (lane 2, Figure 5B) nor the NS3 protease (lane 2, Figure 5A) were affected by the unrelated HBV control mimic. However, the 5B-74 mimic decreased NS3 protease levels by 90% (lane 3, Figure 5A), while tubulin levels remained unchanged (lane 3, Figure 5B), the latter arguing against a non-specific effect or toxicity by the mimics. Quantification of the Western blottings confirmed the strong inhibitory effects of the 5B-46 mimic as shown in Figure 5C.

In the 5B-46 mimic, although most of the stem regions were altered, the sequences in the loops were left unchanged in order to retain secondary structure. Therefore, an antisense effect of the loop regions was still possible. To evaluate this possibility, short single-stranded fragments were prepared spanning the 5B-74 mimic (Figure 6). Analysis by Mfold ver 3.2 did not generate any stem-loop structures corresponding to regions in the native 5B-74 (data not shown). None of these sequences had any significant effects on HCV RNA levels compared to control (Figure 7), supporting the

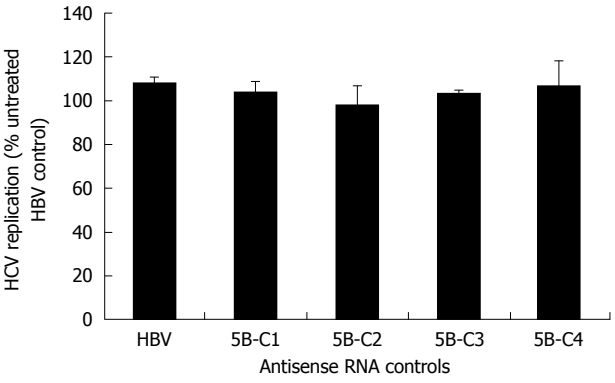

Figure 7 HCV replication after transfection of single-stranded RNA fragments corresponding to specific regions of the 5B-74 mimic in JFH-1-infected Huh7.5 cells. Cells stably infected with JFH-1 HCV were transfected separately with single-stranded fragments spanning the entire length of the 5B-74 mimic. HCV RNA levels were assayed by real-time RT-PCR. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication as percent of unrelated (HBV) control.

conclusion that the 5B-74 and 5B-46 mimics inhibited HCV replication not by sequence complementarity, but by conformational attributes.

DISCUSSION

Previous studies have shown that overexpression of RNA analogs in infected cells can result in sequestration of essential factors. For example, suppression of human immunodeficiency virus-1 replication in cell models has been demonstrated by expression of structural mimics of the TAR element[40], RRE structure[41], and primer binding sequence[42]. HCV RNA has a number of CREs that could potentially be inhibited by structural RNA mimics. Structural mimics based on the HCV IRES were recently shown to inhibit HCV translation *in vitro* and in replicon models[10]. However, no effect on RNA replication was observed in the latter, possibly because of the bicistronic nature of the replicon.

It has been shown previously that double-stranded RNA can induce synthesis of cytokines including interferons which have antiviral effects. However, stimulation of interferon production has been reported to be dependent on the length of the RNA, with duplexes greater than 30 bp having been found to be most efficient[43]. Many studies have shown that smaller siRNAs of 20-21 base pairs (bp) have purely RNAi activity. In the current study, for the 5B-74, 5B-46, and 5B-100 mimics, the longest double-stranded regions were only 9 bp, followed by 8, 6 and 3 bp. Furthermore, the EC mimic was also predicted to have 9, 8, 6 and 3 bp regions. Yet, EC had essentially no significant inhibitory activity. Therefore, while double-stranded RNA stimulation of interferon production cannot be entirely excluded, if it occurs, it is not likely to account for the observed inhibitory effects of the 5B mimics.

Recently, cell culture systems for *in vitro* replication and infectious virus production have been established based on full-length HCV genotype 2a RNA[36,38,44,45]. This infectious HCV possesses 21% differences compared to the HCV 1b subgenomic replicon, BB7. Nucleotide sequences involved in the kissing-loop interaction are conserved between JFH-1 and BB7. However, mutations in other regions may affect this interaction by disrupting the RNA secondary structure[46]. It is interesting to note that small RNAs corresponding to 2 domains of HCV 2a, which differed from those in HCV 1b in 13 of 44 nucleotides and 6 of 48 nucleotides, respectively, inhibited the replication of replicon RNA from pLMH14 (HCV 1b) by 48.3% and 37.1%, respectively indicating that HCV 2a-derived RNAs could cross-interact with HCV 1b components despite the sequence differences between the elements of the 2 viruses[47]. Based on this finding, and the fact that most of the nucleotide substitutions in one domain from HCV 2a were compensatory bp changes, Zhang *et al*[47] hypothesized that the structure rather than the primary sequence was important for its function.

Viral replication has been shown to require the interaction of host factors with viral RNA and/or proteins. For HCV, several RNA-binding proteins, including L autoantigen[48], NS1-associated protein 1[49], polypyrimidine tract binding protein[50] and cyclophilin B[22] have been shown to bind to HCV RNA. Therefore, while we have focused on effects of mimics on HCV replication, because other vital HCV activities also depend on protein-RNA interactions, it is possible that the strategy for inhibition of replication by structural mimicry could be extended to other targets.

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COMMENTS

Background

Chronic hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Current therapeutic options are limited and associated with significant adverse effects. Accordingly, there is a strong impetus to develop novel therapeutic options. Because HCV replicates through the interaction of its RNA-dependent RNA polymerase and its RNA template to generate progeny RNA, physical contact between the enzyme and genomic RNA is required. The authors hypothesized that mimics could be created with sufficient resemblance to the natural genomic structure to compete with the HCV genome, resulting in inhibition of replication.

Research frontiers

Previous studies have shown that overexpression of RNA analogs in infected cells can result in sequestration of essential factors. For example, suppression of human immunodeficiency virus-1 replication in cell models has been demonstrated by expression of structural mimics of the TAR element, RRE structure, and primer binding sequence. HCV RNA has a number of cis-acting replication elements that could potentially be inhibited by structural RNA mimics. Structural mimics based on the HCV internal ribosome entry site were recently shown to inhibit HCV translation *in vitro* and in replicon models.

Innovations and breakthroughs

The novel feature of this research is the discovery that stretches of RNA can

be made that have little sequence similarity to HCV. However, because of the retention of the predicted structure, they have been found to be highly potent and specific agents for inhibition of HCV replication.

Applications

Specific sequences inhibiting HCV replication without substantial HCV sequence identity may be a novel therapeutic option to circumvent the appearance of resistance arising from mutation of the viral genome.

Terminology

Single-stranded RNA structural mimics are a class of polynucleotides that can inhibit replication of the HCV and are designed to mimic the shape of natural viral nucleic acids, without requiring the matching of nucleic acid sequences.

Peer review

The study of Smolic *et al* described the antiviral effect of HCV structural mimics in replicon and infectious models. It is a very straightforward and concise paper. The results provided clear evidence of the inhibition of viral replication by these mimics, likely *via* conformational attributes. However, several issues regarding the mechanism of action should be addressed to further strengthen this study.

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ORIGINAL ARTICLE

Differentiation of focal liver lesions using three-dimensional ultrasonography: Retrospective and prospective studies

Wen Luo, Kazushi Numata, Manabu Morimoto, Akito Nozaki, Michio Ueda, Masaaki Kondo, Satoshi Morita, Katsuaki Tanaka

Wen Luo, Kazushi Numata, Manabu Morimoto, Akito Nozaki, Michio Ueda, Masaaki Kondo, Katsuaki Tanaka, Gastroenterological Center, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama, Kanagawa 232-0024, Japan
Wen Luo, Department of Ultrasound, Xijing Hospital, Fourth Military Medical University, 15th Changle Xi Road, Xi'an 710032, Shaanxi Province, China
Satoshi Morita, Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama, Kanagawa 232-0024, Japan
Author contributions: Luo W and Numata K designed the research; Numata K and Morimoto M performed the research; Luo W, Nozaki A, Kondo M and Tanaka K contributed analytic tools; Luo W, Numata K, Ueda M and Morita S analyzed the data; Luo W and Numata K wrote the paper.
Correspondence to: Kazushi Numata, MD, Gastroenterological Center, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama, Kanagawa 232-0024, Japan. kz-numa@urahp.yokohama-cu.ac.jp
Telephone: +81-45-2615656 Fax: +81-45-2619492
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Abstract

AIM: To differentiate focal liver lesions based on enhancement patterns using three-dimensional ultrasonography (3D US) with perflubutane-based contrast agent.

METHODS: Two hundred and eighty two patients with focal liver lesions, including 168 hepatocellular carcinomas (HCCs), 63 metastases, 40 hemangiomas and 11 focal nodular hyperplasias (FNHs), were examined by 3D US with perflubutane-based contrast agent. Tomographic ultrasound images and sonographic angiograms were reconstructed. Among 282 lesions, enhancement patterns of 163 lesions between January 2007 and October 2007 were analyzed retrospectively. Then from November 2007 to May 2008, compared with contrast-enhanced (CE) 2D US, CE 3D US was performed on 119 lesions for prospective differential diagnosis. Sensitivity, specificity, area under receiver operating characteristic curve (A_z) and inter-reader agreement were assessed.

RESULTS: With the tridimensional view, dominant enhancement patterns were revealed as diffuse enhancement or peripheral ring-like enhancement, followed with washout change for HCCs or metastases, respectively, and peripheral nodular enhancement or diffuse enhancement with spoke-wheel arteries, followed by persistent enhancement for hemangiomas or FNHs, respectively. At CE 3D US, the prospective differentiation of lesions showed sensitivity 92% (mean for two readers), specificity 91% and A_z value 0.95 for HCCs, 84%, 97%, and 0.95 for metastases, 91%, 98%, and 0.98 for hemangiomas and 80%, 99%, and 0.99 for FNHs, respectively, while good to excellent inter-reader agreement was achieved. No significant difference exists between prospective diagnosis accuracy at CE 3D US and that at CE 2D US.

CONCLUSION: CE 3D US provides a spatial perspective for liver tumor enhancement, and could help in differentiating focal liver lesions.

Key words: Three-dimensional; Ultrasonography; Contrast agent; Liver; Neoplasms

Peer reviewer: Mirko D'Onofrio, MD, Assistant Professor, Department of Radiology, GB Rossi University Hospital, University of Verona, Piazzale LA Scuro 10, Verona, 37134, Italy

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INTRODUCTION

Focal liver lesions are increasingly being discovered with the widespread use of diagnostic imaging modalities, and differentiation of various liver lesions is considered to be critical for determining treatment options. Tremendous advancements in imaging techniques, including ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI) have resulted in these modalities being accepted as effective and thus widely used to characterize focal liver lesions[1-5]. This reduces the necessity of invasive histopathological examination.

With the advantages of non-invasiveness, no radiation and flexible operation, contrast-enhanced (CE) US has become increasingly important in the detection and characterization of focal liver tumors over the past several years[6-13]. Microbubbles of ultrasound contrast agent approximately the same size as red blood cells move into vessels but not through the vascular endothelium into the interstitium, which provides exact information about vascularity by reflecting specific signals, while some contrast agents present a postvascular hepato-specific phase probably due to reticuloendothelial system phagocytosis or the adherence of microbubbles to hepatic sinusoids[14-17]. Sonazoid (Daiichi Sankyo, Tokyo, Japan), a newly-developed second-generation ultrasound contrast agent, which consists of microbubbles (mean diameter 2-3 μm) of a perfluorobutane gas stabilized by a phospholipid monolayer shell, allows continuous real-time CE imaging for more than 10 min, and improves the reproducibility and durability of CE US examination[18-20].

Recently, the advent of CE three-dimensional (3D) US, facilitating demonstration of the desired regions with dynamic information in spatial volume, has led to new applications of CE US imaging[21-24]. Different from the 2D images, CE 3D US acquires the data in a volume of interest (VOI) by automatically scanning with a desired angle and allows reconstruction of tomographic images in three orthogonal planes and rendered angiogram-like images. With the feature of illustration in three dimensions, CE 3D US has been proven to offer accurate and detailed visualization of the vascular characteristics of various focal liver tumors[25,26].

To our knowledge, although many studies on differentiation among various focal liver lesions have been conducted using CE 2D US[27-29] and recently a few using CE 2D US with Sonazoid[30], the exact value of CE 3D US with Sonazoid in the differential diagnosis of various focal liver lesions has not yet been clarified. Thus, in this study, in order to examine the potential role of CE 3D US in characterizing focal liver lesions, we retrospectively evaluated the enhancement patterns and the diagnostic criteria established using dominant enhancement patterns were then applied to differentiation among focal liver lesions in a prospective study.

MATERIALS AND METHODS

Patients

Institutional Review Board approval was obtained for this retrospective and prospective study and informed consent was obtained from all patients. The reference standards for focal liver lesions in this study were described as follows: hepatocellular carcinomas (HCCs) with diameters exceeding 2 cm were confirmed by typical findings on both dynamic multi-detector CT and CE MRI[31]; HCCs exceeding 2 cm in diameter without typical radiological findings and those less than 2 cm in diameter, as well as all the liver metastases, were confirmed using histopathological results from percutaneous biopsy or surgery; benign tumors were confirmed by typical appearance on either dynamic multi-detector CT or CE MRI with at least 6-mo follow-up by dynamic multi-detector CT or CE MRI, and those without typical findings were confirmed histopathologically.

Between January 2007 and October 2007, 183 consecutive patients known or suspected to have focal liver lesions were examined using CE 3D US. Among these, 163 patients (90 men and 73 women) with 163 focal liver tumors were retrospectively identified, based on the following enrollment criteria: satisfactory CE 3D US images were acquired without artifacts from the cardiac and respiratory motion, shadows of costal bones, and abdominal gas; the definitive diagnosis of lesions was confirmed according to reference standards; the lesions had not been treated previously. Twenty patients were excluded: 6 with HCCs and 6 with hepatic metastasis lesions lacking a histological diagnosis; 3 HCCs and 3 hemangiomas with serious artifacts on the images; 2 benign lesions suspected on clinical information and imaging follow-up, without typical radiological findings and histological results. For 58 patients with multiple lesions, only the largest one with CE 3D US images and confirmed diagnosis was selected for evaluation. These 163 lesions were reviewed in the retrospective study to summarize the dominant enhancement patterns, which would be the diagnostic criteria in the prospective study.

From November 2007 to May 2008, the prospective study was conducted using CE 3D US on 124 consecutive patients with suspicious focal liver lesions detected by prior conventional US or CT and after that, CE 2D US was performed on each patient. For 40 patients with multiple lesions, CE 3D US examination was performed on the largest one, and if the largest lesion was not suitable for scanning due to its location, the next largest lesion was selected. CE 2D US was performed on the same lesion scanned by CE 3D US. In the prospective study, the exclusion criteria were: patients unable to hold breath; lesions with inappropriate locations for acquiring CE 3D

Table 1 Clinical characteristics of patients and focal liver lesions in retrospective and prospective studies (mean ± SD)

	Retrospective study (n = 163)				Prospective study (n = 119)			
	HCC	Metastasis	Hemangioma	FNH	HCC	Metastasis	Hemangioma	FNH
No. of patients	98	35	24	6	70	28	16	5
No. of lesions	98	35	24	6	70	28	16	5
Age of patients (yr)	69.8 ± 6.5	64.8 ± 13.2	58.9 ± 11.2	54.2 ± 16.7	72.5 ± 7.3	69.2 ± 8.3	55.4 ± 14.1	52.8 ± 18.4
Liver cirrhosis								
Child-Pugh class A	78	0	1	0	52	0	0	0
Child-Pugh class B	20	0	0	0	18	0	0	0
Lesion diameter (mm)	30.1 ± 24.3	28.9 ± 15.5	31.5 ± 18.0	38.4 ± 12.0	29.1 ± 20.4	30.7 ± 21.1	33.7 ± 16.7	37.4 ± 15.0
Final diagnosis								
Surgery	11	15	2	0	6	12	0	0
Biopsy	29	20	0	1	19	16	0	1
Radiological imaging	58	0	22	5	45	0	16	4

HCC: Hepatocellular carcinoma; FNH: Focal nodular hyperplasia.

US images due to artifacts from costal bone shadows, heart motion or abdominal gas; patients with lesions requiring histopathological diagnosis according to a reference standard but in whom surgery or biopsy was not possible due to poor liver function or lack of consent. Finally, 119 patients (67 men and 52 women) with 119 liver lesions were enrolled, while 2 patients with HCCs less than 2 cm in diameter and one with metastases were excluded for lack of consent on biopsy, and one patient with HCC and one with hemangioma were excluded for the obstruction of costal bone shadow.

Final diagnosis and clinical characteristics are shown in Table 1. The metastases in the retrospective study included 17 from colon carcinomas, 6 from pancreatic carcinomas, 5 from intrahepatic cholangiocellular carcinomas (ICC), 4 from rectal carcinomas, 1 from an ileum carcinoma, 1 from a gallbladder carcinoma and 1 from an ovarian carcinoma. Metastases in the prospective study comprised 15 from colon carcinomas, 4 from pancreatic carcinomas, 4 ICCs, 2 from rectal carcinomas, 1 from an ileum carcinoma, 1 from an ovarian carcinoma, and 1 from a gastric carcinoma. In this study, we classified the ICCs as hepatic metastasis because differentiation of ICCs from metastasis is difficult using only radiological imaging enhancement patterns.

Data acquisition

In the retrospective study, using the LOGIQ 7 ultrasound imaging system and 4D3CL volume probe (GE Healthcare, Milwaukee, WI) with a 2.0-5.5 MHz frequency, CE 3D US was performed by one sonographer with 10 years experience in using abdominal CE US. In the prospective study, CE 3D US was performed by the same sonographer in the retrospective study. After 1 or 2 d using the LOGIQ 7 ultrasound imaging system and 3.5 CS convex probe (GE Healthcare, Milwaukee, WI), CE 2D US was performed on each lesion by the same sonographer at CE 3D US. In this study, the acquisition of 3D images was performed with a convex volume 4D3C-L probe which scans automatically with internal sectorial mechanical tilt movement to obtain the data. The scanning angle could be selected from 15° to 84°. In this study we used 30-70° depending on different tumor sizes and the duration for one scanning process varied from 4.5 to 21.3 s (mean time, 12 s).

Prior to both CE 2D US and CE 3D US scanning, all patients received an intravenous bolus injection of 0.2 mL Sonazoid followed by a 2 mL 5% glucose solution and subsequent infusion of 5% glucose solution at 10 mL/min. The coded harmonic angio (CHA) mode (mechanical index = 0.5-0.9) at 8 to 13 frames per second was used for both CE 2D US and CE 3D US scanning. The contrast sonography process included three phases, that is, an early phase (10-40 s after contrast medium injection), a middle phase (80-120 s after contrast medium injection) and a late phase (more than 5 min after contrast medium injection). During each phase, the CE US scanning was performed when the characteristic enhancement of focal liver lesions appeared.

Static 3D and Autosweep 3D functionalities, with which the LOGIQ 7 ultrasound imaging system is equipped, were used for image acquisition. Employing the different engineering features of these two functionalities, in the early phase, both Static 3D and Autosweep 3D were used to acquire 3D data, while in the two other phases only the Autosweep 3D functionality was used. A VOI with an adjustable scanning angle and size was determined before scanning to contain the desired region. The data acquired were stored as cineloops in the hard disk of the ultrasound imaging system.

Image reconstruction

3D image reconstruction was performed using the functionalities with which the LOGIQ 7 ultrasound system was equipped. In the VOI which was presented in an isotropic rectangular coordinate frame, the three orthogonal planes were referred to as plane A which could migrate from front to rear through the VOI, plane B from left to right and plane C from up to down. Tomographic ultrasound images (TUI) with presentation of several parallel slices in three orthogonal planes were reconstructed in three phases, while the number of slices that could be selected was 2, 4, or 6 for images obtained using Autosweep 3D, and 2, 4, or 9 for Static 3D. In

order to show the desired images, the range of distance between two slices in TUI could be adjusted if required. The mean times of these procedures were about 20 s. Sonographic angiogram images were reconstructed in an angio-like view during the early phase by using various rendering modes. The maximum intensity mode displaying the maximum grey values in the VOI, mixed with surface mode displaying the grey value on the surface of objects, was used to visualize tumor vessels and early tumor enhancement, while the average intensity mode displaying the average grey values in the VOI, mixed with surface mode, was employed to describe the tumor based on the predominantly unenhanced areas. The mean times of these procedures were about 45 s. TUI in three dimensions and sonographic angiogram images were stored with volume data in the hard disk of the ultrasound imaging system. In both the retrospective study and the prospective study, the reconstruction of all the images was completed by one operator with 10 years of experience in abdominal US and 1 year in CE 3D US, blinded to the final diagnosis and other related information on patients.

Image evaluation

Before the 3D image evaluations in both the retrospective and prospective studies, all readers were given training in the assessment of various enhancement patterns. Image evaluation was divided into two sections: in the first section, TUI in early, middle and late phases and sonographic angiograms in the early phase were reviewed separately in random order for all the lesions and the enhancement patterns in each phase were classified; in the second section, all images in three phases for each lesion were read consecutively to observe enhancement changes over time. In the prospective study, 2D images were read 2 wk after the 3D image review. All the images were reviewed on the ultrasound imaging system. The zoom function and arbitrary rotation of images are available in both TUI and sonographic angiograms.

In the retrospective study, images were reviewed independently by two readers (Numata K and Morimoto M) with 10 years of experience in liver US and 1.5 years of experience in CE 3D US imaging, who were blinded to final diagnosis and clinical and other radiological information. The readers were asked to classify the enhancement patterns in three phases and enhancement changes over time. In the early phase, the enhancement patterns consisted of tumor enhancement and tumor vessels: four tumor enhancement patterns including diffuse enhancement defined as homogeneous or heterogeneous tumor enhancement, peripheral ring-like enhancement defined as enhancement in the tumor periphery with a ring shape, peripheral nodular enhancement defined as enhancement in the tumor periphery with a nodular shape, and absence of tumor enhancement; four tumor vessel patterns including intratumoral vessels in the central portion of the tumor, peritumoral vessels at the periphery of the tumor, spoke-wheel arteries defined as a centrally located artery with centrifugal stellate branching, and absence of vessels. In the middle phase, four patterns were classified based on tumor enhancement: diffuse enhancement, peripheral ring-like enhancement, peripheral nodular enhancement and perfusion defect. In the late phase, the hypoechoic pattern was defined as all or part of the lesion having low echogenicity as compared to the surrounding liver parenchyma, and the isoechoic pattern as the entire lesion having echogenicity equal to that of the surrounding liver parenchyma. The enhancement changes over time were assessed: washout, early enhancement greater than that of the liver parenchyma and enhancement in the middle or late phase less than that of the liver parenchyma; persistence, whatever the enhancement pattern in the early phase was, enhancement of the lesion in the middle and late phases was at least equal to that of the liver parenchyma; absence of washout and persistence, no distinct enhancement was detected in any of the three phases.

Evaluations of 153 lesions by two readers were in concordance. For 10 lesions, enhancement patterns evaluated by two readers were different but a consensus was finally reached. After the pattern classification, the combination of enhancement patterns in the three phases was summarized and the positive predictive value (PPV) was calculated. The combined enhancement patterns with PPV more than 50% served as diagnostic criteria for the analysis in the prospective study.

In the prospective study, two readers (Nozaki A and Luo W) with 8 and 5 years of experience in CE US of the liver and 1 year of experience in CE 3D US imaging, blinded to the final diagnosis and clinical and other radiological information, read CE 3D US images and differentiated focal liver lesions according to the diagnostic criteria established in the retrospective study. After reading 2D images, these two readers also made a diagnosis of each lesion according to the criteria previously established (Table 2)[27,28,32,33].

On 3D images and 2D images, for each diagnosis, i.e. HCC, metastasis, hemangioma, and focal nodular hyperplasia (FNH), a four-point scale was used to grade diagnostic confidence. For instance, in HCCs, grade 1 was defined as "probably not a HCC lesion", grade 2 as "possibly not a HCC lesion", grade 3 as "possibly a HCC lesion" and grade 4 as "probably a HCC lesion". The confidence level of each reader was compared in consideration of the final diagnosis by two physicians (Kondo M and Tanaka K). True-positive (TP) cases were considered to be those assigned as grade 3 to 4 and verified by reference standards as actually being positive diagnoses. False-positive (FP) cases were considered to be those assigned as grade 3 to 4 but verified as actually being negative diagnoses. True-negative (TN) cases were considered to be those assigned as grade 1 to 2 by the reader, and verified as actually being negative diagnoses. False-negative (FN) cases were considered to be those assigned as grade 1 to 2 but verified as actually being positive diagnoses.

Table 2 Diagnostic criteria for focal liver lesions depicted by CE 2D US

Lesion	Enhancement patterns		
	Early phase	Middle phase	Late phase
HCC	Intratumoral vessels with early homogeneous or heterogeneous enhancement, or intratumoral vessels alone	Homogeneous or heterogeneous enhancement	Hypoechoic lesion
Metastasis	Peritumoral vessels with early peripheral ring like enhancement	Peripheral ring like enhancement or perfusion defect	Hypoechoic lesion
Hemangioma	Peripheral nodular enhancement	Peripheral nodular enhancement, with centripetal progression	Isoechoic lesion, with centripetal progression
FNH	Spoke-wheel arteries with early homogeneous enhancement	Homogeneous enhancement	Isoechoic lesion with central scar

CE: Contrast-enhanced; 2D US: Two-dimensional ultrasonography.

Statistical analysis

The data analysis was performed by using SPSS software (version 11.0; SPSS, Tokyo, Japan). In the retrospective study, SE was defined as the probability of the given enhancement pattern appearing in a reference disease, while SP indicated the probability of the absence of a given enhancement pattern for negative diagnoses. Prior probability was calculated by dividing the numbers of HCCs, metastases, hemangiomas and FNHs by the total number of tumors (163 tumors). According to Bayes theorem, the PPV of the combination of enhancement patterns for each tumor category was calculated based on sensitivity (SE), specificity (SP), and prior probability (PP) using the formula: PPV = SE × PP/[SE × PP + (1 - SP)× (1 - PP)].

In the prospective study, SE was calculated as TP/(TP + FN) and SP was calculated as TN/(FP + TN). The ROC curve was fitted to each reader's confidence level by using a maximum likelihood estimation program (ROCKIT 0.9B; http://www-radiology.uchicago.edu/krl/KRL_ROC). Area under the ROC curve (A_z) with the 95% confidential interval was calculated for each modality to measure the overall diagnostic performance. κ values were used to assess inter-reader agreement in characterizing focal liver tumors. Agreement was graded as poor (< 0.20), moderate (0.20 to 0.40), fair (0.40 to 0.60), good (0.60 to 0.80), or excellent (0.80 to 1.00).

RESULTS

Retrospective study

The enhancement patterns of 163 focal liver lesions are summarized in Table 3. In the early phase, 90% (88 of 98) of HCCs showed diffuse enhancement with intratumoral vessels (Figure 1A and B), 60% (21 of 35) of liver metastases peripheral ring-like enhancement with intratumoral or peritumoral vessels (Figure 2A), 79% (19 of 24) of hemangiomas peripheral nodular enhancement with peritumoral vessels or absence of vessels (Figure 3A and B), and 100% (6 of 6) of FNHs diffuse enhancement with spoke-wheel arteries (Figure 4A-C). In the middle phase, 93% (91 of 98) of HCCs showed diffuse enhancement (Figure 1C) and 69% (24 of 35) of metastases peripheral ring-like enhancement (Figure 2B), 75% (18 of 24) of hemangiomas peripheral nodular enhancement (Figure 3C) and 25% (6 of 24) diffuse enhancement, and 100% (6 of 6) of FNHs diffuse enhancement. In the late phase, 137 of 163 lesions were hypoechoic (Figures 1D, 2C, 2D and 3D), while the others were isoechoic (Figure 4D). Washout of enhancement was detected mainly in 121 of 133 malignant lesions (Figures 1 and 2), while enhancement persistence was detected mainly in 29 of 30 benign lesions (Figures 3 and 4). One HCC and two metastasis lesions were not enhanced from the early to the late phase and were assessed as showing absence of washout and persistence. The combination of enhancement patterns in three phases with PPV above 50% were summarized for each tumor category in Table 4 as the dominant enhancement patterns for establishing diagnostic criteria on 3D images in prospective study.

Prospective study

For CE 3D US, the diagnostic criteria for HCCs resulted in a SE of 93% (mean for two readers), SP of 91% and A_z value of 0.95, while those for metastases showed a SE of 84%, SP of 97% and A_z value of 0.95 in the prospective study. The combined enhancement patterns for hemangiomas as diagnostic criteria resulted in a SE of 91%, SP of 98% and A_z value of 0.98, while the dominant enhancement patterns for FNHs as diagnostic criteria resulted in a SE of 80%, SP of 99%, and A_z value of 0.99. Good to excellent inter-reader agreement was thus achieved for HCCs (κ = 0.86), metastases (κ = 0.83), hemangiomas (κ = 0.79) and FNHs (κ = 0.76).

For CE 2D US, sensitivity, specificity and A_z value of differential diagnosis were shown in Table 5. There were no significant differences between sensitivity, specificity and A_z value on CE 2D US and those on CE 3D US ($P > 0.05$). For HCCs, there was an SE of 92%, SP of 87% and A_z value of 0.95 (mean of two readers), while an SE of 84%, SP of 97% and A_z value of 0.94 were recorded for metastasis. For hemangioma, there was an SE of 84.5%, SP of 98% and A_z value of 0.95. For FNH, an SE of 70%, SP of 98% and A_z value of 0.98 were presented. SE, SP and A_z value of hemangioma and FNH on CE 2D US appeared lower than those on CE 3D US.

Table 3 Enhancement patterns using CE 3D US in three phases: positive predictive value in retrospective study

No.	Enhancement patterns					Positive predictive value			
	Early phase		Middle phase	Late phase	Enhancement changes	HCC	Metastasis	Hemangioma	FNH
	Tumor enhancement	Tumoral vessels	Tumor enhancement	Tumor echogenicity					
1	Diffuse	Intratumoral	Diffuse	Hypoechoic	Washout	0.98 (81)	0.01 (1)	0.01 (1)	0.00 (0)
2	Diffuse	Intratumoral	Diffuse	Isoechoic	Persistence	0.60 (3)	0.00 (0)	0.40 (2)	0.00 (0)
3	Diffuse	Intratumoral	Peripheral ring-like	Hypoechoic	Washout	0.33 (2)	0.67 (4)	0.00 (0)	0.00 (0)
4	Diffuse	Intratumoral	Perfusion defect	Hypoechoic	Washout	0.67 (2)	0.33 (1)	0.00 (0)	0.00 (0)
5	Diffuse	Spoke-wheel arteries	Diffuse	Isoechoic	Persistence	0.00 (0)	0.00 (0)	0.00 (0)	1.00 (6)
6	Peripheral ring-like enhancement	Intratumoral	Diffuse	Isoechoic	Persistence	1.00 (3)	0.00 (0)	0.00 (0)	0.00 (0)
7	Peripheral ring-like enhancement	Intratumoral	Peripheral ring-like	Hypoechoic	Washout	0.14 (1)	0.86 (6)	0.00 (0)	0.00 (0)
8	Peripheral ring-like enhancement	Peritumoral	Peripheral ring-like	Hypoechoic	Washout	0.00 (0)	1.00 (12)	0.00 (0)	0.00 (0)
9	Peripheral ring-like enhancement	Peritumoral	Perfusion defect	Hypoechoic	Washout	0.00 (0)	1.00 (3)	0.00 (0)	0.00 (0)
10	Peripheral nodular	Peritumoral	Diffuse	Isoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (1)	0.00 (0)
11	Peripheral nodular	Peritumoral	Peripheral nodular	Hypoechoic	Persistence	0.00 (0)	0.00 (0)	0.67 (2)	0.00 (0)
12	Peripheral nodular	Peritumoral	Peripheral nodular	Isoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (1)	0.00 (0)
13	Peripheral nodular	Peritumoral	Peripheral nodular	Hypoechoic	Washout	0.00 (0)	0.33 (1)	0.00 (0)	0.00 (0)
14	Peripheral nodular	Absence	Diffuse	Isoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (2)	0.00 (0)
15	Peripheral nodular	Absence	Peripheral nodular	Hypoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (8)	0.00 (0)
16	Peripheral nodular	Absence	Peripheral nodular	Isoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (5)	0.00 (0)
17	Absence	Intratumoral	Diffuse	Isoechoic	Persistence	1.00 (3)	0.00 (0)	0.00 (0)	0.00 (0)
18	Absence	Intratumoral	Diffuse	Hypoechoic	Washout	1.00 (1)	0.00 (0)	0.00 (0)	0.00 (0)
19	Absence	Intratumoral	Peripheral ring-like	Hypoechoic	Washout	0.00 (0)	1.00 (1)	0.00 (0)	0.00 (0)
20	Absence	Intratumoral	Perfusion defect	Hypoechoic	Washout	1.00 (1)	0.00 (0)	0.00 (0)	0.00 (0)
21	Absence	Peritumoral	Peripheral ring-like	Hypoechoic	Washout	0.00 (0)	1 .00(1)	0.00 (0)	0.00 (0)
22	Absence	Peritumoral	Perfusion defect	Hypoechoic	Washout	0.00 (0)	1.00 (3)	0.00 (0)	0.00 (0)
23	Absence	Absence	Perfusion defect	Hypoechoic	Absence	0.33 (1)	0.67 (2)	0.00 (0)	0.00 (0)
24	Absence	Absence	Peripheral nodular	Hypoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (2)	0.00 (0)

Positive predictive values are presented with number of lesions in parentheses.

Table 4 Sensitivity, specificity and A_z value for differential diagnosis based on CE 3D US in prospective study

Focal liver tumors	Diagnostic criteria based on combined enhancement patterns		Sensitivity	Specificity	A_z value
HCC	1, 2, 4, 6, 17, 18, 20	Reader 1	91 (64/70)	90 (44/49)	0.94 (0.88-0.95)
		Reader 2	94 (66/70)	92 (45/49)	0.96 (0.91-0.98)
Metastasis	3, 7, 8, 9, 13, 19, 21, 22, 23	Reader 1	86 (24/28)	97 (88/91)	0.95 (0.89-0.98)
		Reader 2	82 (23/28)	96 (87/91)	0.94 (0.89-0.97)
Hemangioma	10, 11, 12, 14, 15, 16, 24	Reader 1	94 (15/16)	98 (101/103)	0.98 (0.94-1.00)
		Reader 2	88 (14/16)	97 (100/103)	0.97 (0.90-1.00)
FNH	5	Reader 1	80 (4/5)	98 (112/114)	0.99 (0.95-1.00)
		Reader 2	80 (4/5)	99 (113/114)	0.98 (0.87-1.00)

Combined enhancement patterns for diagnostic criteria are summarized according to the serial numbers in Table 3. Sensitivity and specificity data are presented as percentages with numbers of tumors in parentheses, and A_z values are presented with 95% confidence intervals in parentheses.

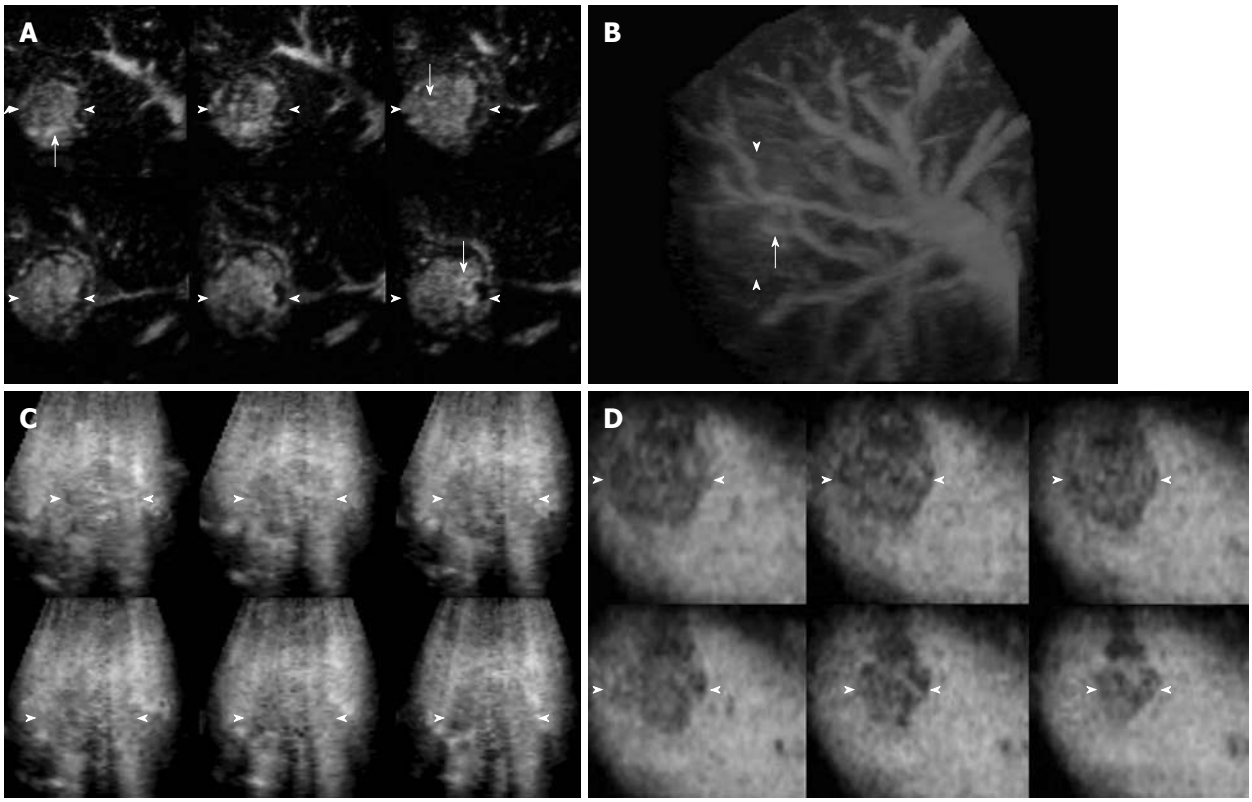

Figure 1 Contrast-enhanced (CE) three-dimensional ultrasonography (3D US) images of the liver in a 57-year-old man with hepatocellular carcinoma (HCC) in the anterior superior segment of the right lobe. A, B: Tomographic ultrasound image with slice distance 1.5 mm in plane A (A) and the sonographic angiogram rendered by maximum intensity with surface mode (B) show diffuse enhancement with intratumoral vessels (arrows) in the early phase; C: Tomographic ultrasound image with slice distance 2.0 mm in plane B shows diffuse enhancement in the middle phase; D: Tomographic ultrasound image with slice distance 2.0 mm in plane C shows hypoechoic pattern in the late phase. Enhancement change of washout was detected in this lesion. Arrowheads indicate the tumor margin.

Table 5 Sensitivity, specificity and A_z value for differential diagnosis based on CE 2D US in prospective study

Lesion	Sensitivity	Specificity	A_z value (95% CI)
HCC			
Reader 1	89 (62/70)	88 (43/49)	0.95 (0.89-0.98)
Reader 2	94 (66/70)	86 (42/49)	0.95 (0.90-0.98)
Metastasis			
Reader 1	86 (24/28)	96 (87/91)	0.93 (0.85-0.97)
Reader 2	82 (23/28)	98 (89/91)	0.95 (0.88-0.98)
Hemangioma			
Reader 1	81 (13/16)	97 (100/103)	0.94 (0.80-0.99)
Reader 2	88 (14/16)	99 (102/103)	0.95 (0.73-1.00)
FNH			
Reader 1	60 (3/5)	97 (111/114)	0.97 (0.74-1.00)
Reader 2	80 (4/5)	98 (112/114)	0.98 (0.66-1.00)

Sensitivity and specificity data are presented as percentages with numbers of tumors in parentheses.

DISCUSSION

Our study explored the potential role of CE 3D US, a newly-developed imaging modality, in presenting various tumor enhancement patterns and further characterizing different types of focal liver lesions. In this study, we used Sonazoid with CHA mode and high mechanical index contrast conditions, which allowed sensitive observation of vessels in the early phase by eliminating microbubbles in microvessels but not those in relatively large vessels, such as tumor vessels and portal veins[19].

In a previous study, we explored the visualization methods of CE 3D US for demonstrating focal liver lesions[24]. Moreover, in a retrospective study, according to the diagnostic criteria based on our experience and that described in the literatures, we assessed the diagnostic value of CE 3D US for characterization of focal liver lesions, in comparison with that of CE 3D CT[22]. Further, in the present study, we performed a retrospective study to clarify the enhanced patterns of focal liver lesions on CE 3D US, and the combined enhancement patterns with PPV more than 50% served as diagnostic criteria. According to these diagnostic criteria, we prospectively evaluated CE 3D US for characterizing liver lesions.

On CE 3D US, sonographic angiograms rendered using maximum intensity mode could be used to delineate tumor enhancement and present the spatial distribution of tumor vessels. Spoke-wheel arteries were regarded as a typical characteristic of FNHs on CE imaging in previous studies[34-36]. CE 3D US facilitated categorization of spoke-wheel arteries and provided a tridimensional view of the stellate branches. With consensus of the two readers, all 6 FNHs in the retrospective study were detected based on spoke-wheel arteries followed by persistent enhancement. In the prospective study, except for one FNH without distinctly detectable of spoke-wheel arteries, 4 of the other 5

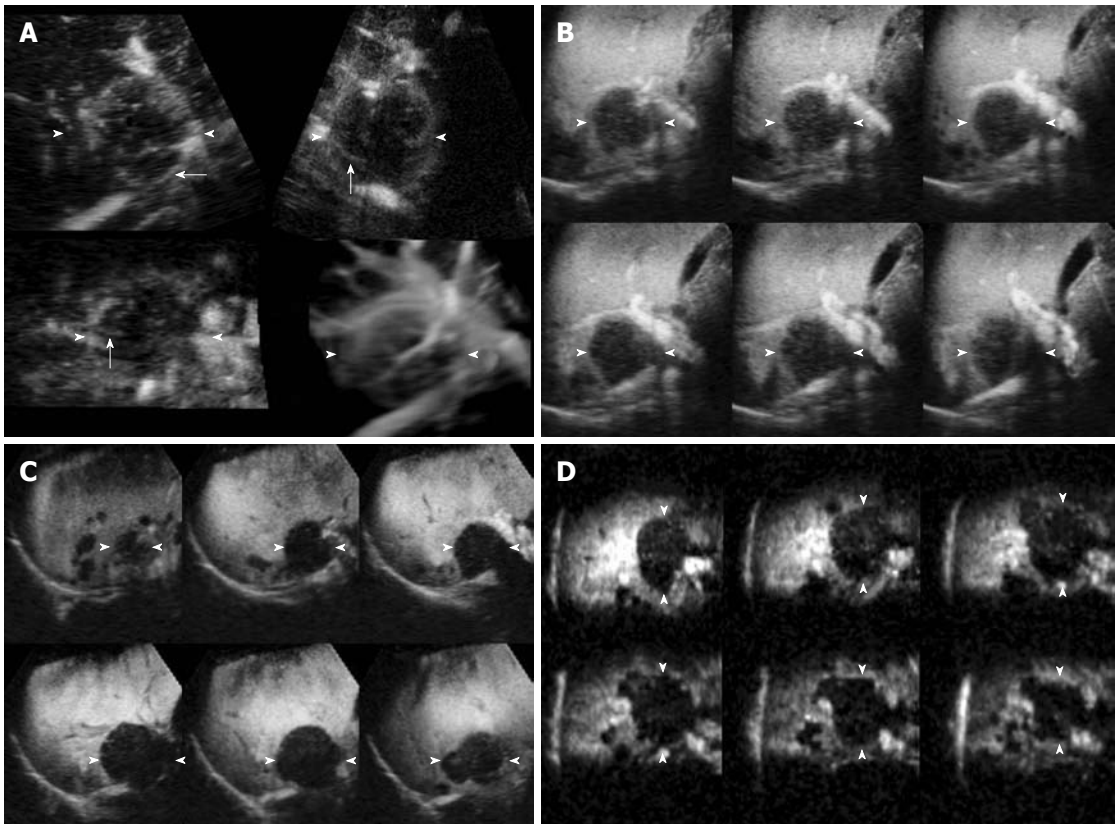

Figure 2 CE 3D US images of the liver in a 64-year-old woman with metastases from pancreatic carcinoma in the anterior superior segment of the right lobe. A: Images in the early phase show peripheral ring-like enhancement with peritumoral vessels (arrows) in plane A (upper left), plane B (upper right), plane C (lower left) and the sonographic angiogram rendered by average intensity with surface mode (lower right); B: Tomographic ultrasound image with slice distance 2.0 mm in plane A shows peripheral ring-like enhancement in the middle phase; C, D: Tomographic ultrasound images (TUI) in plane A with slice distance 2.5 mm (C) and that in plane C with slice distance 2.0 mm (D) show hypoechoic pattern in the late phase. Enhancement change of washout was detected in this lesion. Arrowheads indicate the tumor margin.

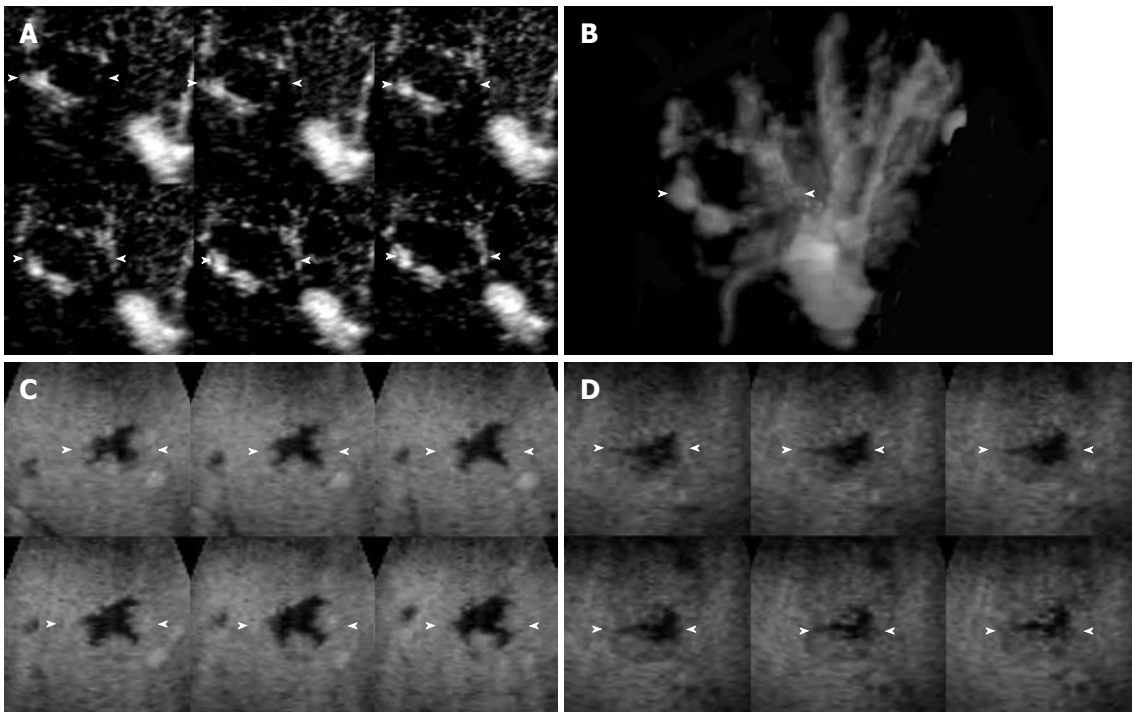

Figure 3 CE 3D US images of the liver in a 59-year-old woman with a hemangioma in the anterior inferior segment of the right lobe. A, B: Tomographic ultrasound image in plane A with slice distance 1.0 mm (A) and sonographic angiogram rendered by average intensity with surface mode (B) show peripheral nodular enhancement without distinct tumor vessels in the early phase; C: Tomographic ultrasound image in plane B with slice distance 1.0 mm shows peripheral nodular enhancement in the middle phase; D: Tomographic ultrasound image with slice distance 1.0 mm in plane B with slice distance 1.0 mm shows hypoechoic pattern in the late phase. Persistent enhancement over time was detected in this lesion. Arrowheads indicate the tumor margin.

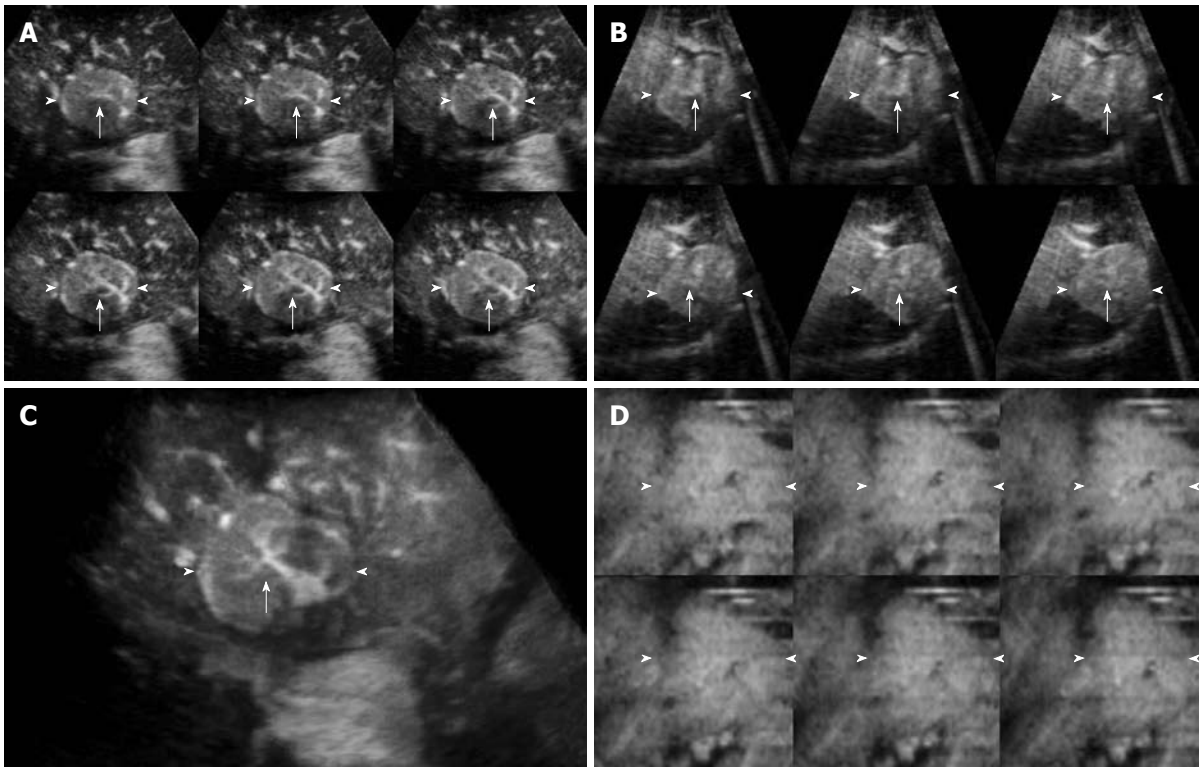

Figure 4 CE 3D US images of the liver in a 77-year-old man with FNH in the medial segment of the left lobe. A-C: TUI in plane A with slice distance 1.5 mm (A) and plane B with slice distance 1.5 mm (B), and the sonographic angiogram rendered by maximum intensity with surface mode (C) show diffuse enhancement with spoke-wheel arteries (arrows) in the early phase; D: TUI in plane C with slice distance 2.5 mm show isoechoic pattern in the late phase and hypoechoic regions within the tumor indicate central scars. Persistent enhancement over time was detected in this lesion. Arrowheads indicate the tumor margin.

lesions were diagnosed correctly. For the lesions with peripheral enhancement, sonographic angiograms rendered using average intensity modes delineated unenhanced portions in the lesions with hypoechoic appearance to make the tumor distinguishable from the surrounding liver parenchyma. Another feature of CE 3D US was the application of tomographic images presented in several parallel slices to multiplanar reconstruction, which allowed the reader to evaluate enhancement patterns in different portions of the lesion simultaneously. In this study, although there were no significant differences between the prospective diagnosis at CE 3D US and that at CE 2D US, CE 3D US created a spatial and easily understood view for both hemodynamic and morphologic evaluation of focal liver tumors, which were formed only in the doctors' imagination by 2D imaging using complex acquisition methods. The good to excellent inter-reader agreement in our previous study about CE 3D US as a means of presenting characteristic enhancement of HCCs has indicated CE 3D US can exhibit the characteristic enhancement of HCC tumors objectively[34].

In the present study, with analysis of combination of the enhancement in three phases at CE 3D US, the dominant patterns were used as the diagnostic criteria for individual category, and prospective differentiation yielded a good sensitivity, specificity, high A_z value, and good to excellent inter-reader agreement, which revealed the potential usage of CE 3D US in differentiating various focal liver lesions.

In the late phase in the retrospective study, 91% of malignant tumors (88 HCCs and 33 metastases) with washout changes appeared hypoechoic, while 53% of benign tumors (10 hemangiomas and 6 FNHs) with persistent enhancement appeared isoechoic. Neither isoechoic metastases nor hypoechoic FNHs were found in the late phase. There were 13 hemangiomas exhibiting hypoechoic findings in the late phase, and 12 of these 13 lesions were detected with incomplete fill-in and persistent enhancement, while one hemangioma was detected with early diffuse enhancement and washout change and was confirmed by the histological results on surgery. Vascularity in the early and middle phases appeared to be important for differentiation of focal liver lesions, and enhancement in the late phase with enhancement changes over time also facilitated characterizing the benign and malignant lesions[28,37].

This study has some limitations. First, CE 3D US on focal liver lesions has shortcomings including artifacts from the heart or respiratory motion, shadows of costal bones, and interference from abdominal gas, and so on. We minimized the influence of artifacts by requiring patients to hold their breath during the scanning procedure, selecting the location of the volume transducer, mediating the size and position of VOI, adjusting the scanning angle, and reconstructing the sonographic angiogram with appropriate rendering modes. Nevertheless, in some cases, artifacts on CE 3D US images might impact the readers' judgments and these lesions were thus excluded.

Second, in each case, only one lesion was evaluated and we did not assess the diagnostic capability and detectability of multiple lesions with CE 3D US. Third, the FNH cases in this study were few because FNHs have a relatively low prevalence and Sonazoid has been available in Japan for less than 2 years. More cases need to be examined in a future investigation.

In summary, our present results indicate CE 3D US with the contrast agent Sonazoid to offer a novel and useful means of presenting the vascularity characteristics three-dimensionally and differentiation of focal liver lesions with good diagnostic capability. These features of CE 3D US are anticipated to be of benefit in clinical diagnosis.

COMMENTS

Background

Recently, contrast-enhanced (CE) ultrasound (US) appeared as an important modality to show the vascularity in the areas of interest, and has been used widely in clinical diagnosis of liver lesions. Three dimensional ultrasonography (3D US) allows three orthogonal planes to spatially demonstrate the features of subjects, which has been frequently used in fetal US. Different from the 2D images, CE 3D US acquires the data in a volume of interest (VOI) by automatically scanning with a desired angle and allows reconstruction of tomographic images in three orthogonal planes and renders angiogram-like images. The combination of 3D US and CE US can present the enhancement of lesions in three dimensions and also in parallel slices by multiple- planar visualization.

Research frontiers

With the advantages of non-invasiveness, no radiation and flexible operation, CE US has become increasingly important in the detection and characterization of focal liver tumors over the past several years. Sonazoid (Daiichi Sankyo, Tokyo, Japan), a newly-developed second-generation US contrast agent, which consists of microbubbles (mean diameter 2-3 μm) of a perfluorobutane gas stabilized by a phospholipid monolayer shell, allows continuous real-time CE imaging for more than 10 min, and improves the reproducibility and durability of CE US examination.

Innovations and breakthroughs

To the authors' knowledge, although many studies on differentiation among various focal liver tumors have been conducted using CE 2D US and recently a few using CE 2D US with Sonazoid, the exact value of CE 3D US with Sonazoid in the differential diagnosis of various focal liver tumors has not yet been clarified. Thus, in this study, in order to examine the potential role of CE 3D US in characterizing focal liver tumors, the authors retrospectively evaluated tumor enhancement patterns, and the diagnostic criteria established using dominant enhancement patterns were then applied to differentiation among focal liver tumors in a prospective study.

Applications

The study's results suggest that CE 3D ultrasound provides a spatial perspective for liver tumor enhancement, and could help in differentiating focal liver tumors.

Terminology

Contrast enhanced ultrasound: the imaging modality of using US scanning while microbubbles serving as contrast agent are injected into vessels in order to improve the signals of red blood cells. Using this modality, the vascularity of VOI was depicted exactly and hemodynamics was demonstrated clearly.

Peer review

The aim of this study is to differentiate focal liver lesions based on enhancement patterns using 3D US with perflubutane-based contrast agent. The paper is written in a good English form, and is properly structured.

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ORIGINAL ARTICLE

Direct *in vivo* injection of ^{131}I-GMS and its distribution and excretion in rabbit

Yu Ma, Yi Wan, Dong-Hui Luo, Li-Geng Duan, Lin Li, Chuan-Qin Xia, Xiao-Li Chen

Yu Ma, Yi Wan, Li-Geng Duan, Xiao-Li Chen, The Research Unit of Hepato-Bilio-Pancreatology and Department of Hepatic Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China
Dong-Hui Luo, Chuan-Qin Xia, College of Chemistry, Sichuan University, Chengdu 610041, Sichuan Province, China
Lin Li, Department of Nuclear Medicine and The National Key Discipline of Medical Imaging and Nuclear Medicine, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Chen XL, Li L, Xia CQ and Ma Y designed the research; Ma Y, Wan Y, Luo DH and Duan LG performed the research; Ma Y and Chen XL analyzed the data and wrote the paper; Xia CQ, Li L, Wan Y and Luo DH wrote part of the paper.
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Correspondence to: Xiao-Li Chen, MD, Professor, The Research Unit of Hepato-Bilio-Pancreatology and Department of Hepatic Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China. zean_z@yahoo.com.cn
Telephone: +86-28-85422868 Fax: +86-28-85534151
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Abstract

AIM: To explore the distribution and metabolism of ^{131}I-gelatin microspheres (^{131}I-GMSs) in rabbits after direct injection into rabbits' livers.

METHODS: Twenty-eight healthy New Zealand rabbits were divided into seven groups, with four rabbits per group. Each rabbit's hepatic lobes were directly injected with 41.336 ± 5.106 MBq ^{131}I-GMSs. Each day after ^{131}I-GMSs administration, 4 rabbits were randomly selected, and 250 μL of serum was collected for γ count. Hepatic and thyroid functions were tested on days 1, 4, 8, 16, 24, 32, 48 and 64 after ^{131}I-GMSs administration. Single-photon emission computed tomography (SPECT) was taken for each group on days 0, 1, 4, 8, 16, 24, 32, 48, 64 after ^{131}I-GMSs administration. A group of rabbits were sacrificed respectively on days 1, 4, 16, 24, 32, 48, 64 after ^{131}I-GMSs administration. Their livers were taken out for histological examination.

RESULTS: After ^{131}I-GMSs administration, the nuclide was collected in the hepatic area with microspheres. The radiation could be detected on day 48 after ^{131}I-GMSs administration, and radiography could be seen in thyroid areas in SPECT on days 4, 8, 16 and 24. One day after ^{131}I-GMSs administration, the liver function was damaged but recovered 4 d later. Eight days after ^{131}I-GMSs administration, the levels of free triiodothyronine and free thyroxin were reduced, which restored to normal levels on day 16. Histological examination showed that the microspheres were degraded to different degrees at 24, 32 and 48 d after ^{131}I-GMSs administration. The surrounding parts of injection points were in fibrous sheathing. No microspheres were detected in histological examination on day 64 after ^{131}I-GMSs administration.

CONCLUSION: Direct *in vivo* injection of ^{131}I-GMSs is safe in rabbits. It may be a promising method for treatment of malignant tumors.

Key words: ^{131}I; Label; Gelatin microspheres; Animal; Rabbit; Hepatic; Direct injection

Peer reviewer: Dr. Serdar Karakose, Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

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INTRODUCTION

The treatment of malignant tumors in hepatic, biliary and pancreatic systems has been and will always be a tough and key part of general surgery[1-3]. Surgical removal has been considered a positive approach for most of malignant tumors in these systems[4]. But the success rate of surgical removal is very low and the prognosis after operation is unsatisfactory[5-8]. For example, the number of cases of primary hepatic carcinoma in China was around 384 119 in 2005[9-12] and the number of deaths was 357 624[13-15], only 10%-30% are related to surgical removal, and 25% of them survived over 5 years[16]. The number of cases of pancreatic cancer in America was around 42 470 and the number of death was around 35 240 in 2009[17]. Surgical procedure has much limitations for treatment of malignant cancers. Many patients with malignant tumors in the three systems at middle to advanced stages are in the urgent need of new and effective non-surgical treatment.

Radionuclide labeled microspheres and seeds are the new progress made in the area of tumor therapy and interventional radiotherapy. It has now become a fast developing sub-field of medicine combining nuclear medicine and oncotherapy. Nuclide microspheres under study over recent years include ^{90}Y glass and resin microsphere[18-20], ^{188}Re glass microsphere[21], ^{32}P glass microsphere[22,23] and ^{166}Ho glass microsphere[24]. The ^{90}Y glass microsphere is being increasingly recognized by the medical community as an important strategy for the treatment of primary and secondary neoplasm, which was officially approved in 1999 in America and Canada for treatment of malignant tumors. Now hundreds of publications have described the treatment of ^{90}Y glass microsphere[25]. The therapeutic practices in thousands of patients with liver cancer in about 80 medical centers around the globe show that hepatic arterial injection of ^{90}Y glass microsphere is a safe and effective method for liver cancer treatment. Carr reports a group of clinical control study involving 65 cases of primary liver cancer. Forty-two cases are Okuda stage 1 with a median survival of 649 d (244 d for the control group), 23 are Okuda stage 2 with a median survival of 302 d (64 d for the control group)[26]. Salem reports another clinical study involving 49 cases of liver cancer with a median survival of 85.9 mo. Twenty-eight patients reached the condition for tumor removal after the treatment and had their tumors removed, and 13 patients had tumor necrosis after the treatment. Their survival rate for 1, 3 and 5 years are 98%, 64% and 57%, respectively[27]. Intratumoral injection of ^{90}Y glass microsphere has also been studied. Tian reported that the tumor was reduced by 92% in 27 cases of primary liver cancer and 6 cases of metastatic liver cancer. α-fetoprotein in 13 cases recovered entirely after intratumoral injection[28]. These studies showed similar clinical therapeutic efficacy of ^{90}Y glass microsphere to that of surgical procedure. The ^{125}I-seeds used for prostate cancer in Europe and America has been tried in intratumoral implantation in China to treat advanced pancreatic carcinoma, which has demonstrated effects in alleviating pains and prolonging life[29-31]. In conclusion, directional radiotherapy using nuclide microsphere or nuclide particle is a potential alternative to treat malignant tumors in hepatic, biliary and pancreatic systems.

China is a country with a large population in the world and has the highest incidence and death rate of primary liver cancer. And the incidence of biliary and pancreatic malignant tumors is increasing. However, most of the patients with middle to advanced stage cancers of the three systems cannot be treated with surgery. Therefore, directional radiotherapy using nuclide microsphere will significantly improve the prognosis of patients with middle to advanced stage cancers. Since ^{90}Y glass microspheres have to be activated in an accelerator to get radioactivity, and when they are activated, they have relatively short half-life. We have been studying the nuclide microspheres for local brachytherapy for hepatic, biliary and pancreatic malignant tumors. In the 1990s, we developed the ^{32}P glass microsphere with relatively long half-life, and treated 40 patients with advanced liver cancer from 1992 to 1994 after the completion of the metabolism tests *in vivo* in tumor-carrying animals[32]. In recent years, we have developed the gelatin microspheres (GMSs) with a diameter of 50-70 μm carrying a high concentration of ^{131}I to treat patients with advanced liver cancer with hepatic arterial transfusion and embolotherapy. Since its half-life is 8.04 d and free ^{131}I in the body is either collected in thyroid tissue or discharged out of the body quickly, ^{131}I is safe and has relatively weak influence on other tissues[33-35]. And the GMS is one of the degradable biomaterials with good biocompatibility, which can also bind ^{131}I nuclide at a high concentration[36]. So in this study, we prepared ^{131}I-GMSs with a diameter of 10-30 μm for intratumoral implantation, which is expected to be easily applied for the hepatic, biliary, pancreatic and other malignant tumors that cannot be removed. Healthy rabbits are used as models to observe the metabolism of ^{131}I-GMSs *in vivo* and the tissue reaction in their livers.

MATERIALS AND METHODS

Materials

Lime-processed gelatin (sigma G-9382) with an isoelectric point of 4.8-5.2 was purchased from Sigma Co. Ltd., USA; ^{131}I-sodium-iodine solution (37 GBq/mL) was purchased from China Nuclear Group Chengdu Gaotong Isotope Co. Ltd., Chengdu, China. All other chemicals were of the highest commercially available purity.

Laboratory animals

This study was approved by the Animal Ethics Committee of Sichuan University. Twenty-eight healthy New Zealand rabbits weighing 1.8-2.5 kg were supplied by the animal experimental center of the Medical School of

Sichuan University and were divided into seven groups (groups 1-7), with four rabbits per group. Half of the rabbits were female and half were male. The rabbits were fed with a particulate (3-5 mm) chow and housed in a layered stainless steel coop. Rabbits had *ad libitum* access to running water. The air humidity and temperature were maintained at 50%-70% and 20-29℃, respectively. Eight days before the operation, four rabbits were randomly selected as control animals to collect serum from their hearts for the measurement of the liver and thyroid function.

Preparation of GMSs

GMSs were produced according to the modified method of Tabata *et al*[37]. Briefly, 10 mL of 10% lime-processed gelatin solution was added dropwise while stirring, to 80 mL of liquid paraffin (Kelong Chemical Reagent Co. Ltd., Chengdu, China), which was preheated to 55℃ with 0.8 mL span-80 (Shenyu Chemical Reagent Co. Ltd., Chongqing, China). The mixture was then stirred at 550 r/min at 55℃ for 15 min to yield a water-in-oil emulsion. The stirring was then continued for 30 min at 4℃. Next, 3 mL of glutaraldehyde (25%, Kermel Chemical Reagent Co. Ltd., Tianjin, China) was added to the mixture after it cooled for 5 min to induce crosslinking and solidification of the microspheres. The resulting microspheres were removed by suction filtration and washed three times with acetone (Changlian Chemical Reagent Industries, Ltd., Chengdu, China) after dehydration by immersion in 30 mL of acetone for 15 min. After air-drying, the GMSs were examined by the Analyzing and Testing Center of Sichuan University and imaged under a scanning electron microscope.

Preparation of ^{131}I-GMS

The ^{131}I was labeled by a modification of the chloramine-T method. Briefly, 50 mg of GMSs were placed in test tubes, rehydrated with 190 μL of phosphate-buffered saline (pH 7.0) for 10 min. Next, 3.4 μL of ^{131}I-sodium-iodine solution (37 GBq/mL) and 200 μL of chloramine-T solution (Bodi Chemical Reagent Co. Ltd., Tianjin, China) (20 mg/mL) were added to each test tube. After the reaction mixture had been incubated for 5-15 min at room temperature, with agitation, 200 μL of sodium pyrosulfite solution (Jiangbei Chemical Reagent Industries, Ltd., Wuhan, China) (15 mg/mL) was added to stop the reaction. The mixture was centrifuged (Eppendorf® 5702R refrigerated centrifuge, Eppendorf Co. Ltd., Hamburg, Germany) at 4400 r/min for 4 min to separate the ^{131}I-GMSs. Finally, the products were washed seven times with normal saline and sterilized by Co60 irradiation.

Implantation of ^{131}I-GMS in the liver

Each rabbit was injected intramuscularly with 0.1-0.2 mL/kg SuMianXin® (Veterinary Institute of the Chinese Military Academy of Medicine, Changchun, China) veterinary injection for anesthesia and placed on an animal operation table. The epigastrium was shaved, disinfected, and covered with a drape. The abdomen was opened with a 5-cm incision along the median line, and the left middle lobe or right middle lobe of the liver was fixed by the left index finger and thumb of the operator. Then, the mixture of 41.336 ± 5.106 MBq of the ^{131}I-GMSs, as measured in a Capintec® CRC-15R (Capintec, Inc., New Jersey, USA) dose calibrator, and 1 mL of 25% glucose solution (Kelun Pharmaceutical Industries, Ltd., Chengdu, China) drawn by a 1 mL syringe with a 4-G needle was slowly injected into the liver, after checking the needle was not inserted into a capillary. Two or three injections of 0.3-0.5 mL of the mixture were administered. When the injection points were not observed to be bleeding after being pressed for approximately 1 min after withdrawing the needle, the abdomen was closed and the operation was finished. The rabbit was immediately administered intramuscularly with 40 000 U gentamycin sulfate (Tianjin Pharmaceutical Co. Ltd., Tianjin, China).

Radioassay of rabbit serum

Four rabbits were chosen randomly for collecting 1 mL of blood from the ear veins between 0 and 24, then at 28, 32, 48 and 64 d after the administration of the GMSs. Those blood were centrifuged at 4400 r/min, and 250-μL aliquots of serum were used for γ counting in a γ counter (No. 262 Industry, Ltd., Xi'an, China).

Single-photon emission computed tomography, hepatic and thyroid functions examinations

The single-photon emission computed tomography (SPECT) scan (Skylight SPECT Camera, Philips Co. Ltd., Amsterdam, Netherlands) was conducted by the Nuclear Medicine Department of West China Hospital at 4 h, and at 1, 4, 8, 16, 24, 32, 48 and 64 d after administration. The animals in group 1, 2, 3, 4, 5, 6 and 7 were sacrificed at 1, 4, 16, 24, 32, 48 and 64 d after the surgery, respectively. Eight milliliters of blood was collected from each deceased animal. On day 8, four rabbits were randomly selected, and a further 8 mL of blood was collected from the heart of each rabbit on this day. Blood samples were used to assess hepatic and thyroid functions, which was conducted at the Biochemical Laboratory and the Hormonal Laboratory of the Experimental Medicine Department of West China Hospital.

Histological examination

Liver samples were fixed in 10% formalin solution (Kelong Chemical Reagent Co. Ltd., Chengdu, China) for 48 h. Liver samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) for histological examination.

Statistical analysis

Results were expressed as mean ± SD and were analyzed by *t* tests using SPSS 11.5 software. The level of significance was regarded at a P value of < 0.05.

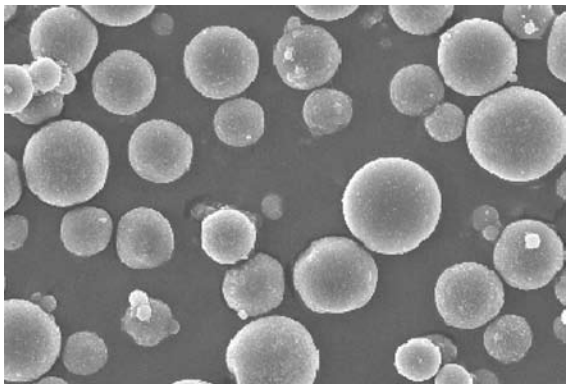

Figure 1 Scanning electron microscopic (20 kV) images of metal-coated gelatin microspheres (original magnification, × 500).

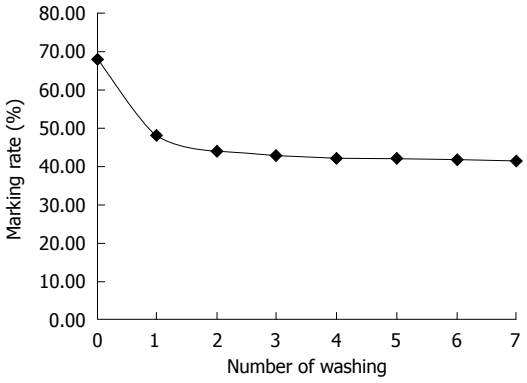

Figure 2 The labeling rate decreases with increasing the number of washes. The slope decreased gradually, nearly reaching a straight line, which demonstrates that the ^{131}I-labeled gelatin microspheres after washing contained very low amounts of nuclides conjugated by physical adsorption.

Table 1 Effect of washing on labeling rate (n = 6)

Number of washes	Labeling rate (%)
0	68.01 ± 2.09
1	47.74 ± 2.26
2	43.68 ± 2.19
3	42.72 ± 2.23
4	42.22 ± 2.27
5	41.91 ± 2.28
6	41.56 ± 2.27
7	41.00 ± 2.29

Table 2 Ratio of radioactivity between the liver and thyroid (n = 6) after the administration of ^{131}I-labeled gelatin microspheres

Time	Liver/thyroid
4 h	15.910 ± 0.740
Day 4	27.197 ± 5.467
Day 8	81.467 ± 24.637
Day 16	91.670 ± 23.278
Day 24	93.601 ± 21.337
Day 32	112.608 ± 12.787
Day 48	162.875 ± 7.955

RESULTS

Morphology of the microspheres

The GMSs were uniform in appearance, with a diameter of 10-30 μm, and a good divergence (Figure 1).

Effect of washing on GMS ^{131}I content

The GMSs labeled with ^{131}I were washed seven times to inhibit physical adsorption. Then, 50 mg of the GMSs labeled with ^{131}I showed decreased radioactivity levels with increasing number of washes. The slope for the relative ^{131}I content decreased gradually until it nearly reached a straight line (Table 1 and Figure 2).

Findings of SPECT imaging

The rabbits showed normal behaviors after the operation. SPECT imaging showed that the radioactive nuclide was concentrated in the liver, in regions surrounding the site of injection at 4 h and at 1, 4, 8, 16, 24, 32 and 48 d after ^{131}I-GMSs administration. However, SPECT did not reveal any nuclide labeling on day 64. The thyroid also showed low levels of nuclide accumulation on days 4, 8, 16 and 24. Furthermore, there was faint nuclide labeling in the bladder of four rabbits before day 24, although this disappeared by day 32. SPECT imaging showed no accumulation of nuclide in other tissues, including the lung, heart, stomach, intestines and kidney in any rabbits for the entire observation period (Figure 3). The radioactive ratios between the injected parts of liver and thyroids was assessed by region of interest analysis and increased with time, from 15.91 ± 0.74 at 4 h after ^{131}I-GMSs administration to 162.875 ± 7.955 at day 48 (Table 2).

Radioactive changes in serum

According to the serum γ counts, the radioactivity level decreased markedly over the first 2 d after ^{131}I-GMSs administration. The decline in radioactivity continued to decline thereafter, but at a slower rate until day 24. At this time, there was no difference in relative radioactivity level compared with the background level (Figure 4).

Hepatic and thyroid function

The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased rapidly, particularly that of ALT, within 1 d after ^{131}I-GMSs administration. The levels of these enzymes then decreased gradually, reverting to the normal level by day 4. The values of alkaline phosphatase and γ-glutamyltransferase were relatively stable. Similarly, the total protein, albumin and globulin did not change markedly during the study period (Table 3 and Figure 5).

The levels of free triiodothyronine (FT3) and free thyroxin (FT4) were significantly decreased on day 8 (P < 0.05), but returned to normal levels at day 16, and remained at the normal level until day 64 (Table 4 and Figure 6). The level of thyrotropic-stimulating hormone (TSH) remained < 0.005 mU/L throughout the study.

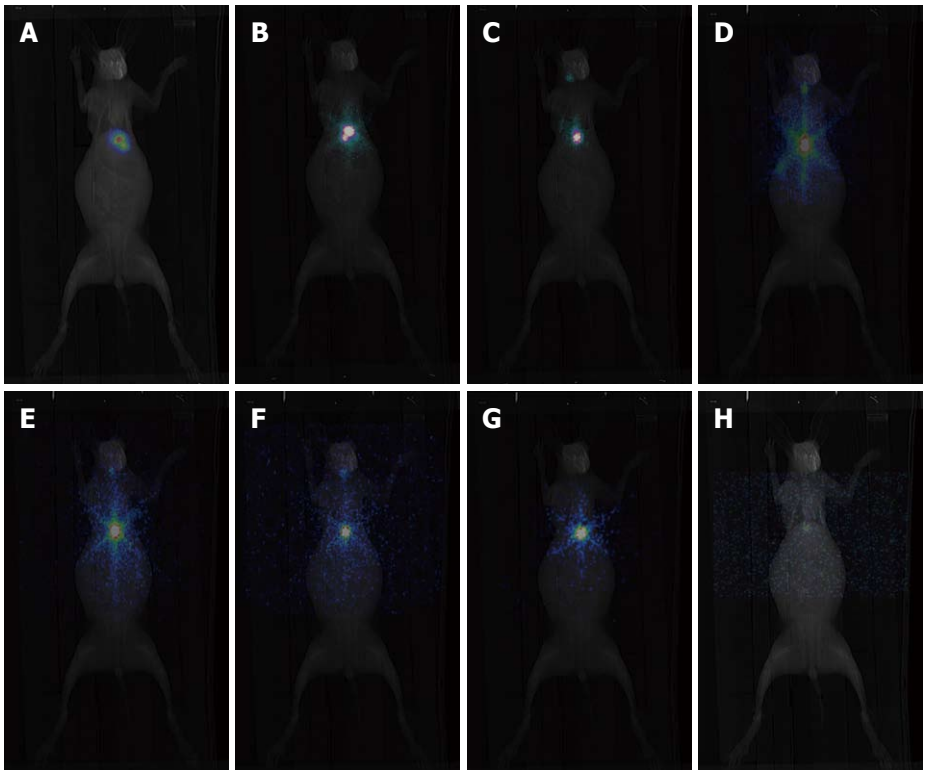

Figure 3 Single-photon emission computed tomography (SPECT) imaging performed at 4 h (A) and 1 (B), 4 (C), 8 (D), 16 (E), 24 (F), 32 (G) and 48 (H) d after the administration of ^{131}I-labeled gelatin microspheres.

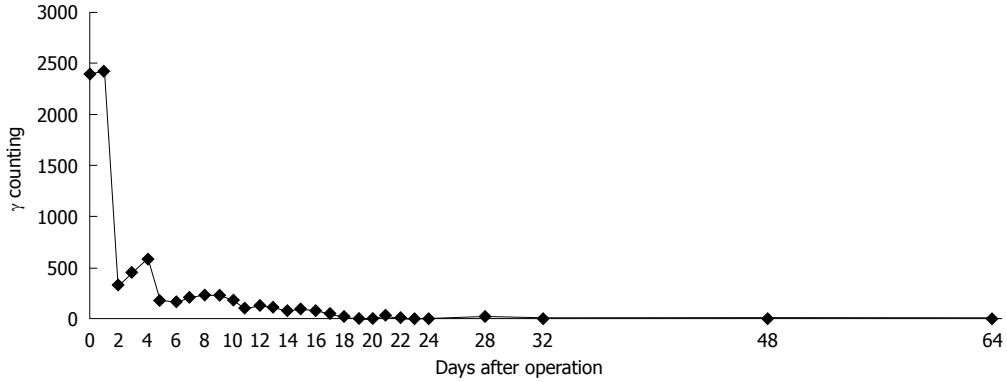

Figure 4 Dynamic changes in serum γ count after the administration of ^{131}I-labeled gelatin microspheres.

Table 3 Effects of the administration of ^{131}I-labeled gelatin microspheres on hepatic function (n = 4)

	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	GGT (IU/L)	TP (g/L)	ALB (g/L)	GLB (g/L)
Before administration	67.75 ± 11.87	55.00 ± 11.87	99.75 ± 14.99	6.00 ± 1.83	54.33 ± 3.16	46.05 ± 2.32	8.28 ± 2.79
After administration							
1 d	291.50 ± 12.79	146.00 ± 13.88	115.00 ± 25.92	9.25 ± 3.30	60.23 ± 1.96	45.65 ± 5.17	15.00 ± 5.34
4 d	46.75 ± 4.79	41.25 ± 10.69	91.00 ± 29.59	7.25 ± 1.26	58.43 ± 8.45	47.55 ± 3.53	12.23 ± 5.03
8 d	48.50 ± 14.39	48.00 ± 17.32	79.75 ± 14.77	7.50 ± 0.58	58.10 ± 4.40	44.63 ± 2.33	13.65 ± 4.33
16 d	37.25 ± 9.32	39.75 ± 11.81	76.00 ± 9.83	9.50 ± 3.11	62.13 ± 5.84	44.38 ± 7.41	19.85 ± 6.79
24 d	46.50 ± 16.01	45.25 ± 9.95	96.25 ± 17.08	8.00 ± 2.83	60.28 ± 4.45	47.60 ± 7.38	14.50 ± 3.54
32 d	52.25 ± 14.29	46.25 ± 8.66	96.75 ± 35.58	12.50 ± 3.87	63.55 ± 4.90	46.40 ± 4.06	18.63 ± 6.72
48 d	43.75 ± 10.01	40.25 ± 14.45	97.25 ± 7.93	9.25 ± 1.71	60.78 ± 4.08	46.83 ± 4.06	14.23 ± 4.22
64 d	45.50 ± 4.80	45.75 ± 3.24	91.75 ± 29.62	10.50 ± 3.42	61.92 ± 8.18	50.15 ± 5.30	15.05 ± 3.89

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase; TP: Total protein; ALB: Albumin; GLB: Globulin.

Histological findings

The histological specimens showed that the ^{131}I-GMSs were quite concentrated, with a few inflammatory cells surrounding the injection sites on day 1 (Figure 7A) and some hepatic cells had died by day 4 (Figure 7B). Fibrous sheaths coating the ^{131}I-GMSs and sequential degradation of the ^{131}I-GMS were observed on days 16, 24 and 32. Most of the hepatic cells around and within the

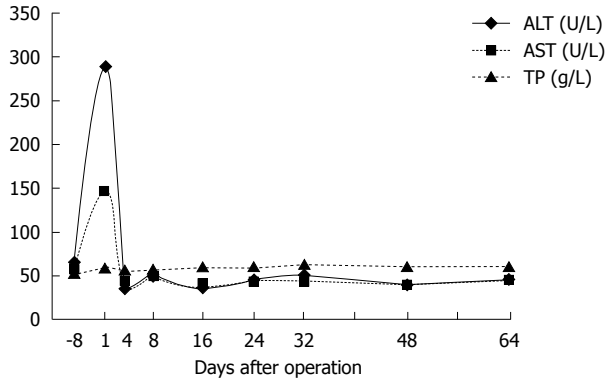

Figure 5 Changes in hepatic function after the administration of ^{131}I-labeled gelatin microspheres.

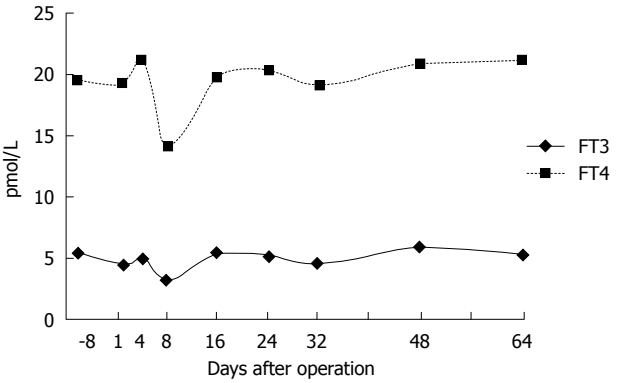

Figure 6 Curves of thyroid functional changes after the administration of ^{131}I-labeled gelatin microspheres.

Table 4 Effects of the administration of ^{131}I-labeled gelatin microspheres on thyroid function (n = 4)

	FT3 (pmol/L)	FT4 (pmol/L)	TSH (mU/L)
Before administration	5.55 ± 0.57	19.61 ± 1.54	< 0.005
After administration			
1 d	4.54 ± 1.53	19.31 ± 6.47	< 0.005
4 d	5.09 ± 1.68	21.31 ± 4.68	< 0.005
8 d	3.29 ± 0.83	14.23 ± 2.81	< 0.005
16 d	5.53 ± 1.01	19.95 ± 2.56	< 0.005
24 d	5.31 ± 0.57	20.39 ± 2.38	< 0.005
32 d	4.66 ± 2.17	19.18 ± 2.98	< 0.005
48 d	6.02 ± 2.77	20.94 ± 10.78	< 0.005
64 d	5.34 ± 1.34	21.23 ± 3.42	< 0.005

FT3: Free triiodothyronine; FT4: Free thyroxin; TSH: Thyrotropic-stimulating hormone.

sheaths died, leaving the hepatic cell cords on days 24 and 32. By day 24, the ^{131}I-GMSs had started to degrade and had an irregular shape (Figure 7C-E). However, the remnant ^{131}I-GMSs could still be histologically identified on day 48 (Figure 7F). No microspheres were found on day 64.

DISCUSSION

In recent years, many radioactive nuclide microspheres and nuclide particles have been successfully developed to target malignant tumors. However, because of the potential toxicity and side-effects associated with ^{90}Y, ^{32}P and other radioactive nuclides on marrow and other tissues, nonbiodegradable glass is often used as a carrier for most nuclides to avoid causing unwanted damage to normal tissues. However, glass has some limitations, including a protracted bioavailability due to its nonbiodegradable characteristics. Furthermore, its high specific gravity may adversely affect its injection and distribution. In this study, we generated degradable GMSs carrying the ^{131}I nuclide. Gelatin has a similar specific gravity to blood, and could be conjugated to many other drugs and nuclides *via* physical adsorption or chemical keys. Gelatin also shows good histocompatibility and degrades gradually *in vivo*[38-40]. Therefore, as a carrier for slow release of drugs, GMSs have been widely used by the medical community[41,42]. Meanwhile, ^{131}I is the most widely used radioactive nuclide in clinical settings, and has shown good anti-tumor effects in many clinical cases[43]. *In vivo*, dissociative ^{131}I mainly accumulates in the thyroid and is excreted *via* the kidney[44-46]. Therefore, ^{131}I that is released by the degradation of ^{131}I-GMS into the serum could result in tissue damage, particularly the thyroid gland. Therefore, detailed evaluation of the metabolic characteristics of ^{131}I-GMS, including its release, distribution and excretion *in vivo*, and the potential damage to the body should be evaluated to comprehensively appraise its safety. The study has provided some insight into these concerns.

The initial labeling rate of ^{131}I includes chemical binding and physical adsorption. When radionuclide-labeled microspheres are injected into the body, the nuclides conjugated to the microspheres *via* physical adsorption are more likely to dissociate. This has been reported to cause a severe de-iodinated state *in vivo*. In this study, we washed the microspheres seven times after the initial labeling, until the dissociative curve flattened, indicating that most of the ^{131}I conjugated *via* physical adsorption had been eluted and the ^{131}I-GMSs mainly exist *via* chemical combination. Although this reduces the labeling index, the *in vivo* de-iodination process is also attenuated, protecting against unwanted de-iodination effects.

Some studies have shown that injecting the microspheres at multiple sites could provide a more even distribution of the microspheres in the tumor tissues[47]. Therefore, in this study, we injected the microspheres in several sites in the liver. SPECT imaging revealed that the nuclides were principally localized to the injection sites at 4 h and at 1, 4, 8, 16, 24, 32 and 48 d after administration. The extrahepatic labeling with ^{131}I was mainly found in the thyroid gland between days 4 and 24 after administration, without any accumulation in other tissues, including the lung, heart, kidney, brain, stomach and intestines. Radioactivity of the serum could be detected for 24 d after the operation; after this time, the serum radioactivity level was not different to that of the background level. Because the thyroid gland is the

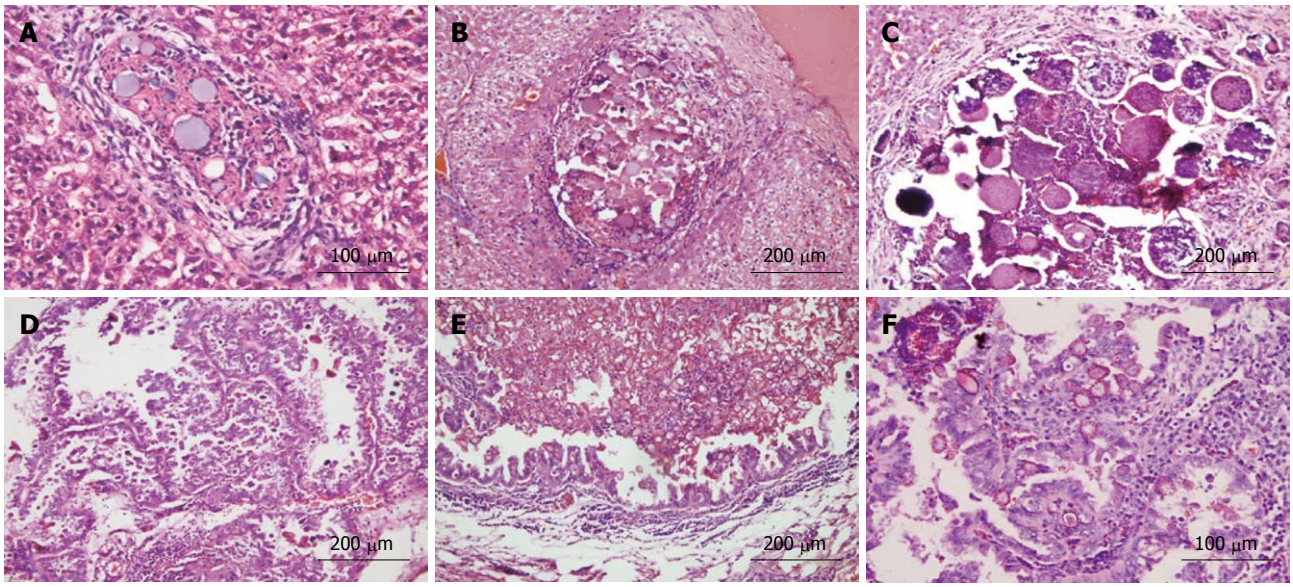

Figure 7 Pathological examination at 1 (A), 4 (B), 16 (C), 24 (D), 32 (E) and 48 (F) d after the administration of ^{131}I-labeled gelatin microspheres (HE stain; original magnification, × 200 in B, C, D and E, and × 400 in A and F).

principle site of iodine absorption and accumulation, it can absorb ^{131}I from the serum into thyroid follicles, which is shown on the SPECT scan. Based on the radioactivity ratio between the site of injection in the liver and the thyroid, it is clear that only a small amount of ^{131}I is absorbed by the thyroid.

SPECT imaging before day 24 revealed low radioactivity levels in the bladder, but not thereafter. This may be related to the full state of the rabbits' bladders when they were scanned, with full bladders showing some radioactivity as a result of excretion *via* the urinary system. Therefore, we believe that the release of ^{131}I from the microspheres mainly occurs within 24 d after administration, but only very small amounts are released.

Assessment of thyroid function revealed that the TSH level was consistently below 0.005 mU/L, while FT3 and FT4 declined on day 8, but returned to normal levels after day 16. This suggests that the thyroid is only subject to transient damage. Because the thyroid has its own repair mechanisms, the damage caused by some radiation doses can be repaired, without causing hypothyroidism[48]. In this study, a small amount of radioactive nuclides released into the blood was absorbed by the thyroid gland, but did not cause permanent damage or long-lasting hypothyroidism.

The assessment of hepatic function revealed that the ALT and AST increased rapidly compared with the normal level within 1 d after ^{131}I-GMSs administration. However, these parameters returned to the normal level 4 d later. This suggests that the administration of 41.336 MBq of ^{131}I caused notable liver damage in these experimental animals; however, because of the liver's capacity for self-repair and compensation, these impairments were transient and resolved within 4 d. This demonstrates the safety profile of radionuclide microspheres in the treatment of liver cancers.

From the pathological examination, we could conclude that the microspheres were gradually surrounded by fibroblasts to form fibrotic sheaths between days 16 and 24. This seemed to delay the degradation of the GMSs and reduced the rate of radionuclide release. This may explain the absence of thyroid radiolabelling from day 24 after surgery. Ohta reported that, after injecting GMSs into the renal artery of rabbits for 2 wk, the microspheres became wrapped with fibrous tissue and the GMSs in the embolism were completely biodegraded within 1 mo[49,50]. However, in this experiment, we used the intra-tissue implantation method, which differs from the arterial embolism method. Indeed, over four half-lives of ^{131}I decay (i.e. 32 d), the GMSs had degraded to varying degrees, but there was no sign of disappearance, with a large number of fiber-coated GMSs present, even by day 48. This difference may be due to the different methods of administering the GMSs. Previous studies have shown that gelatin is degraded in the body by degrading enzymes[51]. In this study, the ^{131}I-GMSs administered into the liver are, on the one hand, treated as a foreign body and induce foreign body reactions, with the activation of inflammatory cells, fibrotic cells and Kupffer cells to encapsulate and phagocytose the GMSs. On the other hand, the radiation will cause cells surrounding the GMSs to die to prevent phagocytosis by macrophages. This may explain why, in this experiment, the GMSs degrade slowly than that in the arterial embolism.

In conclusion, the hepatic administration of ^{131}I-GMSs in rabbits caused marked hepatic damage. Furthermore, there was some ^{131}I accumulation in thyroid tissue, causing slight, but only transient damage to the thyroid tissue. Other tissues showed no radioactive accumulation. Fibrous sheaths formed around the injected GMSs, which likely hampered the degradation of the

GMSs and protracted the release of ^{131}I. Taken together, we believe that it is safe to inject ^{131}I-GMSs into tissues *in vivo*, and these are likely to be effective against malignant tumors.

COMMENTS

Background

Malignant tumors in hepatic, biliary and pancreatic systems are very commonly encountered and treated surgically worldwide. In recent years, the incidence of malignant tumors in these three systems was reported to be rising worldwide. However, the rate of surgical removal of the malignant tumors of the three systems is very low and the prognosis after surgery is very unsatisfactory.

Research frontiers

Internal radiotherapy has become an important facet of clinical therapy for malignant tumors. ^{90}Y, ^{188}Re and ^{166}Ho microspheres have already been shown to be safe and are frequently used to therapy malignant tumors. However, their vehicle material is usually glass, which cannot degrade in the body. Unfortunately, once the nuclides have decayed completely, the vitreous carriers become foreign bodies that persist in the patient's body, and trigger foreign body reactions.

Innovations and breakthroughs

Nuclide microspheres and nuclide particles represent a new generation of tumor therapies and interventional radiotherapies. The authors used gelatin microspheres (GMSs) that can be easily produced to carry radionuclides, and can degrade *in vivo*. This study provides a foundation to show how nuclides attached to GMSs are distributed and metabolized *in vivo*. Healthy rabbits were used as a model to observe the metabolism of ^{131}I-GMSs. Overall, ^{131}I-GMSs were found safe for administration in rabbits.

Applications

By understanding the distribution and metabolism of ^{131}I-GMSs administered in the livers of rabbits, this study supports the use of ^{131}I-GMSs in directional internal radiotherapy for the treatment of hepatic malignant tumors. This approach could be applied to pancreatic, hepatic, biliary and other material malignant tumors that cannot be removed by surgery. This approach could also be considered to treat osteoarthritis.

Terminology

^{131}I-GMSs are protein microspheres containing the radionuclide ^{131}I. The half life of ^{131}I is 8.04 d and free ^{131}I either in the body or accumulates in the thyroid is quickly excreted *via* the urinary system. Compared with glass microspheres, the gelatin microsphere is a biodegradable material with good biocompatibility. In recent years, the authors have developed 50-70 μm nuclide protein microspheres that can be labeled with high concentrations of ^{131}I. These microspheres can be applied for hepatic arterial transfusion and embolotherapy for patients with advanced liver cancer.

Peer review

This is an interesting experimental study.The technique used in this study can be an effective method for treatment of maling tumors in the patients.

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BRIEF ARTICLE

Presence of hepcidin-25 in biological fluids: Bile, ascitic and pleural fluids

Jayantha Arnold, Arvind Sangwaiya, Vijay Manglam, Frank Geoghegan, Mark Thursz, Mark Busbridge

Jayantha Arnold, Arvind Sangwaiya, Vijay Manglam, Department of Gastroenterology, Ealing Hospital NHS Trust, Uxbridge Road, London UB1 3HW, United Kingdom
Frank Geoghegan, Department of Biochemistry, Ealing Hospital NHS Trust, Uxbridge Road, London UB1 3HW, United Kingdom
Mark Thursz, Department of Hepatology, St Mary's Hospital NHS Trust, Imperial College, Praed Street, London W2 1NY, United Kingdom
Mark Busbridge, Department of Clinical Chemistry, Charing Cross and Hammersmith Hospital NHS Trust, Imperial College, Fulham Palace Road, London W6 8RF, United Kingdom
Author contributions: Arnold J designed, supervised, co-authored and proof read the manuscript; Sangwaiya A designed, led laboratory work, wrote the paper and edited the manuscript; Manglam V recruited the patients, participated in research and helped write the manuscript; Geoghegan F helped in analysis of samples and co-authored the manuscript; Thursz M edited and proof read the manuscript; Busbridge M helped with laboratory work and help write the manuscript.
Supported by Grant from Ealing Hospital NHS Trust, Imperial College, United Kingdom
Correspondence to: Jayantha Arnold, Professor, Department of Gastroenterology, Ealing Hospital NHS Trust, Uxbridge Road, London UB1 3HW, United Kingdom. jayanthaarnold@hotmail.com
Telephone: +44-208-9675513 Fax: +44-208-9675083
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Abstract

AIM: To examine body fluids such as ascitic fluid (AF), saliva, bile and pleural effusions for the presence of hepcidin using a novel radioimmunoassay (RIA).

METHODS: Serum samples were collected from 25 healthy volunteers (mean age: 36 ± 11.9 years, 11 males, 14 females). In addition bile was obtained from 12 patients undergoing endoscopic retrograde cholangiopancreatography (mean age: 66.9 ± 16.7 years, M:F = 5:7). Saliva was collected from 17 healthy volunteers (mean age: 35 ± 9.9 years, M:F = 8:9). Pleural and AF were collected from 11 and 16 patients [(mean age: 72 ± 20.5 years, M:F = 7:4) and (mean age: 67.32 ± 15.2 years, M:F = 12:4)], respectively. All biological fluid samples (serum, exudative and transudative fluids) were tested for the presence of hepcidin-25 molecule using RIA.

RESULTS: Hepcidin-25 was detected in all biological fluids tested. The mean ± SD hepcidin-25 in serum was 15.68 ± 15.7 ng/mL, bile 7.37 ± 7.4 ng/mL, saliva 3.4 ± 2.8 ng/mL, exudative fluid 65.64 ± 96.82 ng/mL and transudative fluid 14.1 ± 17.8 ng/mL.

CONCLUSION: We provide clear evidence that hepcidin-25 is present in bile, saliva, pleural and ascitic fluids. Hepcidin is likely to play a role here in innate immunity.

Key words: Hepcidin; Hepcidin assay; Hepcidin in biological fluids; Hepcidin in ascitic fluid; Bile; Exudates; Antimicrobial peptides

Peer reviewers: Alberto Piperno, Professor, Department of Clinical Medicine and Prevention, Clinical Medicine, San Gerardo Hospital, Via Pergolesi 33, 20052, Monza, Italy; Kostas Pantopoulos, Associate Professor, Department of Medicine, McGill University, Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada; Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, Department of Gastroenterology, University Hospital Heraklion, PO Box 1352, 71110 Heraklion, Crete, Greece

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INTRODUCTION

Hepcidin-25 is a cysteine-rich circulating bioactive peptide that is predominantly secreted from the liver and excreted in the urine[1]. It is synthesized as preprohepcidin and undergoes posttranslational processing before release into circulation in an active form[2]. It is a central iron regulator with *in-vitro* evidence of antimicrobial properties. Hepcidin exerts its regulatory effect by preventing the efflux of intracellular iron by binding to ferroportin channels[3]. These ferroportin channels are present on the basal surface of the intestinal enterocytes as well as hepatocytes, macrophages and placental cells and are predominantly involved in the export of iron from these cells[4]. Despite a considerable body of literature on this novel peptide, research has been impeded by the lack of a reliable assay. Initially a semi quantitative assay (immunodot) was described but more recently quantitative assays have been developed using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS)[5] and liquid chromatography tandem MS (LC-MS/MS)[6,7].

We developed and validated a radioimmunoassay (RIA) for hepcidin-25 and measured serum levels of hepcidin-25 in various patient groups[8]. In this manuscript, we describe the use of RIA to demonstrate and measure hepcidin-25 in various biological fluids.

MATERIALS AND METHODS

This was a prospective study performed with approval of our Regional Ethics Committee and written consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki. Patients and healthy volunteers were recruited from a single hospital with mixed ethnicity mainly comprising Caucasians and South Asians living in West London.

Healthy controls

The serum study comprised of 25 healthy controls (HC); 14 were females, mean age was 36 ± 11.9 years (age range: 21-62 years), were hospital colleagues recruited to measure serum hepcidin-25, ferritin and serum prohepcidin. HC with systemic illness (acute or chronic) or any history of regular use of medication including supplemental vitamins were excluded. A fasting venous blood sample was obtained between 08:30-09:00 h. Serum was separated and stored at -20℃ for analysis.

Bile

Bile was collected from 12 patients undergoing endoscopic retrograde cholangiopancreatography (ERCP) for proven or suspected choledocholithiasis, mean age 66.9 ± 16.7 years, M:F = 5:7 (age range: 40-88 years). All patients had ultrasonography (USG) of the abdomen to identify choledocholithiasis, or cholelithiasis. Ten out of 12 patients recruited had cholelithiasis confirmed on USG. Two patients had a dilated common bile duct (CBD) without choledocholithiasis. During ERCP, CBD was cannulated and 2 mL of bile was aspirated before injecting contrast medium for CBD visualisation. Stone extraction or biliary sludge removal including sphincterotomy was done after bile collection to avoid contamination. Bile obtained was stored at -20℃.

Saliva

Saliva was obtained from a separate cohort of 17 healthy volunteers, mean age of 35 ± 9.9 years (age range: 23-60 years), M:F = 8:9. A history of systemic illness or dental or pharyngeal pathology was excluded. Participants were not allowed to eat or drink 30 min before the collection of saliva. Ten minutes before collection, participants had to rinse their mouth with plain water. They had to chew on a cotton swab and return the same with saliva in sterile tube (Salivette, Sarstedt, UK) and stored at -20℃.

Pleural fluid

Eleven patients, mean age 72 ± 20.5 years (age range: 26-95 years), M:F = 7:4 were recruited for pleural fluid (PF) analysis. PF was collected by diagnostic thoracocentesis under sterile conditions and stored in a sterile container at -20℃. PF was analysed and divided into a transudate and exudate by comparing the total protein concentrations in fluid to that in blood as per Light's Criteria[9]. Three patients had transudative fluid (PF-T) (M:F = 2:1). The underlying cause for fluid accumulation in this group was congestive heart failure secondary to ischemic heart disease. Eight patients had exudative fluid (PF-E) (M:F = 5:3). The underlying cause for fluid accumulation in this cohort was parapneumonic (5 cases) and neoplastic [3 cases (1 breast and 2 lung malignancies)].

Ascitic fluid

Ascitic fluid (AF) was collected from 16 patients, mean age 67.32 ± 15.2 years (age range: 42-85 years), M:F = 12:4 under sterile conditions by diagnostic paracentesis as further differentiated by serum/ascites albumin gradient (SAAG)[10]. Twelve patients were identified as having transudative ascites (AF-T) (M:F = 8:4). Alcohol induced liver cirrhosis was the aetiology and comprised majority with 10 patients. The remaining 2 patients had chronic hepatitis B and chronic hepatitis C as etiology. The exudative ascites cohort comprised 2 patients (AF-E) (M:F = 2:0), with aetiology being congestive heart failure secondary to ischemic heart disease and idiopathic.

One patient had transudative AF with spontaneous bacterial peritonitis (SBP) (M:F = 1:0) analysed for presence of hepcidin-25. The cause of ascites was cirrhosis of the liver secondary to alcohol misuse. The AF white cell count was 350/mm^3 (90% polymorphs, 10% lymphocytes) and AF culture had grown *Escherichia coli* (*E. coli*).

On the basis of above, 2 cohorts were obtained, those with Transudative Fluid and those with Exudative Fluid, comprising both pleural and AFs in each respective category. One patient with SBP-ascites was excluded from the transudative fluid group. Thus, there were 10 patients

in the total exudative fluid cohort (M:F = 7:3) and 15 patients in the total transudative fluid cohort (M:F = 10:5).

Assay

Hepcidin-25 was measured using a novel competitive RIA. This was produced in our laboratory and has been described in our previous manuscript (Busbridge *et al*[8]).

The specificity of the antibody was also confirmed by Western blotting analysis of the precipitated proteins, where a single band of < 6 kDa was detected. Western blotting analysis was carried out by using 10 mL normal human plasma, which was first centrifuged and filtered through a 0.22 mm filter (Millipore, MA). After addition of 10-mL dithiothreitol, plasma was again filtered through a 30 kDa filter (Millipore) and the filtrate precipitated using 25% TCA. The precipitated proteins were resuspended in loading buffer and subjected to SDS electrophoresis on a 4%-16% NuPAGE Novex Bis/Tris gel (Invitrogen, UK). Western blotting was performed using an XCell Ⅱ blot module (Invitrogen) and a 0.1 mm pore size nitrocellulose membrane (Sigma Aldrich). Non-specific sites were blocked with 5% BSA in PBS and the membrane probed with purified rabbit anti-hepcidin antibody (1 in 2000 dilution in blocking buffer), followed by incubation with a secondary anti-rabbit antibody conjugated with horseradish peroxidase (Sigma Aldrich). Specific signals were detected with 3,3', 5,5' tetramethylbenzidine liquid substrate (Sigma Aldrich) (Figure 1).

Serum prohepcidin was measured by enzyme linked immunosorbent assay (ELISA) (DRG Diagnostics, UK) as per manufacturer's guidelines. Ferritin, iron and C-reactive protein were measured using Abbott Architect *i*8000 system (Abbott Diagnostics, Ireland).

Statistical analysis

Values are expressed as mean ± SD as indicated. Quantitative variables were compared using unpaired *t*-test; Pearson's rank correlation was used for calculating correlation between the hepcidin-25 and other variables. A value of $P < 0.05$ was considered significant. All statistical analyses were carried out using the statistical package GraphPad Prism version 5.0 for Windows, (GraphPad Software, San Diego California USA, http://www.graphpad.com).

RESULTS

HC

Serum hepcidin in HC was 15.30 ± 15.71 ng/mL with a range of 1.1-55.0 ng/mL. The mean prohepcidin was 236.88 ± 83.68 ng/mL and the mean ferritin was 110.00 ± 128.08 μg/L. There was no correlation between serum prohepcidin and ferritin in healthy volunteers. However, serum hepcidin showed a weak correlation with ferritin ($r = 0.174$, $P < 0.05$).

Bile and saliva cohort

Hepcidin-25 was detected in all bile samples ($n = 12$) and was measured at a mean of 7.37 ± 7.39 ng/mL with a range of < 1.0-23 ng/mL. Hepcidin-25 was detected in saliva with a mean of 3.39 ± 2.83 ng/mL, range of < 0.9-11.8 ng/mL.

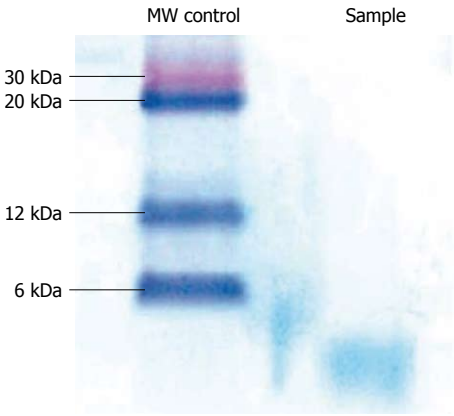

Figure 1 Western blotting analysis of serum proteins using rabbit anti-hepcidin antibody demonstrating the presence of a protein band at < 6 kDa.

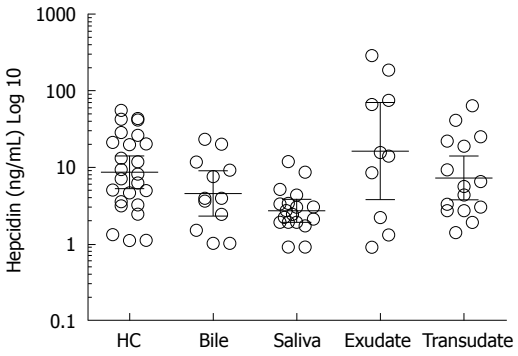

Figure 2 Scatter-dot plot of hepcidin data from healthy control sera (HC) and various body fluids, bile, saliva and pleural fluid both exudates and transudate in nature. Data is presented with geometric mean and 95% CI levels.

Exudative and transudative fluid

Hepcidin-25 level was 65.64 ± 96.82 ng/mL in exudative fluid. The mean total protein level in the fluid was 43.69 g/L. The hepcidin-25 level was 14.10 ± 17.88 ng/mL in transudative fluid. Hepcidin-25 concentration in exudative fluid was higher than in transudative but did not reach statistical significance ($P = 0.05$, student *t*-test). This could be due to the small number of patients involved in the study. We demonstrated hepcidin-25 in all bodily fluids analysed using RIA. To our knowledge this is the first report confirming the presence of hepcidin-25 in these bodily fluids (Figure 2).

DISCUSSION

Hepcidin, as an antimicrobial peptide, was first described as a liver expressed antimicrobial peptide 1[11].

Hepcidin is a novel peptide due to its dual role as an 'iron-hormone' and 'antimicrobial peptide'. However, previous research studies have been hampered due to lack

of a reliable quantitative assay for hepcidin-25. Several factors have been cited for this difficulty including presence of non-biologically active iso-forms of hepcidin-20, hepcidin-22, and the fact that hepcidin-25 is conserved over evolution thus making an immunological response in host species difficult to determine[12]. Several semi quantitative and quantitative assays have recently been developed for hepcidin-25 measurement. The quantitative assays include ELISA[13] and MS. MS methods includes TOF-MS, LC-MS, matrix-assisted laser desorption ionization MS, and SELDI-MS[14,15]. Although very successful, the availability of the MS assay is limited to specialist laboratories as they require strict internal standards and specialised equipment. Two quantitative assays have been described using ELISA techniques. Ganz *et al*[13] have described a competitive quantitative ELISA assay using functional synthetic peptide hepcidin-25 while the method described by Koliaraki *et al*[16] detects hepcidin-25 using a recombinant functional hepcidin-25 peptide. Thus, although a number of methods are available to measure hepcidin-25, there does not appear to be a standardisation amongst various methods. The results of the first international round robin for the quantification of urinary and plasma hepcidin assays were published in an attempt to help standardisation issues[17].

Until recently, hepcidin-25 was shown to be present in urine and serum. However, its expression by various organs such as salivary glands, tonsils, trachea, lung and prostate have also been reported[18]. The exact nature and function of hepcidin expression in these organs remains unclear.

Hepcidin in saliva

This study confirms the presence of hepcidin-25 in saliva. Its exact role in saliva is still uncertain but could contribute to the broad spectrum defence of the oral cavity along with peptides such as cathelicidins[19].

Hepcidin in bile

The presence of hepcidin-25 in bile may be a mechanism of hepcidin-25 excretion apart from urine. It may act along with α-defensins against gut microflora[20]. Further studies are needed to fully understand its role in bile.

Hepcidin in PF and AF

We were able to demonstrate the presence of hepcidin in pleural and AF where its iron regulator function is hard to explain. Hepcidin present in these fluids has unknown biological activity. Hepcidin-25 is a cationic peptide with 4 disulfide bridges[21]. The connection between hepcidin and infection/inflammation became clearer after the initial discovery in 2000. Shike *et al*[22], in 2002 showed that in white bass liver, infection with the fish pathogen *Streptococcus iniae* increased hepcidin mRNA expression 4500-fold. In another study by Nicolas *et al*[23] in 2002 injections of turpentine, a standard inflammatory stimulus, into mice induced hepcidin mRNA 4-fold and led to a 2-fold decrease in serum iron.

Hepcidin maintains a role in host defence by acting as a bridge between immunity and iron metabolism, as indicated by markedly induced hepcidin by infection and inflammation[24-26]. Park *et al*[1] have shown that hepcidin has antimicrobial activity against *E. coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and group B *Streptococcus*. Hepcidin also exhibits antifungal activity against *Candida albicans, Aspergillus niger* and *Aspergillus fumigatus*.

Our study on the presence of hepcidin in bile, saliva and various other biological fluids provides further confirmatory evidence that hepcidin has evolved from being an antimicrobial peptide. Hepcidin may form part of human innate immunity and complement other antimicrobial peptides to provide a wide spectrum of mucosal immunity against pathogens.

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COMMENTS

Background

Until recently iron metabolism had been extensively studied but poorly understood. With the discovery of hepcidin as an iron regulator in the body, there has been a significant growth in the knowledge related to iron metabolism. Crucial research has been done to explore the role of hepcidin in iron deficiency anaemia, anaemia of chronic disease and iron excess disorders.

Research frontiers

Hepcidin is a 25 amino-acid peptide predominantly formed in the liver and is a key component in iron homeostasis. Its major role is to prevent iron export by binding to iron export channels, ferroportin, leading to internalisation and lysosomal degradation of the hepcidin-ferroportin complex. Interestingly, hepcidin is found throughout evolution and in amphibian species has possible antimicrobial properties while in humans its major action is iron metabolism. Prior research showed hepcidin expression in various human organs like heart, intestine and salivary glands. In this study, the authors demonstrate clear evidence that hepcidin is present in various bodily fluids with higher levels found in exudative fluids and could be a potential mechanism for innate immunity preserved over evolution.

Innovations and breakthroughs

Recent studies have highlighted the importance of hepcidin in iron metabolism, particularly anaemia of chronic disease and iron overload. There have also been reports of its expression in various proinflammatory disorders and various organs, linking it to innate immunity and iron metabolism. This is the first study to report that hepcidin is found in various biological fluids. Furthermore, *in-vitro* studies have suggested that hepcidin has antimicrobial activity.

Applications

By understanding how hepcidin is expressed and by blocking its expression, there may be a therapeutic potential in patients with anaemia of chronic inflammation. Further application could be that secondary infection of pleural and ascitic fluid complicating underlying diseases may be better understood by studying hepcidin levels in infected and non-infected patients.

Terminology

Hepcidin is a protein involved in iron metabolism. Hepcidin is formed predominantly in the liver and is released in the circulation where its major function is hypoferremia by binding to iron export channel ferroportin. Hepcidin is expressed by various organs and thus it was conceivable that hepcidin would be found in various biological fluids. The exact role of hepcidin in these fluids remains uncertain and more studies are needed.

Peer review

The authors present a nicely conceived and analysed study. It is well written and offers new information about the presence of hepcidin in human biological fluids.

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BRIEF ARTICLE

Probiotic yeasts: Anti-inflammatory potential of various non-pathogenic strains in experimental colitis in mice

Benoît Foligné, Joëlle Dewulf, Pascal Vandekerckove, Georges Pignède, Bruno Pot

Benoît Foligné, Joëlle Dewulf, Bruno Pot, Institut Pasteur of Lille, Lactic acid Bacteria & Mucosal Immunity, Center for Infection and Immunity of Lille, 1 rue du Pr Calmette, BP 245, F-59019 Lille, France; University of Lille Nord de France, F-59000 Lille, France; Centre National de la Recherche Scientifique, UMR 8204, F-59021 Lille, France; Institut National de la Santé et de la Recherche Médicale, U1019, F-59019 Lille, France
Pascal Vandekerckove, Georges Pignède, Lesaffre International, Direction of Research and Developpement, 147 rue Gabriel Péri, BP 6027, 59700 Marcq-en-Baroeul, France
Author contributions: Foligné B and Dewulf J performed the research; Foligné B, Vandekerckove P and Pignède G designed the research and provided support; Pot B coordinated the research, provided critical reading and revised the manuscript; Foligné B analyzed the results and wrote the paper.
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Correspondence to: Benoît Foligné, PhD, Institut Pasteur of Lille, Lactic acid Bacteria & Mucosal Immunity, Center for Infection and Immunity of Lille, 1 rue du Pr Calmette, BP 245, F-59019 Lille, France. benoit.foligne@ibl.fr
Telephone: +33-320-871192 Fax: +33-320-871192
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Abstract

AIM: To evaluate the *in vitro* immunomodulation capacity of various non-pathogenic yeast strains and to investigate the ability of some of these food grade yeasts to prevent experimental colitis in mice.

METHODS: *In vitro* immunomodulation was assessed by measuring cytokines [interleukin (IL)-12p70, IL-10, tumor necrosis factor and interferon γ] released by human peripheral blood mononuclear cells after 24 h stimulation with 6 live yeast strains (*Saccharomyces ssp.*) and with bacterial reference strains. A murine model of acute 2-4-6-trinitrobenzene sulfonic acid (TNBS)-colitis was next used to evaluate the distinct prophylactic protective capacities of three yeast strains compared with the performance of prednisolone treatment.

RESULTS: The six yeast strains all showed similar non-discriminating anti-inflammatory potential when tested on immunocompetent cells *in vitro*. However, although they exhibited similar colonization patterns *in vivo*, some yeast strains showed significant anti-inflammatory activities in the TNBS-induced colitis model, whereas others had weaker or no preventive effect at all, as evidenced by colitis markers (body-weight loss, macroscopic and histological scores, myeloperoxidase activities and blood inflammatory markers).

CONCLUSION: A careful selection of strains is required among the biodiversity of yeasts for specific clinical studies, including applications in inflammatory bowel disease and other therapeutic uses.

Key words: Yeast; Probiotics; Strain specificity; Experimental colitis; 2-4-6-trinitrobenzene sulfonic acid

Peer reviewer: Dr. Francesco Costa, Dipartimento di Medicina Interna - U.O. di Gastroenterologia Università di Pisa - Via Roma, 67 - 56122 - Pisa, Italy

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INTRODUCTION

Yeasts, as an inevitable part of the microflora of various fermented foods and beverages, are found in a wide range of foods from plant or animal origin, where they have a significant impact on food safety and organoleptic properties. Both baker's and brewer's yeasts [*Saccharomyces cerevisiae* (*S. cerevisiae*)] have been available for the last

decades as dietary supplements because of their high contents of vitamin B, proteins, peptides, amino-acids and trace minerals. Regardless of their non-human origin, such non-pathogenic yeasts fulfill the major criteria for probiotic definition[1]. Interest in probiotic yeasts has increased, especially in relation to animal feed but also for human applications. Hence, yeasts are rarely (if ever) associated with outbreaks or cases of food-borne illness and, through their history, many yeast species are recognized as safe, as confirmed by their Qualified Presumption of Safety status, assigned by the European Food Safety Authority (http://www.efsa.europa.eu). However, some rare cases of *Saccharomyces boulardii* (*S. boulardii*) fungemia have been reported, although they are essentially restricted to immunocompromised patients or associated with patients being contaminated through a central venous catheter[2]. Also, identification of the contaminating yeast strains in some cases could be questioned, as it was often based on cross-reacting anti-yeast mannan circulating antibodies, shared with *Candida* species.

Although *S. boulardii* was originally selected using rather empiric methods, this yeast species has many proven benefits in various gastrointestinal diseases[3-5] and is well recognized as the non-bacterial prototype of a probiotic. Multiple mechanisms have been suggested that explain the broad health-promoting effects of consuming food grade yeasts, ranging from local general trophic effects to action on both innate and/or adaptive immunity[6,7]. The yeast-induced probiotic signals might be either prophylactic or therapeutic in a defined pathologic context or in a specific application.

Most reported effects of yeasts as probiotic organisms in clinical trials stand for alleviation of (1) antibiotic-associated diarrhea; (2) infectious diarrhea [including recurrent *Clostridium difficile* (*C. difficile)* related diseases, CDAD]; (3) irritable bowel syndrome; and (4) inflammatory bowel diseases (IBD). Focusing on IBD, *S. boulardii* efficacy was both observed in Crohn's disease[8] and by favoring remission of ulcerative colitis[9]. Most of the therapeutic approaches in IBD were also using anti-inflammatory drugs such as mesalamine concomitantly and the exclusive use of yeast as possible ecological treatment has not been tested, most probably for ethical reasons. The anti-inflammatory potential of yeasts has been addressed by many *in vitro* studies elaborating on multiple mechanisms[4,7]. Surprisingly, data using yeasts in animal models of colitis are very scarce and the precise mechanism(s) of action by which such intervention may exert its beneficial effects *in vivo* is consequently poorly documented. Dalmasso *et al*[10] have reported a beneficial action of secreted yeast molecules on T cell migration in the chronic model of CD4+CD45RB^{hi} T-lymphocytes transfer into SCID mice. The same authors have also reported the preventive effect of *S. boulardii* in an acute murine 2-4-6-trinitrobenzene sulfonic acid (TNBS) model, characterized by a strong inhibitory activation of NFκB and the reduction of mRNA for pro-inflammatory cytokines together with decreased histological scores of inflammation[11,12]. Consistently, a similar TNBS-induced model of colitis, although developed for a more therapeutic approach in rats, also showed appreciable reduction of histological scores and pro-inflammatory colonic markers by *S. boulardii* (including cytokines but also metabolites of pro-oxidative pathways such as iNOS). The proposed mechanism depended on PPAR activation by the probiotic yeast[13], a regulator previously demonstrated to reduce intestinal inflammation[14]. Finally, yeast-driven therapy in a DSS-induced colitis model was also successfully demonstrated in mice[15], even when this chemically induced colitis was amplified by *Candida albicans* colonization. Because the model is known to affect epithelial crypt cells and destroy barrier integrity without intervention of the adaptive immune system[16], it was suggested that the contribution of yeast-mediated protection was restricted to the switching off of the innate mechanisms of colitis and/or to the enhancement of the mucosal barrier integrity.

Very few yeast strains have been studied as possible biotherapeutics agents and, at the moment, *S. boulardii* is the only yeast commercialized for human use, and consequently the single preparation formally recognized as probiotic. However, other *Saccharomyces spp.* or members of other yeast genera with similar or possibly better therapeutic properties will certainly be isolated in the future[17,18]. Hence, distinct non pathogenic yeast strains may have their own impact on gut homeostasis, sharing some of the common mechanisms of *S. boulardii,* but also other more specific consequences or even novel prophylactic or therapeutic effects.

Considering the widespread use of lactic acid bacteria and bifidobacteria as probiotics, it is now well accepted that all strains are not equally beneficial, that each may have individual mechanisms of action. Furthermore, host characteristics (e.g. flora composition or immune status) may determine which probiotic species or strains may be optimal[19]. Peran *et al*[19] conclude that it would be interesting to compare different probiotics in the same experimental model, in order to establish the best profile in a given setting. Likewise, this reasoning can be extent towards "friendly yeasts" as far as evaluation is conducted for a specific use without generalizations about probiotic effects[20,21].

In this paper, we compared yeast strains in their ability to induce cytokines on human peripheral blood mononuclear cells (PBMC). Our results also demonstrated that some yeast strains exhibit significant anti-inflammatory activities *in vivo*; whereas other may have weaker inflammation lowering effects or no anti-inflammatory effect at all. Thus, as for probiotic bacteria[20], strain-specific differences were seen for yeasts, suggesting that careful selection of strains for therapeutic use is required for preclinical and further clinical studies, including for applications in IBD.

MATERIALS AND METHODS

Microbial strain preparation and growth conditions

All *Saccharomyces* strains used in this study and listed (Table 1), including baker's yeast *S. boulardii* and wine-

Table 1 Bacterial and yeast strains and their origin used in the present study

Strain	Identification	Origin
BB536	*Bifidobacterium longum*	Morinaga Milk Industry Ltd.
Ls33	*Lactobacillus salivarius*	Commercial strain/ Danisco
NCFM	*Lactobacillus acidophilus*	Commercial strain/ Danisco
MG1363	*Lactococcus lactis*	Cheese starter[22]
TG1	*Escherichia coli*	Commensal strain[23]
LV01/ CNCM I-3799	*Saccharomyces boulardii*	Lesaffre collection
LV02/ CNCM I-3856	*Saccharomyces cerevisiae*	Lesaffre collection
LV03	*Saccharomyces pastorianus*	Lesaffre collection
LV04	*Saccharomyces bayanus*	Lesaffre collection
LV06	*Saccharomyces cerevisiae*	Lesaffre collection
LV09	*Saccharomyces cerevisiae*	Lesaffre collection

CNCM: French National deposit Collection of Microorganism Cultures, Institut Pasteur, Paris, France.

related strains, were originally isolated from a proprietary germoplasm bank of *S. cerevisiae* (Societe Industrielle Lesaffre 147 Rue Gabriel Peri, BP 6027, 59700 Marcq-en-Baroeul Cedex, France). They were all provided by Lesaffre as a similar dry form with a cell concentration near to 1×10^{10} colony-forming units (CFU)/g. The different dried yeast products were processed and enumerated as follows. For both *in vitro* and *in vivo* assays, yeast was rehydrated with phosphate buffered saline (PBS) (pH 7.2) 1:10 (w/v) at room temperature, and adjusted to an appropriate concentration in CFU/mL. For this purpose, cellular counts together with viability, expressed in %, were routinely performed using a hematimeter (type Thoma) combined with a Trypan blue exclusion method. More than 95% viability was preserved following rehydration. Enumeration was also confirmed *a posteriori* by plating on *YPD-agar* and allowing growth during 2 d at 30℃ and 37℃.

Some bacterial strains were used as reference strains for immune cell stimulation as previously described[24], also listed (Table 1). *Lactobacillus* strains were grown under limited aeration at 37℃ in MRS medium (Difco) and a *Bifidobacterium* strain was grown anaerobically in MRS supplemented with 0.05% L-cysteine-hydrochloride (Sigma). *Lactococcus lactis* MG1363 was grown at 30℃ in M17 medium supplemented with 0.5% glucose. *Escherichia coli* (*E. coli*) was grown at 37℃ in LB medium (Difco). Bacterial cells were grown until stationary phase, washed and resuspended at 1×10^9 CFU/mL in PBS containing 20% glycerol and stored at -80℃ until required for later assays. Some routine analyses were also performed using a portable photometer (Densimat bioMerieux)[25]. Cells were adjusted to McFarland 3 and stored at -80℃.

In vivo resistance to gastrointestinal tract

Groups contained 10 mice. When needed, fecal samples from 5 mice were collected, pooled, weighed and mechanically homogenized in sterile neutral and isotonic buffer at 50 mg/mL of feces. Dilutions were plated onto the "yeast-selective" tetracycline (150 μg/mL) *YPD-agar* (Yeast extract, Peptone, Dextrose, as described elsewhere[26]. No tetracycline-resistant microorganisms (bacteria or fungi) were detected in non-inoculated mice, indicating no or negligible basal colonization of mice gastrointestinal tract (GIT) by non-pathogenic yeast nor *Candida* species.

PBMC isolation

PBMCs were isolated from peripheral blood of healthy donors as previously described[24]. Briefly, after a Ficoll gradient centrifugation (Pharmacia, Uppsala, Sweden), mononuclear cells were collected, washed in RPMI 1640 medium (Live technologies, Paisley, Scotland) and adjusted to 2×10^6 cells/mL supplemented with gentamicin (150 g/mL), L-glutamine (2 mmol/L), and 10% foetal calf serum (Gibco-BRL) supplementation.

Induction of cytokine release

PBMC (2×10^6 cells/mL) were seeded in 24-well tissue culture plates (Corning, NY). Twenty microliters of the thawed bacterial or yeast suspensions at 10^9 CFU/mL in PBS containing 20% glycerol (bacteria:cell or yeast: cell ratio of 10:1) or standardized at homogeneous cell density, were added. PBS containing 20% glycerol was used as a negative (non-stimulated) control. On the basis of preliminary time-course studies, 24 h stimulation corresponded to the best time point for cytokine responses of bacteria stimulated-PBMCs. After 24 h stimulation at 37℃ in an atmosphere of air with 5% CO_2, culture supernatants were collected, clarified by centrifugation and stored at -20℃ until cytokine analysis. Concerning yeast stimulation, freshly rehydrated powders were used at distinct yeast:cell ratios (from 0.1:1 to 5:1), with or without the presence of fungizone (amphotericin B, 10 μg/mL). Neither medium acidification nor bacterial or fungal proliferation was observed. Cytokines were measured by enzyme-linked immunosorbent assay (ELISA) using BD Pharmingen antibody pairs (BD Biosciences, San Jose, Ca, USA) for interleukin (IL)-10, interferon γ (IFNγ) and IL-12p70, and R&D systems (Minneapolis, Mn, USA) for human tumor necrosis factor (TNF), according to the manufacturer's recommendations.

Induction of colitis and inflammation scoring

BALB/c mice (female, 7 to 8 wk old) were obtained from Charles River (St Germain sur l'Arbresle, France). A standardized murine TNBS colitis model was used in which moderate levels of inflammation were induced[27]. Briefly, a 50 μL solution of 100 mg/kg TNBS (Sigma) in 50 % ethanol was slowly administered in the colon *via* a 3.5 F catheter. Freshly rehydrated yeast suspensions (100 μL), containing 1×10^9 CFU/mL in $NaHCO_3$ buffer were administered intragastrically to mice each day, starting 4 d before until the day of TNBS administration while control mice received the corresponding buffer. Mice were weighed, bled from the retro-orbital venous

plexus and killed 72 h after TNBS administration. Cleared sera were frozen and stored until cytokine assays at -20℃. Colons were removed, washed and opened longitudinally. Inflammation grading was performed by two blinded observers, using the Wallace scoring method[28]. Results are expressed as % protection, corresponding to the reduction of the mean macroscopic inflammation score of treated mice (n = 10) in comparison to the mean score of TNBS-treated control mice ($NaHCO_3$ buffer-treated mice, n = 10)[27]. Histological analysis was performed on May Grünwald-Giemsa stained 5 μm tissue sections from colon samples fixed in 10% formalin and embedded in paraffin and tissue lesions were scored according to the Ameho criteria[29]. Additionally, the degree of polymorphonuclear neutrophil infiltration of the distal colon was assessed by quantifying myeloperoxidase (MPO) - a neutrophil granule enzyme - as reported earlier[30]. A commercial preparation of prednisone (Cortancyl, Sanofi Aventis, France) was used as a positive control of protection and was orally administered for 2 subsequent days at 10 mg/kg starting at the day before TNBS administration. When needed, heparinized whole blood was collected by retro-orbital puncture and, after separation, mice sera samples were stored at -20℃ for subsequent analysis.

Cytokine and serum amyloid A protein assays

Murine IL-6 and serum amyloid A (SAA) protein levels were measured by ELISA using commercial antibodies from Pharmingen antibody pairs (BD Biosciences, San Jose, Ca, USA), and Biosource International (Camarillo, Ca, USA), respectively, with a lower limit of sensitivity of 15 pg/mL for IL-6 and 30 ng/mL for SAA.

Statistical analysis

All analyses were performed as comparison of experimental groups with respective controls by the non-parametric one-way analysis of variance Mann-Whitney U-testing, or by Student-T testing where appropriate. Differences were judged to be statistically significant when the P value was < 0.05. Data are presented as mean ± SE.

Ethical considerations

All experiments were performed in an accredited establishment (number 59-35009; Institut Pasteur de Lille, France) and using approved guidelines according to French Ethical Committee and European Union Normatives (number 86/609/CEE).

RESULTS

Production of cytokines by human PBMCs in response to food-grade yeast strains

To determine the optimal dose of yeast that can induce cytokine production in human PBMCs, immuno-competent cells were first stimulated with three different multiplicity levels of infection (0.1:1, 1:1 and 5:1) of three live yeast strains and compared with immuno-stimulatory effects of reference bacteria (ranging from weak to strong inducers). Substantial amounts of IL-10, and to a lesser extent for TNF were dose-dependently induced by yeast cells (Figure 1A and B, respectively) while no or very low levels of the pro-inflammatory indicators IL-12 p70 and IFNγ could be detected following yeast stimulation (Figure 1C and D). Following these results, the yeast: host cell ratio of 1:1 was retained for further comparative analyses between strains in order to mimic physiological interactions between cells but without saturating the stimulatory capacity. In addition, the variable responsiveness due to individual blood donors was highly minimized by expressing data as % of a reference strain instead of in pg/mL, as shown (Figure 2, panel A and B). PBMCs from 5 donors were stimulated with each of the six yeast strains covering distinct subspecies and origins (Table 1).

All individual strains of yeast induced relatively high levels of the anti-inflammatory IL-10, reaching the values generally obtained with lactobacilli (mean/max values 535/2300 pg/mL). However, in contrast to bacteria, no significant strain-specificity was observed among different strains of yeasts, which all induced similar levels of cytokines (Figure 3A). Analysis of the cell culture supernatants revealed moderate induction of TNF after yeast stimulation, as compared with bacterial stimulation, again without a clear strain dependency (Figure 3B). Yeasts induced extremely low levels of the pro-inflammatory signals IL-12 and IFNγ as compared to some bacteria such as *Lactococcus lactis* MG1363 or *Lactobacillus acidophilus* NCFM (Figure 3C and D). Concerning the intrinsic immunostimulatory capacity of yeast cells *in vitro*, we can conclude that no specific stimulation pattern could be discriminated among the six strains, while all strains favored anti-inflammatory profiles with a high IL-10/IL-12 ratio, low induction of TNF and negligible levels of IFNγ.

Resistance of various yeasts strains to the GIT passage

Next to the effect of yeasts on cytokine induction on PBMCs *in vitro*, we compared the ability of the 3 strains LV01, LV02 and LV09 to survive the digestive tract of mice. Following 0, 1 and 5 consecutive days of intragastric feeding of 10^8 CFU of each of the yeasts; faecal enumeration showed equivalent survival counts for all the strains with a progressive increase with the duration of the treatment (Figure 4). The systematic increase by 2 log units between day 1 and day 5 suggests that proliferation occurs in the gut, rather than simple transit and shedding. Although we cannot speculate on the long term persistence and colonization of the yeast species, a 5-d-treatment clearly leads to a noticeable presence of living yeasts in the colon lumen (up to 10^8 CFU/g for the 3 strains). A significant faecal recovery of yeast colonies from healthy mice was still detectable 10 d after the last feeding (more than 10^5 CFU/g, data not shown). It is noteworthy to mention that neither diarrhoea nor slight changes in faecal character, nor weight disturbance or other deleterious signs were observed in mice treated with those food-grade yeasts.

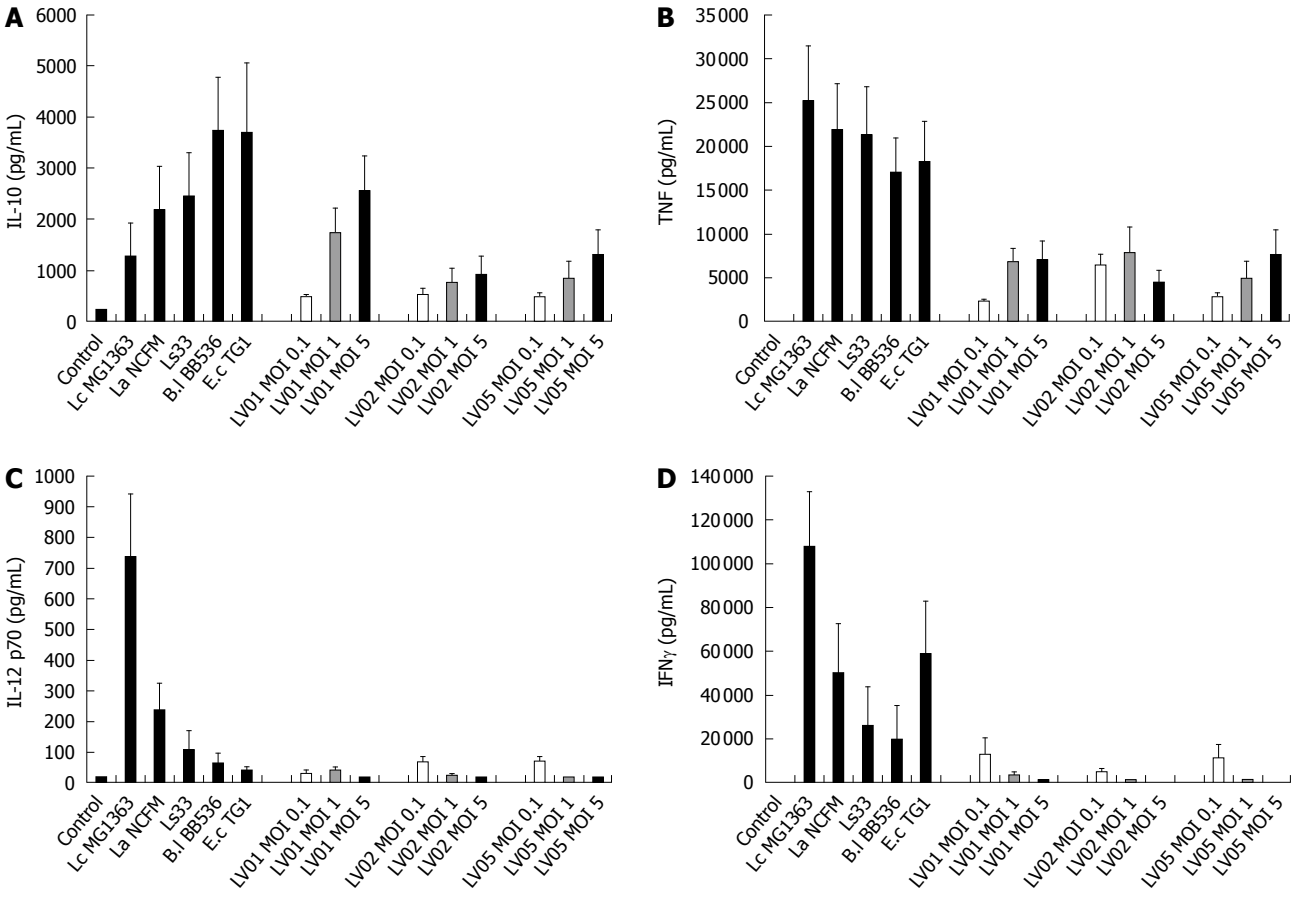

Figure 1 Dose-dependent cytokine production in human peripheral blood mononuclear cells (PBMCs), stimulated with bacterial reference strains and 3 yeast strains at distinct multiplicity levels of infection [multiplicity levels of infection (MOI) 0.1, 1 and 5 yeasts/cells] for 24 h. Cytokine levels were analyzed in supernatants by enzyme-linked immunosorbent assay (ELISA). Panels A, B, C and D respectively show results for interleukin (IL)-10, tumor necrosis factor (TNF), IL-12 p70 and interferon γ (IFNγ), respectively. Results are expressed in pg/mL, mean ± SE, *n* = 3 distinct donors.

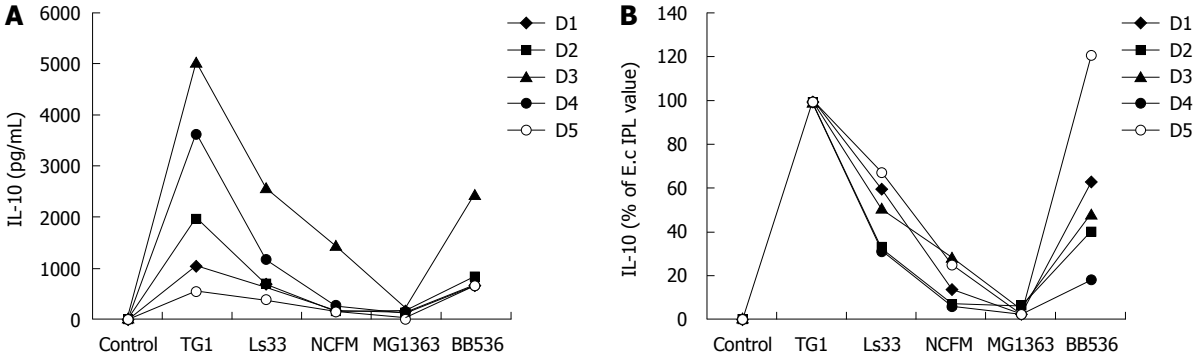

Figure 2 Standardized assessment of cytokine production in human PBMCs from distinct donors. Immuno-competent cells are stimulated with 5 bacterial reference strains for 24 h and cytokine levels were analyzed in supernatants by ELISA. A: IL-10 release for 5 individual donors. Results are expressed in pg/mL; B: Standardized IL-10 release with values expressed as % of *Escherichia coli* (*E. coli*) value for each of the 5 donors.

Comparative preventive capacity of yeasts in alleviating experimental colitis

In order to evaluate whether the administration of distinct yeast strains equally impacted on TNBS-induced colitis, we compared the performance of the three strains LV01, LV02 and LV09 with the anti-inflammatory corticoid drug (prednisone) which we and others previously used in this experimental setting[31,32]. Since some anti-inflammatory effects of lactobacilli were observed both after oral and systemic administration[33,34], we assessed whether intraperitoneal administration of the yeast LV01 could also rescue mice from colitis.

Rectal administration of TNBS causes progressive weight loss in vehicle-treated mice, reaching up to 22.5% of the initial weight 72 h after induction of colitis (Figure 5A and B). As expected, prednisone prevented body weight loss associated with experimental colitis (-3.3%, $P < 0.001$). The oral treatments by the 3 yeast

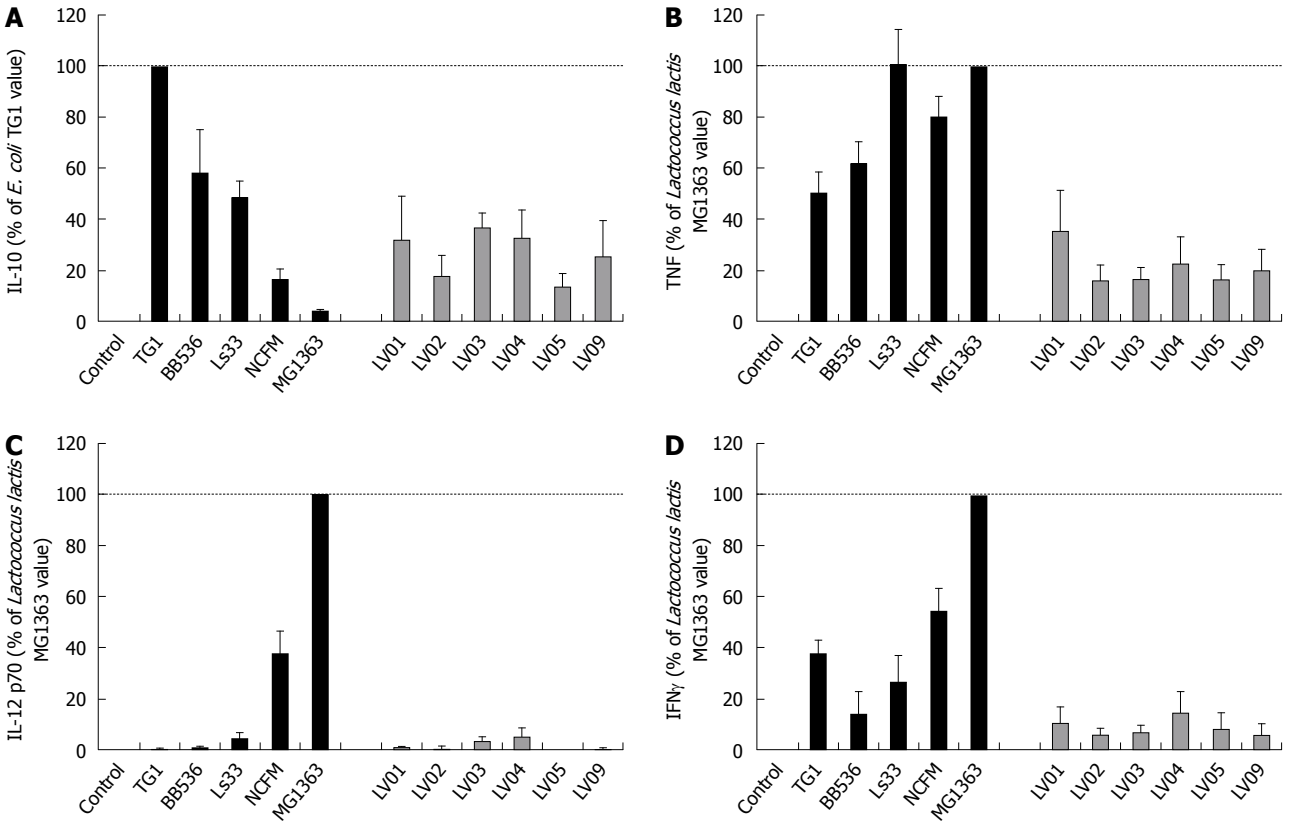

Figure 3 Cytokine production in human PBMCs, stimulated with bacterial reference strains and 6 yeast strains for 24 h. Cytokine levels were analyzed in supernatants by ELISA. A: IL-10; B: TNF; C: IL-12 p70; D: IFNγ. Results are expressed in % of *E. coli* TG1 value for panel A and in % of *Lactococcus lactis* MG1363 for B, C and D, including mean ± SE; *n* = 5.

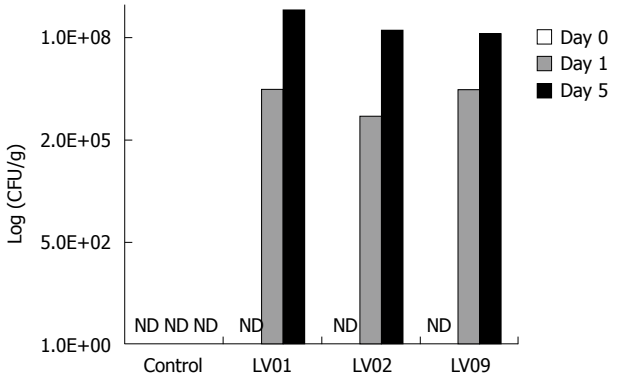

Figure 4 Fecal recovery of the 3 distinct yeast strains in yeast-fed mice, at days 0, 1 and 5. Data were obtained by counting dilutions of stools on appropriate "yeast-selective"-tetracycline agar plates. Results are expressed in Log CFU/g of stools. Results are representative for 2 separate experiments, SD are not represented for clarity reasons. ND: Not detected; CFU: Colony-forming units.

strains also significantly attenuated the deleterious weight changes for the LV01 (-9.9%, $P < 0.01$), and to a lesser extent for LV02 and LV09 (18%, $P < 0.05$). In contrast, the systemic administration of the strain LV01 (LV01-IP) had no additional effect on weight.

After the onset of colitis, anti-inflammatory effects were measured macroscopically for the prednisone-treated as well as for the LV01-treated mice (66% and 27% reduction of macroscopic damage, respectively, $P < 0.01$, Figure 6A). More moderately LV02 reduced the colonic insult (18% of control, $P < 0.05$) while intra-peritoneal injection of LV01 failed to influence the macroscopic scores.

Considering the histological assessment of colitis in various conditions (Figure 6B), both the prophylactic treatment by prednisone and LV01 significantly reduced the microscopic scores of inflammation (by 60.3% and 35%, respectively, $P < 0.05$), while neither LV02 nor LV09 treament could be associated with a significant reduction of the histopathological features observed during active colitis (mucosal erosions, oedema, ulcerations areas and even necrosis). These observations, including thickening of the submucosa and inflammatory infiltrates, were correlated with the colonic myeloperoxidase activities measured (MPO, Figure 6C) and with similar reductions for the steroid drug and LV01 ($P < 0.01$). In contrast, no improvement, and even some aggravation, was depicted for strain LV01 provided by the systemic route, as supported by massive neutrophil infiltrates. Representative aspects of the corresponding colon sections are given (Figure 7). Mouse colons with vehicle-treated TNBS-induced colitis exhibited massive goblet cell depletion and showed disorganized mucosal architecture, including muscle layer necrosis (Figure 7B). By contrast, corticoid-treated animals showed a well-conserved appearance, more similar to normal structures despite a slight thickening of the submucosa. Only minor thickening of submucosa and superficial lesions, mainly

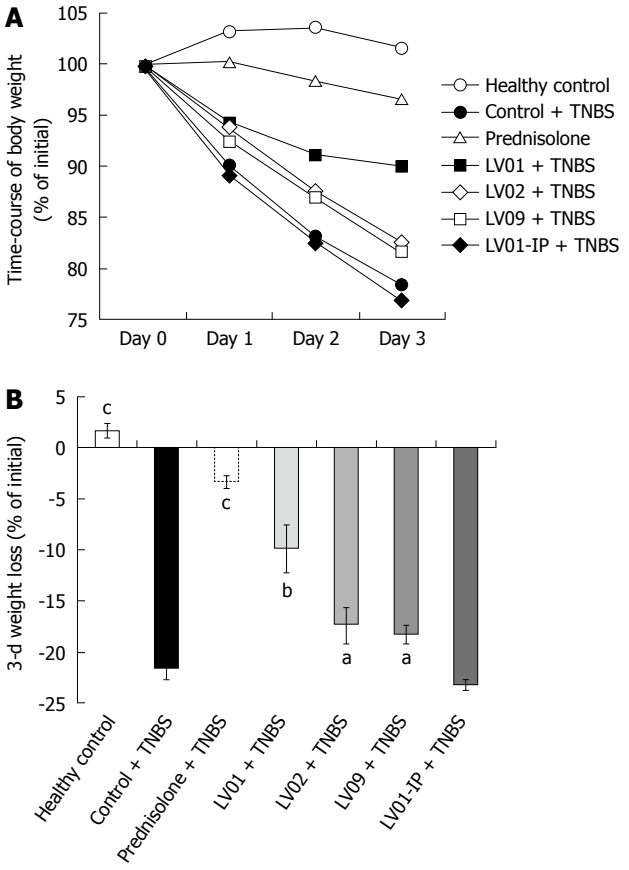

Figure 5 Changes in body weight during the preventive interventional study on experimental colitis. A: Time-course changes of weight for control mice and 2-4-6-trinitrobenzene sulfonic acid (TNBS)-treated mice, covering saline vehicle, prednisone and yeast pre-treated animals. Results are expressed as % of initial weight; SDs are not represented for clarity; B: Final weight loss 3 d after colitis. Results are expressed as mean ± SE (n = 10 per group), $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* TNBS-saline.

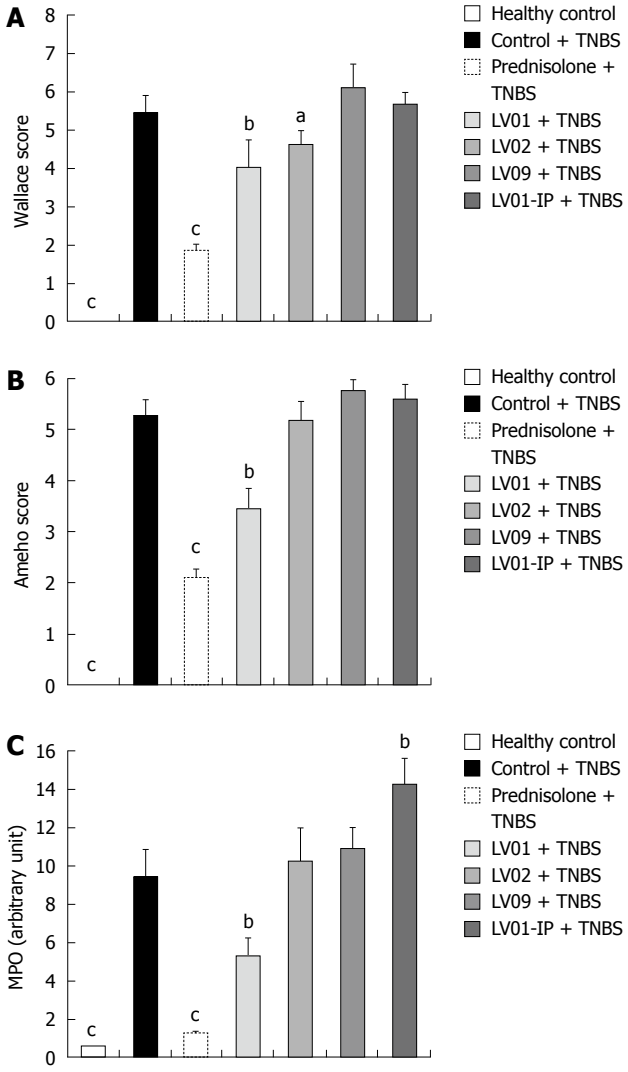

Figure 6 Effects of the various treatments 3 d after the TNBS-induced colitis on colonic macroscopic damage (Wallace) (A), histology scores (Ameho) (B) and myeloperoxidase (MPO) activity (C). Mice received intrarectally either saline or TNBS following oral pre-treatment by prednisone (10 mg/kg, 2 d), by various yeast strains for 5 d or after intra-peritoneal administration (IP) of the yeast at the onset of colitis. Results are expressed as mean ± SE (n = 10 per group), $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* TNBS-saline.

oedema, were seen in mice treated with the yeast LV01, in complete absence of necrosis. Erosions were more important in LV02-pretreatment, although necrosis was restricted to the upper luminal compartment and the crypt's architecture was still identifiable. Treatment with strain LV09 given orally and strain LV01 after intraperitoneal administration were characterized by prominent inflammatory infiltrates and transmural inflammation with necrosis reaching the deeper muscle layers.

The levels of the blood markers of inflammation SAA and IL-6, while significantly increased after TNBS administration, were strikingly reduced by prednisone and LV01 treatments, approaching baseline values ($P <$ 0.01). LV02 strain also minimized the pro-inflammatory cytokine IL-6 in the serum ($P < 0.05$), with a similar trend observed for SAA ($P = 0.055$, Figure 8).

In conclusion, TNBS caused increases in mucosal and systemic inflammatory symptoms that can be prevented by an orally administered anti-inflammatory drug (prednisone) as well as the food-grade yeast LV01. This was substantiated on the basis of reduction of body-weight loss, macroscopic damage, histological scores, colonic inflammatory infiltrates and serum markers. The yeast strain LV02 showed an intermediate protection capacity with sometimes partial and borderline effects in the degree of attenuation, depending on the marker considered. LV09 only lowered the weight loss with no improvement of other colitis-related symptoms. These results support the relevance of yeast for colitis protection, but distinct performance of various strains highly suggests strain-dependent mechanisms.

DISCUSSION

Evidence that yeasts can be used as a probiotic with therapeutic potential in IBD has emerged from clinical trials and experimental models of colitis in animals. However, these studies remained scarce and essentially used the strain *S. boulardii,* formerly *S. cerevisiae* var *boulardii*. In this study we aimed at questioning the differential anti-inflam-

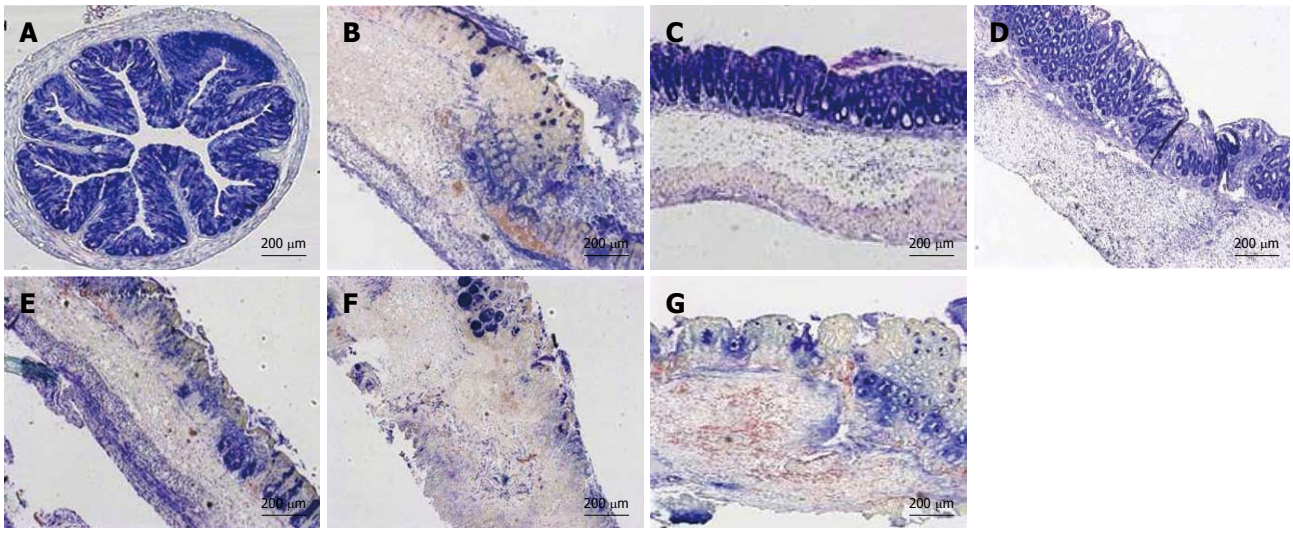

Figure 7 Histological features of yeast-mediated protection. The figure shows representative May-Grünwald and Giemsa-strained colon sections of control healthy mice (A) or day 3 after instillation of TNBS in Balb/c mice pre-treated with either vehicle (B), prednisone (C), LV01 (D), LV02 (E), LV09 (F) and LV01-IP (G). Original magnification, × 20.

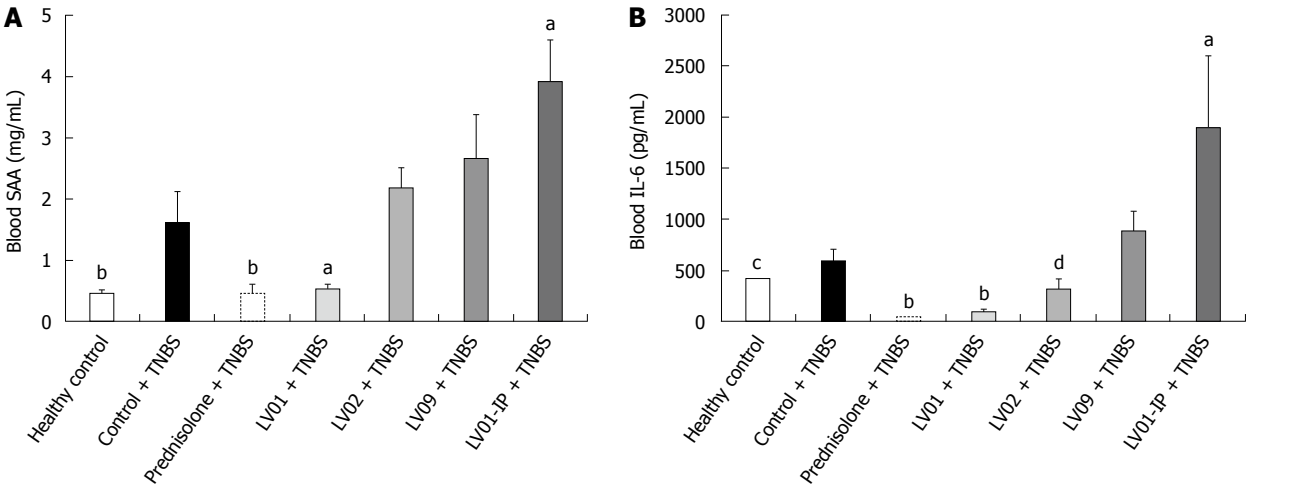

Figure 8 Effects of the various treatments 3 d after the TNBS-induced colitis on blood markers. A: Serum amyloid A protein (SAA); B: IL-6. Mice received intra-rectally either saline or TNBS following oral pre-treatment by prednisone (10 mg/kg, 2 d), by various yeast strains for 5 d or after intra-peritoneal administration (IP) of the yeast at the onset of colitis. Results are expressed as mean ± SE (n = 10 per group), $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $0.05 < {}^{d}P < 0.1$ *vs* TNBS-saline.

matory potential of other yeast strains. Whether food-borne strains of *S. cerevisiae* or other yeast species possess probiotic properties is of fundamental importance in developing, understanding and handling of new potential biotherapeutic agents, both concerning the pharmaceutical and the (functional) food industry. Attempts for such a screening of yeast biodiversity were already carried out *in vitro*[17,35,36], mainly by studying selection criteria such survival at low acid pH and tolerance to bile salts or adhesion on epithelial cells. The predictability for the *in vivo* situation, however, is limited, as the importance of colonization in strain efficacy remains to be determined *in vivo*[37]. An example was recently given with a *Lactobacillus* strain that fulfilled adhesion and survival criteria *in vitro* but was unable to persist in mice, although exerting immunomodulatory effects[38]. To our knowledge, the comparison of distinct yeast strains based on their ability to induce cytokine secretions by immunocompetent cells was never reported.

Results obtained in this study showed that, in contrast to bacteria[22,39,40], *in vitro* immunomodulation did not discriminate among various strains. This can be attributed to the more conserved cell-wall structure of the yeasts. However, yeast cells are not inert from an immunological point of view and substantial induction of IL-10, on PBMCs, associated with weak TNF and no release of IFNγ and IL-12, favor, in general, a more anti-inflammatory profile than bacteria. This is in agreement with very recent studies using germ-free mice[41].

The three strains used in the murine TNBS-induced colitis exhibited similar colonization patterns *in vivo*, thus allowing accurate comparisons. Standardization of experimental settings for all evaluations and, as far as possible into the same study, is highly recommended[42]. The

present work confirms that a yeast strain (*S. boulardii*) is able to significantly prevent symptoms in an acute experimental colitis in mice. The 30% protection observed, lower than the protection obtained by a traditional anti-inflammatory drug, is consistent with previous reports using the same yeast strain in a rat model of TNBS[13]. By contrast, in another study in rats, TNBS-induced inflammation was only weakly lowered with *S. boulardii* with the 18% trend, which did not passed the statistics test, while conventional IBD drugs and other probiotic preparations did show significance[43,44]. Such differences between studies may depend on experimental design including distinct outcomes when considering preventive *vs* therapeutic applications, as reported e.g. for bacteria in the TNBS colitis model[45], in the experimental DSS model[46] or even in clinical studies[47]. Hence, these observations emphasize the necessity to compare distinct treatments and strains in a single study. We enlightened here for the first time that, like probiotic bacteria, probiotic yeasts are not equivalent in their capacity to alleviate colitis, despite a similar colonization profile.

The "main contribution" of yeast-mediated anti-inflammatory effects is suggested to more address the intestinal barrier properties and epithelial cells rather than an immunocompetent cell-based immunity, because (1) protection triggered by yeasts *in vivo* is strain-dependent, whereas together with the immune screening *in vitro*, was poorly discriminating; and (2) the failure of *S. boulardii* to induce protection by the systemic route, in contrast to the oral route. Indeed, in contrast to some beneficial bacteria which are able to exert effects distantly from the inflammation site[33,34,48], emphasizing a possible role of migratory cells such as dendritic cells (DC)[31], yeast-mediated protection seems to take place predominantly in the intestinal mucosa. An intestinal barrier dysfunction in IBD is obvious, although it is not clear yet whether this is a cause or a consequence of the disease[49]. Prophylactic reinforcement and therapeutic restoration of barrier function by changing the luminal environment may successfully stimulate the mucosal barrier. Probiotics are elements of choice to normalize the barrier function, stabilize tight junction components[50] and limit translocation[51]. Trophic effects[52] as well as enhancement of epithelial integrity mediated by yeasts have been extensively described[4,7]. Reduction of bacterial translocation by oral treatment with yeasts in various models of sepsis also confirmed this functional aspect of probiotic-yeasts[53-55].

However, other mechanisms including direct yeast-DC interactions were also evoked to explain how *S. boulardii* exhibits its anti-inflammatory properties[56]. Stimulation may occur in Peyer's patches or in the lamina propria. This mechanism did not seem to be very strain specific and, paradoxically, even *Candida albicans* was recently reported to induce tolerogenic DC and to positively balance inflammation after adoptive transfer in a mice colitis model[57]. Similarly, soluble β-glucans derived from *C. albicans* were able to suppress the production of pro-inflammatory cytokines by PBMCs[58] while mannan from *S. cerevisiae* impaired the ability of macrophages to kill phagocytosed pathogens[59]. On the other hand, colonization of mice by *C. albicans* exacerbated DSS-colitis[60] and delayed healing of TNBS colitis[61]. Finally, intraperitoneal injection of zymosan, a polysaccharide cell wall component from *S. cerevisiae* has been widely used as a (self-resolving) model of acute inflammation[62]. By contrast, yeast extracts administered to mice *per os* resulted in a significant reduction of the *C. difficile*-toxin A-mediated COX-2 increase and in the toxin-mediated disruption of epithelial cells[63]. Whether yeast or yeast-derived molecules will have a pro- or anti-inflammatory role may thus greatly depend on the route of administration and consequently on the target cells and tissues.

Nevertheless, we can speculate that oral yeast-induced immunomodulation predominantly addresses intestinal permeability and, based on published data, the immune function of intestinal cells. This is consistent with clinical findings indicating that *S. boulardii* has a positive effect on the maturation of enterocytes, but only a minor influence on lymphocytes[64]. Our results suggest that further screening for probiotic yeasts may favor the use of epithelial cell or mucosa-integrated models, rather than immune-cell based approaches. This will help us to select yeast or fungal strains with important potential in inflammatory conditions or in a context where inflammation is concomitant with enteropathogens such *C. difficile* and *E. coli*[65]. Understanding how various strains perform *in vivo* will allow us to trace specific mechanisms and select or design optimal strains for specific infectious or inflammatory diseases, or even consider new probiotic applications[66]. As some of the yeast-based probiotic mechanisms seem to be distinct from the bacterial ones, possible synergy might be expected from mixing both types of probiotics[67]. This was previously shown in a preclinical study on traveler's diarrhea, where the combination of yeast and bacteria was found to be more effective than each probiotic alone[68]. Using probiotic *Saccharomyces spp.* alone or in combination with lactic acid bacteria will thus enhance and extend the actual biotherapeutic/probiotic approach in IBD together with conventional anti-inflammatory treatments. Lastly, the strain-specific differences seen in the anti-inflammatory capacity of yeasts may also be extended for clinical use against *Salmonella ssp.*, *C. difficile* and *Candida species*, requiring an optimal and controlled formulation of the probiotic product[69].

ACKNOWLEDGMENTS

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COMMENTS

Background

As for probiotic bacteria, probiotic yeast such *Saccharomyces boulardii*

(*S. boulardii*) can be effective in gastrointestinal pathologies such as inflammatory bowel disease (IBD) and bacterial- or enterotoxin-mediated diarrhea and inflammation. However, very few yeast strains have been studied as possible biotherapeutics agents. The underlying mechanisms of action of probiotics are not fully understood but there is some evidence that they exert their beneficial effect by multiple and specific modes of action depending on the type of the probiotic (bacteria, yeast), the species and strain.

Innovations and breakthroughs

The yeast-mediated capacity to lower inflammation is suggested to mainly involve epithelial cells and barrier function, as demonstrated by (1) the weakness of specific immunomodulatory response *in vitro*; and (2) the lack of protection by *S. boulardii* when administered by the systemic but not the oral route. In contrast with the performance of some probiotic bacteria which the authors previously showed to be directly correlated with their *in vitro* anti-inflammatory potential, the immunoregulatory mechanism of probiotic yeasts fails to directly act on the cytokine level.

Applications

As for probiotic bacteria, strain specificity also matters for yeast as for probiotics, but mechanisms, and consequently screening methods, partly differ between yeast and bacteria. A careful and appropriate selection of strains is then required to screen the biodiversity of yeasts for specific clinical studies including applications in IBD as well as for other dedicated therapeutic uses. This will impact on future product composition to enhance or synergize gastrointestinal related disorders by using probiotics.

Terminology

Probiotics are defined as living microorganisms that may have beneficial effects on health, including mainly food-grade bacteria and non-pathogenic yeasts.

Peer review

The paper by Benoit Foligné and co-workers investigated the anti-inflammatory effects of various yeasts strains in experimental colitis in mice. The overall design of the study is fair and the results are stimulating for future research.

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BRIEF ARTICLE

Prophylaxis for venous thromboembolism after resection of hepatocellular carcinoma on cirrhosis: Is it necessary?

Marco Vivarelli, Matteo Zanello, Chiara Zanfi, Alessandro Cucchetti, Matteo Ravaioli, Massimo Del Gaudio, Matteo Cescon, Augusto Lauro, Eva Montanari, Gian Luca Grazi, Antonio Daniele Pinna

Marco Vivarelli, Matteo Zanello, Chiara Zanfi, Alessandro Cucchetti, Matteo Ravaioli, Massimo Del Gaudio, Matteo Cescon, Augusto Lauro, Gian Luca Grazi, Antonio Daniele Pinna, Department of Surgery and Transplantation, University of Bologna, S. Orsola Hospital, Via Massarenti 9, 40138 Bologna, Italy

Eva Montanari, Institute of Forensic Medicine, University of Bologna, Via Irnerio 49, 40100 Bologna, Italy

Author contributions: Vivarelli M designed and wrote the study; Zanello M and Zanfi C collected the data; Cucchetti A performed the data analysis; Ravaioli M, Del Gaudio M, Cescon M and Lauro A participated in the data collection; Montanari E provided forensic expertise; Grazi GL reviewed the paper; Pinna AD coordinated the study.

Correspondence to: Dr. Marco Vivarelli, Department of Surgery and Transplantation, University of Bologna, S.Orsola Hospital, Via Massarenti 9, 40138 Bologna, Italy. marco.vivarelli@aosp.bo.it
Telephone: +39-51-6363721 Fax: +39-51-397661

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Abstract

AIM: To assess the safety and effectiveness of prophylaxis for venous thromboembolism (VTE) in a large population of patients with hepatocellular carcinoma (HCC) on cirrhosis.

METHODS: Two hundred and twenty nine consecutive cirrhotic patients with HCC who underwent hepatic resection were retrospectively evaluated to assess whether there was any difference in the incidence of thrombotic or hemorrhagic complications between those who received and those who did not receive prophylaxis with low-molecular weight heparin. Differences and possible effects of the following parameters were investigated: age, sex, Child-Pugh and model for end-stage liver disease (MELD) score, platelet count, presence of esophageal varices, type of hepatic resection, duration of surgery, intraoperative transfusion of blood and fresh frozen plasma (FFP), body mass index, diabetes and previous cardiovascular disease.

RESULTS: One hundred and fifty seven of 229 (68.5%) patients received antithromboembolic prophylaxis (group A) while the remaining 72 (31.5%) patients did not (group B). Patients in group B had higher Child-Pugh and MELD scores, lower platelet counts, a higher prevalence of esophageal varices and higher requirements for intraoperative transfusion of FFP. The incidence of VTE and postoperative hemorrhage was 0.63% and 3.18% in group A and 1.38% and 1.38% in group B, respectively; these differences were not significant. None of the variables analyzed including prophylaxis proved to be risk factors for VTE, and only the presence of esophageal varices was associated with an increased risk of bleeding.

CONCLUSION: Prophylaxis is safe in cirrhotic patients without esophageal varices; the real need for prophylaxis should be better assessed.

Key words: Hepatic surgery; Hepatocellular carcinoma; Liver cirrhosis; Postoperative bleeding; Postoperative thromboembolism; Venous thromboembolism prophylaxis

Peer reviewers: Dr. Bijan Eghtesad, Associate Professor, Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, United States; Itaru Endo, MD, PhD, Professor and Chairman, Department of Gastroenterological Surgery, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 2360004, Japan

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INTRODUCTION

Venous thromboembolism (VTE) is a significant cause of morbidity and mortality in patients who have undergone open gastrointestinal surgery, particularly if they were operated on due to malignancy[1,2].

Hepatocellular carcinoma (HCC) is the most frequent primary neoplasm of the liver and often develops as a consequence of chronic liver disease[3]. It is well known that several hemostatic alterations are present in patients with liver disease: primary hemostasis is often impaired due to piastrinopenia and secondary hemostasis can be hampered by the reduced synthesis of coagulation factors that normally takes place in the liver[4]. These alterations can be worsened by the decrease in hepatic volume caused by hepatic resection. A historical series of cirrhotic patients undergoing liver resection reported an 8.4% incidence of hemorrhagic complications following surgery[5].

While there is currently agreement on the need for pharmacological prophylaxis of VTE in surgical patients in general, little is know about the effect of this prophylaxis in patients with chronic liver disease who undergo hepatic resection due to the presence of HCC.

Studies on the coagulative pattern have shown that in patients with chronic liver disease there is decreased production of natural anticoagulant proteins that could result in an increase in the risk of thrombotic events; however, in clinical studies the incidence of VTE in cirrhotic patients seems to be lower than in the general medicine population[6].

Although great advances have taken place in the field of hepatobiliary surgery, resection of a cirrhotic liver remains a challenging procedure and hepatic surgeons may not feel confident in administering antithromboembolic prophylaxis to patients who have an increased risk of bleeding.

In our centre, where a large number of hepatic resections are performed, we have more and more frequently adopted a scheme of prophylaxis with low-molecular-weight heparin, however, we have no clear-cut parameters to refer to when deciding whether to administer prophylaxis or not and a significant number of patients are not given prophylaxis due to the risk of bleeding.

The aim of the present study was to assess the possible effect of different prophylactic strategies in the prevention of venous thrombosis in a large series of cirrhotic patients who underwent hepatic resection for HCC.

MATERIALS AND METHODS

The records of 229 consecutive patients with chronic liver disease who underwent hepatic resection due to the presence of HCC between January 1999 and December 2008 were retrospectively reviewed to ascertain whether there was any difference in the incidence of venous thromboembolic or hemorrhagic complications between those patients who received venous thrombosis prophylaxis and those who did not.

Table 1 Baseline characteristics of the study population (mean ± SD) *n* (%)

Variables	All patients (*n* = 229)	Group A (*n* = 157)	Group B (*n* = 72)	*P* value
Age (yr)	65.0 ± 9.3	65.0 ± 9.8	63.0 ± 9.5	0.080
Gender (M/F)	171 (74.7)/ 58 (25.3)	119 (76.0)/ 38 (24.0)	52 (72.0)/ 20 (28.0)	0.330
Etiology of cirrhosis				0.200
Hepatitis C	148 (64.6)	106 (67.0)	42 (58.3)	
Hepatitis B	37 (16.2)	21 (14.0)	16 (22.2)	
Hepatitis B + C	15 (6.6)	7 (5.0)	8 (11.1)	
Non-viral	29 (12.7)	23 (14.0)	6 (8.3)	
Child-Pugh score				0.005
A	218 (95.2)	154 (98.0)	64 (89.0)	
B	11 (4.8)	3 (2.0)	8 (11.0)	
C	0			
MELD score	8.8 ± 1.5	8.5 ± 1.2	9.3 ± 1.9	0.001
Platelet count (× 10^3)	139 ± 60	150 ± 60	115 ± 51	0.001
Esophageal varices	63 (28.0)	34 (22.0)	29 (40.3)	0.003

MELD: Model for end-stage liver disease.

All patients in the present series had the diagnosis of chronic liver disease confirmed by histology which was carried out on the resected specimen. Etiology of liver disease was predominantly viral (87.3% of cases) (Table 1).

Preoperative Child-Pugh-Turcotte score[7] and model for end-stage liver disease (MELD)[8] score were available for all patients; other parameters that were taken into account to determine the severity of portal hypertension were platelet count and presence of esophageal varices. Two hundred and eighteen patients (95.2%) had class A liver disease according to the Child-Pugh scoring system, while 11 (4.8%) had class B and none had class C disease; the mean MELD score of the patients was 8.8 ± 1.5.

Body mass index (BMI), diabetes mellitus and clinical history of cardiovascular disease were recorded as possible risk factors for thromboembolism. Type of surgical resection (minor when one or less and major when more than one hepatic segment was resected), duration of surgery, intraoperative transfusion of blood and fresh frozen plasma (FFP) were also recorded (Tables 2 and 3). Minor hepatic resection was carried out in 219 cases (95%), while major hepatic resection was performed in 10 cases (5%).

A protocol for general surgical procedures for the prevention of venous thrombosis using low-molecular-weight heparin prophylaxis was always observed in the period considered here. In the case of hepatic resection in cirrhotic patients the decision on whether to use prophylaxis was left to the judgment of the surgeon who performed the resection based on preoperative coagulation tests and intraoperative findings. For those patients

Table 2 Details of surgery performed in the two groups

Variables	All patients (*n* = 229)	Group A (*n* = 157)	Group B (*n* = 72)	*P* value
Type of resection *n* (%)				0.610
Minor	219 (95.0)	150 (95.5)	69 (95.8)	
Major	10 (5.0)	7 (4.5)	3 (4.2)	
Duration of surgery (mean ± SD)	266 ± 99	261 ± 106	275 ± 83	0.320
i.o. blood transfusion [mL, median (range)]	128.2 (0-1500)	124.7 (0-1500)	154.1 (0-1200)	0.346
i.o. FFP transfusion [mL, median (range)]	175.7 (0-1200)	139.5 (0-1200)	266.6 (0-1200)	0.003

i.o.: Intraoperative; FFP: Fresh frozen plasma.

who were administered anticoagulant prophylaxis, this consisted of nadroparin calcium 0.3 mL or enoxaparin sodium 0.4 mL subcutaneously, starting from the day of surgery and continued for at least 7 d or until the patient was actively ambulant.

Prophylaxis was stopped when hemorrhagic complications developed.

Independent of the use of low-molecular-weight heparin, all patients had mechanical prophylaxis by means of anti-embolism stockings.

Patient follow-up included evaluation at the outpatient clinic on days 7 and 30 and months 3, 6, 9 and 12 after discharge from the Hospital.

VTE was defined as the symptomatic or asymptomatic occurrence of deep vein thrombosis confirmed by Doppler ultrasonography or venography; pulmonary embolism was confirmed by helical computed tomography.

Hemorrhagic complications were defined as follows: (1) Blood loss from surgical drainage associated with a significant drop in hemoglobin levels (> 1.5 g/dL from the last control); (2) Intraabdominal fluid collection with density at CT compatible with blood of diameter > 3 cm; and (3) Bleeding from the upper or lower gastrointestinal tract.

The analysis of risk factors for VTE included the following parameters: (1) age; (2) sex; (3) etiology of chronic liver disease; (4) Child-Pugh score; (5) MELD score; (6) presence of esophageal varices; (7) platelet count; (8) BMI; (9) chronic heart disease; (10) diabetes; (11) extent of surgical resection (major when more than 1 hepatic segment was resected); (12) duration of surgery; (13) intraoperative requirement for blood or FFP transfusion; and (14) prophylaxis with low-molecular weight heparin.

Statistical analysis

Continuous variables are reported as mean ± SD or in median and range on the basis of parametric assumption; differences between subgroups were investigated with Levene's test for equality of variances and compared with the Student *t*-test or Mann-Whitney test as appropriate. Categorical variables were reported in a number of cases and prevalence and differences in subgroups were compared using the χ^2 test with Yates correction. Univariate logistic regression analysis was applied in order to investigate risk factors for thrombosis or hemorrhage. *P*-values less than 0.05 were considered statistically significant in all the analyses. Statistical analysis was performed using the SPSS for Windows package (Version 10.0).

Table 3 Specific risk factors for venous thromboembolism in the two groups *n* (%)

Variables	All patients (*n* = 229)	Group A (*n* = 157)	Group B (*n* = 72)	*P* value
BMI > 30 kg/m^2	36 (17.1)	26 (17.8)	10 (15.4)	0.410
Diabetes	47 (20.5)	33 (21.0)	14 (19.4)	0.460
Cardiovascular disease	56 (24.5)	43 (27.0)	13 (18.1)	0.080

BMI: Body mass index.

RESULTS

One hundred and fifty-seven of the 229 (68.5%) patients received antithromboembolic prophylaxis (Group A) while the remaining 72 (31.5%) patients did not (Group B). The proportion of patients who received prophylaxis significantly varied with time: prophylaxis was given to 48 of 99 (48.4%) patients in the period 1999-2003 and to 109 of 130 (83.8%) patients in the period 2004-2008 (*P* = 0.001).

There was no difference in age, gender and etiology of liver disease between the 2 groups, while patients in group B had higher Child-Pugh and MELD scores, lower platelet counts and a higher prevalence of esophageal varices (Table 1).

Extent and duration of the hepatic resection and intraoperative blood transfusions were similar in the 2 groups, while in group B there was a significantly higher requirement for intraoperative transfusion of FFP (Table 2). This latter finding might indicate a higher bleeding tendency observed by the surgeon in the operative field, which resulted in the administration of greater amounts of FFP.

As regards the specific risk factors for venous embolism that were considered, there was no difference between the 2 groups (Table 3).

Two cases (0.87%) of deep venous thrombosis were observed: these were one case of pulmonary embolism secondary to a deep vein thrombosis of the leg in a patient who was receiving prophylaxis and one case of total portal vein thrombosis in a patient who did not receive prophylaxis: this latter patient had a Child score of A6, a MELD score of 13 with a low platelet count (113.000/mL) an INR = 1.3 and no esophageal varices, and underwent wedge resection and died of hepatic failure. The incidence of VTE was therefore 1.38% in patients who did not receive prophylaxis and 0.63% in those patients who were treated with low-molecular-weight heparin (*P* = 0.530).

Six cases of hemorrhagic complications were observed (2.62%); 5 of these cases occurred in those 157

Table 4 Analysis of possible risk factors for venous thromboembolism

Variable	Exp (B)	95% CI	*P* value
Child-Pugh score (score A *vs* B)	0.004	0.00-15.32	0.906
MELD score	1.748	0.96-3.16	0.132
Type of resection (minor *vs* major)	0.004	0.00-17.52	0.914
Platelet count	0.990	0.99-1.00	0.776
Esophageal varices	0.001	0.00-18.22	0.525
BMI	0.001	0.00-11.45	0.687
Diabetes	0.001	0.00-12.45	0.631
Cardiovascular disease	0.001	0.00-19.31	0.570
Duration of surgery	0.997	0.98-1.01	0.720
i.o. blood transfusion (mL)	1.000	0.99-1.00	0.490
i.o. FFP transfusion (mL)	1.001	0.99-1.00	0.518
VTE prophylaxis	0.455	0.02-7.38	0.531

Table 5 Analysis of possible risk factors for hemorrhagic complications

Variable	Exp (B)	95% CI	*P* value
Child-Pugh score (score A *vs* B)	0.004	0.00-23.72	0.742
MELD score	1.280	0.83-1.95	0.252
Type of resection (minor *vs* major)	0.004	0.00-20.75	0.763
Platelet count	0.987	0.99-1.02	0.445
Esophageal varices	5.559	0.99-31.14	0.050
BMI	0.000	0.00-27.45	0.389
Diabetes	0.000	0.00-24.28	0.248
Cardiovascular disease	1.564	0.27-8.77	0.454
Duration of surgery	0.997	0.98-1.00	0.460
i.o. blood transfusion (mL)	1.001	0.99-1.00	0.315
i.o. FFP transfusion (mL)	1.002	0.98-1.00	0.073
VTE prophylaxis	0.443	0.26-20.42	0.387

VTE: Venous thromboembolism.

patients who received prophylaxis (prevalence: 3.18%) and one in patients who did not receive prophylaxis (prevalence: 1.38%); the difference was not significant ($P = 0.380$). The hemorrhagic complications consisted of prolonged blood leakage from the surgical drains requiring blood transfusion (3 cases), intraperitoneal blood collection (2 cases) and gastric bleeding (1 case). Only 1 of the above-mentioned cases required invasive intervention (CT-guided percutaneous drainage of intraperitoneal collection).

None of the considered risk factors proved to be significantly associated with VTE in the univariate analysis, and only the presence of esophageal varices was linked to an increased risk of hemorrhagic complications (Tables 4 and 5).

DISCUSSION

Despite the clinical relevance of this topic, to our knowledge no reports have been published to date on the use of venous thrombosis prophylaxis in cirrhotic patients undergoing hepatic resection for HCC. The absence of clear guidelines for prophylaxis of thromboembolism in the specific setting of hepatic surgery in patients such as those with chronic liver disease who are known to have imbalances in coagulative function may induce those surgeons with less experience in hepatobiliary surgery to avoid prophylaxis for fear of hemorrhagic complications; on the other hand, administering prophylaxis to patients with severe coagulative impairment and severe portal hypertension possibly following an extensive liver resection should be done on more solid grounds than simple adherence to existing guidelines that were drawn up for open abdominal surgery. In both cases, should thromboembolic or hemorrhagic complications develop, then the possibility of a malpractice accusation would be consistent[9].

In our experience, we found a particularly low incidence of deep vein thrombosis in cirrhotic patients who underwent liver resection, which could be partially justified by the absence of specific postoperative screening; however, at our center, HCC patients are always closely monitored with follow-up after resection and we can therefore exclude the fact that major thrombotic complications took place after hospital discharge which do not show up in the clinical records. Of note, all the patients in this report received prophylaxis with a mechanical method (anti-embolism stockings) which, together with the peculiarities of the study population, may have played a role in keeping the rate of VTE particularly low.

Hemorrhagic complications after liver resection are often poorly defined in specific reports, and the impression is that only those that require invasive maneuvers are usually reported; nevertheless, the incidence of these complications after resection in cirrhotic patients ranges between 1% and 8%[5,10,11]. The hemorrhagic complications reported in the present study were mainly represented by prolonged blood leakage from the surgical drains requiring blood transfusion. Although a trend towards a higher incidence of hemorrhage following resection was observed in those patients who received prophylaxis, the difference between the 2 groups was not significant.

Prophylaxis was withheld mainly in those patients who had a higher Child-Pugh or MELD score, lower platelet count and higher prevalence of esophageal varices; however, the overall increase in prophylaxis administration over time reflects an increase in confidence in handling it.

The surgeon performing the hepatic resection can assess some elements such as the bleeding tendency observed in the operative field and the degree of portal hypertension as revealed by the number and size of hepatofugal collateral veins, which can provide information that goes beyond what can be assessed with laboratory and imaging screening. However, the categorization of these elements largely depends on the degree of experience and confidence of the surgeon, and their effective relationship with the risk of bleeding in the postoperative period is difficult to demonstrate.

Similarly, in the cirrhotic patient, the common coagulative tests such as prothrombin time or INR poorly predict the effective risk of bleeding: in fact, these tests

only measure the activity of procoagulant factors while they ignore possible changes in the activity of anticoagulant factors that, in liver disease, are also deficient[12].

In our study, a greater amount of FFP was administered to those patients who did not receive prophylaxis, which might reflect a bleeding tendency that was observed at surgery. We believe that, unless clear guidelines are validated by large clinical trials, antithromboembolic prophylaxis should probably be avoided in those patients who are judged at high risk of bleeding by the surgeon.

Despite their low incidence in this series, the thromboembolic complications seen after surgery were life-threatening, however, we did not experience similar serious hemorrhagic complications. In the absence of more reliable tools to quantify the probability of bleeding it seems reasonable to avoid prophylaxis when portal hypertension is present or significant bleeding is observed at surgery.

Further prospective studies are necessary to establish guidelines for application in this specific setting.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most common primary neoplasm of the liver and often arises in the context of a chronic liver disease that impairs coagulative function. Surgical resection is the best option to cure HCC, however, surgery on cirrhotic liver may increase the risk of bleeding. Despite the clinical relevance of the matter, no guidelines are available on the administration of antithromboembolic prophylaxis in cirrhotic patients undergoing hepatic resection.

Innovations and breakthroughs

The study shows that the risk of venous thromboembolism after hepatic resection in cirrhotic patients is low. A trend towards an increased incidence of postoperative hemorrhage was observed in patients who received prophylaxis. Portal hypertension, as demonstrated by the presence of esophageal varices, is significantly associated with the risk of bleeding.

Applications

The absence of guidelines for prophylaxis of thromboembolism specifically aimed at patients with chronic liver disease undergoing hepatic surgery can lead to the generalized application of prophylactic schemes which are used in the setting of open abdominal surgery, thus increasing the risk of bleeding. Conversely, to withold prophylaxis might increase the risk of thromboembolism. Both these policies can be dangerous and lead to malpractise accusations. Prospective studies are necessary to establish specific guidelines.

Peer review

This is a good manuscript to get published but needs some more information. The manuscript is well written and would be helpful in the field of hepatic surgery.

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BRIEF ARTICLE

Impact of bolus volume on small intestinal intra-luminal impedance in healthy subjects

Nam Q Nguyen, Laura K Bryant, Carly M Burgstad, Robert J Fraser, Daniel Sifrim, Richard H Holloway

Nam Q Nguyen, Carly M Burgstad, Richard H Holloway, Department of Gastroenterology & Hepatology, Royal Adelaide Hospital, Adelaide 5000, Australia
Laura K Bryant, Robert J Fraser, Investigation and Procedures Unit, Repatriation General Hospital, Daw Park 5041, Australia
Daniel Sifrim, Department of Medicine, Centre for Gastroenterological Research, K.U. Leuven, Leuven 3000, Belgium
Author contributions: Nguyen NQ was responsible for study design, data interpretation, statistical analysis and drafting of the manuscript; Holloway RH and Fraser RJ contributed equally to study conception and design, data interpretation and manuscript completion; Bryant LK and Burgstad CM were involved in data acquisition, analysis and manuscript revision; Sifrim D was involved in methodological design and interpretation of data; all authors read and approved the final manuscript.
Supported by The National Health and Medical Research Council of Australia
Correspondence to: Dr. Nam Q Nguyen, Department of Gastroenterology & Hepatology, Royal Adelaide Hospital, North Terrace, Adelaide 5000, Australia. quoc.nguyen@health.sa.gov.au
Telephone: +61-8-82224000 Fax: +61-8-82225885
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Abstract

AIM: To assess the impact of bolus volume on the characteristics of small intestinal (SI) impedance signals.

METHODS: Concurrent SI manometry-impedance measurements were performed on 12 healthy volunteers to assess the pattern of proximal jejunal fluid bolus movement over a 14 cm-segment. Each subject was given 34 boluses of normal saline (volume from 1 to 30 mL) *via* the feeding tube placed immediately above the proximal margin of the studied segment. A bolus-induced impedance event occurred if there was > 12% impedance drop from baseline, over ⩾ 3 consecutive segments within 10 s of bolus injection. A minor or major impedance event was defined as a duration of impedance drop < 60 s or ⩾ 60 s, respectively.

RESULTS: The minimum volume required for a detectable SI impedance event was 2 mL. A direct linear relationship between the SI bolus volume and the occurrence of impedance events was noted until SI bolus volume reached 10 mL, a volume which always produced an impedance flow event. There was a moderate correlation between the bolus volume and the duration of impedance drop (r = 0.63, P < 0.0001) and the number of propagated channels (r = 0.50, P < 0.0001). High volume boluses were associated with more major impedance events (⩾ 10 mL boluses = 63%, 3 mL boluses = 17%, and < 3 mL boluses = 0%, P = 0.02).

CONCLUSION: Bolus volume had an impact on the type and length of propagation of SI impedance events and a threshold of 2 mL is required to produce an event.

Key words: Bolus volume; Health; Impedance; Luminal flow; Small intestine

Peer reviewer: Ming Li, Associate Professor, Tulane University Health Sciences Center, 1430 Tulane Ave Sl-83, New Orleans, LA 70112, United States

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INTRODUCTION

Absorption of nutrients is the main function of the

small intestine and requires not only normal mucosal integrity but also normal motor function[1-4]. Adequate mixing and proper transit of chyme are important in ensuring optimal absorption[3,4]. Accurate assessment of small bowel motor function is important for understanding the physiology of the gastrointestinal tract, as well as assessment of clinical disorders of the intestine[1,5-7]. Currently, several tests are available for the assessment of intestinal motility and transit. Although intra-luminal manometry detects contractile patterns, it provides only indirect data regarding flow[5]. Similarly, although intestinal transit can be measured by conventional scintigraphy and more recently by breath tests, these methods detect only total transit time and not patterns of flow; transit can be affected by gastric emptying rate and conditions such as bacterial overgrowth[5]. Fluoroscopic studies, on the other hand, are able to assess overall intestinal motility and bolus transit, but are greatly limited by the exposure of patients to radiation[5].

In combination with manometry, multiple intra-luminal impedance (MII) has been introduced as a technique to assess motility and bolus transit concurrently[8-10]. Although this combined technique has been validated and applied extensively in the study of oesophageal physiology and disease[11-14], few data are available regarding its use in the evaluation of small bowel motor function[15-17]. Recently, detailed analysis from a study using combined video-fluoroscopy, manometry and MII showed that an impedance drop of > 12% from the baseline which propagated over 5 cm is associated with a flow event as seen on fluoroscopy[17]. Furthermore, various patterns of impedance flow events during fasting and in the postprandial state have been reported in the proximal intestine of healthy subjects[16]. This study, however, did not have concurrent manometric assessment and it is unclear whether the variation in impedance patterns of flow was influenced by intestinal motor activity or volume of the transported bolus or chyme. Knowledge on the relationship between bolus volume and impedance changes in the oesophagus[18] allows investigators to use intra-luminal impedance to assess oesophageal flow and clearance, without the need for radiological assessment. Corresponding data for the small intestine, however, are lacking. The aim of the current study was to assess the impact of bolus volume on the characteristics of small intestinal (SI) impedance signals in healthy humans.

MATERIALS AND METHODS

Subjects

Studies were performed in 12 healthy subjects (6 males; age: 53 ± 6 years; body mass index: 24.5 ± 1.1 kg/m^2) at the Department of Gastroenterology and Hepatology, Royal Adelaide Hospital. Exclusion criteria included previous or current gastrointestinal symptoms or surgery, evidence of acute or chronic illness and medications known to influence gastrointestinal motor function. The protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital and all subjects gave written informed consent.

SI manometry and impedance recording

SI motility and intraluminal electrical impedance were recorded concurrently using (1) a 110-cm perfused multilumen manometric assembly (DentSleeve Pty Ltd., Wayville, Australia); and (2) an impedance catheter (Sandhill Scientific, Highland Ranch, CO, USA).

Intestinal motility was recorded using an assembly comprising 11 pressure recording side holes: eight spaced at 2 cm intervals and the following three at 10 cm intervals, from the catheter tip. All manometric lumina were perfused with degassed distilled water at a rate of 0.15 mL/min, by a pneumo-hydraulic capillary perfusion pump (Dentsleeve).

SI luminal flow was recorded using an electrode with eight impedance rings (4 mm in length) spaced 2 cm apart, enabling 7 consecutive recording segments over a distance of 14 cm (Z1 to Z7; proximal to distal, respectively). Each segment straddled a corresponding manometric side hole and was activated by a high frequency (1 kHz) low amplitude (< 6 μA) alternating current.

Manometric and impedance signals were recorded simultaneously using a specialised computer system (Insight Acquisition, Sandhill Scientific) and displayed and stored on a personal computer for subsequent display and analysis.

Duodenal feeding tube

An 18F feeding tube was used to deliver the liquid boluses. The tube was attached to the manometry-impedance assembly and was positioned so that its tip was 1-cm above the most proximal impedance recording segment, Z1. The assembly configuration is outlined in Figure 1.

Protocol

All subjects were studied after an overnight fast. The assembly was passed into the stomach to a distance of 65 cm, through an anaesthetized nostril. The assembly was then allowed to migrate naturally into the duodenum *via* peristalsis, which was monitored continuously by measurements of the antro-duodenal trans-mucosal potential difference (TMPD). The final catheter position was achieved when the most proximal manometric side-hole was located in the duodenum, confirmed by a TMPD reading ⩾ -15 mV[19]. In this position, the combined manometry-impedance recording segment would be expected to lie in the proximal jejunum and at least 20 cm distal to the pylorus. Radiological confirmation of the catheter position was not performed due to the concern of unnecessary radiation exposure to healthy subjects.

Once the assembly was positioned in the proximal jejunum, subjects were positioned in a 30 degree head-up, supine position. During phase I of the interdigestive migrating motor complex (MMC), a total of 34 boluses of saline (0.9%) were given to each subject *via* the feeding tube. Five boluses for each volume of 1, 2 and 3 mL

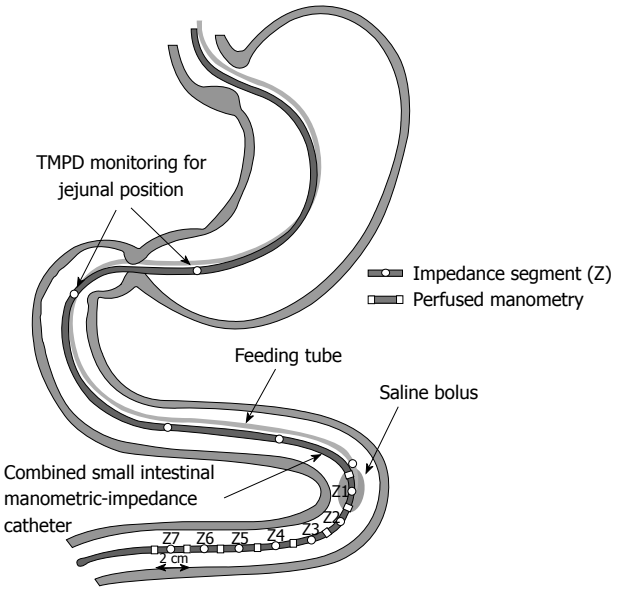

Figure 1 Outline of recording assembly, consisting of combined manometry and impedance catheters and feeding tube, positioned within the proximal small intestine.

were given at 2-min intervals. Five boluses for each volume of 5, 10 and 15 mL were given at 5-min intervals. Two boluses for each volume of 20 and 30 mL were given at 10-min interval. Each bolus was infused at the maximal rate allowed by the assembly lumen.

Data analysis

Manometry: Phase I of MMC was defined as a quiescent period without any intestinal motor activity, and phase II as a period of irregular intestinal motor activity[5,19]. A SI pressure wave was defined as a pressure rise ≥ 6 mmHg from baseline and lasting between 0.8 and 7 s[19]. Pressure waves in adjacent channels were regarded as temporally related if their onsets were within 3 s, and "propagated" if there were ≥ 3 temporally-related sequential pressure waves[19].

Impedance: The recordings were analysed manually using the impedance analysis software (Bioview Analysis, Sandhill Scientific). An impedance event judged to be associated with flow was defined as having an impedance drop of > 12% below the baseline and propagated over 3 or more impedance segments[17]. The impedance baseline for which the percentage drop was based on was the average impedance value over the 5 s immediately before the impedance drop. For each impedance event, the following variables were characterized: (1) the baseline impedance value (5 s) before injection of the bolus; (2) the magnitude of impedance drop, including minimum, average and maximum values; (3) the number of propagated channels; and (4) total bolus clearance time (TBCT), which was defined as the time taken (in seconds) for the bolus to traverse the whole recording segment in the intestine. TBCT was measured from the time the bolus entered the most proximal intestinal recording segment (Z1) until it cleared the most distal recording segment (Z7). Impedance events with TBCT greater than 60 s were classified as 'major' events (Figure 2). For each tested bolus, an impedance event was deemed to be associated with the tested bolus if the event occurred within 10 s from the administration of the bolus and an absence of corresponding motility. Boluses that had the associated impedance signal interfered by movement artefacts, or that coincided with intestinal contractions at the time of bolus administration were excluded from the final analysis as it was not possible to confidently determine whether the impedance changes in these events were related to the bolus administration, the movement artefact or coincident intestinal contractions.

Statistical analysis

Data are presented as mean ± SE. Categorical data were compared by chi-square test with Yates' correction and continuous data by Student's *t*-test, using GraphPad Prism 4 (v 4.02, San Diego, CA, USA) statistical software. The relationships between bolus volume and impedance flow events, magnitude of impedance drop, number of propagated channels and TBCT were determined by repeated measures ANOVA and Pearson's linear regression analysis. Significance was accepted at a P value < 0.05.

RESULTS

Relationship between bolus volume and impedance events

Injection of a saline bolus at the proximal end of the recording segment induced a prompt impedance drop that propagated distally. A minimum volume of 2 mL was required to induce a recognizable intestinal impedance flow event (Figure 3). As the bolus volume increased from 2 to 10 mL, the proportion of detectable intestinal impedance events increased in a linear relationship. A bolus volume of ≥ 10 mL always produced an impedance event.

Relationship between bolus volume and magnitude of impedance drop

The mean baseline impedance value was 389 ± 58 ohms. There were no significant differences in the baseline impedance values among the different bolus volumes. The mean impedance drop for a detectable flow event was 151 ± 4 ohms. The median impedance drop from baseline associated with a detectable flow event was 27% (IQR: 12%-53%). There was a weak relationship between the bolus volumes and the magnitude of impedance drop (mean: $r = 0.33$, $P < 0.0001$; minimum: $r = 0.28$, $P < 0.000$; maximum: $r = 0.30$, $P < 0.01$) (Figure 4A).

Relationship between bolus volume and number of propagated channels

Boluses with a volume of ≥ 5 mL propagated over a longer distance than those with a volume < 5 mL. Once the bolus volume was ≥ 10 mL, an impedance drop always propagated over at least 7 channels (i.e. 14 cm) (Figure 3). Up to a bolus volume of 10 mL, there was

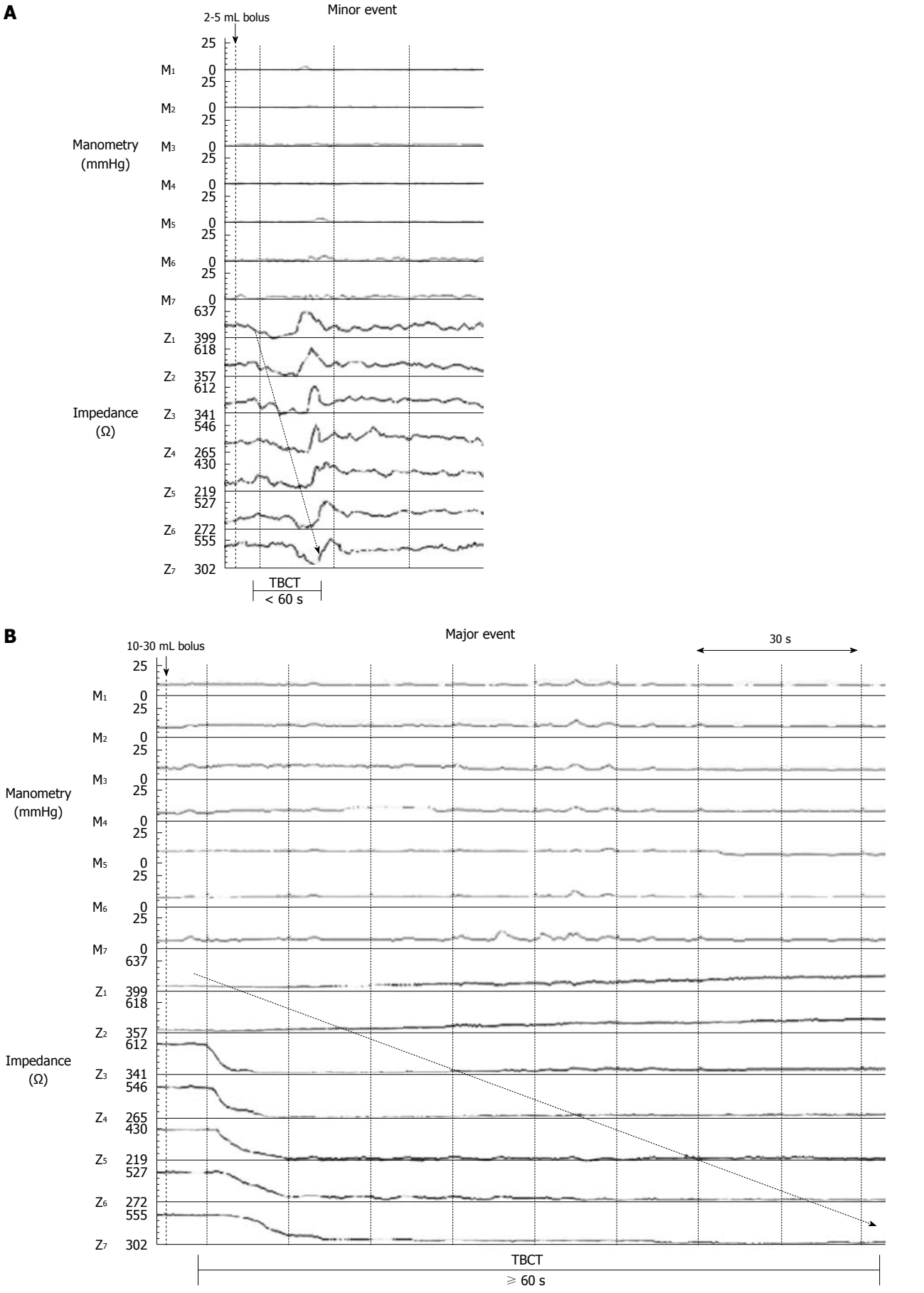

Figure 2 An example of combined manometry-impedance recording, demonstrating the patterns and the definition of total bolus clearance time (TBCT) for minor and major flow events.

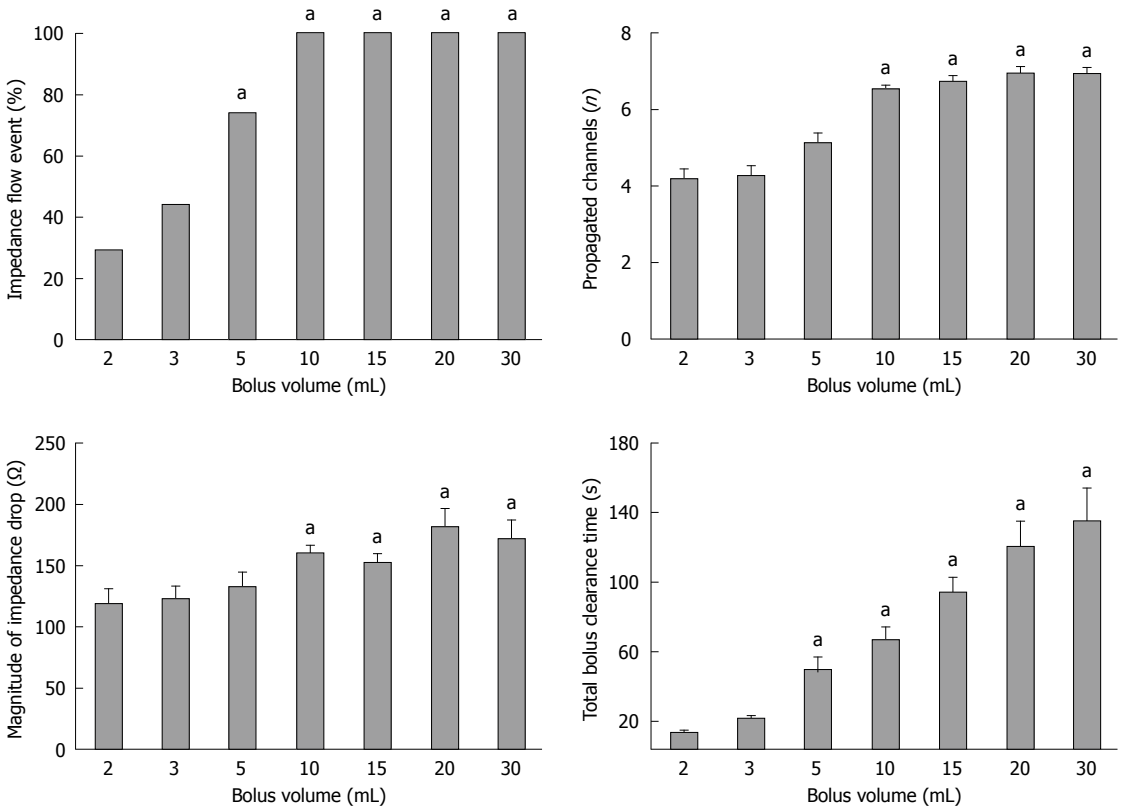

Figure 3 Relationship between bolus volume and characteristics of the associated impedance changes. [a]*P* < 0.05 *vs* 2 mL boluses.

a strong correlation between the bolus volume and the number of channels in which an impedance drop occurred ($r = 0.71$, $P < 0.00001$).

Of the administered test boluses, 48% ± 5% were followed by an intestinal pressure wave that propagated over 6.0 ± 0.1 channels. The duration between the bolus administration and the initiation of the propagated pressure wave was 20 ± 2 s. There was a positive correlation between the bolus volume and proportion of boluses followed by a propagated pressure wave ($r = 0.54$, $P < 0.0001$), which was significantly greater for bolus volumes ⩾ 15 mL (66% ± 12% *vs* 30% ± 11%, $P = 0.04$; *vs* 2 mL, respectively, Figure 4B). The bolus volume was also positively correlated with the duration between the bolus administration and the initiation of the propagated pressure wave ($r = 0.53$, $P < 0.001$).

Relationship between bolus volume and TBCT

The baseline impedance level was recovered after all bolus-induced drops, indicating bolus clearance from the impedance segment. For the majority of boluses ⩽ 5 mL, the impedance drop recovered spontaneously without an intestinal clearance pressure wave, with a TBCT of < 60 s (Figure 3). In contrast, for the majority of boluses ⩾ 10 mL, the recovery of impedance signal was more likely to require the assistance of an intestinal clearance pressure wave. Overall, there was a direct relationship between bolus volume and TBCT ($r = 0.63$, $P < 0.0001$). With increasing bolus volume, the proportion of boluses with a TBCT > 60 s also significantly increased ($P < 0.01$, Figure 4C).

DISCUSSION

This is the first methodological study to examine the relationship between bolus volume and impedance changes in the proximal small intestine. In particular, the study examined the limit of detection, and thus sensitivity, of impedance changes for identifying bolus volume. These data will enable the application of impedance as a technique to assess intestinal flow, without radiology. The main findings were (1) a bolus volume of at least 2 mL is required to generate an impedance event; (2) there is a linear relationship between bolus volume (between 2 and 10 mL) and impedance signal; (3) volumes of 10 mL or greater always generate an impedance event; and (4) bolus volume positively correlates with the magnitude of impedance drop, distance of propagation and clearance time. This modest relationship between bolus volume and intestinal impedance signals may explain, at least in part, the various patterns of flow events associated with chyme transport in the small intestine.

The threshold volume for bolus detection by impedance appears to be higher in the small intestine compared with that previously reported in the oesophagus[18]. However, the relationship between volume of liquid boluses and impedance signal observed in the proximal intestine does not exist in the oesophagus[18]. These differences

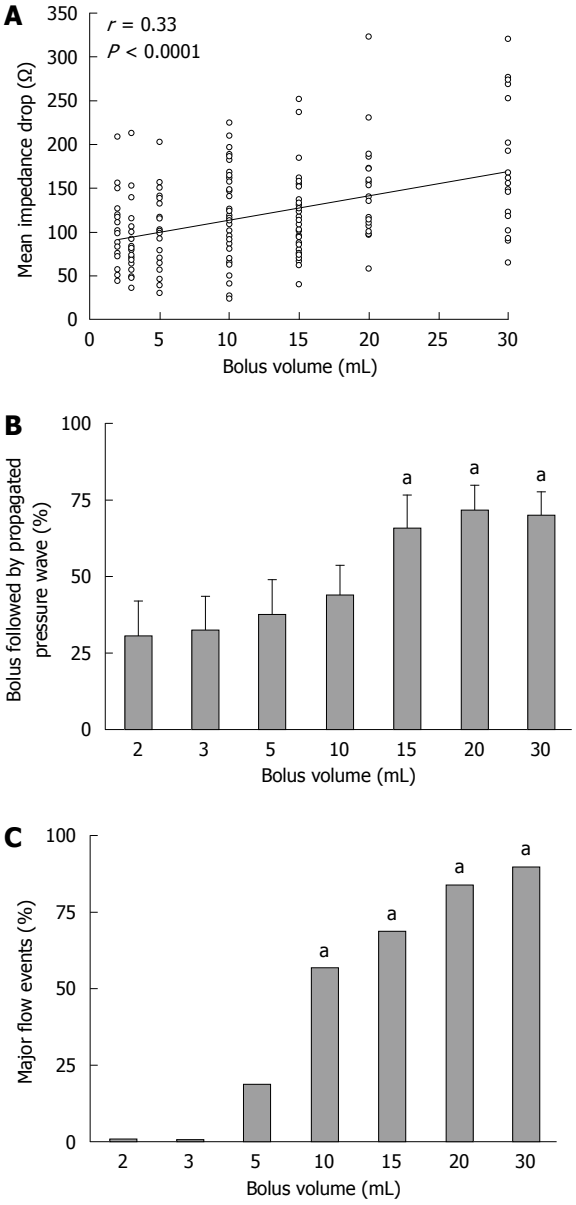

Figure 4 Relationship between bolus volume and the magnitude of impedance drop (A), the proportion of boluses followed by a propagated pressure wave (B) and the occurrence of major flow events (C). $^{a}P < 0.05$ *vs* 2 mL boluses.

may reflect structural and functional differences between the two organs[1-4,17]. The absence of mucosal secretion and possibly a more complete clearance from stronger contraction amplitudes of the oesophagus are likely to explain the higher baseline level of intra-luminal impedance in the oesophagus (approximately 1000 Ω), compared to the intestine (approximately 400 Ω)[16-18]. With a higher baseline signal, liquid boluses of 1 mL or less can induce significant and readily identifiable impedance drops in the oesophagus[18]. In contrast, with a lower baseline impedance signal, possibly caused by a relatively 'wet' lumen from constant intestinal secretions, impedance drops detected in the small intestine are proportionately smaller, and larger volumes are required to induce an identifiable impedance signal. This is supported by the current study, in which a volume threshold of 2 mL was required to induce an intestinal impedance drop. Even with this volume, impedance flow events were only observed in approximately one third of boluses, and were not observed consistently until the volume was at least 10 mL.

The characteristics of impedance changes associated with liquid transit in the small intestine were also influenced by the bolus volume. In the current study, transit of liquid boluses with a volume between 2 and 5 mL was typically associated with a shorter clearance time (less than 60 s) and propagation distance (less than 10 cm). This pattern of flow has been reported to occur during the postprandial period and possibly phase 2 of the MMC[16,17]. In contrast, transit of larger volumes (> 20 mL) were predominantly associated with clearance times greater than 60 s and propagating over 14 cm. The signals of these 'major' impedance events did not spontaneously recover to baseline level until the bolus was cleared by intestinal contraction(s). This pattern of luminal flow is typically seen at the end of phase 3 activity, which may be consistent with the clearance function of the MMC[16,17]. Characteristics of impedance events induced by liquid boluses between 10 and 20 mL varied considerably, particularly in clearance duration. This appears to depend on the timing of intestinal contraction(s) which occur after the bolus is delivered. However, there was also a direct linear relationship between the clearance time and bolus volume. The understanding of the relationship and characteristics between bolus volume and impedance changes will aid the interpretation of various patterns of luminal flow events in the small intestine.

In the current study, impedance changes associated with bolus transit were assessed in the quiescent phase of the MMC cycle, in order to avoid interference from intestinal contraction(s)[17]. Consequently, this relationship is applicable only to bolus transit within a relatively inactive small intestine. However, under normal physiological conditions of digestion, frequent intestinal contractions would be expected and may shorten the clearance time, but not propagation distance.

Furthermore, the current study used saline as a test medium, rather than chyme, which may not reflect the true transit of intestinal contents during normal digestion in humans. Nevertheless, although there are no human data that have evaluated spontaneous flow events in the small intestine, the bolus volumes used in the present study are similar to those reported during the pulsatile flow of gastric emptying in pigs[20]. However, chyme or digested food in the small intestine is typically more viscous than saline, and it is well known that viscosity significantly influences the characteristics of impedance flow events in the oesophagus[21]. Further investigation is required to assess the flow volume of chyme and its relationship with impedance signals. Due to the small diameter of the feeding tube, evaluation of a viscous medium was not possible in the current study.

A potential limitation of this study is the lack of fluoroscopic confirmation of bolus flow. However, fluoroscopy would have exposed the volunteers to excessive

radiation exposure due to the prolonged recording period. More importantly, the criteria used in this study to define an impedance "flow event" in the small intestine has already been established and validated against fluoroscopy[17]. Flow could thus be reasonably inferred from the pattern of impedance changes with evidence of propagated clearance from successive impedance segments.

In conclusion, there is a strong relationship between bolus volume and changes in impedance signals within the small intestine. Bolus volume has an impact on both the type and length of propagation of flow events and a threshold volume of 2 mL is required to produce a flow event.

COMMENTS

Background

Fluoroscopy and scintigraphy are the currently available techniques to assess intestinal motility and bolus transit, but their utilities are greatly limited by radiation exposure. Although combined intraluminal manometry and impedance has been recently used to assess flow in the small intestine, the relationship between bolus volume and impedance changes has not been assessed.

Research frontiers

The ability to use a combined intraluminal manometry-impedance technique to assess bolus transit in the small intestine will not only improve the safety of the procedure, but also its utilization in clinical practice.

Innovations and breakthroughs

This is the first methodological study to demonstrate a linear relationship between bolus volume and impedance changes in the proximal small intestine. Bolus volumes of at least 2 mL are required to generate an impedance signal that can be recognised as a flow event. Volumes of 10 mL or greater will always generate an impedance drop. These findings may explain the various patterns of flow that are associated with chyme transport in the small intestine.

Applications

These findings assist the interpretation of various patterns of bolus transit or flow of chyme in the small intestine assessed by a manometry-impedance technique, without the need for fluoroscopy or scintigraphy.

Terminology

Intra-luminal impedance is a new technique designed to detect intraluminal flow without the use of radiation. The impedance technique measures changes in resistance (in Ohms) to alternating electrical current when a bolus passes a pair of metallic rings mounted on a catheter. In an empty tubular organ (i.e. small intestine), the electrical current is conducted by the few ions present in and on the intestinal mucosa. Liquid boluses with an increased number of ions have a higher conductivity and when entering the measurement segment will lower the impedance to a nadir value. Measurement of flow at multiple sites allows for determination of bolus direction based upon temporal differences in bolus entry and exit.

Peer review

This is a high quality manuscript, presenting a series of experimental results which demonstrates, for the first time, the relationship between bolus volume and impedance changes in the proximal small intestine.

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BRIEF ARTICLE

Treatment outcome of localized *Helicobacter pylori*-negative low-grade gastric MALT lymphoma

Hyung Soon Park, Yu Jin Kim, Woo Ick Yang, Chang Ok Suh, Yong Chan Lee

Hyung Soon Park, Yu Jin Kim, Yong Chan Lee, Department of Internal Medicine, Yonsei University College of Medicine, 120-752 Seoul, South Korea
Woo Ick Yang, Department of Pathology, Yonsei University College of Medicine, 120-752 Seoul, South Korea
Chang Ok Suh, Department of Radiation Oncology, Yonsei University College of Medicine, 120-752 Seoul, South Korea
Author contributions: Park HS analyzed the data and wrote the manuscript; Yang WI performed the histopathological analysis; Suh CO and Lee YC designed the study; Kim YJ and Lee YC edited and reviewed the manuscript.
Correspondence to: Yong Chan Lee, MD, PhD, Department of Internal Medicine, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, 120-752 Seoul, South Korea. leeyc@yuhs.ac
Telephone: +82-2-22281960 Fax: +82-2-3936884
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Abstract

AIM: To investigate treatment outcome of *Helicobacter pylori* (*H. pylori*)-negative low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphoma.

METHODS: In this study, we retrospectively reviewed the clinical outcome and clinicopathologic factors of stage I E *H. pylori*-negative low-grade gastric MALT lymphoma cases from August 1998 to June 2009.

RESULTS: A total of eleven patients with *H. pylori*-negative low-grade gastric MALT lymphoma were enrolled in the study and received anti-*H. pylori* eradication treatment and/or radiotherapy or excisional therapy. Complete remission (CR) of gastric MALT lymphoma was achieved in all patients. The time to CR was 1-66 mo (median, 1 mo).

CONCLUSION: Eradication therapy may be offered as an initial treatment option even in cases of localized *H. pylori*-negative gastric MALT lymphoma.

Key words: Anti-bacterial agents; *Helicobacter pylori*; Mucosa-associated lymphoid tissue lymphoma; Radiotherapy; Stomach

Peer reviewer: Philippe Lehours, MD, PhD, University Bordeaux 2, INSERM U853, Bat 2B RDC Zone Nord, 146 rue Léo Saignat, Bordeaux cedex, 33076, France

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INTRODUCTION

Mucosa-associated lymphoid tissue (MALT) lymphoma, first described in 1983 by Isaacson and Wright[1], has been recently re-classified as extranodal marginal zone lymphoma of MALT-type, in the Revised European-American Classification of Lymphoid Neoplasms (REAL)/World Health Organization (WHO) Classification of Lymphoid Neoplasms. An important feature of MALT lymphoma is the presence of lymphoepithelial lesions formed by the invasion of individual glands by aggregates of lymphoma cells[2].

In previous studies, *Helicobacter pylori* (*H. pylori*) infection was suggested to be causally associated with primary gastric MALT lymphoma[3,4]. Indeed, *H. pylori* provides the antigenic stimulus, which is mediated by mucosal T cells, for sustaining the growth of gastric MALT lymphoma[5]. The regression of gastric MALT lymphoma after the eradication of *H. pylori* was first reported in 1993 by Wotherspoon *et al*[3]. *H. pylori* eradication led to complete remission in 80% of stage I E, low-grade gastric MALT lymphoma patients, with a yearly recurrence rate of approximately 5%[6]. Curr-

ently, eradication of *H. pylori* is considered the accepted initial therapy in cases of localized, stage I E low-grade gastric MALT lymphoma[7].

However, there are no treatment guidelines for the management of patients who are unresponsive to antibiotic treatment or for the subset of patients who present with stage I E low-grade gastric MALT lymphoma but are *H. pylori*-negative and understandably do not respond to antibiotic treatment[8]. In these cases, treatment choices include the known, conventional therapeutic approaches such as surgery, chemotherapy or radiotherapy[9]. Because of the ambiguity of treatment in *H. pylori*-negative patients, further studies are required to clarify a treatment strategy for *H. pylori*-negative MALT lymphoma. In this study, we evaluated the treatment outcome of eleven *H. pylori*-negative low-grade gastric MALT lymphoma patients to offer therapeutic guidelines for treating *H. pylori*-negative low-grade gastric MALT lymphoma.

MATERIALS AND METHODS

Patients

We retrospectively studied eleven patients with *H. pylori*-negative low-grade gastric MALT lymphoma diagnosed at the Severance Hospital from August 1998 to June 2009. All patients were confirmed by pathology to have low-grade gastric MALT lymphoma without a diffuse large B-cell lymphoma (DLBCL) component. Histological diagnosis of MALT lymphoma was performed according to the criteria outlined in the WHO classification, and histological assessment was performed by a reference pathologist (Yang WI). Immunological phenotyping of paraffin sections was performed to demonstrate light chain restriction and the $CD20^{+}CD5^{-}CD10^{-}cyclinD1^{-}$ phenotype which was microscopically consistent with the presence of low-grade MALT lymphoma. The study was approved by our institutional review board, and informed consent was not required.

Examination of H. pylori infection and endoscopic findings

None of the patients had evidence of *H. pylori* infection as judged by histology, a urea breath test, a rapid urease test (CLO™, Delta West, Bentley, Western Austria) and serological testing. The gross phenotype for each patient was classified into five types according to endoscopic features: (1) ulcerative: one or more ulcerations; (2) protruding: elevated or polypoid; (3) granular: small nodules on the lesion; (4) infiltrative: mucosal infiltration; and (5) mixed.

Staging work-up

Determination of the stage of disease included a detailed physical examination, chest X-ray, abdominal computed tomography (CT), endoscopic ultrasonography (EUS), bilateral bone marrow examination, and ^{18}F-FDG PET scan.

Therapeutic approach

Treatment modalities included anti-*H. pylori* eradication therapy, radiotherapy, and excisional therapy (one endoscopic mucosal resection and one subtotal gastrectomy). The most common treatment was radiotherapy (six patients), followed by anti-*H. pylori* eradication only (three patients). Prior to radiotherapy, three of the six radiotherapy patients received anti-*H. pylori* eradication therapy which consisted of amoxicillin (2 × 1000 mg/d), clarithromycin (2 × 500 mg/d), and esomeprazole (2 × 40 mg/d) or pantoprazole (2 × 20 mg/d) for 7 or 14 d. Radiotherapy was performed at a total dose ranging from 30 Gy to 36 Gy on an outpatient basis. Two patients underwent local excision therapy, including one subtotal gastrectomy and one endoscopic mucosal resection (EMR).

Response assessment

The median time for follow-up after remission was 25 mo (range: 5-76 mo). Complete remission (CR) was defined as the total disappearance of clinical evidence for lymphoma and an absence of histologic evidence for lymphoma on biopsy specimens. Partial remission (PR) was defined as a tumor reduction of at least 50%, and stable disease (SD) was defined as variation within either a 50% decrease or 25% increase in tumor size. In cases with complete remission, endoscopic examinations and biopsies were performed at regular intervals.

RESULTS

Baseline characteristics

The male to female patient ratio in this study was 1:1.2. The mean age of the patients was 55.7 (36-73) years. Four patients were symptomatic at presentation indicating abdominal pain (two cases), abdominal discomfort (one case), and GI bleeding (one case). Lymphoma was most often localized in the body and the antrum in 36% of the patients. Endoscopic lesions were characterized as ulcerative (five cases), infiltrative (one case), protruding (two cases), granular (one case) and mixed (two cases). Initial endoscopic findings are summarized in Table 1. Initial clinical staging with EUS and/or CT scans and BM examinations revealed all the cases to be stage I E.

Treatment outcome

Complete remission (CR) of gastric MALT lymphoma was achieved in all patients (Table 2). The time to CR ranged from one to 66 mo (median, 1 mo). Anti-*H. pylori* eradication therapy was performed in six of the eleven patients. Three of six patients, who completed the follow-up endoscopic examination 1 or 2 mo later, had complete remission of gastric MALT lymphoma. However, two patients refused to wait for the treatment response evaluation and subsequently underwent radiotherapy for definitive treatment. The remaining patient had stable disease for 2 mo before being referred for radiotherapy. All three of these patients showed complete remission of gastric MALT lymphoma 1 mo after the cessation of radiotherapy based on histological evidence. None of the 11 patients showed

Table 1 Baseline characteristics of *H. pylori*-negative low-grade gastric MALT lymphoma *n* (%)

	H. pylori negative MALT lymphoma (*n* = 11)
Age (yr, mean)	55.7 (36-73)
Sex	
Men	5 (45)
Women	6 (55)
Site	
Cardia	1 (9)
Fundus	1 (9)
Body only	3 (27)
Body & antrum	4 (36)
Diffuse	2 (18)
Endoscopy	
Ulcerative	5 (45)
Infiltrative	1 (9)
Protruding	2 (18)
Granular	1 (9)
Mixed	2 (18)

H. pylori: *Helicobacter pylori*; MALT: Mucosa-associated lymphoid tissue.

Table 2 Individual characteristics of 11 cases of *H. pylori*-negative low-grade gastric MALT lymphoma

	Age (yr)/ sex	Stage	Treatment	Response	Time to CR (mo)	Follow up time after remission (mo)
Case 1	54/F	I E	Eradication	CR	1	6
Case 2	60/F	I E	Eradication	CR	1	27
Case 3	52/F	I E	Eradication	CR	2	25
Case 4	36/M	I E	RTx	CR	1	76
Case 5	58/F	I E	RTx	CR	2	17
Case 6	69/M	I E	RTx	CR	1	47
Case 7	41/F	I E	Eradication + RTx	CR	1	7
Case 8	62/M	I E	Eradication + RTx	CR	1	8
Case 9	55/M	I E	Eradication -> RTx	CR	1	5
Case 10	73/F	I E	STG	CR	66	54
Case 11	53/M	I E	EMR	CR	2	69

CR: Complete remission; RTx: Radiotherapy; STG: Subtotal gastrectomy; EMR: Endoscopic mucosal resection.

local or distant recurrence after a median follow-up time of 25 mo (range: 5-76 mo).

DISCUSSION

The prevalence of *H. pylori* in patients with low-grade gastric MALT lymphoma is variable[10]. It is possible that a reduced number of *H. pylori* organisms present in the infection may account for the negative *H. pylori* diagnostic test result in some cases, and false-negative results may also be obtained when only one diagnostic method is employed[11]. The European Guidelines generally take the gold standard to be represented by at least two tests[12]. When appropriate diagnostic methods are used, the prevalence of *H. pylori* infection in low-grade MALT lymphoma is high, at nearly 90%[10]. In our study, four diagnostic tests were performed and showed that true *H. pylori*-negative patients were included. However, additional tests, such as culture with polymerase chain reaction, would allow the detection and identification of other infective organism[13].

Due to the excellent clinical outcome of *H. pylori* eradication treatment, the eradication of *H. pylori* with antibiotics should be employed as the sole initial treatment in the localized form (confined to the stomach) of gastric MALT lymphoma[14]. However, in advanced stages with *H. pylori*-positive gastric MALT lymphoma and in *H. pylori*-negative lymphoma, definitive treatment guidelines are not yet available despite the numerous published clinical research papers[8,14,15].

Antibiotic treatment for *H. pylori*-negative gastric MALT lymphoma has been described in a limited number of patients. We collectively reviewed a series of four published studies including patients treated solely with the anti-*H. pylori* antibiotic regimen as the initial treatment for *H. pylori*-negative stage I E gastric MALT lymphoma (Table 3). A total of 31 patients were included in these studies, and ten patients (32%) were shown to respond to this treatment alone. Raderer *et al*[16] reported that five of six patients with *H. pylori*-negative gastric MALT lymphoma responded to antibiotic treatment (one partial remission and four complete remissions). However, Steinbach *et al*[17] and Ye *et al*[18] experienced a 0% response rate. In other words, marked variation in treatment response rate was noted. Nevertheless, instituting anti-*H. pylori* treatment showed satisfactory clinical outcomes and allowed for gastric preservation in a significant number of cases with *H. pylori*-negative gastric MALT lymphoma. Although seemingly contradictory, anti-*H. pylori* treatment may be advised as the first-line therapy in *H. pylori*-negative localized gastric MALT lymphoma. Unfortunately, the effectiveness of anti-*H. pylori* treatment could not be confirmed in this study, as only four out of the six patients who received the anti-*H. pylori* eradication regimen waited long enough to receive the follow-up endoscopic examination which was used to confirm the complete remission status of MALT lymphoma. The other two patients proceeded to radiation therapy due to various clinical and psychosocial issues. In addition, previous studies also included patients with advanced stages of disease, including ⅡE$_1$ (Table 3). However, it was not possible from the available data to categorize lymphoma regression according to tumor stage (I E *vs* ⅡE$_1$). Therefore, additional studies are needed to define the role of anti-*H. pylori* eradication therapy in advanced stages of gastric MALT lymphoma such as stages ⅡE, ⅢE or Ⅳ.

There is still no explanation for the effectiveness of anti-*H. pylori* treatment in *H. pylori*-negative gastric MALT lymphoma[16]. However, it has been speculated that another infective organism, *Helicobacter heilmannii*[19], might be involved in the development of gastric MALT lymphoma. Also, one cannot rule out the possibility that unknown bacterial agents capable of surviving in the stomach might play a role in the development of rare *H. pylori*-negative

Table 3 Efficacy of eradication treatment in *H. pylori*-negative gastric MALT lymphoma reported in the literature *n* (%)

Author	Yr	No. of patients	Stage	Follow-up after treatment (mo)	Response rate
Steinbach *et al*[17]	1999	6	I E	5 or more	0 (0)
Ye *et al*[18]	2003	5	I E	4-12	0 (0)
Raderer *et al*[16]	2006	6	I E	12-19	5 (83)
Stathis *et al*[23]	2009	14	I E	Not described	5[1] (35)
Ruskone-Fourmestraux *et al*[24]	2001	10	I E, II E$_1$	2-21	0 (0)
Nakamura *et al*[25]	2006	7	I E, II E$_1$	1-15	2 (29)
Akamatsu *et al*[21]	2006	9	I E, II E$_1$	6 or more	1 (11)
Terai *et al*[26]	2008	4	I E, II E$_1$	Not described	1 (25)

[1]3 presented a local relapse.

Table 4 Efficacy of radiotherapy for *H. pylori*-negative gastric MALT lymphoma reported in the literature

Author	Yr	No. of patients	Stage	Follow-up after treatment, median (mo)	Response rate (%)
Schechter *et al*[27]	1998	12	I E, II E	Not described	100
Ye *et al*[18]	2003	1[1]	I E	12	100
Akamatsu *et al*[21]	2006	12 (5[1])	I E, II E$_1$	Not described	100
Chung *et al*[22]	2009	4	I E, II E	12-39	100

[1]Failure of anti-*H. pylori* treatment as the initial therapy.

gastric MALT lymphomas[16]. Another possibility is that low bacterial counts[20] and urease-negative *H. pylori* mutant strains may escape detection by the diagnostic tests that are currently available. If this assumption is correct, one might expect the noted *H. pylori*-negative gastric MALT lymphoma patient response to broad-spectrum antibiotic therapy. Also, MALT lymphoma is to some extent an immunologically driven disease, therefore an additional hypothesis may involve the potential immunomodulatory effects of the antibiotic agents used[16].

Disease control using radiotherapy alone has been previously reported in the literature, which supports the use of a modest dose of involved-field radiotherapy for patients with stages I E-II E MALT lymphoma of the stomach without evidence of *H. pylori* infection[14]. Table 4 summarizes these previously reported cases. Including our cases, 35 of 35 cases responded to radiotherapy. One of the 35 patients reported by Akamatsu *et al*[21] had a partial remission, while the others all had a complete remission. Based on these results, it is prudent to say that radiotherapy is suitable in early stage (I E or II E) *H. pylori*-negative gastric MALT lymphoma. In addition, excisional treatments such as endoscopic mucosal resection or laparoscopic gastric resection may represent another therapeutic option in the case of localized forms of gastric MALT lymphoma in individuals who are not suitable for, or who refuse, standard therapeutic approaches[15,20,22]. In the past, surgical treatment has often been advocated to establish accurate diagnosis, staging, and management of early-stage gastric lymphoma, which has about a 90% 5-year survival rate, but leads to significant morbidity[22]. In our study, two patients received endoscopic mucosal resection and subtotal gastrectomy, respectively. There has been no local or distant recurrence during the follow-up period of 54 to 69 mo.

In our series, therapeutic measures such as anti-*H. pylori* eradication, radiotherapy and excisional therapy achieved complete remission in all cases. Nevertheless, antibiotic treatment is simple, inexpensive, less harmful and *H. pylori* negative low grade MALT lymphoma shows a favorable long-term outcome[22]. Therefore we suggest that *H. pylori* eradication therapy may be an initial treatment option for localized *H. pylori*-negative gastric MALT lymphoma. In addition, further studies on eradication therapy are required to help in the establishment of strategies for patients with localized *H. pylori*-negative gastric MALT lymphoma.

COMMENTS

Background

Eradication of *Helicobacter pylori* (*H. pylori*) is a well-accepted initial therapy in cases of localized (stage I E) low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphoma associated with *H. pylori* infection. However, there are no treatment guidelines for the management of *H. pylori*-negative low-grade gastric MALT lymphoma.

Research frontiers

Previous studies revealed the effectiveness of radiotherapy for localized *H. pylori*-negative low grade gastric MALT lymphoma. However, *H. pylori* eradication therapy is still a controversial treatment modality, although this treatment is simple and less harmful than other treatments.

Innovations and breakthroughs

With the exception of this study, only 31 *H. pylori*-negative patients have received eradication therapy (response rate 32%). However, treatment response was variable. The authors' study enrolled a small number of patients, but they all had complete remission.

Applications

The findings from this study suggest that *H. pylori* eradication therapy may be an initial treatment option for localized *H. pylori*-negative gastric MALT lymphoma. Their study adds information which helps in the establishment of strategies for patients with localized *H. pylori*-negative gastric MALT lymphoma.

Peer review

This is an interesting paper that may be beneficial for patients with *H.pylori*-negative gastric MALT lymphoma.

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BRIEF ARTICLE

Serum VEGFR-3 and survival of advanced gastric cancer patients treated with FOLFOX

Xue-Feng Ni, Chang-Ping Wu, Jing-Ting Jiang

Xue-Feng Ni, Chang-Ping Wu, Jing-Ting Jiang, Department of Oncology, The Third Affiliated Hospital, Soochow University, Changzhou 213003, Jiangsu Province, China
Author contributions: Ni XF performed the experiments and wrote the paper; Wu CP and Jiang JT supervised the research.
Correspondence to: Dr. Xue-Feng Ni, Department of Oncology, The Third Affiliated Hospital, Soochow University, Juqian Road 185, Changzhou 213003, Jiangsu Province, China. nxf2010@hotmail.com
Telephone: +86-519-86180000 Fax: +86-519-86180000
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Abstract

AIM: To explore if vascular endothelial growth factor receptor-3 (VEGFR-3) and carcinoembryonic antigen (CEA) can predict overall survival in advanced gastric cancer.

METHODS: VEGFR-3 level was assessed by enzyme-linked immunosorbent assay, and CEA was assessed by chemiluminescence immunoassay in the sera of 81 advanced gastric cancer patients before treatment with oxaliplatin plus 5-fluorouracil and folinic acid.

RESULTS: Median survival time in patients with a low serum VEGFR-3 level was significantly longer than in those with a higher VEGFR-3 level (15.4 mo *vs* 7.7 mo, $P < 0.001$). Patients with a low CEA level had a longer survival than those with a higher CEA level (15.8 mo *vs* 8.6 mo, $P < 0.001$). Thirty-nine patients with low VEGFR-3 and low CEA levels had a median survival of 19.7 mo ($P = 0.0006$). The hazard ratio for patients with a high VEGFR-3 level was 2.443 ($P = 0.002$).

CONCLUSION: High serum VEGFR-3 level is correlated significantly with poor survival. In patients with a high serum level of VEGFR-3, alternative chemotherapy regimens should be considered.

Key words: Stomach neoplasms; Vascular endothelial growth factor receptor-3; Carcinoembryonic antigen; Oxaliplatin plus 5-fluorouracil and folinic acid protocol; Survival

Peer reviewer: Masahiro Iizuka, MD, PhD, Director, Akita Health Care Center, Akita Red Cross Hospital, 3-4-23, Nakadori, Akita, 010-0001, Japan

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INTRODUCTION

According to global estimates, gastric cancer is the second most frequent cancer-related cause of death. The incidence of gastric cancer is estimated to be 934 000 cases, with 56% of the new cases occurring in East Asia, 41% in China and 11% in Japan[1]. In 2005, there were approximately 400 000 new cases and 300 000 deaths from gastric cancer in China[2].

Great efforts are being made to develop serological markers that are noninvasive and can easily reflect the dynamic status of the tumor. Among these, carcinoembryonic antigen (CEA) has been used widely as a serological marker in patients with gastrointestinal malignancies[3-5]. CEA was first described by Gold and Freedman in 1965 as an antigen that is expressed by gastrointestinal carcinoma cells, which is secreted in blood or body fluids. Several studies have focused on the utility of CEA and carbohydrate antigen 19-9 (CA19-9) measurement in cancer progression, recurrence, and prognosis in patients with gastric carcinoma[4,6,7].

There has been a dramatic increase in the number of studies of the mechanisms of associated lymphangio-

genesis and lymphatic metastasis. It has been recognized that lymphangiogenic growth factors promote the spread of cancer cells to regional lymph nodes[8-10], and one of the most important ones is vascular endothelial growth factor receptors (VEGFR).

Regional lymph node metastasis is an important indicator of tumor aggressiveness, as well as a known prognostic factor[11]. Therefore, it is important to estimate the degree of lymphatic system invasion and lymphangiogenesis in the evaluation of biological tumor aggressiveness and patient outcome. VEGFRs1, 2 and 3 are endothelial-specific receptor tyrosine kinases that are regulated by members of the VEGF family. VEGFR-3 expression has been demonstrated in a variety of human malignancies[12]. The role of the VEGF-C, D and/or VEGFR-3 axis in various types of cancer has been investigated by many research groups[12]. In clinical studies, a negative correlation between VEGF-C, D and/or VEGFR-3 and patient survival time has been reported in non-small cell lung cancer, colorectal carcinoma, endometrial carcinoma, epithelial ovarian carcinoma and primary breast cancer[13-17].

Advanced gastric cancer patients need chemotherapy, but there is currently no established standard regimen; oxaliplatin plus 5-fluorouracil and folinic acid (FOLFOX) is well tolerated. Recently, several phase Ⅱ studies have yielded a median time to progression (TTP) of 5.4-6.5 mo and a median overall survival (OS) of 9.8-12.6 mo[18-21].

A reliable factor is necessary to predict objectively the effectiveness of some special chemotherapy protocols. Recently, two studies have investigated if gene polymorphisms and mRNA can predict the TTP and OS in advanced gastric cancer treated with FOLFOX[22,23]. For oxaliplatin-associated chemotherapy of advanced gastric cancer, seeking more reliable predictive values for chemotherapy is of great importance in research and clinical settings.

We hypothesized that secreted CEA reflects the tumor burden, and VEGFR-3 is associated with poor survival. The purpose of this study is to find out the prognostic value of serum levels of VEGFR-3 and CEA in gastric cancer patients receiving FOLFOX chemotherapy.

MATERIALS AND METHODS

Patients

Patients with histologically proven locally advanced or metastatic gastric cancer and Eastern Cooperative Oncology Group (ECOG) performance status (PS) $\leqslant 2$ were included in the study. Clinical stage and histological type of gastric cancer were evaluated according to AJCC criteria (the sixth edition). All patients received FOLFOX chemotherapy after resection of primary tumors as follows: oxaliplatin 130 mg/m^2 on day 1, plus folinic acid 200 mg/m^2 as a 2-h infusion, followed by a 22-h infusion of 5-fluorouracil (5-FU) 450 mg/m^2 on days 1-5, every 3 wk. Survival was calculated from the date of diagnosis to the date of last follow-up or death from any cause.

All patients gave their signed informed consent, and the study was approved by the Institutional Ethics Review Boards.

Quantitative detection of VEGFR-3 and CEA

Sera from 40 healthy volunteers (20 females and 20 males ranging in age from 25 to 60 years) and gastric cancer patients were collected using a serum separator tube and kept frozen at -80℃ until assay.

Enzyme-linked immunosorbent assay for serum VEGFR-3 and CEA

Serum was collected before chemotherapy. VEGFR-3 was analyzed with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). An ELISA component kit that measures the extracellular (soluble) domain of VEGFR-3 was employed. Serum VEGFR-3 assays were calibrated against recombinant proteins that consisted of the full-length extracellular domain of the respective receptors. A 96-well microplate was coated with diluted capture antibody, and incubated overnight. After washing, the plate was blocked by adding diluent reagent. Plate preparation was finished. Samples or standards were added, then the plates were washed, detection antibody was added, and washing was repeated. Streptavidin-horseradish peroxidase was added to each well. After washing, substrate solution was added to each well. Finally, stop solution was added to each well. The plate was tapped gently. The optical densities of each well were quantified within 30 min at dual wavelengths of 450 nm corrected to 540 nm using a micro-plate reader.

CEA level of all serum samples was analyzed by chemiluminescence immunoassay (CEA Regent Kit, Abbott Diagnostics). Assays were carried out according to the manufacturer's instructions using the machine of ARCHITECT i2000 SR.

Statistical analysis

Spearman's Rho method was used to correlate levels of VEGFR-3 and CEA. The maximal χ^2 method of Miller *et al*[24] and Halpern[25] was adapted to determine which cutoff value can best dichotomize the patients into low- and high-expression CEA and VEGFR-3 subgroups; the Tree method[26] was then applied to optimize these cutoff values. The final cutoff values were confirmed by recursive partitioning and amalgamation using S-Plus software, version 6.1 (Statistical Sciences, Seattle, WA, USA). Cumulative survival rates were determined using the Kaplan-Meier method, and the difference between each group was evaluated by the log-rank method. The cases lost to follow-up were treated as censored data for the analysis of survival rates. A univariate Cox model with overall survival as the dependent variable was constructed and categorized with two factors levels as independent variables, and the factors that were significant in the univariate analysis were included in a multivariate Cox proportional hazards model for survival. Differences were considered significant at $P < 0.05$. All statistical calculations were performed using the Statistical Package for the Social Sciences, version 13 (SPSS Inc., Chicago, IL, USA).

Table 1 Clinical characteristics associated with overall survival of the patients

Characteristics	*n* (%)	MST (mo) (95% CI)	*P* Log-rank test
Age (yr) (median: 59, range: 28-73)			
< 59	37 (45.7)	13.900 (8.075-19.725)	0.610
⩾ 59	44 (54.3)	12.600 (6.595-18.605)	
Gender			
Male	55 (67.9)	12.600 (7.674-17.526)	0.384
Female	26 (32.1)	15.300 (6.017-24.583)	
ECOG			
0-1	64 (79.0)	14.300 (9.194-19.406)	0.034
2	17 (21.0)	9.800 (5.307-14.293)	
Lymph node			
Negative	13 (16.0)	35.100 (5.695-64.505)	0.004
Positive	68 (84.0)	9.900 (7.480-12.320)	
Initial staging			
Ⅲ	38 (46.9)	17.800 (14.187-21.413)	0.017
Ⅳ	43 (53.1)	8.600 (6.157-11.043)	
Grading			
G2	23 (28.4)	14.500 (2.761-26.239)	0.584
G3	58 (71.6)	12.600 (7.823-17.377)	
Site of tumor			
Proximal stomach	30 (37.0)	10.300 (5.283-15.317)	0.806
Distal stomach	44 (54.3)	15.400 (9.380-21.420)	
Whole stomach	7 (8.6)	10.800 (4.439-17.161)	

MST: Median survival time; ECOG: Eastern Cooperative Oncology Group.

RESULTS

Patient characteristics

A total of 81 advanced gastric adenocarcinoma patients were included in the study. The median age was 59 years; 55 patients were male and 64 patients had ECOG PS 0-1; and 13 patients had no lymph node involvement. Thirty-eight (46.9%) patients had stage Ⅲ, and 43 (53.1%) had stage Ⅳ disease at the time of diagnosis. Patient characteristics are summarized in Table 1.

Levels of VEGFR-3 and CEA

Serum VEGFR-3 and CEA levels were detected in all the patients and healthy donors. Serum VEGFR-3 level varied in healthy donors, and the median level was 24.5 ng/mL [range: 0.3-40.3 ng/mL, 95% confidence interval (CI): 19.2-31.7]. In gastric cancer, the median VEGFR-3 level was 41.8 ng/mL (range: 17.2-385.5 ng/mL, 95% CI: 52.5-114.6). A highly significant difference was found in the median VEGFR-3 level between gastric cancer patients ($P < 0.01$) and healthy donors.

Serum CEA level was variable in healthy donors, and median level was 2.3 ng/mL (range: 0.1-6.2 ng/mL, 95% CI: 1.7-3.9). In gastric cancer, the median CEA level was 13.7 ng/mL (range: 2.2-301.7 ng/mL, 95% CI: 18.3-57.8). A highly significant difference was found in the median CEA level between gastric cancer patients ($P < 0.01$) and healthy donors.

Using a cutoff value of 70.6, 52 (64.2%) patients had low VEGFR-3 expression levels and 29 (35.8%) patients had higher VEGFR-3 levels. Using a cutoff value of 23.5, 57 (70.4%) patients had low CEA expression levels and 24 (29.6%) had higher levels. There was no significant association between VEGFR-3 and CEA levels ($P = -0.136$, $P = 0.227$). No significant association was detected between VEGFR-3 or CEA and clinical parameters.

Table 2 Serum VEGFR-3 and CEA levels and survival in advanced gastric cancer patients

Factors	*n*	MST (mo) (95% CI)	*P*[1]
VEGFR-3 (ng/mL)			
Low ⩽ 70.6	52	15.400 (11.799-19.001)	< 0.001
High > 70.6	29	7.700 (5.691-9.709)	
CEA (ng/mL)			
Low ⩽ 23.5	57	15.800 (11.223-20.377)	< 0.001
High > 23.5	24	8.600 (7.219-9.981)	

[1]Adjusted *P*-value based on log-rank statistics after 1000 bootstrap simulations. VEGFR-3: Vascular endothelial growth factor receptor-3; CEA: Carcinoembryonic antigen; CI: Confidence interval.

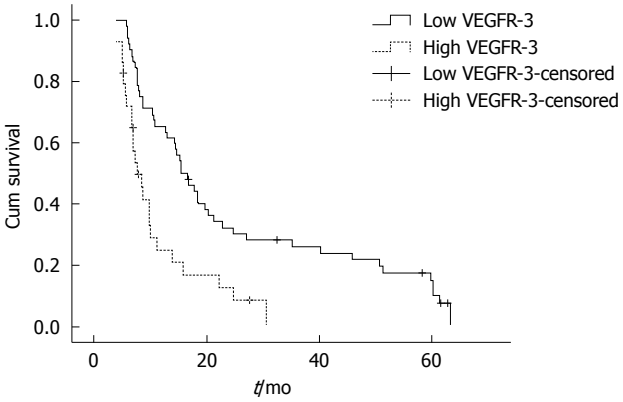

Figure 1 Kaplan-Meier estimates of overall survival by vascular endothelial growth factor receptor-3 (VEGFR-3) levels (*n* = 81, low VEGFR-3: 52; high VEGFR-3: 29).

Survival time

The median survival time for all patients was 13.0 mo (95% CI: 8.820-17.180). A significant association was observed between survival and ECOG PS ($P = 0.034$), lymph node involvement ($P = 0.004$) and initial staging ($P = 0.017$). No other association between clinical characteristics and survival was found (Table 1).

A significant association was also observed between survival and levels of VEGFR-3 ($P < 0.05$) and CEA ($P < 0.05$). Median survival for patients with low VEGFR-3 levels was 15.4 mo (95% CI: 11.799-19.001) compared with 7.7 mo (95% CI: 5.691-9.709) for those with higher VEGFR-3 levels ($P < 0.001$) (Table 2 and Figure 1). Median survival for patients with low CEA levels was 15.8 mo (95% CI: 11.223-20.377) compared with 8.6 mo (95% CI: 7.219-9.981) for those with higher CEA levels ($P < 0.001$, Table 2).

Among the 24 patients with high CEA levels, 13 patients with low VEGFR-3 levels had a median survival of 13.0 mo (95% CI: 5.954-20.046) while 11 patients with higher VEGFR-3 levels had a median survival of 7.2 mo (95% CI: 4.622-9.778) ($P = 0.003$, Figure 2A).

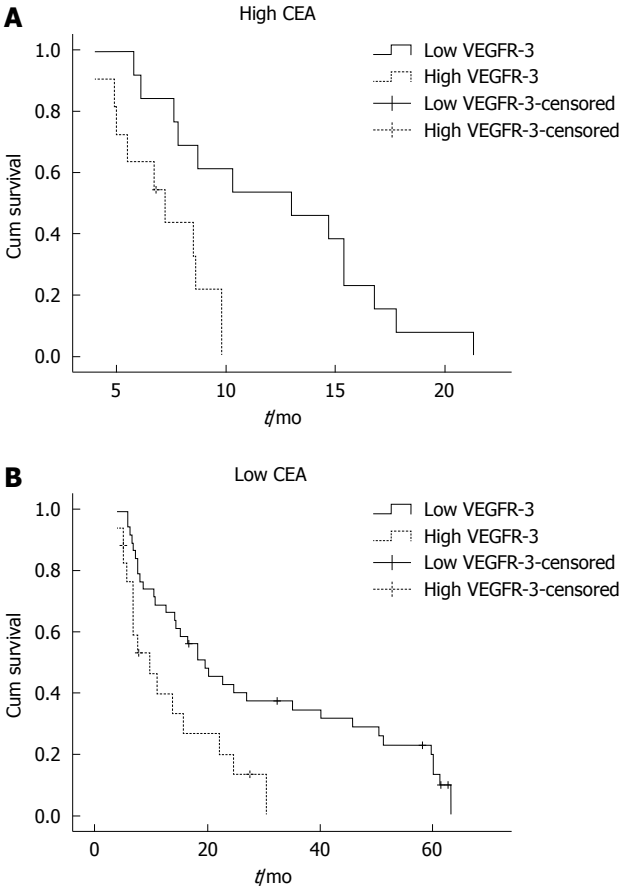

Figure 2 Kaplan-Meier estimates of overall survival according to VEGFR-3 levels in patients. A: Patients with high carcinoembryonic antigen (CEA) levels (n = 24, high CEA and low VEGFR-3: 13, high CEA and high VEGFR-3: 11); B: Patients with low CEA levels (n = 57; low CEA and low VEGFR-3: 39; low CEA and high VEGFR-3: 18).

Among the 57 patients with low CEA levels, 39 patients with low VEGFR-3 levels had a median survival of 19.7 mo (95% CI: 12.53-26.87) while 18 patients with higher VEGFR-3 levels had a median survival of 9.9 mo (95% CI: 4.651-15.149) (P = 0.006, Figure 2B).

Multivariate analysis identified VEGFR-3 levels [hazard ratio (HR) = 2.443, P = 0.002], initial staging (HR = 1.844, P = 0.018), and ECOG PS (HR = 2.396, P = 0.011) as independent markers for survival (Table 3), whereas CEA levels (HR = 1.255, 95% CI: 0.721-2.184, P = 0.121) were not an independent marker for survival.

DISCUSSION

In this study of serum levels of VEGFR-3 and CEA in advanced gastric cancer, multivariate analysis identified VEGFR-3 levels, initial staging, and ECOG PS as independent markers for the survival of the patients who received the FOLFOX regimen. CEA was not an independent marker for survival.

VEGF-C and VEGFR-3 are associated with lymphatic metastasis, mainly *via* tumor lymphangiogenesis in animal models and human tumors[9,27]. Many previous studies have investigated the association between expression of VEGFR-3 and tumor histology. VEGFR-3 is an independent prognostic factor for survival; the expression of VEGFR-3 is correlated with lymphatic invasion, lymph node metastasis, and poor prognosis for survival[28-30]. In the present study, the median survival time in patients with low serum VEGFR-3 levels was significantly longer than in those with higher VEGFR-3 levels (15.4 mo *vs* 7.7 mo, $P < 0.001$). Patients with low VEGFR-3 and CEA levels had a median survival of 19.7 mo (P = 0.0006). The HR for patients with a high VEGFR-3 level was 2.443 (P = 0.002).

Table 3 Multivariate analysis of factors associated with overall survival

Factors	*n*	Hazard ratio (95% CI)	*P*
ECOG PS			
0-1	64	1 (ref.)	
2	17	2.396 (1.219-4.708)	0.011
Lymph node			
Negative	13	1 (ref.)	
Positive	68	1.846 (0.871-3.912)	0.110
Initial staging			
Ⅲ	38	1 (ref.)	
Ⅳ	43	1.844 (1.109-3.066)	0.018
VEGFR3			
Low ≤ 70.6	52	1 (ref.)	
High > 70.6	29	2.443 (1.374-4.345)	0.002

PS: Performance status.

Although great efforts have been devoted to improve early detection of gastric cancer, the majority of patients are diagnosed at an advanced stage. The median OS has been shown to be 9.8-12.6 mo after FOLFOX chemotherapy[18-21]. Identification of patients with potentially poor prognosis after FOLFOX chemotherapy would help us to optimize another treatment protocol for patients with advanced gastric cancer.

These findings are similar to those with metastatic malignant melanoma, for which, a high pretreatment serum VEGFR-3 level is correlated significantly with poor prognosis. Patients with a low serum VEGFR-3 level had a higher median disease-free survival than those with a high serum VEGFR-3 level (16.2 mo *vs* 10.8 mo, χ^2 = 3.85, P = 0.022). Median serum VEGFR-3 levels were significantly higher in patients with a high tumor burden than those with a low tumor burden (P = 0.013)[31].

There are few reports on biomarkers with a high and reliable predictive value for chemotherapy. Recently, two studies have investigated predictive factors for FOLFOX chemotherapy in advanced gastric cancer. In one study of genetic polymorphism, the glutathione S-transferase M1 positive genotype showed a significantly longer survival time compared with negative genotype in advanced gastric cancer treated with FOLFOX[22]. In a study of mRNA, the median survival time in patients with low levels of mammalian excision repair *via* complementing protein ERCC1 was significantly longer than in those with higher levels (15.8 mo *vs* 6.2 mo, $P < 0.0001$) in advanced gastric cancer treated with FOLFOX chemotherapy[23].

However, in the present study, the difference in the survival of the patients with a low level and a high level of VEGFR-3 was much more striking (15.4 mo *vs* 7.7 mo, $P < 0.001$), with an HR of 2.443. The determination of relative serum VEGFR levels by ELISA and electrochemiluminescence is considered currently to be easier than by immunohistochemistry and quantitative PCR.

It has been shown that VEGFR-3 is expressed not only in lymphatic endothelial cells, but also in tumor cells, and it has been seen in the cytoplasm, along the nuclear and cell membranes, which underlines its potential role in tumor growth[15,32,33]. Despite vast amounts of literature on VEGFR-3 expression in tissues (quantitative PCR and immunohistology)[32,34-37], there are few data about serum levels of VEGFR-3, which is the major axis specific for lymphangiogenesis.

To address the biological and clinical significance of pre-chemotherapeutic serum VEGFR-3 levels in advanced gastric cancer, we compared serum VEGFR-3 levels with clinicopathological parameters. A significant association was observed between survival and ECOG PS, lymph node involvement and initial staging. We found a significant association between the pre-chemotherapeutic serum level of VEGFR-3 and lymph node status. In contrast, there was no correlation between survival and age, sex, tumor grading, or tumor site.

In a recent study of anti-angiogenic treatment, DePrimo *et al*[38] found that the decrease in the soluble variant of VEGFR-3 could be a marker of sunitinib activity in patients with metastatic renal cell carcinoma. Similar to our results, Rini *et al*[39] have shown that a high baseline VEGFR-3 level is related to non-response to treatment and shorter progression-free survival in renal cell cancer that is refractory to bevacizumab, when the patients are treated with sunitinib. The presence of high levels of circulating VEGFR-3 in advanced gastric cancer patients might prospectively identify high-risk patients undergoing FOLFOX chemotherapy with a worse prognosis and shorter survival, and special target medicine is needed for therapy.

It is also not known in which way VEGFR-3 contributes to tumor angiogenesis, lymphangiogenesis, tumor progression and metastasis[40,41]. However, our results were from serum, and further investigations are necessary to confirm our observations.

A high CEA level has been shown to be a negative factor for survival in breast, gastric and colorectal cancer[42-47]. In patients with metastatic colorectal cancer receiving cetuximab plus FOLFIRI or FOLFOX-4 chemotherapy, serum CEA could predict progression-free survival time. Survival time in responders assessed by changes in serum CEA was significantly longer than that in non-responders ($P = 0.0091$)[48].

In another study, a preoperative CEA level was an independent prognostic factor in patients with node-positive Dukes' C colorectal cancer treated with 5-FU-based adjuvant chemotherapy[49]. However, the role of CEA in predicting chemosensitivity remains controversial. In a prospective study, by multivariate analysis, serum CA19-9 level ($P < 0.001$) was found to be an independent prognostic factor, whereas pretreatment serum CEA level was not considered to be a significant prognostic indicator in patients with metastatic colorectal cancer treated with 5-FU-based chemotherapy[50].

In the present study, patients with lower CEA levels had a longer survival than those with higher CEA levels, especially in patients with higher VEGFR-3 levels. VEGFR-3 over-expression increases proliferation of MCF7 cells, but proliferation of BT474 cells is reduced drastically when endogenous VEGFR-3 is down-regulated[51]. Producing a plausible explanation of why a meaningful number of patients with low VEGFR-3 and CEA levels had the longest survival is one of the aims of the present study.

Irinotecan and taxane-based regimens have been used in the treatment of advanced gastric cancer patients, with a similar survival to those attained with FOLFOX[52-54]. Irinotecan or taxane-based regimens could be the better alternative for patients with high VEGFR-3 levels. A randomized customized trial is warranted in this setting.

Targeting the VEGF-C/VEGFR-3 axis may be therapeutically significant for certain types of tumors. Thus, the continued discovery and characterization of factors that regulate VEGF-C or VEGFR-3 are essential for developing new therapies that limit the spread of cancer. In a recent study of gastric tumors, the target drug Ki23057 inhibited the phosphorylation of VEGFR-3 in lymphatic endothelial cells. The degree of lymphatic invasion and lymphangiogenesis was significantly ($P < 0.05$) lower in the gastric tumors treated by Ki23057[55]. Therefore, the target medicine is required to be developed in the future. In particular, new drugs that block the VEGFC/VEGFR-3 signaling pathway may provide useful anticancer therapeutics by mechanisms other than the blockage of lymphangiogenesis.

In conclusion, the data from our study indicate that serum VEGFR-3 level is the most significant prognostic indicator of patients with advanced gastric cancer. It is recommended that stratification for further clinical trials of patients with advanced gastric cancer should be carried out according to serum VEGFR-3 levels. Combined analysis of the VEGF-C/VEGF-D/VEGFR-3 system might be useful for identifying patients with an unfavorable clinical outcome, thereby helping refine therapeutic decisions in gastric cancer.

In our study, we only detected the levels of VEGFR-3 and CEA before chemotherapy. Changes in these levels after chemotherapy, especially with regard to differences between the responding and non-responding groups, need further researches.

COMMENTS

Background

Carcinoembryonic antigen (CEA) has been used widely as a serological marker in patients with gastrointestinal malignancies. Several reports have focused on the utility of CEA measurements in cancer progression, recurrence, and prognosis in patients with gastric carcinoma. Vascular endothelial growth factor

receptor-3 (VEGFR-3) expression has been demonstrated in a variety of human malignancies. CEA reflects the tumor burden, and VEGFR-3 is associated with tumor progression. For oxaliplatin-associated chemotherapy of advanced gastric cancer, seeking a more reliable predictive marker for chemotherapy has been of great interest in research and clinical settings.

Research frontiers

In this study, serum VEGFR-3 and CEA levels were assessed in advanced gastric cancer. The authors observed that VEGFR-3 and CEA could help predict survival in advanced gastric cancer patients treated with oxaliplatin plus 5-fluorouracil and folinic acid (FOLFOX).

Innovations and breakthroughs

High serum VEGFR-3 levels are correlated significantly with poorer survival. In patients with a higher serum VEGFR-3 level, alternative chemotherapy regimens should be considered.

Applications

The results of this study will help predict survival in advanced gastric cancer patients treated with FOLFOX chemotherapy.

Peer review

In this paper, the authors analyzed the clinicopathological parameters, serum CEA, serum VEGFR-3 levels and survival of advanced gastric cancer patients treated with FOLFOX. Based on the results of the study, the authors concluded that a high serum VEGFR-3 level is significantly correlated to poorer survival of the patients.

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BRIEF ARTICLE

Peroxisome proliferator-activated receptor-γ 34C>G polymorphism and colorectal cancer risk: A meta-analysis

Yong-Liang Lu, Gai-Ling Li, Hui-Lian Huang, Jing Zhong, Li-Cheng Dai

Yong-Liang Lu, Department of Surgery, Huzhou Teachers College School of Medicine, Huzhou 313000, Zhejiang Province, China
Gai-Ling Li, SIP Healo & Halo Life Science Research Center, Suzhou 215000, Jiangsu Province, China
Hui-Lian Huang, Jing Zhong, Li-Cheng Dai, Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou 313000, Zhejiang Province, China
Author contributions: Li GL, Huang HL and Zhong J collected the materials and analyzed the data; Lu YL and Dai LC designed the study and wrote the manuscript.
Supported by The National Natural Science Foundation of China, No. 30772534
Correspondence to: Li-Cheng Dai, Professor, Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou 313000, Zhejiang Province, China. dlc@hzhospital.com
Telephone: +86-572-2033020 Fax: +86-572-2033020
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Abstract

AIM: To investigate the association between peroxisome proliferator-activated receptor-γ (*PPAR-γ*) gene polymorphism 34 C>G and colorectal cancer (CRC), a meta-analysis review was performed in this report.

METHODS: A systematic literature search and selection of eligible relevant studies were carried out. Nine independent studies with a total number of 4533 cases and 6483 controls were included in the meta-analysis on the association between polymorphism 34 C>G and CRC.

RESULTS: There was no evidence for the association between PPAR-γ 34 C>G and CRC if all of the subjects in the nine studies were included. However, CG + GG showed a marginally significant difference from CC (OR = 0.84, 95% CI: 0.69-1.01, *P* = 0.07) in random-effect model. Stratified meta-analysis indicated that PPAR-γ 34 C>G was associated with colon cancer (OR = 0.8, 95% CI: 0.65-0.99, *P* = 0.04) in random-effect model, and the G allele decreased colon cancer risk. No significant association was observed between PPAR-γ 34 C>G and rectal cancer.

CONCLUSION: PPAR-γ 34 C>G is associated with colon cancer risk, but not associated with CRC and rectal cancer risk.

Key words: Peroxisome proliferator-activated receptor-γ; Colorectal cancer; Polymorphism; Meta-analysis

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INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of cancer death in developed countries, with over 9500 new cases in Netherlands in 2002 alone, for instance[1]. In 2003, it was estimated that about 147 500 new cancer cases and 57 100 deaths were caused by CRC in the USA[2]. With more than 71 000 new occurrences per year, the incidence and mortality rate of CRC in Germany is almost the highest all over the world[3]. Epidemiological and experimental evidences attributed CRC to both genetic and experimental factors were involved. Accumulating evidences suggested that the peroxisome

proliferator-activated receptor-γ (*PPAR-γ*) gene is related to CRC, which has been implicated in the pathogenesis of CRC in animal models and clinical studies. PPAR-γ is a member of the nuclear hormone receptor super-family, and plays a pivotal role in regulating adipocyte differentiation, glucose and lipid metabolism, insulin sensitivity, atherogenesis and immune[4]. A proline to alanine substitution has been detected in the *PPAR-γ* gene which is a common structural polymorphism (34 C>G, rs1801282) located at codon 12 (Pro12Ala) of PPAR-γ2-specific exon B. Although many studies were performed to investigate the relationship between the polymorphism 34 C>G of *PPAR-γ* gene and CRC, results were contradictory. Our study aims to confirm the former data of the association between *PPAR-γ* gene 34 C>G and CRC.

MATERIALS AND METHODS

Identification and eligibility of relevant studies

A systematic literature search in PubMed and Google was carried out in January 2010 using '*PPAR* gene', 'association' and 'CRC' with restriction to 'human' or 'homo sapiens'. Additional articles were identified through references cited in retrieved articles. Publications containing the same or overlapping data from the same authors were excluded. Studies were considered as eligible for the meta-analysis if the frequency of relevant genotypes was reported in both CRC cases and CRC-free controls, or in both colon cancer cases and CRC-free controls, as well as in both rectal cancer cases and CRC-free controls. Moreover, all of them were case-control studies or cohort studies. Nine articles reported on the analysis of the association between PPAR-γ Pro12Ala and CRC[1,2,4-10], four of which focused on the association between the polymorphism PPAR-γ Pro12Ala and colon cancer risk or rectal cancer risk[4-7].

Data extraction and statistical analysis

For each study, information was gathered about the first author, year of publication, country where the study was conducted and the distribution of each PPAR-γ 34 C>G genotype in cases and controls (Table 1). Some calculated data collected from the original data of the articles were applied in the subsequent meta-analysis.

Percentage of GG genotype in controls of each study was calculated, followed by Hardy-Weinberg Equilibrium (HWE) test in controls to determine the reliability of data, using a Chi-squared Goodness-of-fit Test by SPSS 13.0. Analysis was also conducted on inter-ethnicity difference in minor allele frequency. One-way ANOVA was used to compare more than two independent groups, while two-tailed *t* test was used to compare two independent groups by SPSS 13.0 software.

To investigate the effect of each allele, the ORs of G allele were calculated, referenced by C. Subsequently, pairwised combinations of genotypes were used to determine the hereditary models, including GG *vs* CC, CG *vs* CC, and GG *vs* CG, CG *vs* CC + GG, GG *vs* CC + CG and CG *vs* GG + GC, and the later genotype was used as a reference in each pair.

We also conducted meta-analyses for a combination of CG and GG genotypes *vs* CC genotype in each subgroups (European and USA population). In addition, stratified analyses were performed based on the case collection, including meta-analyses on the association between PPAR-γ 34 C>G and colon cancer risk and rectal cancer risk.

Heterogeneity among studies was tested to estimate which effect model, the fixed-effect one or the random-effect one, should be used. With a $P > 0.05$, the included studies were considered homogeneous and the fixed-effect model should be selected, otherwise, random-effect model should be used.

All of the meta-analyses above were conducted using Review Manager 4.2 software. The two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Nine studies published from 2003-2007 were about the analysis on the relationship between PPAR-γ 34 C>G polymorphism and CRC risk, with a total number of 4533 cases and 6483 controls (Table 1). Seven studies (3870 cases/5028 controls) were conducted in Western countries, including 4 in Europe[1,5,9,10] and 3 in the USA[2,6,8]. Another two studies were performed in Asian countries[4,7]. There were four studies concerning colon cancer or rectal cancer with a total number of 2073 cases/3735 controls and 1321 cases/2765 controls, respectively[4-7].

The genotype distribution in the control groups in each study did not depart from the HWE with $P > 0.05$, except for two studies, in which HWE test could not be performed because of the incomplete data (Table 1).

The G allele frequency of PPAR-γ 34 C>G was 0.13 in the control group (626 cases/4694 controls) and 0.12 in the case group (424 cases/3600 controls), respectively. No statistical significance was found between case group and control group ($P = 0.381$). The G allele frequency of PPAR-γ 34 C>G in the Western controls (seven studies) was 0.14, the same as in the European controls (four studies). And in USA controls of three studies, G allele frequency of PPAR-γ 34 C>G was 0.13. In conclusion, there is no inter-ethnicity difference in minor allele frequency ($P = 0.968$).

Overall and subgroup-specific summary ORs and 95% CIs for the relationship between PPAR-γ 34 C>G and CRC risk are summarized in Table 2. For G *vs* C allele, GG *vs* CC, CG *vs* CC, GG *vs* CG, CG *vs* CC + GG, GG *vs* CC + CG and CG + GG *vs* CC genotypes of the overall study population, the fixed-effect and the random-effect ORs (95% CIs) were listed, respectively. Test for heterogeneity indicated that studies in the analyses for G *vs* C allele, CG *vs* CC, CG *vs* CC + GG, CG + GG *vs* CC genotypes, respectively were heterogeneous with $P < 0.05$. Hence, random-effect models were selected. And

Table 1 Study characteristics

No.	Ref.	Yr	Country	Number of cases					Number of controls					34G allele frequency in control	P^1
				CC	CG	GG	Total	CG + GG	CC	CG	GG	Total	CG + GG		
1	Landi *et al*[5]	2003	Spain (W, E)	311	46	3	360	49	243	61	5	309	66	0.11	0.773
2	Gong *et al*[2]	2005	USA (W)	129	30	4	163	34	153	52	7	212	59	0.16	0.743
3	Murtaugh *et al*[6]	2005	USA (W)	1840	-	-	2371	531	2283	-	-	2972	689	-	-
4	Jiang *et al*[4]	2005	India (A)	240	57	4	301	61	230	57	4	291	61	0.11	1.000
5	Siezen *et al*[1]	2006	Netherlands (W, E)	160	40	1	201	41	325	71	2	398	73	0.09	0.783
6	Koh *et al*[7]	2006	Singapore (A)	345	-	-	362	17	1075	-	-	1164	89	-	-
7	Gunter *et al*[8]	2006	USA (W)	153	41	4	198	45	146	37	1	184	38	0.11	0.838
8	Theodoropoulos *et al*[9]	2006	Greek (W, E)	164	48	10	222	58	118	70	12	200	82	0.24	0.950
9	Vogel *et al*[10]	2007	Denmark (W, E)	252	96	7	355	103	550	190	13	753	203	0.14	0.816

[1]*P* value for Hardy-Weinberg equilibrium (HWE) for peroxisome proliferator-activated receptor-γ (PPAR-γ) 34C>G polymorphism among controls. W: Western country; E: European country; A: Asian country; -: No data was shown in reference.

Table 2 Overall and group-specific summary statistics for PPAR-γ 34 C>G and colorectal, colon and rectal cancers

	No. of studies	Polymorphisms	P^1	Fixed-effect OR (95% CI), P^2	Random-effect OR (95% CI), P^3
Colorectal cancer					
Total	7	G *vs* C	0.010	0.88 (0.77, 1.00), 0.060	0.86 (0.69, 1.08), 0.210
	7	GG *vs* CC	0.720	0.85 (0.53, 1.35), 0.480	0.83 (0.52, 1.33), 0.440
	7	CG *vs* CC	0.020	0.86 (0.74, 1.00), 0.050	0.83 (0.65, 1.07), 0.160
	7	GG *vs* CG	0.990	1.10 (0.68, 1.79), 0.690	1.09 (0.67, 1.78), 0.720
	7	CG *vs* CC + GG	0.020	0.86 (0.74, 1.00), 0.060	0.84 (0.66, 1.07), 0.150
	7	GG *vs* CC + CG	0.830	0.91 (0.57, 1.44), 0.680	0.89 (0.56, 1.43), 0.630
	9	CG + GG *vs* CC	0.009	0.90 (0.82, 0.99), 0.030	0.84 (0.69, 1.01), 0.070
Western	6	G *vs* C	0.006	0.87 (0.75, 1.00), 0.050	0.85 (0.65, 1.11), 0.230
	6	GG *vs* CC	0.600	0.83 (0.51, 1.36), 0.470	0.81 (0.49, 1.34), 0.420
	6	CG *vs* CC	0.009	0.84 (0.72, 1.00), 0.040	0.81 (0.60, 1.09), 0.170
	6	GG *vs* CG	0.970	1.12 (0.67, 1.87), 0.670	1.11 (0.66, 1.86), 0.700
	6	CG *vs* CC + GG	0.010	0.85 (0.72, 1.00), 0.050	0.82 (0.61, 1.09), 0.160
	6	GG *vs* CC + CG	0.730	0.90 (0.55, 1.47), 0.680	0.88 (0.54, 1.45), 0.630
	7	CG + GG *vs* CC	0.006	0.91 (0.82, 1.01), 0.070	0.85 (0.68, 1.06), 0.140
USA	3	CG + GG *vs* CC	0.320	0.95 (0.84, 1.07), 0.360	0.94 (0.80, 1.11), 0.450
Europe	4	CG + GG *vs* CC	0.002	0.84 (0.70, 1.00), 0.060	0.79 (0.52, 1.19), 0.260
Colon cancer	4	CG + GG *vs* CC	0.030	0.83 (0.72, 0.96), 0.010	0.80 (0.65, 0.99), 0.040
Rectal cancer	4	CG + GG *vs* CC	0.050	0.98 (0.82, 1.18), 0.860	0.84 (0.58, 1.22), 0.370

[1]*P* value for test for heterogeneity; [2]*P* value for fixed-effect model; [3]*P* value for random-effect mode.

for the rest ones with $P > 0.05$, fixed-effect model was used. As a result, no statistical significance was observed among the above analyses. However, the data of CG + GG *vs* CC genotypes was marginally significant with OR = 0.84 (95% CI: 0.69-1.01, $P = 0.07$).

Limited results in studies from Western countries, the fixed-effect and random-effect ORs (95% CIs) for G *vs* C allele, GG *vs* CC, CG *vs* CC, GG *vs* CG, CG *vs* CC + GG, GG *vs* CC + CG and CG + GG *vs* CC genotypes are listed in Table 2, respectively. Same to the former analyses of the total study population, there was no evidence for the association between PPAR-γ 34 C>G and CRC risk.

Further subgroup-specific analyses were performed in the European and USA studies. In studies from European countries, summary ORs (95% CIs) for CG + GG *vs* CC genotypes in fixed-effect model and random-effect model were 0.84 (0.70, 1.00) and 0.94 (0.80, 1.11), respectively. In studies from the USA, the corresponding ORs (95% CIs) were 0.79 (0.52, 1.19) and 0.95 (0.84, 1.07), respectively. No evidence was found for the association between PPAR-γ 34 C>G and CRC risk in each of the two study populations.

However, when stratified analyses were performed, the results were different. As shown in Table 2, four studies were involved in the meta-analyses for the association of PPAR-γ 34 C>G with colon cancer risk and rectal cancer risk. In the four studies, only the data for CG + GG *vs* CC genotypes was sufficient enough for the analyses. As *P* values of the heterogeneity test in the two meta-analyses were less than 0.05, random-effect model was used. Summary ORs (95% CIs) for CG + GG *vs* CC genotypes in colon cancer studies and rectal cancer studies were 0.80 (0.65, 0.99) and 0.84 (0.58, 1.22),

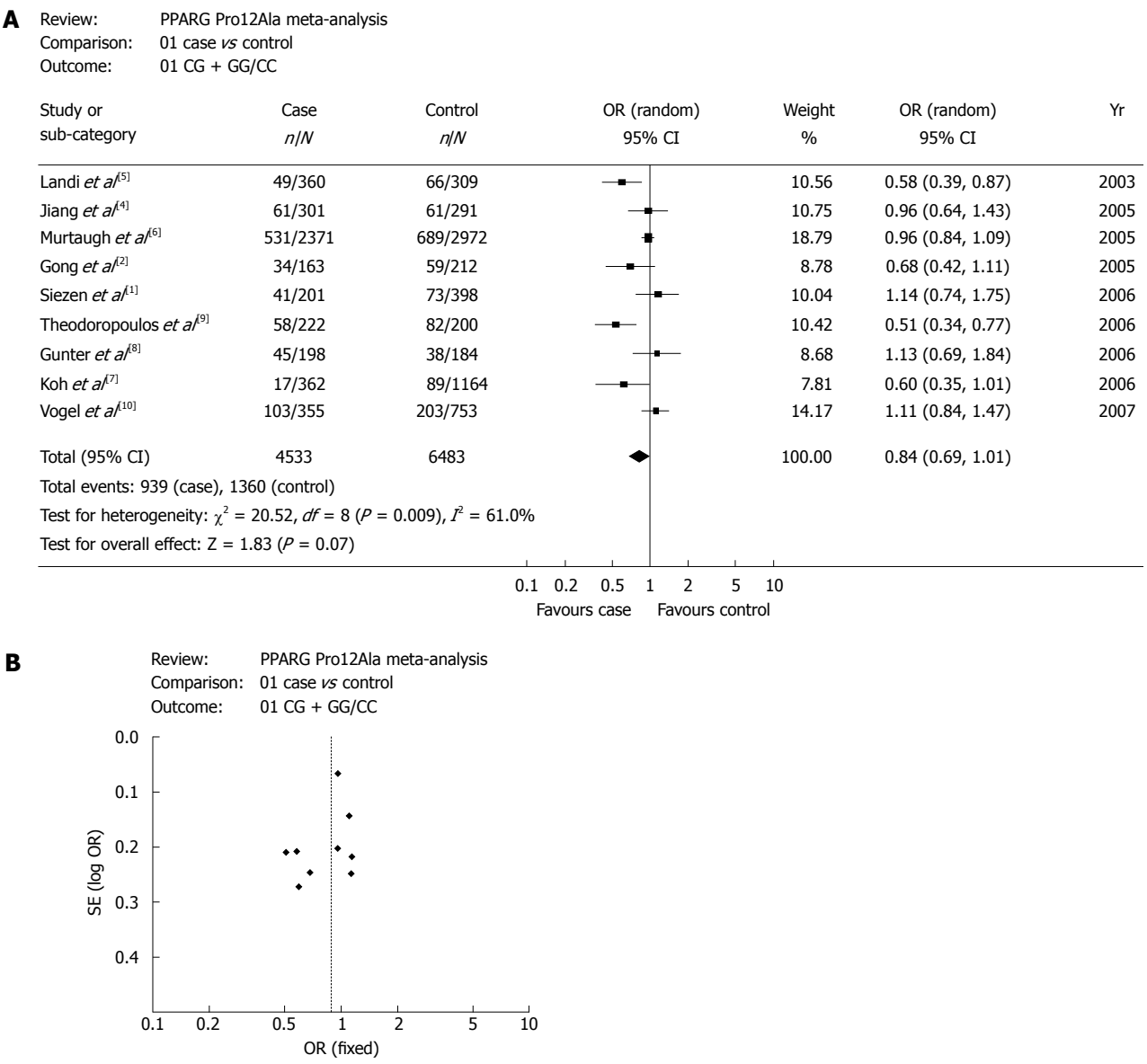

Figure 1 Association between peroxisome proliferator-activated receptor-γ (PPAR-γ) 34 C>G and colorectal cancer risk (CG + GG *vs* CC). A: Forest plot-studies are sorted in order of publication year; B: Funnel plots for the associations.

respectively. Statistical significance was observed in the association meta-analysis between PPAR-γ 34 C>G and colon cancer risk (P = 0.04), indicating that PPAR-γ 34 C>G was associated with colon cancer risk, and G allele decreased the colon cancer risk. However, there was no evidence for the association between PPAR-γ 34 C>G and rectal cancer risk (P = 0.37).

Figure 1A shows the forest plots of meta-analysis for CG + GG *vs* CC genotypes to confirm the association between PPAR-γ 34 C>G and CRC risk in the overall study population. The association between PPAR-γ 34 C>G and CRC risk had a marginally statistical significance. Figure 1B is the funnel plot, suggesting that there was no publication bias in the studies.

Figure 2A displays the forest plots of the study on the association between PPAR-γ 34 C>G and colon cancer risk. Obviously, there was a significant association of PPAR-γ 34 C>G with colon cancer risk. Publication bias was not found in this study as shown in Figure 2B. Other forest plots and funnel plots of meta-analyses on the association between PPAR-γ 34 C>G and CRC risk, colon cancer risk and rectal cancer risk were not shown. However, the results are presented in Table 2.

DISCUSSION

CRC is one of the leading causes of cancer death in the developed countries[11,12]. Both sporadic and hereditary CRC is caused by a set of molecular events[13]. Accumulated evidences indicate that lipid metabolism, especially the one involved in the arachidonic acid (AA)-pathway, appears to play a critical role in the development of colorectal tumor[14]. *PPARγ* gene, one of the most important components of the AA-pathway, has been veri-

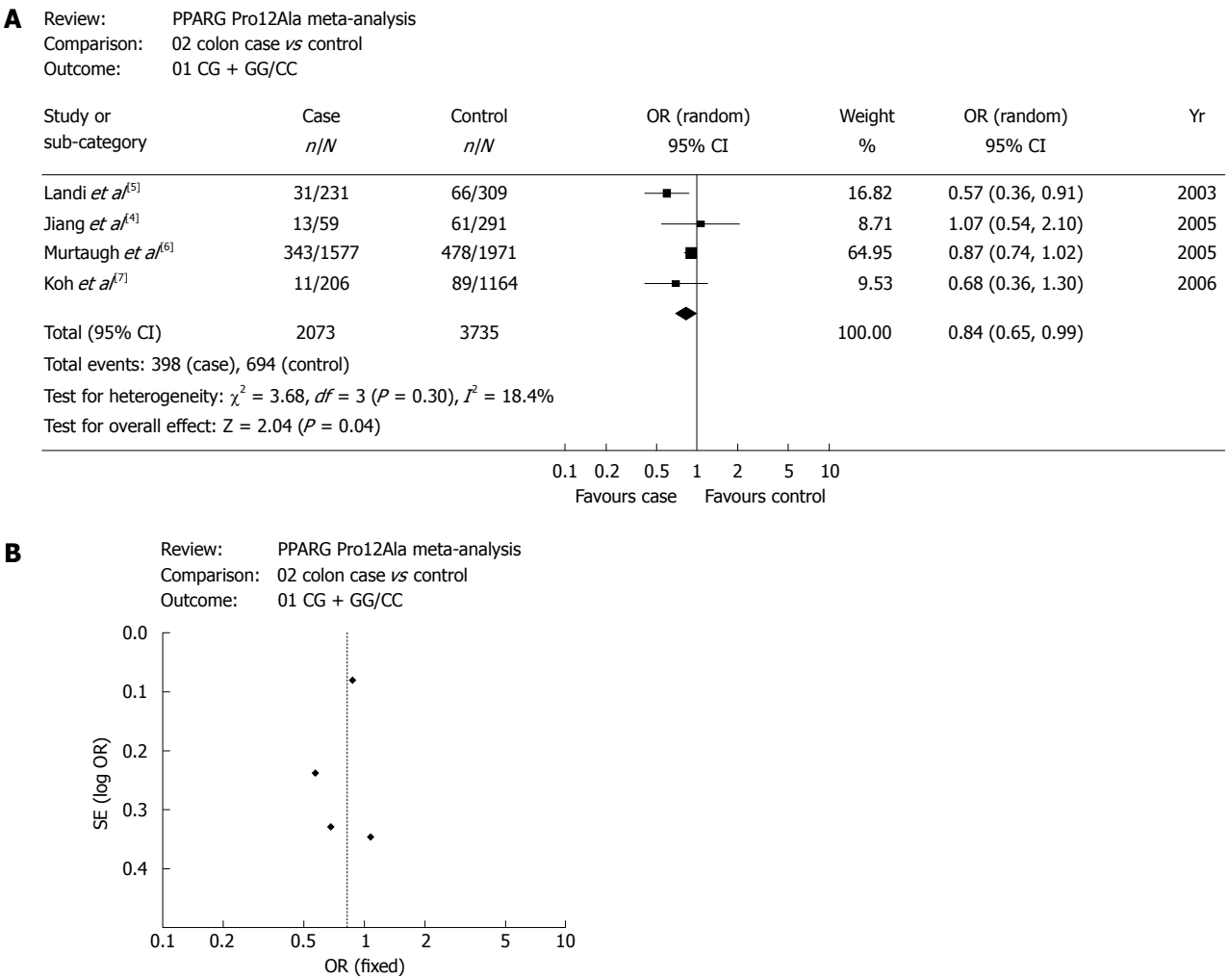

Figure 2 Association between PPAR-γ 34 C>G and colon cancer risk (CG + GG *vs* CC). A: Forest plot-studies are sorted in order of publication year; B: Funnel plots for the associations.

fied to express in a variety of tumor cells. And it will lead to either inhibition of cell proliferation or induction of apoptosis after bonding with ligands[15,16]. Many studies reported that PPAR-γ was also expressed in colon tumors, normal colon mucosa and colon cancer cell lines[17-19]. Genomics research showed that there was a polymorphism in the coding region 34 C>G in PPAR-γ which resulted in the amino acid change of Pro12 Ala[20]. So far, many studies have been performed on the association between PPAR-γ 34 C>G polymorphism and CRC risk, but produced controversial results.

According to our search of references, no systematic review has been published on the analysis of the association between PPAR-γ 34 C>G and CRC. In order to confirm the data on the associations between *PPAR-γ* gene polymorphism and CRC, we did a meta-analysis based on nine studies from Europe, Asia and the USA.

Our results showed that there was no evidence for the association between PPAR-γ 34C>G and CRC if all of the subjects in the nine studies were included. Subgroup-specific meta-analyses also indicated that there was no association between PPAR-γ 34C>G and CRC in European, Asian and the USA studies. As shown in our analysis, G allele might decrease CRC risk, although statistical difference was not significant in these meta-analyses.

Many studies have indicated that both genetic and environmental factors are involved in the development of colorectal tumor[21]. Environmental factors include ethnicity, gender, diet, age, NSAIDS use, BMI, smoking, drinking, family history of disease and so on. It was inferred that there must be interaction between the environmental factors and *PPAR-γ* gene, which had been proved in many researches, including the nine studies. Therefore, interaction is one of the factors of the meta-analyses. Further analysis should be conducted to confirm the influence of PPAR-γ 34 C>G on CRC risk.

Stratified meta-analysis indicated that PPAR-γ 34 C>G was associated with colon cancer, and the G allele decreased the colon cancer risk. No evidence was observed for the association between PPAR-γ 34 C>G and rectal cancer.

In conclusion, no evidence was observed for the association between PPAR-γ 34 C>G and CRC risk and rectal cancer risk. However, PPAR-γ 34 C>G is associated with colon cancer risk, which is meaningful to early

diagnosis, prevention and individual-based treatment of colon cancer. Furthermore, 34 C>G of *PPAR-γ* gene might be a potential therapeutic target for colon cancer.

COMMENTS

Background

Colorectal cancer (CRC) is one of the major causes of cancer death in the developed countries. Accumulated evidences suggest that the peroxisome proliferator-activated receptor-γ (*PPAR-γ*) gene is related to CRC.

Research frontiers

Accumulated evidences indicate that lipid metabolism, especially the one in the arachidonic acid (AA)-pathway, appears to play a critical role in the development of CRC. *PPAR-γ* gene, one of the most important components of the AA-pathway, has been verified to express in a variety of tumor cells. A proline to alanine substitution in the *PPAR-γ* gene was detected, which might be associated with CRC.

Innovations and breakthroughs

Many studies have been performed about the association between the polymorphism 34 C>G of *PPAR-γ* gene and CRC, but got conflicting results. In order to confirm the data, meta-analyses, as a better statistical analysis technique, were performed in this report.

Applications

In this report, the association between *PPAR-γ* gene polymorphism 34 C>G and colon cancer risk was observed, and the G allele decreased the colon cancer risk, which is meaningful to early diagnosis, prevention and individual-based treatment of colon cancer. Furthermore, 34 C>G of *PPAR-γ* gene might be a potential therapeutic target for colon cancer.

Terminology

Susceptibility to colon cancer differs according to genotype of PPAR-γ 34 C>G, and people with G allele might have a lower colon cancer risk.

Peer review

This is an interesting meta-analysis on the association between PPAR-γ 34C>G polymorphism and CRC risk. The main finding of the paper was that PPAR-γ 34 C>G was weakly associated with colon cancer risk, but was not associated with CRC and rectal cancer risk. The methods are properly used; authors report a high quality meta-analysis.

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BRIEF ARTICLE

Aberrant expression of nuclear matrix proteins during HMBA-induced differentiation of gastric cancer cells

Guang-Jun Jing, Dong-Hui Xu, Song-Lin Shi, Qi-Fu Li, San-Ying Wang, Fu-Yun Wu, Hai-Yan Kong

Guang-Jun Jing, Song-Lin Shi, Qi-Fu Li, San-Ying Wang, Fu-Yun Wu, Hai-Yan Kong, Key Laboratory of Ministry of Education for Cell Biology & Tumor Cell Engineering, School of Life Science, Xiamen University, Xiamen 361005, Fujian Province, China
Dong-Hui Xu, Department of Hepatic Biliary Pancreatic Vascular Surgery, First Affiliated Hospital of Xiamen University, Xiamen 361003, Fujian Province, China
Author contributions: Jing GJ, Xu DH, Wang SY and Li QF designed the research; Jing GJ, Xu DH and Shi SL performed the research; Jing GJ, Li QF, Wu FY and Kong HY wrote the paper.
Supported by National Natural Science Foundation of China, No. 30871241
Correspondence to: Qi-Fu Li, Professor, Key Laboratory of Ministry of Education for Cell Biology & Tumor Cell Engineering, School of Life Science, Xiamen University, Xiamen 361005, Fujian Province, China. chifulee@xmu.edu.cn
Telephone: +86-592-2185363 Fax: +86-592-2181015
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Abstract

AIM: To investigate the aberrant expression of nuclear matrix proteins in human gastric cancer cells before and after hexamethylene bisacetamide (HMBA) treatment.

METHODS: Proteomics analysis of differential nuclear matrix proteins was performed by two dimensional electrophoresis polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The expression levels of three nuclear matrix proteins were further confirmed by Western blotting and their locations in nuclear matrix filament were observed by quantum dots-based immunofluorescence.

RESULTS: Proteomics analysis showed that 43 protein spots were significantly changed due to HMBA treatment. Fifteen proteins were identified in the HMBA-induced differentiation of gastric tumor cells. Eight proteins spots were down-regulated while seven were up-regulated. Among these proteins, prohibitin, nucleophosmin and hnRNP A2/B1 were significantly decreased in HMBA-treated human gastric cancer cells, and their locations in nuclear matrix were altered by HMBA. Our results proved the alteration of specific nuclear matrix proteins during the differentiation of human gastric cancer cells. And the aberrant expressions of nuclear matrix proteins were of significance in revealing the regulatory mechanism of tumor cell proliferation and differentiation.

CONCLUSION: The aberrant expressions and intracellular redistributions of nuclear matrix proteins before and after HMBA treatment indicated that nuclear matrix proteins play a pivotal role in the differentiation of gastric cancer cells.

Key words: Human gastric tumor cell; Hexamethylene bisacetamide; Differentiation; Nuclear matrix; Proliferation

Peer reviewers: Dr. Jianyuan Chai, PhD, MS, BS, Assistant Professor, Research (09-151), VA Long Beach Healthcare System, 5901 E. 7th St, Long Beach, CA 90822, United States; Dr. Mukaddes Esrefoglu, Professor, Department of Histology and Embryology, Inonu University, 44280, Malatya, Turkey

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INTRODUCTION

Gastric cancer is the most common malignant gastrointestinal cancer, which accounts for 25% of cancer

deaths[1]. Researches on gastric cancer cell differentiation will not only contribute to the diagnosis of gastric cancer, but also the regulatory mechanism of tumor cell differentiation. Studies have been carried out on the mechanism of induced differentiation of gastric cancer cells. But reports about the proteins related to the differentiation and proliferation of gastric cancer are rare. It is important to elucidate the regulatory mechanism of gastric cancer cell differentiation. Nuclear matrix is a filamentous protein framework for eukaryotic cell chromatin. Nuclear matrix proteins closely relate to DNA duplication and transcription[2]. Some regulatory proteins or enzymes are nuclear matrix proteins or nuclear matrix-binding proteins[3], and they regulate cell differentiation. Protein components of nuclear matrix are dynamic throughout cell proliferation and differentiation. Protein composition of nuclear matrix in tumor cells differ from normal tissue-derived cells[4]. In our previous study we found that the protein compositions of nuclear matrix were changed during the induced-differentiation of various tumor cells[5,6], suggesting that analysis on the aberrant expression of nuclear matrix proteins was of great importance for an in-depth investigation in the mechanism of cell carcinogenesis and reversal. Hexamethylene bisacetamide (HMBA) is a hybrid bipolar compound and originally developed as a potent inducer of cell differentiation[7]. Hereby, based on the induced effects of HMBA on the differentiation of human gastric cancer cells[8,9], we extended our study to determine the aberrant expression of nuclear matrix proteins in HMBA-treated human gastric adenocarcinoma BGC-823 cells. We aimed to identify specific nuclear matrix proteins related to gastric cancer cells and provide further scientific evidences for the mechanism of gastric cancer cell proliferation and differentiation.

MATERIALS AND METHODS

Cell culture

BGC-823 cells, obtained from China Center for Type Culture Collection, were routinely cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone) and 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL kanamycin at 37℃ in a humidified atmosphere containing 5% CO_2.

Whole cell and nuclear matrix lysates preparation

To prepare the whole cell extracts, cells were washed in phosphate buffered saline (PBS) and then lysed in ice-cold lysis buffer {7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1.5% Triton X-100, 1% pharmalyte (pH 3-10 Bio-rad), 65 mmol/L DL-Dithiothreitol (DTT), 40 mmol/L Tris, 5 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 2 mmol/L phenylmethanesulfonyl fluoride (PMSF), and 5 mmol/L ethylenediaminetetraacetic acid (EDTA)}. The suspension was then sonicated for 20 min at 0℃ and centrifuged at 8000 × *g* for 30 min.

Nuclear matrix proteins were prepared using a method described by Michishita *et al*[10]. After washed in ice-cold PBS twice, BGC-823 cells were suspended in cytoskeleton (CSK) buffer [100 mmol/L KCl, 3 mmol/L $MgCl_2$, 5 mmol/L ethylene glycol tetraacetic acid, 10 mmol/L piperazine-N,N'-bis(2-ethanesulfonic acid), 300 mmol/L sucrose, 0.5% Triton X-100, and 2 mmol/L PMSF, pH 6.8] for 10 min at 0℃. After being centrifuged at 1000 × *g* for 5 min, the pellet was resuspended in digestion buffer (identical to CSK buffer except for 50 mmol/L NaCl instead of KCl) containing 400 mg/mL DNase I for 30 min at room temperature and centrifuged at 1000 × *g* twice. Cold ammonium sulfate at a final concentration of 0.25 mol/L was used to precipitate proteins. After centrifugation, the pellet was dissolved in lysis buffer [7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 1.5% Triton X-100, 1% Pharmalyte (pH 3-10 Bio-rad), 65 mmol/L DTT, 40 mmol/L Tris, 5 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 2 mmol/L PMSF, and 5 mmol/L EDTA] and then sonicated at 0℃ for 20 min. Finally, the suspension was centrifuged at 10000 × *g* at 4℃ for 30 min and the supernatants were used as nuclear matrix extracts.

Protein concentrations were determined by BCA assay.

Two dimensional electrophoresis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis and protein identification

2-D polyacrylamide gel electrophoresis (PAGE) was performed as follows. Protein lysates were diluted in sample buffer with 2% Immobiline™ DryStrip gel (IPG) buffer, pH 3-10, nonlinear (GE Heathcare). The samples were applied to 18 cm, immobilized pH gradient strips of pH 3-10 (IPG Drystrips, GE Healthcare). After isoelectric focusing was completed, the strips were equilibrated and the second dimensional electrophoresis was carried out overnight at 3 W/gel at 20℃. The triplicate sets of silver-stained gels were scanned using a UMAX POWER LOOK Ⅲ photometer and analyzed with the PD Quest 8.0 software (Bio-rad). The digitalized two dimensional electrophoresis (2-DE) gel images were compared by matching method. Differentially expressed spots were analyzed and annotated.

The spots were cut and digested using 12.5 ng/μL trypsin (Promega, Madison, WI, USA) in 50 mmol/L ammounium bicarbonate (pH 8.0, Sigma). After that the samples were eluted with 2 μL of matrix solution containing 10 mg/mL α cyano-4-hydroxy cinnamic acid (CHCA, Sigma) and were submitted to Bruker Ⅲ matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. The spectra were internally calibrated using the trypsin autolysis products [842.51 (M + H) and 2211.11 (M + H)] by Flex Analysis software and searched against Swiss-prot and NCBI database using the Mascot tool from matrix science. All the searches were analyzed with a 50 ppm mass tolerance.

Western blotting

For Western blotting experiments, 20 μg cell lysates were

Table 1 Differentially expressed nuclear matrix proteins identified by mass spectrum

Spot number	Protein name	Accession number	Mol. mass calc (Da)	pI (calc)	Score
Up-regulated proteins					
L1	Zinc finger protein 268	Q14587	108373	9.14	57
L2	Calcium-binding protein CaBP5	Q9NP86	19812	4.46	61
L3	MAGUK p55 subfamily member 3	Q13368	66168	6.27	45
L4	Vacuolar protein sorting 33B	Q9H267	70570	6.29	54
L5	Ras-related protein Rab-30	Q15771	23058	4.91	64
L6	Vimentin	57471646	49623	5.19	48
L7	Calnexin (Precursor)	4262127	10638	5.30	89
Down-regulated protein					
L8	Peroxiredoxin 2	P32119	21892	5.66	72
L9	Transcriptional repressor CTCFL	Q8NI51	75668	8.58	67
L10	Prohibitin	P35232	29859	5.57	79
L11	Keratin 1, type Ⅱ, cytoskeletal	7428712	65455	6.03	86
L12	PACAP protein	18204192	20681	5.37	161
L13	hnRNP A2/B1	P22626	36600	8.67	97
L14	C-type lectin	Q2K157	22130	5.47	68
L15	Nucleophosmin	Q96EA5	32726	4.64	76

loaded and separated on polyacrylamide gles and then transferred to positively-charged nylon membranes (Millipore, Bedford, MA) according to standard protocol. These blots were blocked for 1 h at room temperature in 5% skim milk. The target proteins were probed with primary antibodies and horseradish peroxidase-labeled secondary antibodies (Santa Cruz). β-actin was used as an indicator for equality of lane loading. Antibody positive bands were visualized using ECL Western blotting detection reagents (Pierce). The X-ray film was scanned and the band density was calculated using the ImageJ software[11].

Quantum dots-based sample preparation for fluorescence microscopy

The NM-IF system was prepared according the methods described by Liang *et al*[12]. After being prefixed with 4% paraformaldehyde at room temperature for 10 min, the cover slips were blocked with 3% bovine serum albumin for 1 h and then incubated with nucleophosmin (NPM), prohibitin (PHB) and hnRNP A2/B1 primary antibodies at 37℃ for 1 h. After washing, the cover slips were rinsed in biotin-labeled secondary antibodies for 45 min at 37℃, washed with tris-buffered saline Tween-20, and incubated with streptavidin-conjugated quantum dots (size scale, 605 nm) for 1 h at 37℃. After that, the cover slips were enveloped with 90% glycerol and observed under fluorescence microscope.

RESULTS

Proteomics analysis of BGC-823 cells before and after HMBA treatment

For proteomics analysis, cell lysates from control and HMBA-treated cells were submitted to 2D-PAGE followed by silver-staining of the gel. The procedures were independently repeated three times and the representative gel images are shown in Figure 1A. And Figure 1B displayed the enlarged maps of changed expression of nuclear matrix proteins from BGC-823 cells. PD Quest 8.0 software (Bio-rad) detected about 342 ± 10 proteins spots on the silver-stained gels of control group while 280 ± 13 protein spots of HMBA-treated group, suggesting that the result of 2-DE PAGE had a high repeatability. Forty-three changed spots were excised and digested with trypsin, and were identified by mass spectrometry. Fifteen proteins were identified in the HMBA-induced differentiation of gastric tumor cells. The identified proteins are listed in Table 1. The expressions of Zinc finger protein 268, calcium-binding protein CaBP5, MAGUK p55 subfamily member 3, vacuolar protein sorting 33B, and Ras-related protein Rab-30, Vimentin, Calnexin (Precursor) were up-regulated while peroxiredoxin 2, transcriptional repressor CTCFL, PHB, Keratin 1 type Ⅱ cytoskeletal, PACAP protein, hnRNP A2/B1, C-type lectin and NPM were down-regulated. Another 28 protein spots were not identified for their low abundances. We next focused on PHB, NPM and hnRNP A2/B1, which were also identified as aberrantly expressed proteins in our previously studies[12-15].

Immunoblotting confirmation of differentially expressed nuclear matrix proteins

To further verify the aberrant changes of PHB, NPM and hnRNP A2/B1, Western immunoblotting was employed to confirm the expression levels of these proteins in cells before and after HMBA treatment, and the intensities of protein bands were densitometrically quantified as described by Sheffield JB[11]. Results showed that signals of PHB, NPM and hnRNP A2/B1 in HMBA-treated cells were much lower than that in control cells, suggesting that the expressions of these three proteins at 7 d of treatment were significantly inhibited by HMBA (Figure 2). All the Western blotting results were consistent with the proteomic analysis.

Localization of PHB, NPM and hnRNP A2/B1 in NM-IF system before and after HMBA treatment

Proteomics analysis showed that PHB, NPM and hnRNP

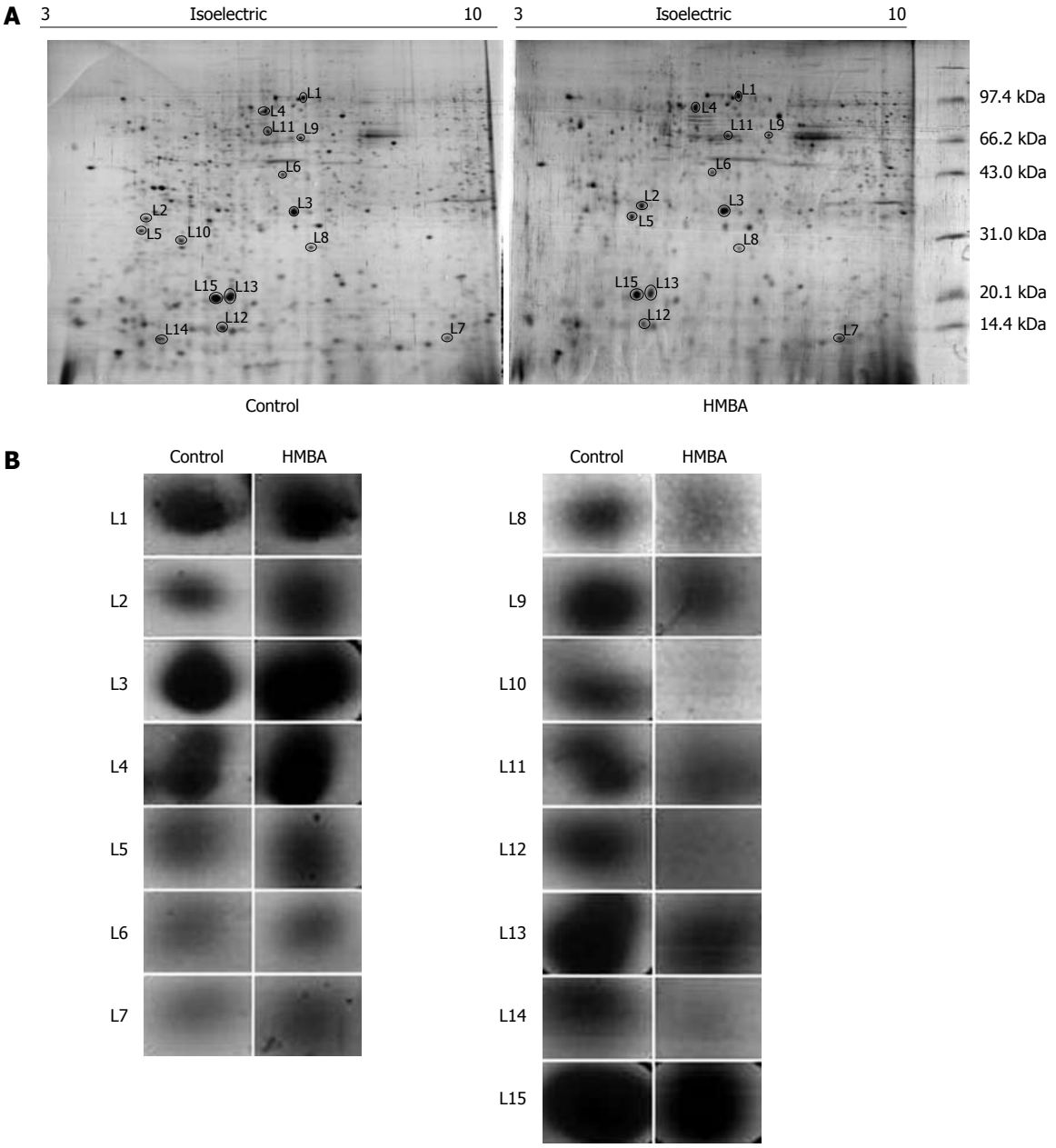

Figure 1 Two-dimensional protein profiles from the nuclear matrix of BGC-823 cells. A: Representative two-dimensional protein profiles from the nuclear matrix of BGC-823 cells before and after hexamethylene bisacetamide (HMBA) treatment. The differentially expressed proteins are marked with circular symbols on the gels. L1-L7 spots indicate the up-regulated proteins while L8-L15 indicates the down-regulated proteins; B: Enlarged portions from 2-DE gels.

A2/B1 were nuclear matrix proteins. QDs-based location experiments were conducted to corroborate the alteration of the physical distribution of these three proteins in nuclear matrix-intermediate system. Clearly altered distribution patterns for NPM and hnRNP A2/B1 were observed in the nucleus while PHB in at the cytoplasm. The red fluorescence representing PHB mainly accumulated at the nucleus periphery in control cells (Figure 3C), while PHB was uniformly distributed in the whole cytoplasm after HMBA treatment, suggesting that HMBA obviously altered the location of PHB (Figure 3D). Highly intensified NPM fluorescence mainly localized in the residue of nucleoli in correspondence with the fact that NPM was an established marker for the granular components[16], with very subtle expression in the cytoplasm (Figure 3A). HMBA treatment resulted in the low intensity of fluorescence in nucleolus and faint fluorescence in cytoplasm derived from NPM, suggesting that NPM had a tendency of translocation from nucleoli regions to nuclear matrix (Figure 3B). When referred to hnRNP A2/B1, a similar distribution pattern to NPM was observed. In control cells, the fluorescence representing hnRNP A2/B1 was mainly detected in nuclear region with subtle signals in cytoplasm region (Figure 3E), whereas after HMBA treatment, a clear translocation appeared from nuclear to cytoplasm region, and hnRNP A2/B1was uniformly distributed in the cytoplasm but not in the nucleus (Figure 3F).

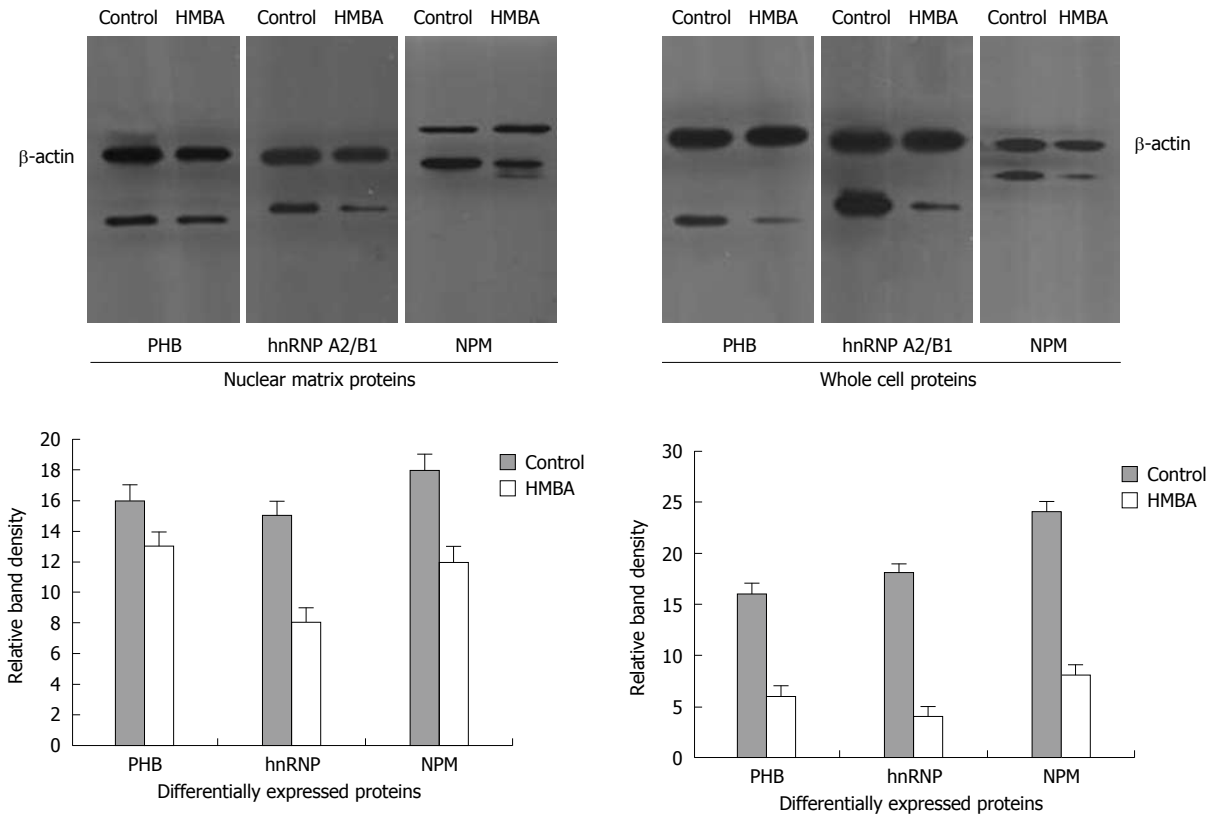

Figure 2 Determination of prohibitin (PHB), nucleophosmin (NPM) and hnRNP A2/B1 at nuclear matrix proteins and whole cell proteins by Western immunoblotting. All of these three protein signals were found to be down-regulated at whole cell level and nuclear matrix level after HMBA treatment.

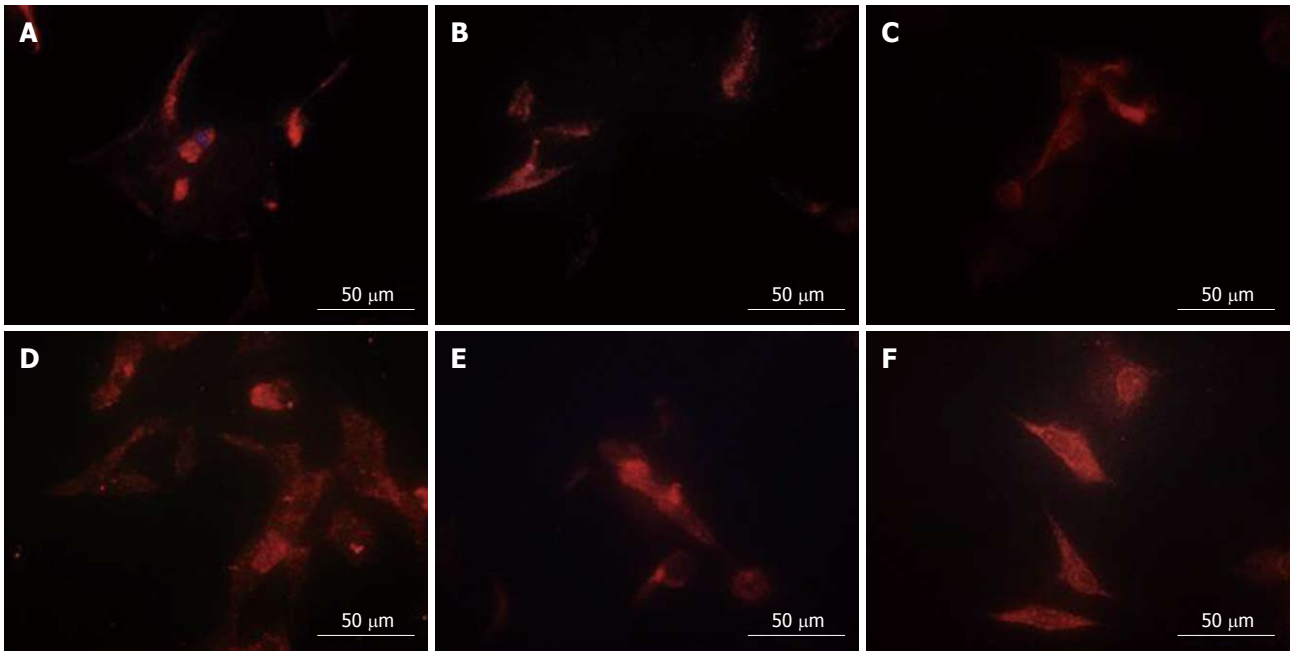

Figure 3 Effects of HMBA treatment on the localization of differentially expressed nuclear matrix proteins in NM-IF system as shown by a quantum dots-based immnuofluorescence. Typical changes of localization of NPM (A, B), PHB (C, D) and hnRNP A2/B1 (E, F) are observed after treatment with 5 mmol/L HMBA. The figure is composed of representative pictures taken from three independent experiments. A, C, E: Control; B, D, F: HMBA.

DISCUSSION

The alteration of nuclear matrix components is closely related to cell carcinogenesis. Configuration and protein composition of nuclear matrix in tumor cells differ from normal tissue-derived cells. For further investigating the effects of HMBA on the protein components of nuclear matrix of human gastric cancer cells, proteomics

analysis was employed to screen the aberrant nuclear matrix proteins of human gastric cancer cells before and after HMBA treatment. And 15 differentially expressed proteins were identified, seven of which, including Zinc finger protein 268, nebulin, *etc.* were up-regulated while the other eight proteins, including PHB, KeHMBAtin 1 type Ⅱ, cytoskeletal, hnRNP A2/B1, NPM, *etc.* were down-regulated. Getzenberg *et al*[17] reported that nuclear matrix profiles of bladder cancer, liver cancer and colon cancer were significantly altered in comparison with that of the normal tissues; moreover, differential nuclear matrix proteins were tumor-specific[17-19]. In our previous studies, we found variational nuclear matrix profiles of tumor cells[5,6]. HMBA could change protein compositions of gastric cancer cell nuclear matrix. The alteration of nuclear matrix protein expressions during the differentiation of gastric cancer cells was closely related to their functional transformation.

In this article we focused on the aberrant expression of nuclear matrix proteins in the induced-differentiation of gastric cancer cells. Among all the differentially expressed proteins, the down-regulated proteins, PHB, NPM and hnRNP A2/B1, were also identified in our previous studies on the induced differentiation of gastric cancer MGC80-3 cells, suggesting that PHB, NPM and hnRNP A2/B1 were common aberrantly expressed nuclear matrix-specific proteins in different tumor cell lines[8,12-15]. Proteomics analysis showed that expressions of PHB, NPM and hnRNP A2/B1 were down-regulated both in the whole cell lysates and nuclear matrix lysates, and the expression changes were further confirmed by Western blotting. Quantum dots-based immunofluorescent assay was performed to investigate the localization of specific nuclear matrix proteins in nuclear matrix-intermediate filament system. And results displayed that PHB, NPM and hnRNP A2/B1 were localized in nuclear matrix fibers, and their locations were altered by HMBA treatment. Our data further proved that PHB, NPM and hnRNP A2/B1 were common differential nuclear matrix proteins. And the aberrant expression and relocation of these three proteins were closely related to their regulatory roles in the process of tumor cell differentiation.

The importance of differentially expressed nuclear matrix proteins was embodied not only by their locations in nuclear matrix fibers but also their regulations on cell differentiation and apoptosis. For example, PHB presented anti-proliferation activities by inhibiting the activity of nuclear estrogen receptors, and it was usually over-expressed in tumor tissues[20]. PHB was also a biomarker protein for differentiating benign prostate hyperplasia from prostate cancer[21]. NPM was an abundant nucleolar phosphoprotein, and it could cause the suppression of cell differentiation and anti-apoptosis in human carcinogenesis. Inhibiting the activity of NPM led to the over-expression of P53[22]. Cell proliferation could be inhibited by blocking the shuttle of NPM from nuclear to cytoplasm[23]. hnRNP A2/B1 played a pivotal role in DNA damage repair[24]. The expression and location of PHB, NPM and hnRNP A2/B1 were changed in gastric tumor cells before and after HMBA treatment, suggesting their pivotal biological functions in the differentiation of gastric tumor cells. Continued investigation on specific nuclear matrix proteins will contribute to better understanding the regulatory mechanisms of nuclear matrix proteins in gastric carcinogenesis and reversal.

COMMENTS

Background

Gastric cancer is the most common malignant gastrointestinal cancer and accounts for 25% of cancer deaths. Nuclear matrix proteins are closely related to DNA duplication and transcription. Researches on nuclear matrix proteins of gastric tumor cells will contribute to the diagnosis and prevention of gastric cancer.

Research frontiers

It is important to identify specific nuclear matrix proteins related to gastric cancer cells and provide further scientific evidences for the mechanism of gastric cancer cell proliferation and differentiation.

Innovations and breakthroughs

Forty-three protein expressions were significantly changed in hexamethylene bisacetamide (HMBA)-treated human gastric cancer cells. Moreover, the locations of three proteins in nuclear matrix fibers were obviously altered after HMBA treatment.

Applications

The differentially expressed nuclear matrix proteins might be intracellular target proteins of HMBA and the tumor markers for gastric cancer.

Peer review

This study examined the effects of HMBA on nuclear matrix protein expression in gastric cancer cell BGC-823. Forty-three proteins were affected significantly by the treatment, among them 15 were identified including 7 up-regulated and 8 down-regulated ones. The study is neatly designed and the figures are presented with good quality. The manuscript is highly interesting, well-written and valuable.

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CASE REPORT

Refractory gastric ulcer with abundant IgG4-positive plasma cell infiltration: A case report

Takayoshi Fujita, Takafumi Ando, Masatoshi Sakakibara, Waki Hosoda, Hidemi Goto

Takayoshi Fujita, Masatoshi Sakakibara, Department of Gastroenterology, Aichi Cancer Center Aichi Hospital, Okazaki City, Aichi 444-0011, Japan
Takafumi Ando, Hidemi Goto, Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya City, Aichi 466-8550, Japan
Waki Hosoda, Department of Pathology and Molecular Diagnostics, Aichi Cancer Center, Nagoya City, Aichi 464-8681, Japan
Author contributions: Fujita T and Sakakibara M saw the patient; Hosoda W worked on the pathological study; Fujita T, Sakakibara M, Ando T and Goto H worked on the treatment decision; Fujita T, Ando T and Goto H wrote the paper.
Correspondence to: Takafumi Ando, MD, PhD, Associate Professor, Department of Gastroenterology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya City, Aichi 466-8550, Japan. takafumiando-gi@umin.ac.jp
Telephone: +81-52-7442144 Fax: +81-52-7442175
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Abstract

We describe a 77-year-old man with refractory gastric ulcer that worsened after *Helicobacter pylori* eradication therapy. Pathology showed marked infiltration of IgG4-positive plasma cells in the gastric lesions, which led us to suspect IgG4-related sclerosing disease. To the best of our knowledge, this is the first report of IgG4-related gastric ulcer without the main manifestation of autoimmune pancreatitis.

Key words: Sclerosis; Gastric ulcer; IgG4; Plasma cells

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INTRODUCTION

Gastric ulcer is now recognized as an infectious disease that results from infection with *Helicobacter pylori* (*H. pylori*). *H. pylori* causes continuous gastric inflammation with abundant neutrophil infiltration, and eradication therapy is standard treatment in gastric ulcer[1]. We encountered a 77-year-old man with gastric ulcer of 5 years' duration, which worsened after *H. pylori* eradication therapy. Pathologically, gastric lesions show marked infiltration of plasma cells, and immunohistochemical study revealed frank IgG4-positive plasma cell infiltration. This clinical picture suggested refractory gastric ulcer that was suspected of being IgG4-related sclerosing disease.

CASE REPORT

A 72-year-old man visited the gastroenterology department of our hospital in June 2004 complaining of abdominal discomfort and appetite loss. He had been well until 1 mo earlier, when he experienced discomfort in the upper abdomen and decreased appetite. He had no abdominal pain, diarrhea, fevers, night sweats or body weight loss. He had no allergies, and was not taking any medication. He had undergone appendectomy at age 21 years. He had no history of tobacco smoking and drank alcohol socially. There was no family history of cancer or autoimmune disease. On examination, he appeared comfortable, with a temperature of 37.3℃, blood

pressure 110/70 mm Hg, and pulse 128 beats/min. The abdomen was soft, without tenderness or distension. The remainder of the examination was normal.

Esophagogastroduodenoscopy (EGD) revealed multiple ulcers in the stomach, while the rapid urease test (RUT) was negative (Figure 1A). Biopsy specimens revealed no malignant cells. Treatment with famotidine was initiated at a single daily dose of 20 mg, which resulted in the gradual resolution of symptoms. EGD at 2 mo revealed multiple gastric ulcer scars. At 14 mo, he remained well, and EGD revealed multiple gastric ulcer scars and biopsy revealed gastritis.

At 26 mo, he remained well, and again EGD revealed multiple gastric ulcer scars and biopsy specimens revealed gastritis (Figure 1B). However, urea breath testing was positive for *H. pylori* infection. Elimination with lansoprazole (30 mg twice daily), amoxicillin (750 mg twice daily) and clarithromycin (200 mg twice daily) for 7 d was successful, and was maintained with rabeprazole (10 mg daily).

At 37 mo, he remained well, but EGD revealed severe active inflammation (edematous mucosa with exudates, multiple ulcers and stricture) at the body of the stomach (Figure 1C). Biopsy revealed no neoplastic infiltrate and RUT was negative. Laboratory results in serum included lactate dehydrogenase 122 IU/L (reference range: 124-226 IU/L), soluble interleukin 2 receptor 325 U/mL (reference range: 190-650 U/mL), carcinoembryonic antigen 3.3 ng/mL (normal value: ≤ 5.0) and amylase 181 IU/L (reference range: 58-167 IU/L). Serum Epstein-Barr antiviral-capsid-antigen IgG titer was 160. Cytomegalovirus (CMV) antigenemia assay and the *Treponema pallidum* hemagglutination test were negative. Additional investigation on reference to other facilities precluded the presence of malignant tumor and inflammatory bowel disease, whereas ultrasonography of the abdomen showed slight enlargement of the pancreatic head. Serum IgG4 at this time was 165 mg/dL (reference range: 4.8-105 mg/dL). The presence of autoimmune pancreatitis (AIP) was suspected.

At 49 mo, he remained well on maintenance treatment with rabeprazole. EGD revealed multiple ulcers and ulcer scars, and worsening of the stricture of the upper stomach (Figure 1D). At 54 mo, in February 2009, he remained well and completely asymptomatic. Repeated EDG disclosed multiple ulcer scars with stenosis of the upper body and well-defined ulcers at the lesser curvature (Figure 2). Pathological examination of gastric lesion biopsies revealed intense infiltration of plasma cells that contained IgG4 (Figure 3). Serum IgG was 1909 mg/dL (reference range: 870-1700 mg/dL); IgG4 was 203 mg/dL (reference range: 4-108 mg/dL); complement component C3 was 114 mg/dL (reference range: 80-140 mg/dL); complement component C4 was 25.9 mg/dL (reference range: 11.0-34.0 mg/dL); and amylase was 167 IU/L (reference range: 58-167 IU/L). Antinuclear antibody, rheumatoid factor, anti SS-A antibody, and anti SS-B antibody were negative. Computed tomography scanning of the abdomen showed that the pancreas was normal, with no evidence of enlargement. Magnetic resonance imaging of the abdomen with magnetic resonance cholangiopancreatography revealed normal biliary and pancreatic ducts, and confirmed the lack of pancreatic enlargement.

Although the high serum IgG4 level strongly suggested AIP, the clinical imaging studies showed no pancreatic enlargement. Corticosteroid therapy and the management of side effects were discussed, but the patient, who was asymptomatic, declined this option. At 64 mo (December 2009), he remains well and stable under maintenance therapy with a proton-pump inhibitor.

DISCUSSION

AIP is a specific type of pancreatitis that is thought to have an autoimmune etiology, and typically shows infiltration of IgG4-positive plasma cells and increased levels of serum IgG4[2,3]. IgG4-positive plasma cells may involve not only the pancreas, but also other organs including the bile duct, gallbladder, salivary gland, thyroid gland, lungs, stomach, colon, liver, retroperitoneum, kidney, prostate and lymph nodes. In 2003, Kamisawa *et al*[4,5] proposed a new clinicopathological entity, IgG4-related sclerosing disease, and suggested that AIP is a pancreatic lesion that reflects this systemic disease. Shinji *et al*[6] described a relationship between gastric ulcer and AIP in 2004, and detected gastric ulcer in eight of 23 AIP patients, and significantly more abundant IgG4-positive plasma cell infiltration in the gastric lesions of AIP patients. They have concluded that AIP is closely associated with gastric ulcer with abundant IgG4-bearing plasma cell infiltration.

A common cause of gastric ulcer is *H. pylori* infection. However, the gastric lesions in the present patient worsened after successful eradication of *H. pylori* with triple therapy. He had no history of non-steroidal anti-inflammatory drug use, which is another important cause of gastric ulcer. Pathological and serological findings precluded the possibility of gastric cancer of the scirrhous type, malignant lymphoma arising from mucosa-associated lymphoid tissue, Crohn's disease, reactivation of CMV infection, chronic active EBV infection, and syphilis. Given his elevated serum levels of IgG4 and abundant infiltration of IgG4-positive plasma cells, we consider that the gastric lesions in this patient were associated with infiltration of IgG4-positive plasma cells, and that the most likely diagnosis is IgG4-related sclerosing disease. Since some IgG4-related sclerosing disease patients relapse and improve spontaneously, worsening of this gastric lesion after *H. pylori* eradication was assumed to be independent of *H. pylori* infection. From recent reports that suggest that IgG4-related sclerosing disease is associated with cancer, our treatment goals in this asymptomatic 77-year-old man are the prevention of gastric cancer and gastric obstruction[7,8]. Since corti-

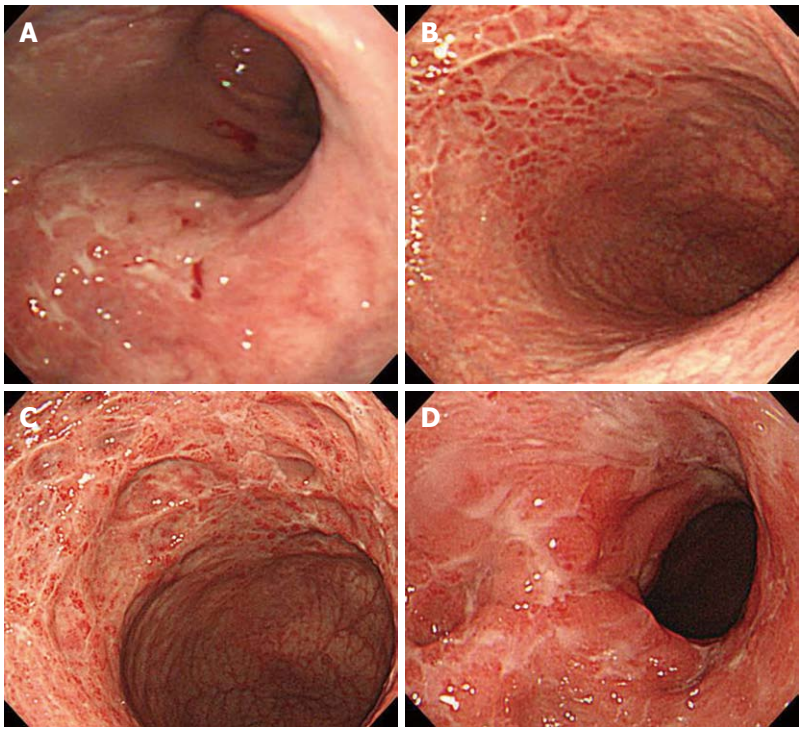

Figure 1 Endoscopic findings show chronic persistent inflammation of the stomach. A: Multiple active ulcers and edematous mucosa in June 2004; B: Multiple scarring ulcers and erythematous mucosa in August 2006; C: Multiple healing and scarring ulcers in July 2007; D: Multiple active ulcers and edematous mucosa with luminal stenosis in July 2008.

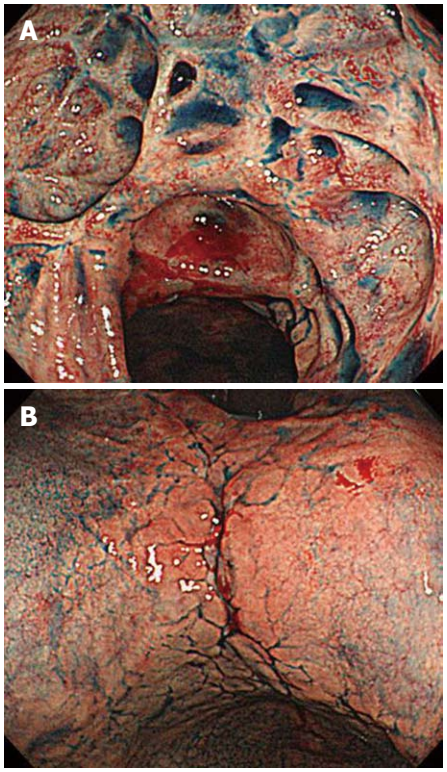

Figure 2 Endoscopic view after indigo carmine dye spray showed luminal deformity and active ulcers in February 2009. A: Multiple scarring ulcers appearing like pseudo-diverticulum in the upper body of the stomach and stenosis of the upper body due to annular scarring ulcers; B: Linear ulcers on the lesser curve of the middle body of the stomach.

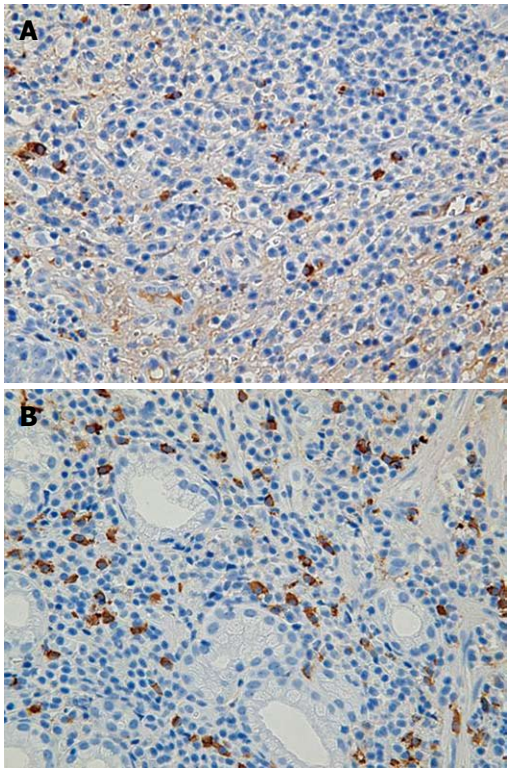

Figure 3 Histopathological findings of biopsy specimens of the stomach. A: Mild infiltration of IgG4-positive plasma cells at the scarring ulcer (15/high power field); B: Severe infiltration of IgG4-positive plasma cells at the active ulcer (50/high power field).

costeroid therapy is effective in IgG4-related sclerosing disease, it may be considered the first treatment option, albeit with consideration to its many side effects, particularly in elderly patients, and uncertainty over optimal dosages. This patient, who was asymptomatic, declined this option and was placed instead on maintenance therapy with a proton-pump inhibitor. Treatment of this

rare gastric ulcer will be advanced by multicenter trials in patients with gastric ulcer and IgG4-related sclerosing disease.

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CASE REPORT

Late retroperitoneal recurrence of hepatocellular carcinoma 12 years after initial diagnosis

Tiffany Cho-Lam Wong, Ka-Fai To, Simon See-Ming Hou, Sidney Kam-Hung Yip, Chi-Fai Ng

Tiffany Cho-Lam Wong, Simon See-Ming Hou, Sidney Kam-Hung Yip, Chi-Fai Ng, Department of Surgery, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, China
Ka-Fai To, Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin, Hong Kong, China
Author contributions: Wong TCL drafted the manuscript; To KF was responsible for the histological figure and genetic assessment of the specimens; Ng CF supervised and assisted the manuscript preparation; Hou SSM and Yip SKH were involved in revising the manuscript critically; all authors have approved the final manuscript to be published.
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Correspondence to: Chi-Fai Ng, Professor, MBChB, FRCSEd, FCSHK, FHKAM (Surgery), Department of Surgery, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, China. ngcf@surgery.cuhk.edu.hk
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Abstract

Hepatocellular carcinoma (HCC) is an aggressive tumor with poor long-term prognosis. Here, we present an unusual patient with a solitary recurrence of HCC in the right kidney 12 years after the initial diagnosis. This illustrates the importance of considering late recurrence in patients with a history of HCC and the management of these metastases.

Key words: Hepatocellular carcinoma; Late recurrence; Metastasis; Retroperitoneal

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Wong TCL, To KF, Hou SSM, Yip SKH, Ng CF. Late retroperitoneal recurrence of hepatocellular carcinoma 12 years after initial diagnosis. *World J Gastroenterol* 2010; 16(17): 2187-2189 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i17/2187.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i17.2187

INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive tumor with poor long-term prognosis. The reported 5-year recurrence rate ranges from 75% to 100%[1]. Therefore, extrahepatic metastasis of HCC is uncommon due to the highly malignant nature of the primary tumor. However, with advances in different treatment modalities for HCC, the incidence of extrahepatic metastasis appears to be increasing[2]. Nevertheless, most recurrences occur relatively early after the initial diagnosis and treatment. Here, we report a patient who presented with a renal tumor 12 years after the initial treatment of primary HCC, which turned out to be a solitary recurrence of the initial HCC. The management of these distant metastases is also highlighted.

CASE REPORT

A 61-year-old man who was a chronic hepatitis B carrier with Child's A liver cirrhosis, first presented 12 years ago with hemoperitoneum secondary to ruptured HCC. Emergency laparotomy for hemostasis was performed. Non-anatomical resection of the liver tumor was subsequently performed 1 mo later after his condition was optimized. Pathology showed an 8 cm × 6 cm × 4 cm, moderately differentiated HCC with a clear resection

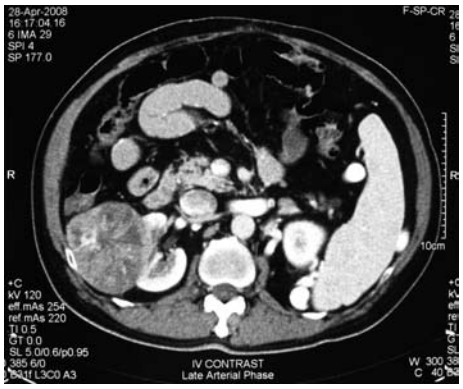

Figure 1 Computed tomography showing the contrast-enhanced mass in the right kidney.

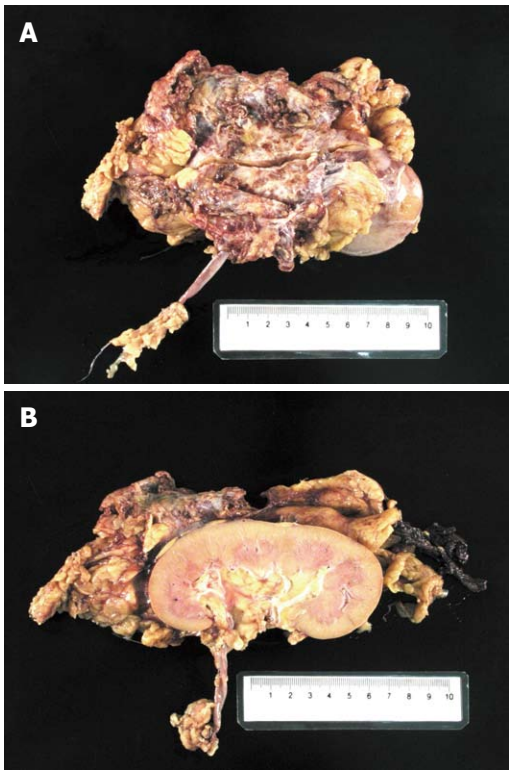

Figure 2 Gross specimen of tumor showing its relationship with the right kidney. A: External appearance; B: From cut section of kidney.

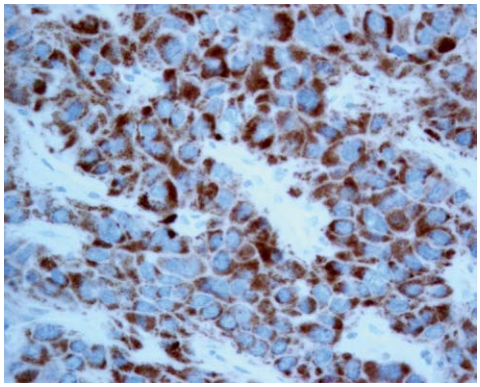

Figure 3 Immunological stain of the tumor with HepPar, confirmed the tumor as hepatocellular carcinoma.

margin. The postoperative serum α fetoprotein (AFP) level dropped from 1500 to 2 ng/mL.

He was followed up regularly, 3-monthly for the first 2 years, followed by half-yearly follow-up. Serum AFP was measured during each follow-up and abdominal ultrasound was also performed during each follow-up for the first 5 years and then yearly during subsequent follow-up. There was no evidence of recurrence and the patient remained well clinically until 12 years later, when surveillance ultrasound revealed a right renal mass. Subsequent computed tomography showed a 5.3 cm pedunculated, well-circumscribed, contrast-enhanced soft tissue tumor in the right kidney, suspicious of renal cell carcinoma (RCC) (Figure 1). There was no space-occupying lesion in the liver or ascites. The tumor was asymptomatic and was not found on the last ultrasound performed 1 year ago. Serum AFP level was also increased from 3 (6 mo previously) to 27 ng/mL. However, in view of the long recurrence-free period and no evidence of local recurrence, the treatment plan was to closely monitor the raised AFP level and proceed to treatment of the renal mass.

With the past history of laparotomy and hepatectomy, retroperitoscopic nephrectomy was planned. During the operation, a large vascular, friable and exophytic tumor was found to have developed in the right kidney. However, the tumor was quite adhesive to the surrounding tissue, such that it inadvertently ruptured despite careful dissection. The surgery was then converted to an open procedure. The operation lasted 195 min with an estimated blood loss of 3 L. The post-operative course was uneventful and he was discharged 7 d after the operation.

However, the subsequent unexpected pathology was found to be extra-hepatic metastasis of HCC at Gerota's fascia. Macroscopically, the tumor was in the perinephric fat with no invasion to the renal capsule (Figure 2). Microscopic examination showed trabeculae of polygonal eosinophilic tumor cells, with epithelial cohesion, marked pleomorphism, associated with sinusoidal-type vascular channels and bile canaliculi were also identified. The kidney and pelvi-calyceal system were intact. When compared with the previously resected HCC 12 years ago, they both demonstrated similar microscopic characteristics and both were immunoreactive to polyclonal carcinoembryonic antigen and HepPar (Figure 3), but not to markers for RCC. Both the primary and recurrent HCC demonstrated the same TP53 mutation in exon 7 (R249S AGG > AGC). This result by itself was not confirmatory, but supported the clonal relationship of the primary and recurrent HCC in this case.

After nephrectomy, the patient's serum AFP level dropped to 1 ng/mL, i.e. normal. As there was no evidence of other systemic recurrence, no adjuvant chemotherapy was administered. The patient remained well 18 mo after the nephrectomy and serum AFP remained normal. The latest positron-emission tomography per-

formed at 1 year after nephrectomy showed no evidence of recurrence.

DISCUSSION

In the past, extrahepatic metastasis of HCC was considered a relatively uncommon phenomenon, owing to the highly malignant nature of the primary tumor. Nonetheless, with advances in treatment for HCC, its overall prognosis is improving and the incidence of extrahepatic metastasis appears to be increasing[3]. The commonest sites of extrahepatic metastases are lung, lymph node and bone followed by adrenal glands[4-6]. Extrahepatic metastasis can occur *via* three routes: hematogenous (56%), lymphogenous (27%) and direct invasion (22%) and more than one mode of spread can occur[7]. It is uncommon for HCC to spread to kidney and most believe that tumor spread to the kidneys is *via* the hematogenous route[8]. In our patient, the metastasis spread to the perinephric tissue without direct invasion of kidney parenchyma. It was postulated that the initial rupture of the primary liver tumor resulted in some seeding of tumor cells in the retroperitoneal region giving rise to subsequent tumor recurrence.

However, it is still extremely rare to have recurrent HCC 12 years after the initial diagnosis and treatment. The reported median duration between initial treatment for HCC to extrahepatic spread is 23.2 mo[1]. While there was a report suggesting that HCC can recur more than 6 years after initial management[9], there are no reports of recurrence occurring 10 years after initial treatment. It is difficult to explain the exceptionally long time lapse between treatment of the primary tumor and the current recurrence in our patient. It can not be explained even by shed cells belonging to a very low malignant clone of tumor cells.

Although there is no standard management protocol for patients with extrahepatic metastasis of HCC, it is generally believed that aggressive treatment for extrahepatic metastasis can prolong survival in selected patients with good performance status and limited volume of recurrence. In a review of patients with both intrahepatic and extrahepatic recurrent HCC, patients who received an aggressive combination of metastatectomy and locoregional therapy, with or without systemic therapy, had a better survival rate compared to those who received only non-surgical treatment (median survival after recurrence was 44.0 mo in the aggressive treatment group *vs* 10.6 mo in the non-surgically treated group)[2]. For patients with lung metastasis, it was also shown that a longer disease-free interval from primary treatment and a smaller number of metastasis (less than three) were associated with better prognosis[10]. In our patient, the solitary nature and long disease-free interval were relatively good prognostic factors.

Certainly, a more frequent follow-up protocol, especially imaging, may help to detect the lesion at an earlier stage. However, a careful balance between the yield and medical cost is needed, as late recurrence of HCC after a disease-free period of 5 years is still a very rare condition.

Our case illustrates that, in managing patients with a past history of HCC who present with new mass lesions, the diagnosis of extra-hepatic metastasis should always be suspected. Although the final treatment might not be altered, the diagnosis of metastatic HCC would allow better surgical planning and counseling for the patient.

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CASE REPORT

Partial intestinal obstruction secondary to multiple lipomas within jejunal duplication cyst: A case report

Xin-Yue Wan, Tao Deng, He-Sheng Luo

Xin-Yue Wan, Tao Deng, He-Sheng Luo, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
Author contributions: Deng T performed the double-balloon endoscopy; Wan XY wrote the paper; Luo HS revised the paper.
Correspondence to: Tao Deng, MD, Department of Gastroenterology, Renmin Hospital of Wuhan University, No. 99 Ziyang Road, Wuhan 430060, Hubei Province, China. wanxy8366@hotmail.com
Telephone: +86-27-88041911 Fax: +86-27-88042292
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Abstract

Lipoma within jejunal duplication presenting as abdominal bloating and partial intestinal obstruction is an exceptional clinical entity. We report a case of 68-year-old man complaining of abdominal bloating for 10 d due to multiple lipomas arising from jejunal duplication cysts. Only a few cases of a single lipoma within a Meckel's diverticulum giving rise to this clinical scenario have been reported in the English language literature. However, no case of multiple lipomas within jejunal duplication cysts has been reported. We present a case in which double-balloon endoscopy revealed a small intestinal structure changed into Meckel's diverticulum-like cavities containing several lipomas. This case highlights intestinal lipoma as an uncommon cause of adult intussusceptions, which should be included in the differential diagnosis of small intestinal obstruction and appropriate examinations should be chosen.

Key words: Duplication cyst; Lipoma; Double-balloon endoscopy; Jejunum; Intestinal obstruction

Peer reviewer: Roberto de Franchis, Professor of Medicine (Gastroenterology), Department of Medical Siences, University of Milan, Head, Gastroenterology 3 Unit, IRCCS Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena Foundation, Via Pace 9, 20122 Milano, Italy

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INTRODUCTION

Lipoma of the small intestine is a benign tumor of mesenchymal origin which is mostly found by chance during gastrointestinal investigation. Invaginations account for 2/3 of small bowel occlusion caused by up to 80% of tumors and the lipoma is the most frequent benign tumor that causes invagination in its submucous polypoid and it is in more or less scissile form[1].

However, multiple lipomas within the intestinal duplication canal as a predominant cause of partial intestinal obstruction is an exceptional clinical scenario. We report a case of a 68-year-old man with a 10-d history of abdominal bloating after meals caused by intussusception due to a small intestine lipomatous lesion located in the jejunal duplication cyst. Although the same complications such as intestinal obstruction and intussusception may crop up in the duplication patients, we still consider the multiple lipomas as the main cause of the symptom.

CASE REPORT

A 68-year-old man was referred to our department complaining of gradual abdominal bloating after meals for the last 10 d, but without abdominal pain and vomiting. He had a bowel movement every 2 d despite a history of chronic constipation for 6 years. The patient refused eating for 2 d and lost approximately 5 kg. There was no

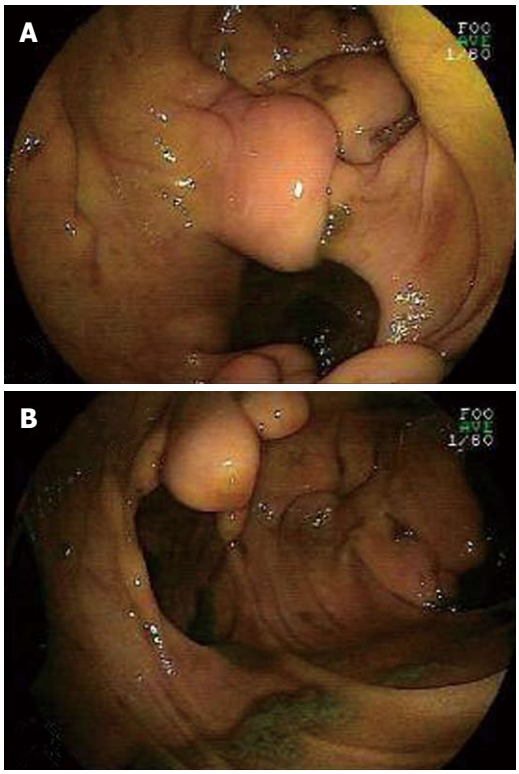

Figure 1 Double-balloon endoscopy (DBE) by the oral approach revealing several rounded protuberances in the jejunum (B) and a diverticulum-like hole (A).

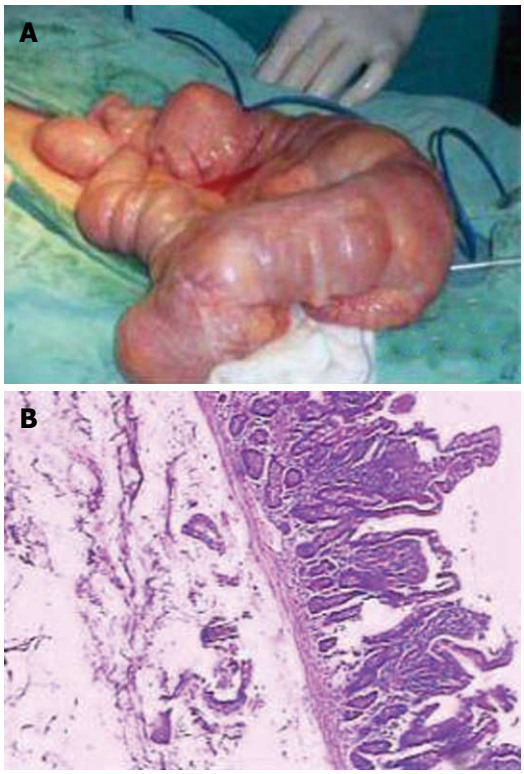

Figure 2 Resected specimen of jejunal tissue. A: Macroscopic appearance of the resected specimen with seven remarkably dilated cysts; B: Microscopic appearance of resected tissue showing multiple lipomas lining within intestinal wall (HE, × 20).

more relevant past medical, family or surgical histories. Physical examination revealed abdominal bulge with visible intestinal peristalsis, and active bowel sounds. Abdominal palpation revealed a firm, round and fixed mass of 7 cm × 4 cm over the left umbilical region. Examination of other systems was unremarkable with insignificant laboratory results.

Abdominal plain X-ray film showed air-fluid levels while colonoscopy failed to reveal any lesions which might be responsible for the symptoms. However, double-balloon endoscopy (DBE) revealed small intestinal structures which changed into Meckel's diverticulum-like cavities at 30 cm beyond the ligament of Treitz from the oral route (Figure 1A). Several yellow spherical or stalactite-like protuberances of unequal size rose into the jejunal cavity in these cystic formations with smooth surface (Figure 1B). We also found scar tissues in the partial jejunal cavity (Figure 1A). A possible diagnosis of jejunal duplication with multiple lipomas was established and the patient underwent exploratory laparotomy. One remarkably dilated intestinal canal with seven duplication cysts of about 7.5 cm × 6 cm on the mesenteric aspect of the jejunum of around 55 cm was found intraoperatively (Figure 2A). All the cyst-like dilatation had independent vascular supply, some of them were connected to the intestinal cavity while some did not. The end of intestinal tract invaginating into the low intestinal canal appeared dark red and edematous, while the upper segment was dilated significantly. Excision of the cystic lesion and an end-to-end anastomosis of the jejunum were performed. Pathological examination of the resected specimen revealed jejunal duplications having seven cysts ranging from 5.5 cm × 6 cm to 8.5 cm × 7.5 cm, with multiple submucosal lipomas lining on its wall (Figure 2B). The biggest lipoma was 32 mm in diameter. The patient had an uneventful postoperative recovery and was discharged on the seventh post-operative day. He remained well 14 mo after surgery without any symptoms of recurrence.

DISCUSSION

Alimentary tract duplications are rare congenital malformations, which in most of cases are diagnosed in infancy and childhood, although they can remain undetected until older age[2]. In contrast to Meckel's diverticulum, duplication cysts are located on the mesenteric aspect and share a common smooth muscle wall and vascular supply[2,3]. Most of the patients are asymptomatic and remain undiagnosed for years. When symptomatic, they manifest as bleeding, constipation and obstruction as a result of direct compression, volvulus or intussusception[4,5]. Our patient had a history of chronic constipation as a presenting symptom of jejunal duplication. Although it seemed to be the complication of duplication cyst, the right diagnosis was only made after 6 years.

Lipoma is a benign tumor of mesenchymal origin which is uncommon to be localized in the small bowel.

It is mostly encountered incidentally during the investigation of the gastrointestinal tract for other reasons, since they are usually asymptomatic[6]. Generally, lipoma is defined to originate in the submucosal layer and usually solitary (85%-90%), with variable sizes ranging from 1 to 30 cm[7]. Zografos *et al*[8] reported that lesions smaller than 1 cm are generally unable to cause symptoms, while 75% of those > 4 cm might give rise to gastrointestinal symptoms. Intestinal occlusion is one of the major results, which is caused by direct pressure by the lipoma or due to intestinal intussusception. Duplication cysts and lipomas can both lead to intussusception or obstruction, but in this case, we speculated that the small bowel lipomas should be the main reason. Firstly, more than 60% of the gastrointestinal duplications are diagnosed prenatally or in the first 2 years of life[9], making it rare to be symptomatic in adults. Ileocecal valve makes cystic duplications easier to cause obstruction of the colon than jejunal duplication. Secondly, several lipomas in the lesion are greater than 3 cm in diameter which might be the cause of intussusception and partial intestinal obstruction in our patient. Direct compression of cystic duplication led to intestinal stenosis, prolonging the time for chyme passage. Consequently, jejunal lipomas continued to be under progressive inflammatory stimulation.

Preoperative diagnosis of duplication cyst or small intestinal lipoma is difficult. Data from our hospital shows that, from 1998 to 2008, only 14.3% of duplication cysts have been correctly diagnosed before surgery. Abdominal computed tomography and ultrasonography were helpful in identifying large lesions but not in small intestinal lesions. However, the availability of DBE[10] and wireless capsule endoscopy made it possible to directly examine the whole digestive tract. In this case, we found multiple small intestinal lipomas arising from the spherical type duplication cysts of the jejunum using DBE. From April 2007 to May 2009, 214 enteroscopic studies were carried out, and 165 patients underwent entire digestive tract examinations. We found other two cases of tubular duplications of the ileum.

Although it is a rare entity, malignant transformation has been reported, mostly to adenocarcinoma[11]. From our patient, we have found out that jejunal duplication may induce the growth of lipoma, hence causing symptoms of intestinal obstruction. We consider that surgical removal of the lesion in asymptomatic cases is necessary because of the risk of malignant degeneration.

Multiple lipomas within jejunal duplication cysts are rare and difficult to diagnose before surgery. This case highlights intestinal lipoma as an uncommon cause of adult intussusceptions, which should be included in the differential diagnosis of small intestinal obstruction. Such a case found by DBE is an example to remind doctors to be vigilant in diagnosing patients with intestinal obstruction and in choosing appropriate examinations.

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BOOK REVIEW

Rectal prolapse: Diagnosis and clinical management

Randa Mohamed Mostafa

Randa Mohamed Mostafa, Department of Basic Medical Sciences, College of Medicine, Sharjah University, PO Box 27272, Sharjah, United Arab Emirates
Author contributions: Mostafa RM solely contributed to this book review.
Correspondence to: Randa Mohamed Mostafa, MD, PhD, Professor and Head, Department of Basic Medical Sciences, College of Medicine, Sharjah University, PO Box 27272, Sharjah, United Arab Emirates. mostafaranda@sharjah.ac.ae
Telephone: +971-6-5057204 Fax: +971-6-5585879
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Abstract

The exact cause of rectal prolapse is not well addressed, but it is often associated with long standing constipation, advanced age, chronic obstructive pulmonary disease and some neurological disorders. Rectal prolapse is usually only a symptom, which needs a focus on discovery of the underlying pathology or disorder. Three different clinical presentations are often combined and called rectal prolapse. Rectal prolapse can be divided into full thickness rectal prolapse where the entire rectum protrudes beyond the anus, mucosal prolapse where only the rectal mucosa (not the entire wall) prolapses, and internal intussuception wherein the rectum collapses but does not exit the anus. Although constipation and straining may contribute to the development of rectal prolapse, simply correcting these problems may not improve the prolapse once it has developed. There are many different approaches to surgical correction of rectal prolapse.

Key words: Rectal Prolapse; Procidentia; Complete prolapse

Peer reviewer: Luca Stocchi, MD, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, United States

Mostafa RM. Rectal prolapse: Diagnosis and clinical management. *World J Gastroenterol* 2010; 16(17): 2193-2194 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i17/2193.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i17.2193

INTRODUCTION

Rectal prolapse, or procidentia or "complete prolapse", defined as a protrusion of the rectum beyond the anus[1], occurs at the extremes of age. In the pediatric population, the condition is usually diagnosed by the age of 3 years, with an equal sex distribution. In the adult population, the peak incidence is after the fifth decade and women are more commonly affected, representing 80%-90% of patients with rectal prolapse[2].

Complete rectal prolapse is a disabling condition that has been reported even since the Egyptian and Greek civilizations[3]. Throughout the history of medicine, multiple approaches to the treatment of rectal prolapse have been described. In the past century, its management has evolved a great deal due to accumulation of knowledge obtained from physiologic investigations and follow up of surgical series[4].

The surgical landscape for rectal prolapse has expanded to include new treatment modalities such as the STARR and EXPRESS procedures. However, technical details, indications, and outcomes of these new techniques are not widely understood. The richness and variety of choices for treating rectal prolapse may become confusing, and controversial. These are some reasons why many surgeons feel the need for one articulate and comprehensive volume that present an all inclusive understanding of the pathophysiology of rectal prolapse and state of the art surgical treatment for it.

BOOK REVIEW

The book "Rectal Prolapse: Diagnosis and Clinical Management, edited by Altmomare and Filippo Pucciani (PP 206), illustrated, Springer Publisher (2008), ISBN:

978-88-470-0688-6" offers a body of information encompassing all aspects of rectal prolapse. It deals comprehensively with the various forms of prolapse, including external prolapse, rectal intussusception and genital prolapse. The chapters on etiology and investigation set out in detail the present position regarding the value of the advancements in clinical practice. Beside the classic operations, new treatment modalities such as the STARR and EXPRESS procedures are dealt with, and their indications are considered in relation to the clinical presentation and the various other options. Function following surgery receives considerable attention and the difficult problems that may be posed by recurrence after surgery are dealt with. Non surgical treatment and rehabilitation are also described in this book.

The book, is beautifully laid out with very clear illustrations including high quality operative color photographs and line drawings. It is important not only to surgeons but also to gastroenterologists, physiologists and radiologists.

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Shahab Abid, Dr., Associate Professor, Department of Medicine, Aga Khan University, Stadium Road, PO Box 3500, Karachi 74800, Pakistan

Fausto Catena, MD, PhD, Department of General, Emergency and Transplant Surgery, St Orsola-Malpighi University Hospital, Via Massarenti 9, Bologna 40139, Italy

Justin MM Cates, Dr., MD, PhD, Department of Pathology, Vanderbilt University Medical Center, Medical Center North, C-3322, 1161 21st Avenue South, Nashville, TN 37232, United States

Giuseppe Currò, MD, University of Messina, Via Panoramica, 30/A, 98168 Messina, Italy

Gianlorenzo Dionigi, MD, FACS, ESES, ETA, Asscociate Professor of Surgery, Department of Surgical Sciences, University of Insubria, Ospedate di Circolo, v. guicciardini, 21100 Varese, Italy

Robert JL Fraser, Associate Professor, Investigations and Procedures Unit, Repatriation General Hospital, Daw Park, Australia

Bhupendra Kumar Jain, Dr., MS, Professor of Surgery and Head, Department of Surgery, GTB Hospital & University College of Medical Sciences, Delhi 110 095, India

Rashmi Kaul, PhD, Assistant Professor, Department of Biochemistry and Microbiology, Oklahoma State University- Center for Health Sciences, 1111 W. 17th St., Tulsa, OK 74107, United States

I-Rue Lai, Assistant Professor, Department of Anatomy and Cell Biology, Medical College, National Taiwan University, 7, Chun-San S. Rd, Taipei 106, Taiwan, China

Yuk-Tong Lee, MD, Department of Medicine and Therapeutics, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, China

Peter L Moses, MD, FACG, AGAF, Professor, University of Vermont College of Medicine Section of Gastroenterology & Hepatology,111 Colchester Avenue, Smith 237B, MCHV, Burlington, VT 05401, United States

Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

Arturo Panduro, MD, PhD, Head of the Department of Molecular Biology in Medicine, Civil Hospital of Guadalajara Fray Antonio Alcalde/University of Guadalajara, Hospital No. 278 S.H., Guadalajara, Jalisco, 44280, Mexico

Nagarajan Perumal, Dr., Compliance veterinarian, Center for life science, IACUC OFFICE, National University of Singapore, 117456, Singapore

Mark Pines, PhD, Institute of Animal Sciences, Volcani Center, PO Box 6, Bet Dagan 50250, Israel

Gerardo Rosati, MD, Medical Oncology Unit, "S. Carlo" Hospita, Via Potito Petrone, 1, Potenza 85100, Italy

Ross C Smith, Professor, Department of Surgery, University of Sydney, Royal North Shore Hospital, St Leonards, New South Wales 2065, Australia

Yuk Him Tam, Dr., Department of Surgery, Prince of Wales Hospital, Shatin, Hong Kong, China

Wei-Dong Tong, Dr., MD, MS, BS, Associate Professor, Department of General Surgery, Veterans Affairs Medical Center, Medical college of Wisconsin, Milwaukee, WI 53295, United States

Debbie Trinder, PhD, School of Medicine and Pharmacology, University of Western Australia, Fremantle Hospital, PO Box 480, Fremantle 6959, Western Australia, Australia

De Aretxabala Xabier, Professor of Surgery, Universidad de Chile, Santos Dumont 999, Santiago, 8380000, Chile

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Meetings

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January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

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Boston, MA, United States
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September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
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The 7th Biannual International Symposium of Society of Coloproctology

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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