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Contents

Contents

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Contents

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EDITORIAL

Prediction of severe acute pancreatitis: Current knowledge and novel insights

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Abstract

Acute pancreatitis (AP) is a common and potentially lethal acute inflammatory process with a highly variable clinical course. It is still unclear why some patients progress to organ failure and others do not. Ability to predict which patients will develop severe disease is limited. Routine clinical and laboratory data and multi-factorial clinical scores measured on admission and during the first 48 h of hospitalization are currently the standards of care used to estimate the magnitude of the inflammatory response to injury. Current literature highlights several common environmental, metabolic and genetic factors that increase the risk of AP development and subsequent adverse sequelae. Several cytokines have been found to play a critical role in the pathogenesis of AP by driving the subsequent inflammatory response, to include tumor necrosis factor-α (TNF-α), Interleukin-1 (IL-1), IL-6 and monocyte chemotactic protein-1 (MCP-1). Large, prospective studies are still needed to address these questions by identifying AP risk factors and serum biomarkers of severe disease.

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Key words: Acute pancreatitis; Prediction; Severity; Monocyte chemotactic protein-1

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INTRODUCTION

Acute pancreatitis (AP) is a common and potentially lethal acute inflammatory process with a highly variable clinical course. It accounts for greater than 300 000 emergency room visits annually in the US, which is steadily increasing, with a mean length of hospital stay of 7 d[1].

Approximately 20% of affected individuals will develop a severe clinical course in association with the development of a systemic inflammatory response syndrome (SIRS), multiple organ failure (MOF), and on occasion death. Despite substantial animal model research[2], it is still unclear as to why some patients progress to organ failure and others do not, or at what step in the inflammatory cascade will an intervention have an impact upon disease progression. Predictive disease severity scoring systems are widely used in clinical practice; but in reality they reflect the inflammatory response rather than the severity of the insult experienced by the pancreatic parenchyma.

Several clinical and molecular pre-AP susceptibility and severity factors have been identified which may modify an individual's predisposition to AP, and the associated risk of severity. Obesity is one such important factor. An elevated BMI ($\geq$ 30 kg/m^2) significantly increases the extent of AP severity (OR, 2.6; 95% CI, 1.5-4.6) and is implicated in both local and systemic complications[3]. The severity risk increases at an OR of 1.2 per 5 units of BMI. Severe AP is associated with android fat distribution, increased waist-hip ratio (> 1.0) and appears to correlate with an "overactive" immune response.

Alcohol consumption is another risk factor associated with severe AP as it lowers the threshold for intrapancreatic trypsin activation and shifts pancreatic acinar cell death from apoptosis to necrosis as demonstrated in alcohol-fed animals[4]. Our group reaffirmed this finding in human subjects consuming two or more alcoholic drinks per day[5]. Furthermore, active tobacco smoking has been suggested as a susceptibility

factor for AP (RR, 2.14; 95% CI, 1.48-3.09)[6].

In preliminary genetic susceptibility factor studies, the presence of a single nucleotide polymorphism in the gene of a potent chemokine, named monocyte chemotactic protein-1 (MCP-1), at position -2518 A/G predicted that the inflammatory response to AP would be systemic and associated with death[7]. The G allele was present in 86% of severe pancreatitis cases, 46% of mild pancreatitis cases and 43% of controls. The presence of the G allele increased the risk of developing severe AP seven fold (OR, 7.7; 95% CI, 1.6-100).

Routine clinical and laboratory data and multifactorial clinical scores measured on admission and during the first 48 h of hospitalization are currently the standards of care used to estimate the magnitude of the inflammatory response to injury, and to predict whether or not intensive care support is needed to address inflammation-associated complications. Admission hematocrit, C-reactive protein (CRP) at 48 h, Ranson's criteria and the Acute Physiology and Chronic Health Evaluation (APACHE-Ⅱ) scores are the most popular. In addition, a variety of cytokines, chemokines, and other markers of the inflammatory response have been evaluated as predictors of severe AP, as well as markers of development of specific organ-system failure.

Collectively, the literature highlights several common environmental, metabolic and genetic factors that are predisposing factors increasing the risk of AP development and subsequent adverse sequelae. The mechanisms by which such factors increase the risk of severe disease, and whether or not they directly interact with or potentiate one another remains speculative. Knowledge of the inflammatory cascade is important in recognizing when the peak response occurs for various cytokines and inflammatory mediators.

Several reports have evaluated patients with endoscopic retrograde cholangiopancreatography (ERCP) induced pancreatitis, and studied post-ERCP cytokine profiles. Cytokines play a critical role in the pathogenesis of AP by driving the subsequent inflammatory response. Patients with post-ERCP AP have an amylase and lipase increase within the first hour reaching a maximum value between 4 h and 12 h following ERCP[8]. Interleukin-6 (IL-6) increases to a maximal concentration at 24-48 h, and the highest CRP concentrations are established 72 h following an ERCP. In another study, in patients who developed post-ERCP pancreatitis, the serum levels of these cytokines including tumor necrosis factor-α (TNF-α), IL-1, IL-6, IL-8, and IL-10 rose significantly at 8 and 24 h but not at 1 h and 4 h when compared to patients without pancreatitis[9]. These data suggest that serum markers (amylase/lipase) are detected early, but that the acute inflammatory response does not fully develop until at least 8-12 h after the initial pancreatic insult. These data may be useful for determining the extent of pancreatic injury, the timing of the acute inflammatory response and for assessing such inflammatory markers in equation-based models.

TNF-α

TNF-α is a pleiotropic cytokine expressed in acinar cells, and is a key regulator of other pro-inflammatory cytokines and leukocyte adhesion molecules which acts as a priming activator of immune cells[10]. It is also a cell death signal through the TNF-α-related apoptosis induced ligand (TRAIL) receptor pathway, with the potential to cause severe tissue damage. TNF-α plays a pivotal role in severe AP, acting early in the disease course, and is quickly cleared. As a result of its rapid clearance, TNF-α serum levels are less useful as biomarkers of early events than downstream cytokines (e.g. IL-6). To limit the systemic effect of TNF-α, the body releases TNF-α inhibitors. The soluble TNF receptor (sTNFR) attenuates the effects of TNF-α by binding to TNF-α in the serum and thus acts as an anti-inflammatory molecule. sTNFR levels have been found to predict severity in AP with an accuracy of 96%, and also to have a high sensitivity for mortality[11].

IL-1

IL-1 is another major pro-inflammatory cytokine that can drive the SIRS response. It has recently been shown to be the major cytokine mediating inflammation in sterile necrosis[12], which is often problematic in severe AP. In contrast to TNF-α, IL-6 does not directly cause pancreatic damage[13]. It has been used as a biomarker of disease severity and has similar accuracy to IL-6 in predicting severe AP on admission (82% *vs* 88%)[11]. IL-1 receptor antagonist (IL1-RA) levels also correlate with the inflammatory response and severity in AP and may in fact be superior to IL-6 or CRP within the first 48 h.

IL-6

IL-6 is a multifunctional cytokine released by macrophages in response to tissue injury and constitutes the principal mediator in the synthesis of acute-phase proteins, in addition to transitioning the acute inflammatory response to a chronic response. It is an accurate early predictor of severity in AP, with a sensitivity range of 89% to 100% and 90% accuracy within the initial 24 h[11]. It has also been shown to be superior to CRP and the APACHE-Ⅱ score at 24 h following admission.

MCP-1

MCP-1 is a potent chemokine which is released early in the inflammatory process. MCP-1 serum concentrations have been shown to display a dramatic increase in patients with AP who develop local complications or remote organ failure. A close correlation has also been found between the incidence of remote organ failure and the degree of MCP-1 level elevation[7,14]. As highlighted earlier, a common single nucleotide polymorphism on the MCP-1 gene is shown to predispose to severe AP. Macrophage migration inhibitory factor (MIF) is a unique chemokine; that participates in inflammation, immune response and cell growth. Serum MIF levels

have been found to be higher in patients with severe AP than patients with mild disease[11].

Although altering the inflammatory response in animals translates into a possible benefit, the potential translational benefit to humans has not been confirmed to date. For example, the platelet activating factor (PAF) inhibitor, Lexipafant displayed early promise. However, it was not deemed to be an effective treatment in a large, multi-national study of 1500 patients[15]. Although IL-10 decreases the severity of AP in mouse models, and could be of potential benefit in humans, sufficiently powered human studies have yet to be reported in the literature.

The discriminatory power of general prediction schemes improved considerably in the early 1990's. Indeed, Ranson's criteria and APACHE Ⅱ score achieved reasonable discrimination with receiver-operating characteristic curve (ROC) area under the curve (AUC) values approaching 0.8 in most validation studies. Yet, these classification tools are designed to predict ICU mortality and not potentially preventable complications; they are, therefore, least useful in the middle prediction range where the clinician needs most support and information to direct management. Although these tools are of assistance in medical decision making at the extreme end of the prediction range, their use has been confined to a global ICU performance assessment and criteria for clinical trial enrolment.

Successful prediction of individual outcomes is undoubtedly one of the holy grails in the care of the critically ill. Remarkably, although progress has been made along all those fronts in risks and markers for severe AP, little has been achieved in translating data and quantitative tools into clinically useful and appealing predictive knowledge for physicians managing patients with AP.

Large, prospective studies are needed to address these questions by identifying AP risk factors and serum biomarkers of severe disease. Such data could be potentially used to develop patient-specific predictive algorithms of AP risk and to guide the treatment decision-making process early in the disease course. Such studies could aim to: firstly, determine the role of demographic, environmental, genetic and physiological variables on the initiation, progression, severity and clinical outcomes of AP; secondly to identify biomarkers that reflect the extent of pancreatic injury and the acute inflammatory response which are critical in the assessment of the activity of potentially pathologic cascades; thirdly to build advanced statistical models based on pre-injury risk factors and biomarkers of pancreatic injury and inflammation to accurately predict primary and secondary outcomes of AP, including organ failure, complications and death; and finally to guide the research on inflammatory cascade blocking agents administered early in the disease course based on patient-specific predictive algorithms.

REFERENCES

1 **Whitcomb DC**. Clinical practice. Acute pancreatitis. *N Engl J Med* 2006; **354**: 2142-2150
2 **Steinberg WM**, Schlesselman SE. Treatment of acute pancreatitis. Comparison of animal and human studies. *Gastroenterology* 1987; **93**: 1420-1427
3 **Martinez J**, Sanchez-Paya J, Palazon JM, Suazo-Barahona J, Robles-Diaz G, Perez-Mateo M. Is obesity a risk factor in acute pancreatitis? A meta-analysis. *Pancreatology* 2004; **4**: 42-48
4 **Wang YL**, Hu R, Lugea A, Gukovsky I, Smoot D, Gukovskaya AS, Pandol SJ. Ethanol feeding alters death signaling in the pancreas. *Pancreas* 2006; **32**: 351-359
5 **Papachristou GI**, Papachristou DJ, Morinville VD, Slivka A, Whitcomb DC. Chronic alcohol consumption is a major risk factor for pancreatic necrosis in acute pancreatitis. *Am J Gastroenterol* 2006; **101**: 2605-2610
6 **Lindkvist B**, Appelros S, Manjer J, Berglund G, Borgstrom A. A prospective cohort study of smoking in acute pancreatitis. *Pancreatology* 2008; **8**: 63-70
7 **Papachristou GI**, Sass DA, Avula H, Lamb J, Lokshin A, Barmada MM, Slivka A, Whitcomb DC. Is the monocyte chemotactic protein-1 -2518 G allele a risk factor for severe acute pancreatitis? *Clin Gastroenterol Hepatol* 2005; **3**: 475-481
8 **Messmann H**, Vogt W, Holstege A, Lock G, Heinisch A, von Furstenberg A, Leser HG, Zirngibl H, Scholmerich J. Post-ERP pancreatitis as a model for cytokine induced acute phase response in acute pancreatitis. *Gut* 1997; **40**: 80-85
9 **Chen CC**, Wang SS, Lu RH, Lu CC, Chang FY, Lee SD. Early changes of serum proinflammatory and anti-inflammatory cytokines after endoscopic retrograde cholangiopancreatogr aphy. *Pancreas* 2003; **26**: 375-380
10 **Papachristou GI**, Clermont G, Sharma A, Yadav D, Whitcomb DC. Risk and markers of severe acute pancreatitis. *Gastroenterol Clin North Am* 2007; **36**: 277-296, viii
11 **Malleo G**, Mazzon E, Siriwardena AK, Cuzzocrea S. Role of tumor necrosis factor-alpha in acute pancreatitis: from biological basis to clinical evidence. *Shock* 2007; **28**: 130-140
12 **Chen CJ**, Kono H, Golenbock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* 2007; **13**: 851-856
13 **Denham W**, Yang J, Fink G, Denham D, Carter G, Bowers V, Norman J. TNF but not IL-1 decreases pancreatic acinar cell survival without affecting exocrine function: a study in the perfused human pancreas. *J Surg Res* 1998; **74**: 3-7
14 **Rau B**, Baumgart K, Kruger CM, Schilling M, Beger HG. CC-chemokine activation in acute pancreatitis: enhanced release of monocyte chemoattractant protein-1 in patients with local and systemic complications. *Intensive Care Med* 2003; **29**: 622-629
15 **Johnson CD**, Kingsnorth AN, Imrie CW, McMahon MJ, Neoptolemos JP, McKay C, Toh SK, Skaife P, Leeder PC, Wilson P, Larvin M, Curtis LD. Double blind, randomised, placebo controlled study of a platelet activating factor antagonist, lexipafant, in the treatment and prevention of organ failure in predicted severe acute pancreatitis. *Gut* 2001; **48**: 62-69

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EDITORIAL

Hypnosis and upper digestive function and disease

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Abstract

Hypnosis is a therapeutic technique that primarily involves attentive receptive concentration. Even though a small number of health professionals are trained in hypnosis and lingering myths and misconceptions associated with this method have hampered its widespread use to treat medical conditions, hypnotherapy has gained relevance as an effective treatment for irritable bowel syndrome not responsive to standard care. More recently, a few studies have addressed the potential influence of hypnosis on upper digestive function and disease. This paper reviews the efficacy of hypnosis in the modulation of upper digestive motor and secretory function. The present evidence of the effectiveness of hypnotherapy as a treatment for functional and organic diseases of the upper bowel is also summarized, coupled with a discussion of potential mechanisms of its therapeutic action.

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Key words: Hypnosis; Hypnotherapy; Gastric emptying; Small bowel transit; Functional dyspepsia; Functional esophageal disorders; Functional bowel disorders

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INTRODUCTION

Hypnosis can be defined as an altered state of consciousness, different from both sleep and normal wakefulness, characterized by highly focused attention and heightened compliance with suggestion[1]. As a rule, the onset of this state is facilitated by eye closure. A number of other phenomena are often described as associated with hypnosis, including altered perception of passage of time, partial or complete amnesia for the events experienced, and attenuation of stress experiences[1-3]. In addition, subjects may show enhanced compliance to suggestion given during hypnosis meant to influence favorably their behavior after the trance state has been terminated (post-hypnotic suggestion)[1]. Furthermore, a more contentious property of hypnosis is either increased access to memories, feelings, and perceptions which are normally kept below the level of conscious awareness, or *vice versa* enhanced suppression of these from the conscious mind[4-6].

Clinical hypnosis is the method of deliberately inducing the state of hypnosis in a patient through verbal guidance, and making use of its characteristic properties for targeted therapeutic purposes. The possibilities of hypnosis as a healing method stem principally from the heightened responsiveness to suggestion in this altered mental state. Hypnotic and post-hypnotic suggestions can be used to facilitate desired therapeutic changes in feelings, behavior and physiology, and this can be useful not only for mental health purposes, but also in medicine[1]. Although a single hypnosis session targeting a simple symptom or bodily function can sometimes yield useful results, treatment of complex psychological and somatic conditions with hypnosis typically requires a structured form of therapeutic intervention, hypnotherapy, administered in a series of several therapy sessions[1].

Hypnosis has a long history of application as a clinical tool in medicine, dating back to the early 18th

Century, when it was used with considerable success for the purpose of inducing anesthesia during surgery in thousands of cases, predominantly by British physicians. Only the availability of chemical anesthesia with ether and chloroform in 1846 and 1847 made this application obsolete[7].

In the latter half of the 19th century, hypnosis became prominently utilized in the treatment of psychiatric conditions like hysteria by some of Europe's foremost authorities in neurology and psychiatry of that time, such as Sigmund Freud in Austria and Jean-Martin Charcot in France[8]. Ever since then, hypnosis has been more widely recognized as a treatment aid for mental health problems than for physical ailments. However, medical uses of hypnosis continued, and sufficient experience with various advantageous medical applications gradually accumulated for the technique of clinical hypnosis to earn formal acceptance in mainstream medicine[7,8]. Hypnosis gained official approval as a medical treatment, first by the British Medical Association in 1955 and then by the American Medical Association in 1958, in a report that stated that hypnosis had "definite and proper applications in medicine and dentistry", and recommended that physicians should receive training in the technique[7,8]. However, even today, most medical school curricula in the U.S. and elsewhere provide no training or education in hypnosis. Although clinical hypnosis is currently practiced by thousands of health professionals in many Western countries, it is practiced by a variety of professional disciplines, including psychologists, counselors, clinical social workers, dentists, nurses and nurse practitioners, but relatively few physicians[7,8]. In many places, the great majority of practitioners providing hypnosis are mental health professionals who rarely use it to treat physical conditions. Additionally, hypnosis services are commonly offered also by large numbers of lay hypnotherapists without any qualifications or formal education in treating medical problems[8]. These limitations, as well as myths, misconceptions and apprehensions that still linger in the public's mind from the exploitation and inaccurate portrayal of hypnosis in stage shows, movies and other popular media, has continued to hamper a widespread proper medical use of hypnosis.

Nonetheless, several medical applications of clinical hypnosis have been sufficiently investigated and considered effective in multiple formal studies. A review by a 1995 National Institutes of Health panel in the U.S. concluded that there is "strong evidence for the use of hypnosis in alleviating pain associated with cancer"[9]. Published systematic reviews of randomized clinical trials have also deemed hypnosis to be effective for treating nausea and vomiting associated with cancer chemotherapy[10] as well as the most promising psychological treatment for controlling procedure-related pain and distress in children and adolescents[11]. Furthermore, three separate systematic reviews published in the past three years[12-14] have concluded that hypnotherapy is an effective treatment for irritable bowel syndrome.

Research on the use of hypnosis for gastrointestinal disorders began with a randomized placebo-controlled study of hypnotherapy for treatment-refractory irritable bowel syndrome (IBS) in England, published in the Lancet in 1984[15]. In this study, by Peter Whorwell and colleagues in Manchester, England, the investigators randomly allocated 30 patients with IBS which was refractory to standard medical care, to either seven sessions of hypnotherapy or to the same amount of supportive psychotherapy plus placebo pills. The hypnosis approach used was a structured intervention developed by this Manchester team called gut-focused hypnotherapy. This technique aims primarily to normalize disordered bowel function, but additionally provides relaxation, coping skills, and ego-strengthening suggestion[16]. After the treatment, the patients in the hypnosis group showed substantial improvement in all cardinal IBS symptoms, and were significantly more improved on all outcome variables than the supportive psychotherapy group[15]. In a later paper, the investigators reported that the benefits of hypnotherapy in the same group of patients persisted up to 18 mo[17].

This study, albeit small, was a landmark trial, demonstrating for the first time the substantial possibilities that hypnosis offers for ameliorating gastrointestinal symptoms. Since then, positive results on the efficacy of hypnotherapy as a treatment for IBS have been reported by independent investigators both in uncontrolled and controlled trials (Table 1)[18-22]. The Manchester group has created a Hypnotherapy Unit, where this mode of therapy is routinely offered to functional GI patients who do not gain satisfactory benefit from more conventional medical treatment[16]. This group recently reported the long-term outcomes of the first 250 IBS patients treated in their clinic[22,23]. The results show an impressive 71% overall response rate to treatment, more than 50% average reduction in bowel symptom severity, and with four out of five treatment responders maintaining the full therapeutic benefit for one to five years after treatment termination[22,23].

The Manchester group has also expanded their experience from IBS therapy to other functional bowel disorders[16]. They demonstrated that functional esophageal disorders and functional gastroduodenal disorders are also suitable targets for hypnotherapy, with equally satisfactory results (Table 1)[24,25]. A small, but significant group of papers now provides evidence that hypnosis and hypnotherapy may effectively influence upper digestive function and disease. The aim of this review is to focus on this literature and to highlight the potential of hypnotherapy as a treatment option for upper digestive functional disorders.

HYPNOSIS AND UPPER DIGESTIVE FUNCTION

Gastric acid production is the bowel function where the influence of hypnosis was first investigated[26,27]. In the past, gastric acid secretion was an important research domain for gastroenterologists and its responsiveness to

Table 1 Randomized controlled trials of hypnosis treatment for severe functional bowel disorders

Authors & yr	No. of patients	Control treatment	Positive outcome	Follow-up (mo)
A: Irritable bowel syndrome				
Whorwell *et al* (1984)	30	Psychotherapy plus Placebo Pill	Hypnosis 100% $P < 0.0001$	12
Galovski & Blanchard (1998)	11	Waiting List	Hypnosis 82% $P = 0.016$	2
Palsson *et al* (2002)	24	Waiting List	Hypnosis 87% $P = 0.002$	10
B: Functional Dyspepsia				
Calvert *et al* (2002)	126	Psychotherapy plus Placebo Pill or Ranitidine 300 mg daily	Hypnosis 73% *vs* Placebo $P < 0.02$ *vs* Ranitidine $P < 0.01$	14
C: Non-cardiac chest pain				
Jones *et al* (2006)	28	Psychotherapy plus Placebo Pill	Hypnosis 80% $P = 0.008$	4

Note: Randomized controlled studies run in primary care are not reported for different patient population.

emotions and psychological stress were documented[28,29]. This interest was driven by the belief that peptic ulcer disease was a psychosomatic disease, and excess gastric acid secretion the pathophysiological mechanism linking emotion to the disease[30,31]. As a consequence, acid secretion was an attractive parameter to attempt to influence by hypnosis. A few studies were published in the nineteen sixties and early seventies examining the gastric secretory responses to hypnotic conditions, where either food-related (hunger-eating) or emotion-related (sleep-relaxation) suggestions were provided[27-29]. These early studies were flawed by small samples and questionable research methodology, and produced contradictory results. In 1989, however, Klein and Spiegel published a well-designed trial investigating the ability of hypnosis to modulate gastric acid secretion in highly hypnotizable healthy volunteers, as defined by accepted scales of trance depth[32]. The study was conducted in two centers, by two experienced hypnotherapists using two different hypnosis induction techniques. After naso-gastric intubation, gastric secretion was measured both basally and after pentagastrin stimulation in two separate studies. In the first study (acid stimulation test), acid secretion was collected in 28 subjects (13 females, age range 18-60 years) after hypnotic instructions to visualize and eat the most delicious meal possible. All the sensory aspects of the eating process, including food appearance, aroma, texture and taste, were explored and reinforced by hypnotic suggestions from the therapist. The second study consisted of two separate sessions that were held in random order. In the no-hypnosis session, the peak acid output (PAO) was obtained after maximal pentagastrin stimulation in 17 subjects (7 females, age range 18-60 years), but hypnosis was not provided. The procedure for the hypnosis sessions was the same, but deep muscle relaxation and intense imagery to divert one's attention from eating were provided. Imagery involved either lying on a beach, watching a sunset, or meeting a friend somewhere else. In both studies, none of the subjects reported difficulty in following the hypnotic suggestion or adverse side effects. Hypnotic suggestion of eating significantly increased gastric acid output compared to basal conditions[32]. In addition, the pentagastrin-stimulated PAO was significantly lowered in the averting-food hypnosis condition compared to the no-hypnosis session[32]. The authors concluded that gastric acid secretion may be modulated by hypnosis in highly hypnotizable subjects. Treatment mechanisms of action were left unexplored. But, the authors postulated that hypnosis influenced cognitive processing within the central nervous system[32]. Since the relevance of gastric secretion in peptic ulcer disease has diminished, no other centers have tried to replicate these positive results.

Two additional studies have evaluated the influence of hypnosis on upper digestive transit. In 1991 Beugerie *et al* studied the ability of hypnosis to modulate the orocaecal transit time of 10 g lactulose in six healthy volunteers[33]. Orocaecal transit time was measured by the hydrogen breath test. Oral ingestion of a poorly absorbable carbohydrate (lactulose) results in a sustained rise in breath hydrogen, which occurs within minutes of the substrate entering the cecum[34]. The orocaecal transit time is the interval elapsing from the ingestion of the substrate to the evidence of a persistent increment in breath hydrogen concentration[34]. It is commonly considered a non-invasive, reliable index of small bowel transit, particularly when lactulose is included in a caloric meal to securely interrupt the fasting motility pattern of the small bowel[35]. The subjects in this trial were recruited irrespectively to their hypnotizability, but two of them had previously been hypnotized. Orocaecal transit was evaluated on three occasions in random order: (A) control session without hypnosis; (B) hypnotic session with suggestion of deep relaxation; (C) hypnotic session with visualization of a cascading waterfall to promote transit acceleration[33]. All hypnosis sessions were started just before orocaecal transit and maintained till the transit time elapsed. The mean orocaecal transit time was significantly longer during the hypnotic relaxation session compared to the control session[33]. On the contrary, the hypnotic acceleration session did not result in significant modification of small bowel transit time[33]. The small sample size and the limited breath technique used (lactulose not administered together with a caloric test meal) did flaw the results of this study. However, it was the first study showing an influence of hypnosis on upper digestive function in individuals not selected for high hypnotizability.

The potential influence of hypnosis on gastric emptying rates has been evaluated only recently, by Chiarioni and coworkers in Italy[36]. In this study, the gastric emptying rate of a typical Mediterranean meal

(pasta with meat sauce, cheese, bread) was tested by a non-invasive ultrasonography technique. Real-time ultrasonography was used to measure the diameters of the gastric antrum in the sagittal plane passing through the aorta. Serial measurements were taken before the meal, immediately after eating and at 30 min intervals thereafter to obtain total emptying time of the meal. The total emptying time of the meal has been validated as reliable index of gastric motor function both in health and in disease when compared with total emptying time measured by gastric scintigraphy[37]. Gastric emptying rates and epigastric sensations were evaluated in 11 healthy volunteers from the hospital staff and in 15 patients with severe functional dyspepsia unresponsive to standard care under three conditions according to a fixed schedule to avoid a carry-over effect: (A) basal session, (B) prokinetic drug session (cisapride 10 mg po 30 min before meal), and (C) hypnosis session (90 min hypnosis session 30 min after finishing meal). An additional session was run in eight healthy volunteers while listening to relaxing music, to address the potential influence of both repeated testing and posture. Cisapride is a prokinetic agent that has been shown to significantly improve both gastric emptying and symptoms in functional dyspepsia compared to placebo, before being withdrawn from the market for its cardiovascular side-effects[38,39]. The method of progressive relaxation by verbal suggestion was used for hypnosis induction. Techniques to deepen the hypnosis included induction of limb heaviness and warmth. The hypnotically warmed hand was then placed over the epigastrium to associate suggestion of improved well being and gastric function mediated by the warmth of the hand. Imagery was provided of water flowing in a river and in a waterfall. This was related to suggestions for improved well-being and gastric function, derived from the gut-oriented suggestions developed by the Manchester group to treat irritable bowel syndrome[16]. The hypnosis session was completed by the classic Hartland's ego-strengthening technique, providing direct and broad hypnotic suggestions to increase the patient's confidence[40]. In patients with functional dyspepsia, gastric emptying was significantly shortened by cisapride and even more by hypnosis compared to the basal session[36]. In healthy volunteers, gastric emptying was significantly accelerated by hypnosis, but not by cisapride, compared to the basal session[36]. The relaxing music session did not influence gastric emptying rates. Epigastric sensations (i.e. fullness and discomfort) were significantly improved by hypnosis in the dyspeptic patients, but not by cisapride[36]. Interestingly, symptomatic improvement did not correlate with improved gastric motor function, leaving the mechanism/s of action of hypnosis unexplained[36]. Limitations of the study were lack of randomization and the highly selected study population.

HYPNOTHERAPY TO TREAT UPPER DIGESTIVE DISEASES

Hypnotherapy delivered as a structured, multi-session focused intervention has been most extensively used to treat IBS according to the protocols of the Manchester group or the North Carolina group[13,16]. However, the Manchester group has also provided experimental evidence to support the use of hypnotherapy in some upper digestive diseases. The first of these was a controlled study to prevent relapse of peptic ulcer[41]. The investigation was published in 1988 when peptic ulcer was considered to be a psychosomatic disorder caused by increased gastric secretion[30,31]. Thirty patients with frequently relapsing duodenal ulcer were randomized to receive either seven sessions of gut-focused hypnotherapy plus ranitidine 150 mg twice daily or seven routine consultations at a GI clinic without hypnosis plus the same ranitidine dosage over a 10-wk interval[41]. Hypnosis was induced with an arm-levitation technique followed by a combination of standard deepening procedures. The subject was then asked to place her/his hand over the abdomen, feel a sense of warm beneath the hand, and relate this to the control of gastric secretions. Reinforcement by visualization was used depending on the patient's ability. Patients were also given an audio tape for daily autohypnosis. At one year follow-up, all the subjects in the no-hypnosis group had relapsed while only 53% in the hypnotherapy group showed endoscopic evidence of relapsing duodenal ulcer[41]. The authors concluded that hypnotherapy is helpful in maintaining remission in those patients with peptic ulcer who are prone to relapse[41]. Shortly after the study, consensus developed that Helicobacter pylori infection of the stomach is the primary cause of peptic ulcer disease, and hypnotherapy was, therefore, not pursued further as potential treatment for peptic disease[42]. Nonetheless, this remains the first study to investigate the efficacy of hypnotherapy to treat upper digestive diseases.

Recently, the Manchester group assessed the efficacy of hypnotherapy for upper digestive functional diseases in two controlled trials; one on functional dyspepsia (FD) and the other for non-cardiac chest pain (NCCP)[24,25]. Functional dyspepsia refers to symptoms thought to originate in the gastroduodenal region in the absence of any organic or metabolic disease that is likely to explain the symptoms[43]. Postprandial fullness, early satiety, epigastric pain and/or burning may be reported as symptoms in FD[43]. Delayed gastric emptying, abnormal gastric tone, altered visceral perception, and autonomic imbalance have all been considered as potential etiologic factors[43,44]. In addition, comorbidity with psychiatric disorders, especially anxiety disorders, is reported to be high in FD[45]. Up to 30% of people in the community report having dyspeptic symptoms each year[43,45]. Symptomatic drug treatment, especially proton pump inhibitor medications, are often used for FD symptoms. But, the results are unsatisfactory[43,44,46]. To investigate the efficacy of hypnotherapy in FD, Calvert and coworkers randomly assigned 126 FD patients to receive either 12 hypnotherapy sessions, supportive therapy plus placebo tablets, or medical treatment with ranitidine 150 mg twice daily[24]. Patients underwent a 16-wk

treatment phase followed by a 40-wk follow-up phase where no further study interventions were undertaken. Hypnosis was induced using eye fixation followed by progressive muscular relaxation and deepened by standard procedures. The patients were then asked to place their hands on their abdomens and imagine a reduction of all symptoms. Suggestions of improvement in gastric motor function, sensitivity and gut secretion activity were also given. Reinforcement by appropriate visualization processes were administered as well. At the short term follow-up (16 wk), hypnotherapy significantly ameliorated symptoms compared to both the supportive therapy and the medical treatment groups[25]. Analogous improvements were observed when quality of life scores (QOL) were considered. Anxiety scores were lower after hypnotherapy; but there was no correlation between improvement in anxiety and FD symptom improvement[24]. No differences were evident between groups in terms of depression scores. Improvement in FD symptoms and QOL were well-maintained at long term follow-up (56 wk)[24]. In addition, patients in the hypnotherapy group were significantly less likely to consult the referring physician and to establish additional drug treatments than were the subjects in the other two groups[24]. The authors concluded that hypnotherapy is an effective treatment for functional dyspepsia both in the short and long term, but the mechanism/s of action remained speculative[24]. Hypnotherapy seems also to be cost-effective for the observed reduction in medication use and consultation rate at long term follow-up. This study was methodologically sound by most standards: (A) study design and sample size were both adequate, and (B) the double placebo control condition plus the standard care arm were likely to have produced a high expectation of therapeutic effect. Replication of these positive results by independent investigators is eagerly awaited.

Recently, the Manchester group has extended the application of hypnotherapy to non-cardiac chest pain, a condition later redefined as functional chest pain of presumed esophageal origin by the Rome Ⅲ Committee[25,47]. This functional disorder refers to relapsing episodes of unexplained chest pain that is usually located in the midline of the chest and of visceral quality[47]. The pain involved may be similar in nature to the one reported by angina patients, and by those affected by other esophageal disorders including achalasia and gastro-esophageal reflux disease (GERD)[47]. To diagnose functional chest pain, heart disease needs to be excluded as well as structural esophageal diseases, GERD, and esophageal motility disorders with defined histopathologic bases (i.e. achalasia, scleroderma of the esophagus)[47]. Epidemiology of functional chest pain is ill defined; but one should consider that 15%-30% of coronary angiograms performed for chest pain are negative for ischemic heart disease[47,48]. Disordered esophageal motility, altered visceral perception, and abnormal central signal processing with secondary errors in autonomic response have all been reported, alone or in combination, as potential causative factors[47]. In addition, overrepresentation of psychiatric disorders, particularly depression, anxiety and somatization disorders, have been described in functional chest pain of presumed esophageal origin[49]. Quality of life is impaired in continued pain and spontaneous recovery is rare[47]. In these patients, a therapeutic trial with proton pump inhibitors is mandatory to exclude symptomatic reflux disease[50]. Antidepressants may be of help, but their continuous use is associated with a high rate of side effects[47,51].

To address the effect of hypnotherapy in NCPP, the Manchester group randomized 28 patients with functional chest pain to receive either 12 sessions of individualized hypnotherapy or 12 sessions of supportive listening plus placebo tablets to control for expectancy and equalize the amount of time spent with a clinician[25]. All the patients were referred by the local cardiothoracic center after negative coronary angiography for angina-like chest pain. Reflux disease as a potential causative factor of chest pain was excluded in all subjects either by normal 24 h pH monitoring or by non-responsiveness to a proton pump inhibitor trial. Hypnosis was induced by eye closure, followed by progressive muscle relaxation and deepened by standard techniques. Suggestions focused on improved esophageal functioning and sensitivity were then introduced by using both imagery and conditioning techniques. In addition, direct suggestions of reduced pain and improved general health were given on a repetitive basis at each session. After treatment, 80% of patients in the hypnotherapy group described their chest pain as completely better or moderately better, compared to only 23% of patients in the control group[25]. This benefit persisted long-term (2 years), as reported by the authors in a follow-up paper[52]. Hypnotherapy also resulted in a significantly greater reduction in pain intensity scores, greater improvements in quality of life, and a greater reduction in medication usage when compared to the control treatment[25]. There were no significant differences between treatment groups in terms of improvement of either anxiety or depression scores as assessed by the Hospital anxiety and depression scale[25].

Limitations of this trial include the small sample size and the high patient selection. As in previous studies, the mechanism of action of hypnosis was left unexplored in this study. However, it remains the only randomized trial to show that hypnotherapy is effective treatment for functional chest pain, a disabling disorder that responds poorly to conventional care. Therefore, additional larger studies evaluating the effect of hypnotherapy on functional chest pain of presumed esophageal origin should be pursued.

MECHANISM OF ACTION OF HYPNOSIS AND HYPNOTHERAPY

The mechanism of action of hypnosis is ill defined; but we may speculate that many factors possibly contribute to its influence on physiological function and symptoms in the upper digestive tract. Abnormal motor activity and

altered autonomic function have both been reported in functional gastroduodenal and esophageal diseases[43,47]. In functional dyspepsia, delayed gastric emptying seems particularly common in patients complaining of nausea, fullness and vomiting; but this is controversial[43,53]. Other disturbances of gastroduodenal motility have been described in functional dyspepsia (e.g. antral hypomotility, gastric dysrhythmia, reduced frequency of interdigestive migrating motor complexes); but their relationship to the symptoms is less documented[43]. On the contrary, evidence of increased gastric visceral perception (so-called hypersensitivity) in a subset of functional dyspepsia patients is well documented in the literature[43,54]. This altered perception may be mediated by the autonomic imbalance both on a cortical and a peripheral level often described in functional bowel disorders[55]. Hypnosis induces a state of profound relaxation consistent with a generalized decrement in sympathetic nervous system activity[1,3]. This relaxation response is not specific to hypnosis, but may be induced by different techniques such as autogenic training, yoga, and meditation[3]. The physiological changes of the relaxation response include simultaneous lowering of blood pressure, heart and respiratory rates, which are opposite to those induced by stressful events[3]. These changes are actually distinct from those observed during sleep and characterize a wakeful hypometabolic state[56]. In addition, the relaxation response seems to last longer than the actual hypnosis interval[3]. A distinct feature of the relaxation response is that its action seems to be mediated through a reduction in epinephrine end-organ responsivity[3]. Stress has been shown to increase gastric acid secretion, and it used to be considered a risk factor to developing peptic ulcer disease[30,57]. We may speculate a potential influence of hypnosis on gastric secretion through modulation of the sympathetic tone. In addition, experimental stress delays gastric emptying and increases plasma levels of noradrenaline plus accelerating small bowel transit[58-60]. Therefore, the capability of a single session of hypnosis either to accelerate gastric emptying or to slow small bowel transit may be secondary to the relaxation response. However, a recent study investigating hypnosis mechanisms of action showed that hypnotherapy did not change cardiovascular responses in IBS[23]. The only parameter of sympathetic tone that was significantly decreased by hypnotherapy was skin conductance, a measure reflecting sweat gland responses to stress[23].

The effect of hypnosis in gastric visceral sensitivity has not been investigated. However, in IBS the influence of hypnotherapy on rectal perception has been evaluated with controversial results. The Manchester group provided experimental evidence that hypnosis improved rectal sensitivity; but this was not confirmed by a recent study by the North Carolina group[23,61]. In addition, one should consider that significant symptom improvement has been reported in functional bowel diseases without correlating with gut sensorimotor functioning modifications[23,36,12]. Therefore, the symptomatic improvement observed after hypnosis should also be related to some modulation of perception at a cortical level. Brain imaging studies have shown a variety of alterations in cortical activation pattern to visceral sensitive stimulation (rectal distension, esophageal distension and acid perfusion) in patients with functional bowel disorders compared with controls[62]. However, a consistent finding has been the reported excessive activation of the anterior cingulated cortex where the affective response to pain is elaborated[62]. It has also been shown that non-painful esophageal distension activated the somatosensory and anterior cingulated cortex while visual stimulation activated a different central area (visual cortex), thus postulating a more specific response to visceral stimulation[63]. Studies on somatic pain have shown that hypnosis is capable of decreasing reported pain sensation in response to pain-inducing stimuli, while the neurophysiological reactions of spontaneous and evoked EEG were unaffected (i.e. cerebral potentials were modified as the subject was actually feeling pain)[64]. Further supporting evidence has been given by studies on somatic pain analgesia where hypnosis reduced activity of the anterior cingulate cortex, but not that of the somatosensory cortex[65]. This dissociation of sensory and affective components of pain under hypnosis would also be consistent with the new "dissociation theory" to explain the effectiveness of hypnotherapy in psychopathology[1,8,66]. There is growing evidence that patients with IBS, functional chest pain and probably functional dyspepsia show increased levels of vigilance toward gut pain related sensations, easily interpreting them as symptoms of disease as a consequence[62,67,68]. Modulating the affective component of pain ratings may be one of the therapeutic mechanisms of hypnotherapy in functional bowel disease.

An additional reason for the effectiveness of hypnotherapy in functional bowel diseases could be related to the focus of many protocols on reducing the catastrophising cognitions commonly present in these patients[67,68]. Gonsalkorale and coworkers reported that hypnotherapy improved symptom-related cognitions in IBS by using a dedicated cognitive scale[69]. In this study, improved cognitive scores correlated with symptomatic improvement[69]. Finally, the role of the placebo effect of hypnotherapy needs to be considered in producing the beneficial hypnotherapy outcomes observed. In many hypnotherapy trials patients affected by severe, unremitting symptoms of functional bowel disorder have been included[13,24,25,36]. The motivation to undergo a new treatment and therapy expectancy in these patients are predicted to be high[70]. In addition, the most powerful placebo effect is to be expected in patients suffering from chronic pain syndromes[70]. In this context, the placebo effect is stronger when complex interventions such as hypnotherapy are provided[70]. Unfortunately, to undertake a double blind controlled trial of treatments such as hypnotherapy is almost impossible because the recipient will know what treatment is provided and establishing a sham hypnosis therapy is not doable[16]. Therefore, appropriate control treatments (e.g. supportive

listening and placebo pills) are desirable options when designing a meaningful trial of hypnotherapy[13,16]. However, the results of such placebo-controlled studies conducted so far on hypnosis for IBS[15,24] and FD, using a powerful double placebo of inert pills combined with supportive listening, suggest that the placebo effect only plays a small role in the therapeutic impact of hypnotherapy on these conditions.

LIMITATIONS OF HYPNOTHERAPY IN UPPER DIGESTIVE DISEASES

Only two studies, coming from the same center, have tested the efficacy of hypnotherapy in the treatment of upper digestive functional diseases[24,25]. These studies provide encouraging evidence that hypnotherapy is effective treatment for functional dyspepsia and functional chest pain of presumed esophageal origin[19,20]. However, these results need to be replicated in less selected populations, and by independent investigators before a more widespread use of hypnotherapy to treat upper digestive dysfunction can be recommended. In addition, hypnotherapy is a time consuming, labor intensive and costly treatment, and the number of health care providers trained in hypnosis is limited. Non-medical qualified hypnotherapists and hypnosis audiotapes may reduce costs, but the effectiveness of these alternative delivery methods on outcomes have not been thoroughly investigated[13,16]. Specific knowledge in gut-directed hypnosis is required to obtain successful outcomes in treating gastrointestinal disorders, and such training has not been widely available[13,16]. In an effort to overcome this problem, some centers are providing gut-focused hypnosis scripts to treat IBS[13]. Finally, skepticism by some patients and physicians about the use of a psychological intervention for a gut disease may deter them from trying this treatment option. This may be particularly true for hypnosis because of the aura of magic and mystery associated with it.

CONCLUSION

Hypnosis is an altered state of consciousness characterized by highly focused attention and heightened compliance with suggestion[1]. Clinical hypnosis can be used to treat a range of complex psychological or somatic diseases, but this generally requires a structured form of hypnotherapy intervention consisting of several sessions[1,8]. Hypnosis has a long history of applications in medicine, and is now formally recognized as a valuable aid for various medical problems. However, a limited number of health professionals offer hypnotherapy for medical problems, and it has traditionally been hampered by misconceptions shrouding this psychological intervention[8,16]. Yet, sufficient evidence has amassed over the years to firmly support the effectiveness of hypnotherapy for various pain problems, as well as to treat IBS, a complex and prevalent functional disorder of the lower bowel[12,13,16]. Recently, a few studies have addressed the potential influence of both single-session hypnosis and a course of hypnotherapy on upper digestive function and diseases with encouraging results.

Hypnosis delivered on a single session by an expert therapist has been shown capable of modulating gastric secretion and accelerating gastric emptying in healthy volunteers[32,36]. In addition, hypnosis has improved gastric emptying and epigastric sensations in severe functional dyspepsia[36]. Small bowel transit may also be influenced by hypnosis[33].

In the past, hypnotherapy has been used with a successful outcome to decrease the relapsing rate of peptic ulcer disease[41]. More recently, two randomized controlled trials have shown hypnotherapy to be a highly effective treatment for functional dyspepsia and functional chest pain of presumed esophageal origin unresponsive to standard care[24,25]. In both of these upper gastrointestinal diseases, clinical benefits were well maintained at long-term follow-ups[24,52]. However, both of these studies were carried out by the same research team -- the Manchester group in England[16]. Additional well designed studies from independent investigators are eagerly awaited to substantiate the efficacy of hypnotherapy in this domain.

REFERENCES

1 **Heap M**. The nature of hypnosis. *Eur J Gastroenterol Hepatol* 1996; **8**: 515-519
2 **von Kirchenheim C**, Persinger MA. Time distortion--a comparison of hypnotic induction and progressive relaxation procedures: a brief communication. *Int J Clin Exp Hypn* 1991; **39**: 63-66
3 **Benson H**. Hypnosis and the relaxation response. *Gastroenterology* 1989; **96**: 1609-1611
4 **Wickramasekera I**. How does biofeedback reduce clinical symptoms and do memories and beliefs have biological consequences? Toward a model of mind-body healing. *Appl Psychophysiol Biofeedback* 1999; **24**: 91-105
5 **Lynn SJ**, Nash MR. Truth in memory: ramifications for psychotherapy and hypnotherapy. *Am J Clin Hypn* 1994; **36**: 194-208
6 **Erickson MH**. The interspersal hypnotic technique for symptom correction and pain control. *Am J Clin Hypn* 1966; **8**: 198-209
7 **Forrest DW**. Hypnotism: A History. London, UK: Penguin, 1999
8 **Waxman D**. Hartland's Medical and Dental Hypnosis, 3rd Edition, London, UK: Harcourt Brace and Company Limited, 1998
9 **Integration of behavioral and relaxation approaches into the treatment of chronic pain and insomnia**. NIH Technology Assessment Panel on Integration of Behavioral and Relaxation Approaches into the Treatment of Chronic Pain and Insomnia. *JAMA* 1996; **276**: 313-318
10 **Richardson J**, Smith JE, McCall G, Pilkington K. Hypnosis for procedure-related pain and distress in pediatric cancer patients: a systematic review of effectiveness and methodology related to hypnosis interventions. *J Pain Symptom Manage* 2006; **31**: 70-84
11 **Uman LS**, Chambers CT, McGrath PJ, Kisely S. Psychological interventions for needle-related procedural pain and distress in children and adolescents. *Cochrane Database Syst Rev* 2006; CD005179
12 **Wilson S**, Maddison T, Roberts L, Greenfield S, Singh S. Systematic review: the effectiveness of hypnotherapy in the

management of irritable bowel syndrome. *Aliment Pharmacol Ther* 2006; **24**: 769-780
13 **Whitehead WE**. Hypnosis for irritable bowel syndrome: the empirical evidence of therapeutic effects. *Int J Clin Exp Hypn* 2006; **54**: 7-20
14 **Tan G**, Hammond DC, Joseph G. Hypnosis and irritable bowel syndrome: a review of efficacy and mechanism of action. *Am J Clin Hypn* 2005; **47**: 161-178
15 **Whorwell PJ**, Prior A, Faragher EB. Controlled trial of hypnotherapy in the treatment of severe refractory irritable-bowel syndrome. *Lancet* 1984; **2**: 1232-1234
16 **Whorwell PJ**. Review article: The history of hypnotherapy and its role in the irritable bowel syndrome. *Aliment Pharmacol Ther* 2005; **22**: 1061-1067
17 **Whorwell PJ**, Prior A, Colgan SM. Hypnotherapy in severe irritable bowel syndrome: further experience. *Gut* 1987; **28**: 423-425
18 **Harvey RF**, Hinton RA, Gunary RM, Barry RE. Individual and group hypnotherapy in treatment of refractory irritable bowel syndrome. *Lancet* 1989; **1**: 424-425
19 **Galovski TE**, Blanchard EB. The treatment of irritable bowel syndrome with hypnotherapy. *Appl Psychophysiol Biofeedback* 1998; **23**: 219-232
20 **Vidakovic-Vukic M**. Hypnotherapy in the treatment of irritable bowel syndrome: methods and results in Amsterdam. *Scand J Gastroenterol Suppl* 1999; **230**: 49-51
21 **Palsson OS**, Turner MJ, Johnson DA, Burnett CK, Whitehead WE. Hypnosis treatment for severe irritable bowel syndrome: investigation of mechanism and effects on symptoms. *Dig Dis Sci* 2002; **47**: 2605-2614
22 **Gonsalkorale WM**, Miller V, Afzal A, Whorwell PJ. Long term benefits of hypnotherapy for irritable bowel syndrome. *Gut* 2003; **52**: 1623-1629
23 **Gonsalkorale WM**, Houghton LA, Whorwell PJ. Hypnotherapy in irritable bowel syndrome: a large-scale audit of a clinical service with examination of factors influencing responsiveness. *Am J Gastroenterol* 2002; **97**: 954-961
24 **Calvert EL**, Houghton LA, Cooper P, Morris J, Whorwell PJ. Long-term improvement in functional dyspepsia using hypnotherapy. *Gastroenterology* 2002; **123**: 1778-1785
25 **Jones H**, Cooper P, Miller V, Brooks N, Whorwell PJ. Treatment of non-cardiac chest pain: a controlled trial of hypnotherapy. *Gut* 2006; **55**: 1403-1408
26 **Eichhorn R**, Tracktir J. The effect of hypnotically induced emotions upon gastric secretion. *Gastroenterology* 1955; **29**: 432-438
27 **Eichhorn R**, Tracktir J. The effect of hypnosis upon gastric secretion. *Gastroenterology* 1955; **29**: 417-421
28 **Kehoe M**, Ironside W. Studies on the experimental evocation of depressive responses using hypnosis. III. The secretory rate of total gastric acid with respect to various spontaneous experiences such as nausea, disgust, crying, and dyspnea. *Psychosom Med* 1964; **26**: 224-249
29 **Stacher G**, Berner P, Naske R, Schuster P, Bauer P, Starker H, Schulze D. Effect of hypnotic suggestion of relaxation on basal and betazole-stimulated gastric acid secretion. *Gastroenterology* 1975; **68**: 656-661
30 **Piper DW**, Greig M, Shinners J, Thomas J, Crawford J. Chronic gastric ulcer and stress. A comparison of an ulcer population with a control population regarding stressful events over a lifetime. *Digestion* 1978; **18**: 303-309
31 **Nasiry RW**, McIntosh JH, Byth K, Piper DW. Prognosis of chronic duodenal ulcer: a prospective study of the effects of demographic and environmental factors and ulcer healing. *Gut* 1987; **28**: 533-540
32 **Klein KB**, Spiegel D. Modulation of gastric acid secretion by hypnosis. *Gastroenterology* 1989; **96**: 1383-1387
33 **Beaugerie L**, Burger AJ, Cadranel JF, Lamy P, Gendre JP, Le Quintrec Y. Modulation of orocaecal transit time by hypnosis. *Gut* 1991; **32**: 393-394
34 **Bond JH Jr**, Levitt MD, Prentiss R. Investigation of small bowel transit time in man utilizing pulmonary hydrogen (H2) measurements. *J Lab Clin Med* 1975; **85**: 546-555
35 **La Brooy SJ**, Male PJ, Beavis AK, Misiewicz JJ. Assessment of the reproducibility of the lactulose H2 breath test as a measure of mouth to caecum transit time. *Gut* 1983; **24**: 893-896
36 **Chiarioni G**, Vantini I, De Iorio F, Benini L. Prokinetic effect of gut-oriented hypnosis on gastric emptying. *Aliment Pharmacol Ther* 2006; **23**: 1241-1249
37 **Benini L**, Castellani G, Sembenini C, Bardelli E, Caliari S, Volino C, Vantini I. Gastric emptying of solid meals in achalasic patients after successful pneumatic dilatation of the cardia. *Dig Dis Sci* 1994; **39**: 733-737
38 **Jian R**, Ducrot F, Ruskone A, Chaussade S, Rambaud JC, Modigliani R, Rain JD, Bernier JJ. Symptomatic, radionuclide and therapeutic assessment of chronic idiopathic dyspepsia. A double-blind placebo-controlled evaluation of cisapride. *Dig Dis Sci* 1989; **34**: 657-664
39 **Nightingale SL**. New warnings added to cisapride labeling. *JAMA* 1998; **280**: 410-412
40 **Hartland J**. Further observations on the use of "ego-strengthening" techniques. *Am J Clin Hypn* 1971; **14**: 1-8
41 **Colgan SM**, Faragher EB, Whorwell PJ. Controlled trial of hypnotherapy in relapse prevention of duodenal ulceration. *Lancet* 1988; **1**: 1299-1300
42 **Levi S**, Beardshall K, Swift I, Foulkes W, Playford R, Ghosh P, Calam J. Antral Helicobacter pylori, hypergastrinaemia, and duodenal ulcers: effect of eradicating the organism. *BMJ* 1989; **299**: 1504-1505
43 **Tack J**, Talley NJ, Camilleri M, Holtmann G, Hu P, Malagelada JR, Stanghellini V. Functional gastroduodenal disorders. *Gastroenterology* 2006; **130**: 1466-1479
44 **Locke GR 3rd**. Nonulcer dyspepsia: what it is and what it is not. *Mayo Clin Proc* 1999; **74**: 1011-1014; quiz 1015
45 **Talley NJ**, Boyce P, Jones M. Dyspepsia and health care seeking in a community: How important are psychological factors? *Dig Dis Sci* 1998; **43**: 1016-1022
46 **Moayyedi P**, Delaney BC, Vakil N, Forman D, Talley NJ. The efficacy of proton pump inhibitors in nonulcer dyspepsia: a systematic review and economic analysis. *Gastroenterology* 2004; **127**: 1329-1337
47 **Galmiche JP**, Clouse RE, Balint A, Cook IJ, Kahrilas PJ, Paterson WG, Smout AJ. Functional esophageal disorders. *Gastroenterology* 2006; **130**: 1459-1465
48 **Chambers J**, Bass C. Chest pain with normal coronary anatomy: a review of natural history and possible etiologic factors. *Prog Cardiovasc Dis* 1990; **33**: 161-184
49 **Clouse RE**, Carney RM. The psychological profile of non-cardiac chest pain patients. *Eur J Gastroenterol Hepatol* 1995; **7**: 1160-1165
50 **Numans ME**, Lau J, de Wit NJ, Bonis PA. Short-term treatment with proton-pump inhibitors as a test for gastroesophageal reflux disease: a meta-analysis of diagnostic test characteristics. *Ann Intern Med* 2004; **140**: 518-527
51 **Jackson JL**, O'Malley PG, Tomkins G, Balden E, Santoro J, Kroenke K. Treatment of functional gastrointestinal disorders with antidepressant medications: a meta-analysis. *Am J Med* 2000; **108**: 65-72
52 **Miller V**, Jones H, Whorwell PJ. Hypnotherapy for non-cardiac chest pain: long-term follow-up. *Gut* 2007; **56**: 1643
53 **Sarnelli G**, Caenepeel P, Geypens B, Janssens J, Tack J. Symptoms associated with impaired gastric emptying of solids and liquids in functional dyspepsia. *Am J Gastroenterol* 2003; **98**: 783-788
54 **Rhee PL**, Kim YH, Son HJ, Kim JJ, Koh KC, Paik SW, Rhee JC, Choi KW. Evaluation of individual symptoms cannot predict presence of gastric hypersensitivity in functional dyspepsia. *Dig Dis Sci* 2000; **45**: 1680-1684
55 **Tougas G**. The autonomic nervous system in functional bowel disorders. *Gut* 2000; **47** Suppl 4: iv78-iv80; discussion iv87

56 **Wallace RK**, Benson H, Wilson AF. A wakeful hypometabolic physiologic state. *Am J Physiol* 1971; **221**: 795-799

57 **Goldman MC**. Gastric secretion during a medical interview. *Psychosom Med* 1963; **25**: 351-356

58 **Thompson DG**, Richelson E, Malagelada JR. Perturbation of gastric emptying and duodenal motility through the central nervous system. *Gastroenterology* 1982; **83**: 1200-1206

59 **Stanghellini V**, Malagelada JR, Zinsmeister AR, Go VL, Kao PC. Stress-induced gastroduodenal motor disturbances in humans: possible humoral mechanisms. *Gastroenterology* 1983; **85**: 83-91

60 **Cann PA**, Read NW, Cammack J, Childs H, Holden S, Kashman R, Longmore J, Nix S, Simms N, Swallow K, Weller J. Psychological stress and the passage of a standard meal through the stomach and small intestine in man. *Gut* 1983; **24**: 236-240

61 **Lea R**, Houghton LA, Calvert EL, Larder S, Gonsalkorale WM, Whelan V, Randles J, Cooper P, Cruickshanks P, Miller V, Whorwell PJ. Gut-focused hypnotherapy normalizes disordered rectal sensitivity in patients with irritable bowel syndrome. *Aliment Pharmacol Ther* 2003; **17**: 635-642

62 **Mayer EA**, Naliboff BD, Craig AD. Neuroimaging of the brain-gut axis: from basic understanding to treatment of functional GI disorders. *Gastroenterology* 2006; **131**: 1925-1942

63 **Gregory LJ**, Yaguez L, Williams SC, Altmann C, Coen SJ, Ng V, Brammer MJ, Thompson DG, Aziz Q. Cognitive modulation of the cerebral processing of human oesophageal sensation using functional magnetic resonance imaging. *Gut* 2003; **52**: 1671-1677

64 **Meier W**, Klucken M, Soyka D, Bromm B. Hypnotic hypo- and hyperalgesia: divergent effects on pain ratings and pain-related cerebral potentials. *Pain* 1993; **53**: 175-181

65 **Faymonville ME**, Laureys S, Degueldre C, DelFiore G, Luxen A, Franck G, Lamy M, Maquet P. Neural mechanisms of antinociceptive effects of hypnosis. *Anesthesiology* 2000; **92**: 1257-1267

66 **Hilgard ER**, Morgan AH, Macdonald H. Pain and dissociation in the cold pressor test: a study of hypnotic analgesia with "hidden reports" through automatic key pressing and automatic talking. *J Abnorm Psychol* 1975; **84**: 280-289

67 **Levy RL**, Olden KW, Naliboff BD, Bradley LA, Francisconi C, Drossman DA, Creed F. Psychosocial aspects of the functional gastrointestinal disorders. *Gastroenterology* 2006; **130**: 1447-1458

68 **Palsson OS**, Whitehead WE. Hypnosis for non-cardiac chest pain. *Gut* 2006; **55**: 1381-1384

69 **Gonsalkorale WM**, Toner BB, Whorwell PJ. Cognitive change in patients undergoing hypnotherapy for irritable bowel syndrome. *J Psychosom Res* 2004; **56**: 271-278

70 **Musial F**, Klosterhalfen S, Enck P. Placebo responses in patients with gastrointestinal disorders. *World J Gastroenterol* 2007; **13**: 3425-3429

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REVIEW

Interstitial cells of Cajal in the gut - A gastroenterologist's point of view

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Abstract

Alterations of normal function of interstitial cells of Cajal (ICC) are reported in many intestinal disorders. Diagnosis of their involvement is rare (infrequent), but necessary to propose a specific treatment. This article reviews the place of ICC in the pathogenesis of achalasia, gastroesophageal reflux disease, infantile hypertrophic pyloric stenosis, chronic intestinal pseudo-obstruction and slow transit constipation. Moreover we discuss the role of the Cajal cells in the development of stromal tumors of the gastrointestinal tract.

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Key words: Cajal cells; c-kit; Intestinal motility; Achalasia; Gastrointestinal stromal tumor

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INTRODUCTION

Digestive motility is highly coordinated and consists of local, non-propulsive mixing (segmental) and propulsive (peristaltic) movements. Mixing movements are produced by intrinsic pacemakers generating rhythmic contractions and peristalsis by intrinsic excitatory and inhibitory neural reflex pathways[1,2].

Even in the absence of stimulation, most regions of the gastrointestinal tract can generate some spontaneous electrical and mechanical activity. Recordings made from isolated muscle cells in the gastrointestinal tract show a regular discharge recorded as plateau and slow potentials. These pacemaker potentials are generated by a specialized population of cells, known as interstitial cells of Cajal (ICC)[3].

Together with the enteric nervous system, composed of both the myenteric (inter-muscular) plexus and the submucosal plexus, the ICC plays a major role in gastrointestinal motility[4]. The ICC was firstly described by Cajal SR in 1911[5]. He characterized "interstitial neurons" as "primitive accessory components that could modify smooth muscle contraction, subject themselves to regulation from principal neurons". Cajal provided detailed pictures of methylene blue-stained networks of interstitial cells, which were described as spindle shaped or stellate cells with long, ramified cell processes and large, oval, nuclei with sparse perinuclear cytoplasm, and intercalated between autonomic nerve endings and smooth muscle cells[5].

ICC constitutes networks that are widely distributed within the submucosal, intra-muscular and inter-muscular layers of the gastrointestinal tract from the lower esophagus to the internal anal sphincter.

These cells are defined by the expression of the CD117 (c-kit) protein which is a membrane receptor with tyrosine kinase activity[3,4,6].

In the past decade, knowledge of the role of ICC in the digestive physiology and pathology has progressed. In this review, we highlight some of these advances which could have clinical impact either in pathogenesis or treatment.

ESOPHAGUS

Achalasia

Achalasia is characterized by relaxation failure of lower

esophageal sphincter (LES) and lack of peristaltic contraction of esophageal body[7]. The etiology of this disorder is unknown and may be "idiopathic" or secondary to malignancy (local invasion or a paraneoplastic manifestation).

In primary or idiopathic achalasia, the failure of deglutitive inhibition is responsible for aperistalsis. This dysfunction is due to a loss of inhibitory nerves and progressive degeneration of ganglion cells containing vasoactive intestinal peptide (VIP) and nitric oxide (NO). Hypertensive LES is thought to result from a combination of the lack of tonic inhibitory nitrergic influence and an unopposed cholinergic activity.

The mechanism of inflammatory process responsible of these alterations is unclear. It is suggested to be an autoimmune disorder induced by a viral or food antigen in a patient genetically predisposed to the disease[8,9]. ICC involvement in achalasia is debated[10,11].

Electronic microscope studies of muscle coat of LES in seven patients with achalasia showed that muscle wall components (nerve endings, smooth muscle cells, ICC and connective tissue) were modified. ICC ultrastructure was altered, namely clear cytoplasm, fewer mitochondria, and scarce smooth endoplasmic reticulum. A reduced number of contacts between nerves and ICC were reported. Specific changes in smooth muscle cells were also documented, whereas the nerve endings had a normal ultrastructure. Alterations in older patients were more pronounced[12]. Since the LES components specifically altered in achalasia are the nerve endings and ICC, they are regarded as principally responsible for abnormal motility[12].

Achalasia is uncommon among pediatric population and some authors consider it as a different entity. Rare familial forms, combining early onset achalasia, alacrymia, ACTH insensitivity and dysautonomia, are known as Allgrove's syndrome or "four A" syndrome. Allgrove's syndrome is inherited in an autosomal recessive mode and may express in adulthood. Massive loss of neural elements and neuronal NO synthase as well as a marked fibrotic process of the muscle layers of the cardia have been observed in this syndrome[13]. ICC in cardia was also markedly decreased or absent while ICC (and neural structures) were preserved in pylorus[13].

Gastro-esophageal reflux disease (GERD)

GERD is a highly prevalent condition. Typical symptoms of heartburn and acid regurgitation are encountered in 15%-20% of the general population[14].

GERD represents the most common cause of esophagitis that may be complicated with esophageal ulcers, peptic stenosis and Barrett's esophagus, which carries a high risk of esophageal adenocarcinoma[14].

The role of the ICC in inhibitory transmission in the LES is still discussed.

In W/W_v mutant mice (lack of ICC) LOS pressure was lower than wild-type mice but a normal swallow still induced LOS relaxation, arguing against the role of ICC in inhibitory transmission[15]. Another study demonstrated that in W/W_v animals, cholinergic and nitrergic neurotransmission is greatly reduced pleading for the role of ICC in mediating neural inputs[16]. However enteric neurons, varicose processes, and the ability to release neurotransmitters are not reduced, and smooth muscle cells demonstrate responsiveness to exogenous transmitters[16].

Loss of ICC during development or in pathologic conditions would significantly compromise the ability of GI muscles to generate typical motor reflexes[17].

Esophagitis itself may be at the origin of an alteration of normal function of the Cajal cells: in advanced stages of GERD, inflammatory changes in the esophageal wall will also involve the ICC. That way, the more severe the esophagitis, the more severe is the ICC impairment. This destruction leads to loss of effective contraction of esophagus, maintaining reflux and thus aggravating the symptoms[18].

STOMACH

Gastroparesis

Delayed gastric emptying can be secondary to muscular, neural, humoral causes or use of anticholinergic and opiates medicines. In the absence of an identified cause, gastroparesis is termed as idiopathic[19]. Clinical features of gastroparesis are frequently indistinguishable from true mechanical obstruction and severity of symptoms is variable. Most patients present with early satiety, nausea, and abdominal pain. In some cases, symptoms can be highly incapacitating: chronic abdominal pain and vomiting leading to dehydration, electrolyte imbalance, nutritional impairment and weight loss[20,21].

ICC is involved in regulation of gastric emptying by generating slow waves.

A decrease in ICC density ranged from 60% to 100% depending on the area investigated was demonstrated in histologic studies of stomach of type 1 diabetic patients[6]. The number of immunopositive cells for c-kit was significantly decreased in the corpus and antrum of the gastroparesis patients compared with control tissues[21]. The loss of intra muscular ICC and associated nerves in the gastric fundus could explain the low basal gastric tone and increased compliance of the stomach. The hypomotility of the antrum can also be explained by the absence of slow wave generation by the ICC[21,22].

Infantile hypertrophic pyloric stenosis

This is a congenital disorder characterized by functional gastric-outlet obstruction. Dysfunction of pyloric inhibition has been implicated in the pathophysiology of hypertrophic pyloric stenosis. Normal inhibition process is mediated by peptidergic and NO enteric nerves and also may involve ICC. Although myenteric neurons appear normal, those innervating the circular-muscle layer of the pyloric sphincter lack NO synthetase[21]. In children with hypertrophic pyloric stenosis, there was a significant decrease in the number of ICC[22,23]. The following observations were made using electron microscopy in gastric specimens from patients with pyloric stenosis versus normal controls[24]. Muscle cells were primarily in a

proliferative phase and exhibited very few gap junctions between smooth muscle cells or ICC: (1) Near absence of nerve fibers containing large granular vesicles in the circular muscle layer; (2) Fewer nerve cell bodies in the myenteric plexus and lower total number of ganglia; (3) Decreased number of ICC. These findings may plead for a role of ICC in the pathogenesis.

SMALL INTESTINE AND COLON

Idiopathic chronic intestinal pseudo-obstruction (CIIP)

CIIP is characterized by defective gastrointestinal propulsion together with symptoms and signs of bowel obstruction in the absence of any lesions or mechanical obstacle[25]. CIIP is regarded as a neuropathy, myopathy or both[26,27].

A possible role played by the ICC is demonstrated by the alterations in ICC network reported in patients with CIIP. Electron microscopy and immunochemistry studies showed a decreased number of ICCs along with structural abnormalities such as loss of processes and damaged intracellular cytoskeleton and organelles[28].

Slow transit constipation (STC)

This is a very prevalent motility problem, but its mechanisms are unclear. Studies found that ICC density in the colon of patients with constipation was significantly decreased compared with those of normal patients[29]. Expression of *c-kit* mRNA and c-kit protein was significantly decreased in the colon of STC, suggesting that the c-kit signal pathway may play an important role in ICC reduction in STC[30-32].

Since slow-transit constipation is secondary to problems with the ENS, ICC, or smooth muscle cells, replacement of the missing or defective cells would be an attractive way of treatment[31]. Growing precursors of the defective cells from stem cells should be easy, but the distribution of the cells to their proper locations is still problematic[31,32]. For the moment this is a promise of genetic treatment.

TUMORS OF GASTROINTESTINAL TRACT

Gastrointestinal stromal tumors (GISTs)

GISTs have been recognized as a biologically distinctive tumor type, different from smooth muscle and neural tumors of the gastrointestinal tract. They constitute the majority of gastrointestinal mesenchymal tumors[33].

GISTs originate from the ICC. Their origin from the ICC has been proven by their immunophenotypic (CD117 positive) and ultrastructural resemblance and also by the presence of an embryonic smooth muscle myosin similar to the one present in the ICC[33-36] (Table 1). Approximately 80% of GISTs also express CD34.

Annual incidence of clinically detected new cases of GISTs in the United States has increased to 5000-6000 per year due to better diagnosis, and incidence is rising. Uncommonly, GISTs arise in families, and in these patients germline mutations of c-kit have been identified particularly in exons 11 and 13. A diffuse hyperplasia of the ICC, which is regarded as a pre-neoplastic lesion is noted in these patients[33]. The patients with exon 11 mutations develop cutaneous mastocytosis with or without cutaneous hyperpigmentation, but those with exon 13 mutations do not have these features[33,34]. The tumors under 3 cm in diameter are mostly benign, but all GISTs have a malignant potential[35].

Table 1 Immunohistochemical analysis of GI mesenchymal tumors[35,36]

Tumor	Positive immunohistochemical staining
GIST	CD 117
	CD 34
Malignant GIST	Ki 67
Smooth muscle tumor	Smooth muscle actin
	Desmin
Schwannoma	S100
Glomus tumor	Smooth muscle actin
	Vimentin

The majority of GISTs occurs in the stomach (60%-70%), small intestine (20%-30%) and only 10% or less in the esophagus, colon and rectum, and they affect mainly middle aged patients. Similar tumors, sometimes known as extra-gastrointestinal stromal tumors (E-GIST), may arise in the omentum, mesentery, or retroperitoneum and at least one case of pancreatic tumor was described[37,38]. The presence of ICC in normal pancreas was demonstrated recently[39].

The symptoms may vary from none or slight abdominal discomfort to brisk gastrointestinal hemorrhage, perforation or obstruction.

Imatinib mesylate, a synthetic tyrosine kinase inhibitor developed for the use in the management of interferon resistant chronic myeloid leukemia (CML), was shown to be effective against a number of other tyrosine kinases including c-kit and platelet derived growth factor (PDGF) and now it is considered to be the drug of choice for metastatic and inoperable GIST's[33,34].

CONCLUSION

Knowledge on the role of ICC in gastrointestinal disorders is increasing. However, with the exception of GIST's, no major breakthrough has been made in treatment. Further studies may provide new treatments.

REFERENCES

1 **Stevens RJ**, Publicover NG, Smith TK. Induction and organization of Ca2+ waves by enteric neural reflexes. *Nature* 1999; **399**: 62-66
2 **Wood JD**. Mixing and moving in the gut. *Gut* 1999; **45**: 333-334
3 **Ward SM**. Interstitial cells of Cajal in enteric neurotransmission. *Gut* 2000; **47** Suppl 4: iv40-iv43; discussion iv52
4 **Takaki M**. Gut pacemaker cells: the interstitial cells of Cajal (ICC). *J Smooth Muscle Res* 2003; **39**: 137-161
5 **Cajal SR**. Histology of the nervous system of man and

vertebrates (translated by N Swanson and LW Swanson), New York: Oxford University Press, 1995: 891-942
6 **Long QL**, Fang DC, Shi HT, Luo YH. Gastro-electric dysrhythm and lack of gastric interstitial cells of cajal. *World J Gastroenterol* 2004; **10**: 1227-1230
7 **Sifrim D**, Janssens J, Vantrappen G. Failing deglutitive inhibition in primary esophageal motility disorders. *Gastroenterology* 1994; **106**: 875-882
8 **Robertson CS**, Martin BA, Atkinson M. Varicella-zoster virus DNA in the oesophageal myenteric plexus in achalasia. *Gut* 1993; **34**: 299-302
9 **Metman EH**, Lagasse JP, Pic P, Picon L, Danquechin Dorval E, Goudeau A. [Varicella and primary achalasia of the lower esophageal sphincter] *Gastroenterol Clin Biol* 1996; **20**: 1138-1139
10 **Ward SM**, Morris G, Reese L, Wang XY, Sanders KM. Interstitial cells of Cajal mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. *Gastroenterology* 1998; **115**: 314-329
11 **Sanders KM**, Ward SM, Daniel EE. ICC in neurotransmission: hard to swallow a lack of involvement. *Gastroenterology* 2002; **122**: 1185-1186; author reply 1186-1187
12 **Faussone-Pellegrini MS**, Cortesini C. The muscle coat of the lower esophageal sphincter in patients with achalasia and hypertensive sphincter. An electron microscopic study. *J Submicrosc Cytol* 1985; **17**: 673-685
13 **Metman EH**, Debbabi S, Negreanu L. Troubles moteurs de l' œsophage, Encyclopédie medico-chirurgicale. *Elsevier* 2006; **4**: 1-19
14 **Richter JE**. Gastrooesophageal reflux disease. *Best Pract Res Clin Gastroenterol* 2007; **21**: 609-631
15 **Dickens EJ**, Edwards FR, Hirst GD. Selective knockout of intramuscular interstitial cells reveals their role in the generation of slow waves in mouse stomach. *J Physiol* 2001; **531**: 827-833
16 **Ward SM**, Beckett EA, Wang X, Baker F, Khoyi M, Sanders KM. Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. *J Neurosci* 2000; **20**: 1393-1403
17 **Ward SM**, Sanders KM. Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. I. Functional development and plasticity of interstitial cells of Cajal networks. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G602-G611
18 **Shafik A**, El-Sibai O, Shafik I, Shafik A. Electroesophagogram in gastroesophageal reflux disease with a new theory on the pathogenesis of its electric changes. *BMC Surg* 2004; **4**: 13
19 **Forster J**, Damjanov I, Lin Z, Sarosiek I, Wetzel P, McCallum RW. Absence of the interstitial cells of Cajal in patients with gastroparesis and correlation with clinical findings. *J Gastrointest Surg* 2005; **9**: 102-108
20 **Hirst GD**, Edwards FR. Role of interstitial cells of Cajal in the control of gastric motility. *J Pharmacol Sci* 2004; **96**: 1-10
21 **Ibba Manneschi L**, Pacini S, Corsani L, Bechi P, Faussone-Pellegrini MS. Interstititал cells of Cajal in the human stomach: distribution and relationship with enteric innervation. *Histol Histopathol* 2004; **19**: 1153-1164
22 **Ordog T**, Redelman D, Horvath VJ, Miller LJ, Horowitz B, Sanders KM. Quantitative analysis by flow cytometry of interstitial cells of Cajal, pacemakers, and mediators of neurotransmission in the gastrointestinal tract. *Cytometry A* 2004; **62**: 139-149
23 **Vanderwinden JM**, Rumessen JJ. Interstitial cells of Cajal in human gut and gastrointestinal disease. *Microsc Res Tech* 1999; **47**: 344-360
24 **Langer JC**, Berezin I, Daniel EE. Hypertrophic pyloric stenosis: ultrastructural abnormalities of enteric nerves and the interstitial cells of Cajal. *J Pediatr Surg* 1995; **30**: 1535-1543
25 **De Giorgio R**, Sarnelli G, Corinaldesi R, Stanghellini V. Advances in our understanding of the pathology of chronic intestinal pseudo-obstruction. *Gut* 2004; **53**: 1549-1552
26 **Coulie B**, Camilleri M. Intestinal pseudo-obstruction. *Annu Rev Med* 1999; **50**: 37-55
27 **De Giorgio R**, Guerrini S, Barbara G, Cremon C, Stanghellini V, Corinaldesi R. New insights into human enteric neuropathies. *Neurogastroenterol Motil* 2004; **16** Suppl 1: 143-147
28 **Feldstein AE**, Miller SM, El-Youssef M, Rodeberg D, Lindor NM, Burgart LJ, Szurszewski JH, Farrugia G. Chronic intestinal pseudoobstruction associated with altered interstitial cells of cajal networks. *J Pediatr Gastroenterol Nutr* 2003; **36**: 492-497
29 **Basilisco G**, Gebbia C, Peracchi M, Velio P, Conte D, Bresolin N, Nobile-Orazio E. Cerebellar degeneration and hearing loss in a patient with idiopathic myenteric ganglionitis. *Eur J Gastroenterol Hepatol* 2005; **17**: 449-452
30 **Tong WD**, Liu BH, Zhang LY, Xiong RP, Liu P, Zhang SB. Expression of c-kit messenger ribonucleic acid and c-kit protein in sigmoid colon of patients with slow transit constipation. *Int J Colorectal Dis* 2005; **20**: 363-367
31 **Schiller LR**. New and emerging treatment options for chronic constipation. *Rev Gastroenterol Disord* 2004; **4** Suppl 2: S43-S51
32 **Rao SS**. Constipation: evaluation and treatment. *Gastroenterol Clin North Am* 2003; **32**: 659-683
33 **D'Amato G**, Steinert DM, McAuliffe JC, Trent JC. Update on the biology and therapy of gastrointestinal stromal tumors. *Cancer Control* 2005; **12**: 44-56
34 **de Silva CM**, Reid R. Gastrointestinal stromal tumors (GIST): C-kit mutations, CD117 expression, differential diagnosis and targeted cancer therapy with Imatinib. *Pathol Oncol Res* 2003; **9**: 13-19
35 **Hwang JH**, Kimmey MB. The incidental upper gastrointestinal subepithelial mass. *Gastroenterology* 2004; **126**: 301-307
36 **Ando N**, Goto H, Niwa Y, Hirooka Y, Ohmiya N, Nagasaka T, Hayakawa T. The diagnosis of GI stromal tumors with EUS-guided fine needle aspiration with immunohistochemical analysis. *Gastrointest Endosc* 2002; **55**: 37-43
37 **Nakagawa M**, Akasaka Y, Kanai T, Yamashita T, Kuroda M, Takayama H, Miyazawa N. Extragastrointestinal stromal tumor of the greater omentum: case report and review of the literature. *Hepatogastroenterology* 2003; **50**: 691-695
38 **Yamaura K**, Kato K, Miyazawa M, Haba Y, Muramatsu A, Miyata K, Koide N. Stromal tumor of the pancreas with expression of c-kit protein: report of a case. *J Gastroenterol Hepatol* 2004; **19**: 467-470
39 **Popescu LM**, Hinescu ME, Ionescu N, Ciontea SM, Cretoiu D, Ardelean C. Interstitial cells of Cajal in pancreas. *J Cell Mol Med* 2005; **9**: 169-190

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REVIEW

Current status of intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma (ICC) is a rare primary liver cancer with a global increasing trend in recent years. Symptoms tend to be vague and insidious in development, often are diagnosed at an advanced stage when only palliative approaches can be used with a median survival rate of months. Comparing with HCC, ICC tends to spread to lymph nodes early, and is rarely limited to the regional lymph nodes, with a frequent postoperative recurrence. Surgery is the only choice of curative therapy for ICC, but recently no consensus has been established for operation. Thus, more data from multiple centers and more cases are needed. Generally speaking, current adjunctive therapy cannot clearly improve survival. Further research is needed to find more effective radio- and chemotherapeutic regimens.

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Key words: Intrahepatic cholangiocarcinoma; Lymph node metastasis; Liver transplantation; Adjunctive therapy

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EPIDEMIOLOGY

Intrahepatic cholangiocarcinoma (ICC) is a rare malignant tumor which arises from the epithelial cells of intrahepatic bile ducts (beyond the second order bile ducts). The incidence of ICC is reported to be only about 10% of primary liver cancers. But, recent studies from several countries have indicated that the incidence of ICC is increasing which cannot be solely explained by reclassification and improved detection[1-10]. The rate of ICC for males is greater than that for females; but ICC is less distinct than hepatocellular carcinoma and usually occurs after the sixth decade of life[1-10]. A recent study reported that in addition to the established risk factors such choledochal cysts, chronic cholangitis, inflammatory bowel disease, primary sclerosing cholangitis (PSC) parasitic infections, drug or toxin exposure, and genetic risks, other conditions such as biliary cirrhosis, cholelithiasis, alcoholic liver disease, nonspecific cirrhosis, are significantly associated with ICC[11]. The incidence of diabetes, thyrotoxicosis, chronic pancreatitis, obesity, chronic nonalcoholic liver disease, HCV/HBV infection, chronic typhoid carrier state and smoking, is increasing, suggesting that these conditions might partly explain the trends of ICC in incidence[12]. However, many tumors arise in the absence of any known predisposition[13-17]. Despite the global increase, regional, racial, ethnic, gender and age variations occur. Moreover, it was reported that the incidence of ICC has decreased in Denmark[18,19]. ICC has the worst prognosis of any tumor arising in the liver; its 5-year survival is poor, and accompanied by a high recurrence rate. The overall 5-year survival rate ranges 13%-42%[20-22]. Chu *et al*[23] showed that the median survival after conservative therapy and hepatic resection is 1.8 mo and 12.2 mo, respectively.

DIAGNOSIS

Recent advances have been made in diagnosis of ICC with MRCP combined MRI, CT, positron-emission tomography scanning (PET) with [F-18] fluorodeoxyglucose (FDG), virtual three-dimensional images and optical coherence tomography (OCT), a high-resolution imaging technique that produces cross-sectional images *in vivo*[24,25], endoscopic retrograde cholangiography (ERCP) with brush cytology and biopsy, endoscopic ultrasound with guided fine-needle aspiration, advanced cytological tests including fluorescent *in situ* hybridization or

digital image analysis (DIA), cholangioscopy (peroral cholangioscopy, percutaneous cholangioscopy, transpapillary cholangioscopy)[26,27]. Sandwich enzyme-linked immunosorbent assay can show a 71% sensitivity and 90% specificity for new tumor markers in serum and bile including genomic and proteomic markers [such as CA199, CEA and mucin5, subtypes A and C (MUC5AC)][28]. On the other hand, most patients present too late to be diagnosed at an advanced stage when only palliative approaches can be used with a median survival of months.

Macroscopic aspect

ICC is defined as a kind of tumor originating from the second branch (segmental branch) or the proximal branch of bile duct[29] and further classified into hilar type and peripheral type. The former arises from the large intrahepatic biliary epithelium (segmental branches) having histological features of a papillary epithelial component or a large tubular component. The latter arises from small biliary epithelium (smaller than segmental branches) with histological features of small-sized glands in a fibrotic background, closely packed, somewhat distorted small ducts, and cordlike structure, but lacking large glands, and Shinichi Aishima, *etc*. It was recently reported that ICC is associated with different predispositions when arising from different levels of the biliary tree and likely to show an aggressive course even in cases of a small tumor arising from the large biliary duct[30,31].

Histological aspect

ICC, arising from cholangiocytes, is a moderately- to well-differentiated tubular adenocarcinoma. Papillary adenocarcinoma, signet-ring carcinoma, squamous cell or muco-epidermoid carcinoma and lymphoepithelioma-like forms are rare histological variants. The most outstanding histological feature is the presence of abundant desmoplastic stroma in ICC compared with HCC, leading to a low diagnostic yield of random biopsies. Desmoplasia may also cause capsular retraction. According to the degree of stromal desmoplasia in the tumor and Kajiyama, Kiyoshi, *etc*, ICC is microscopically categorized into scirrhous-type (SICC) and nonscirrhous-type (NSICC). The frequencies of lymphatic permeation, perineural invasion and the proliferative activity measured by MIB-1 immunostaining, monoclonal antibody specific for Ki-67 [a nuclear antigen expressed throughout the cell cycle (G_1, S-G_2 and M), but absent in quiescent cells (G_0)], were significantly higher in SICC than in NSICC, and serosal invasion, vascular invasion, lymph nodes metastases also tend to be more frequent in SICC and are closely related to the prognosis[32].

Classification

Three types of ICC have been established using the TNM staging system and the classification system established by the Liver Cancer Study Group of Japan: mass-forming type (MF), periductal-infiltration type (PI) and intraductal growth type (IG). MF type forms a definite round-shaped mass with an expansive growth pattern, but without fibrous capsule, and locates in the liver parenchyma. The border between cancerous and non-cancerous portions is distinct, and this type of ICC does not invade the major branch of the portal triad. PI type is defined as a mass extending longitudinally along the bile duct, occasionally involves the surrounding blood vessels and/or hepatic parenchyma, often resulting in dilatation of the peripheral bile duct. IG type proliferates towards the lumen of bile duct papillary or like a tumor thrombus, occasionally involving superficial extension. This type of ICC is usually detected in a thick bile duct[29].

Table 1 Staging system for ICC proposed by Liver Cancer Study Group of Japan

T1: Meet requirements (single nodule, tumor 2 cm or less and no portal vein, hepatic vein and serous membrane invasion)			
T2: Meet two of the three requirements			
T3: Meet one of the three requirements			
T4: Meet none of the three requirements			
N1: No metastasis to lymph node			
N2: Metastasis to any lymph nodes			
M0: No distant metastasis			
M1: Positive distant metastasis			
Stage Ⅰ	T1	N0	M0
Stage Ⅱ	T2	N0	M0
Stage Ⅲ	T3	N0	M0
Stage ⅣA	T4	N0	M0
	Or any T	N1	M0
Stage ⅣB	Any T	Any N	M1

Staging system

ICC is a rare type of primary liver cancer, accounting only for 5%-10% of all liver cancers, and has a low resectability rate. So the International Union against Cancer (UICC) defined the TNM staging system solely from clinical experience in treating HCC[33]. Based on the distinct difference in the mechanism and biologic behavior between HCC and ICC, the Liver Cancer Study Group of Japan has proposed a new TNM staging system for the MF type of ICC (Table 1)[34]. Serosal invasion is not a T-factor component in the UICC tumor staging system. Uenishi *et al*[35] retrospectively analyzed sixty-three patients who underwent hepatic resection for mass-forming intrahepatic cholangiocarcinoma between January 1983 and December 2003, and found that that serosal invasion has no impact on survival of patients after hepatic resection for MF type of ICC. Another staging system used for MF type of ICC, defined a solitary tumor without vascular invasion as stage Ⅰ, a solitary tumor with vascular invasion as stage Ⅱ, multiple tumor with or without vascular invasion as stage ⅢA, tumor with regional lymph node metastasis as stage ⅢB, tumor with distant metastases as stage Ⅳ. In this system, tumor size is excluded from T factor. It is likely that the influence of tumor size on its prognosis cannot be evaluated because the number of small tumors is too small[36].

Table 2 Lymph node groups by tumor location

	N1	N2	N3
Right lobe	Hepatoduodenal ligament	Along left gastric artery Along common hepatic artery Along celiac artery Posterior surface of pancreas head	Distant
Left lobe	Right cardiac region	Along left gastric artery	
	Lesser curvature of stomach	Along common hepatic artery	
		Along celiac artery	Distant
	Hepatoduodenal ligament	Posterior surface of pancreas head	

N3 distant: Abdominal aorta, root of the mesentery, inferior vena cava, *etc.*

Spreading mode

The spreading modes of ICC, such as sinusoidal invasion, spreading along duct walls and periductal tissue, growth replacing the biliary epithelium or intraductal growth, spreading along Glisson's sheath (lymphatic involvement, perineural or intraneural invasion, permeation of the portal connective tissue and vascular involvement) have been reported[37-40]. Nakajima *et al*[37] reported that sinusoidal invasion and portal vein invasion are the most frequent mode of intrahepatic spread. Different macroscopic types of ICC have different modes of spread. The MF type of ICC tends to invade the liver *via* the portal vein system and Glisson's sheath when the tumor increases in size with a frequent remnant hepatic recurrence. The PI type of ICC has a tendency to infiltrate making it difficult to get clear margins during hepatectomy, and to spread along Glisson's sheath *via* lymphatic vessels, thus invading connective tissue and major vessels at the hilum and hepatoduodenal ligament. The IG type of ICC has an extremely favorable prognosis after surgical resection compared with the other two types. Moreover, this type of ICC has a lower rate of lymphatic or intrahepatic metastasis and recurrence after curative surgical resection[37-39]. Yamamoto *et al*[40] suggested that anatomic and extensive hepatectomy is a rational procedure for MF type of ICC, and hepatectomy with extrahepatic duct excision and hilar lymph node resection is a rational procedure for IP and MF types of ICC with biliary invasion. The Liver Cancer Study Group of Japan has proposed a criterion for the invasion degrees of ICC: (1) no tumor invasion of the portal vein, hepatic vein, or bile duct; (2) tumor invasion distal to the second branch of the portal vein or bile duct and/or invasion of a branch of the hepatic vein; (3) tumor invasion of the second branch of the portal vein or the bile duct, the major hepatic veins and/or the short hepatic veins; (4) tumor invasion of the first branch of the portal vein or of the bile duct and tumor invasion of the inferior vena cava[29].

Lymph node metastasis

The most outstanding pattern of ICC compared with HCC is early lymphatic spread. The findings in the majority recent literature indicate that lymph node status is an important prognostic factor for patients undergoing hepatic resection[41-47]. Yet nodal status does not affect survival after aggressive surgical treatment in patients with ICC[48], and some long-term survivors with positive lymph nodes have also been reported[49-51]. It was reported that the rate of metastasis for ICC to hilar lymph nodes is about 50%[41,42,52]. Nakagawa *et al*[43] reported that the positive rate of lymph nodes in patients with lymph node dissection is 47%, 33%, 17%, 13%, 10%, 3%, respectively. Regarding the pattern of lymph node spread, the Liver Cancer Study Group of Japan has proposed a classification of regional lymph nodes in liver cancer (Table 2) and three major routes of lymphatic spread of ICC: hepatoduodenal route, cardiac route (through the lesser omentum to the cardiac portion of the stomach and the gastric lesser curvature), and diaphragmatic route[48]. Hepatoduodenal ligament is the most common site of nodal metastasis in ICC patients irrespective of the tumor location. Almost all patients are involved in positive lymph nodes of the hepatoduodenal ligament or along the common hepatic artery, lymph nodes are also found in about half of the patients involving the left lobe of liver[43,44,53-56]. Nozaki *et al*[57] reported that extensive lymph node metastasis was observed in most patients, only 3 (20%) of 15 patients with lymph node metastasis had regional lymph node metastasis. Shimada *et al*[47] has reported the similar observations, suggesting that lymph node metastasis of ICC is rarely limited to the regional lymph nodes.

Surgical treatment

Surgery is the only choice of curative therapy for ICC. However, only a few patients are suitable for surgery. Good results depend on comprehensive preoperative evaluation, patient selection and discreet operation.

EVALUATION

Assessment of resectability

Tumors that are medically fit for hepatic resection must be completely resected with negative histological margins, no evidence of metastases, disseminated disease, and extensive lymphadenopathy. The following factors must be considered. (1) Biliary tract invasion: bilateral involvement of hepatic ducts to the level of the secondary biliary radicals, atrophy of one liver lobe with contralateral secondary biliary radical involvement is a contraindication to resection. (2) Lymph node metastasis: Inoue *et al*[46] reported that the outcome of 16 patients with lymph node metastasis, accounting for 31.4% of all patients, was quite poor. Their median survival time was 14.1 mo and none of them survived 5 years except for one patient with the IG type of ICC, suggesting that the presence of lymph node metastasis in the MF type of ICC is a sign of non-curability. However, a longer survival time (over 5 years) in ICC patients with lymph node metastasis has been described[54,58]. (3) Vessel invasion: Based on some

centers' support for resection of ICC with vascular reconstruction *en bloc*, involvement of the main hepatic artery or portal vein is the relative contraindication to resection[40,59]. (4) Intrahepatic metastasis: Intrahepatic metastasis in the remaining liver is considered unfit for hepatic resection, and disseminated disease should not undergo hepatectomy. (5) Hepatic functional reserve: It is important to accurately estimate liver reserve function before hepatectomy to avoid postoperative liver failure. Methods to evaluate liver function, including routine examinations of aminotransferase, bilirubin, albumin, prothrombin time, Child-Pugh classification, and hepatic imaging providing volumetric information, indocyanine green (ICG) test. The indocyanine green retention rate at 15 min [ICG (R15)] has recently been considered a sensitive marker for liver reserve function. Nevertheless, it remains imperfect. Moreover, how to evaluate the maximal hepatic resection volume according to liver reserve function remains controversial. Trimethadione (TMO) tolerance test can show the Child-Pugh score in evaluation of cirrhosis. Hepatic 99mTc-diethylenetriamine pentaacetic acid-galactosyl-human serum albumin (99mTc-GSA) clearance test can show postoperative hepatic function and liver stiffness assessed quantitatively with a tactile sensor. The combination of Child-Pugh score, presence of ascites, serum bilirubin levels, indocyanine green retention (ICG R15) value, and remnant liver CT volumetry as well as age, diabetes, cardiopulmonary function, and general performance need to be taken into consideration preoperatively. Other factors affecting respectability include the size and extent of the tumor[60].

Evaluation modalities

Ultrasound can diagnose biliary dilatation and suspected cholangiocarcinoma by localizing the site of obstruction and excluding gallstones. Color Doppler can detect tumor-induced compression/thrombosis of the portal vein or hepatic artery. However, ultrasound is non-specific, often misses small perihilar, extrahepatic, and periampullary tumors, and is not good at defining the extent of tumor.

CT can detect biliary dilatation, intrahepatic cholangiocarcinoma greater than 1 cm in diameter, small liver metastases, lymphadenopathy, biliary obstruction, suspected perihilar tumor or tumor involving the portal venous/arterial system. However, CT can only establish the resectability in 60% of cases[61], and cannot usually define the extent of cholangiocar-cinoma because of the sclerosis and fibrosis of surrounding tissue. In addition, CT cannot accurately differentiate ICC from PSC[62].

MRI along with MRCP can detect ICC and assess preoperative ICC patients by investigating all involved structures, such as the bile ducts, vessels, and hepatic parenchyma, which are important factors for prognosis. Some new tissue-specific MR contrast agents with hepatobiliary and reticuloendothelial cell affinity, such as gadobenate, and ultra-small iron-oxide (USPIO) particles contrast agents with lymph node specificity can be used to detect and assess tumor invasion[63-65]. MRCP may have some potential advantages over CT in identifying intrahepatic mass lesions, and can provide a three-dimensional computerized reconstruction of the biliary tree allowing assessment of bile ducts both above and below a stricture. The non-invasively acquired cholangiographic images obtained by MRCP are comparable with invasive cholangiographies (ERCP and PTC), high positive and negative predictive values for detecting the level and features of biliary obstructions[61,66-68]. Owing to its intrinsically high tissue contrast and multiplanar capability, MRCP is superior to ERCP for defining the anatomy of tumor and assessing its respectability. However, the tendency of MRCP to understage the extent of cholangiocarcinoma has been reported[69]. MRI is not superior to CT in identifying lymph node metastasis.

PET scanning with the focal accumulation of nucleotide tracer 18-fluorodeoxyglucose (FDG) is an emerging staging technique for many cancers. This technique can detect nodular cholangiocarcinoma as small as 1 cm in diameter, but is less sensitive to infiltrating tumors[70]. FDG-PET has a higher specificity for lymphadenopathy than CT, although there is no difference in sensitivity between them[71,72]. In a retrospective study, 21 patients with ICC underwent CT, MRI and PET for lymph node metastasis, which were concordant in 16 patients and discordant in 5 patients (positive FDG-PET in three, positive CT and MRI in two). Moreover, PET may has some superiority over CT and MRI in detecting distant metastases[71].

The above non-invasive techniques may be complementary and sometimes are all necessary as part of surgical assessment depending on the clinical situation. Furthermore, invasive modalities are also needed sometimes to assess the resectability and predict the prognosis. In most cases, ERCP/PTC is replaced by MRCP, but ERCP with OCT can provide more information for surgical plan[21]. Another advantage of these techniques over MRCP is that washing, brushing and intraductal biopsies can be obtained for cytopathologic analysis, adding some new cytological tests, such as DIA, fluorescent *in situ* hybridization, so that the sensitivity increases, especially to patients with PSC or apparent biliary obstruction[73,74]. But, negative cytology from brushings does not exclude malignancy. Preoperative (percutaneous choledochoscope) and intraoperative vholedochoscope with biopsy can help to make an early diagnosis. Blood vessel involvement is an important prognosis factor. As a means of evaluating vascular invasion, hypovascular or hypervascular lesion, concomitant vascular resection and reconstruction, angiography should be reserved in some cases. Percutaneous transhepatic portography (PTP) and retrograde selective hepatic venography should be selected. Virtual 3D is a new kind of technique for constructing three-dimensional virtual images of the portal vein, hepatic artery, and bile ducts. On account of it, accurate knowledge of partial anatomy can be gotten. Preoperative planning for complex biliary

surgery especially lesions invading the hepatic hilum may be improved[75]. ICC in patients with lymphadenopathy is often missed on preoperative imaging. Endoscopic ultrasound can be useful in identifying local lymph node enlargement and allows a good view of distal extrahepatic biliary tree and vasculature[26]. The sensitivity of fine needle aspiration of the tumor mass or its surrounding lymph nodes and endoscopic ultrasound is greater than ERCP with brushings in detecting malignancy[26,27,76]. Endoscopic ultrasound-guided regional lymph node sampling can be performed in early disease to assess the respectability or eligibility for transplantation[77]. However, endoscopic aspiration of hilar masses is not recommended because of the potential of tumor seeding. Laparoscopy is gradually replaced by ultrasonography and other imaging studies, but has identified a third case of peritoneal and superficial liver metastases[51,78,79].

OPERATION

Hepatectomy

It was recently reported that aggressive surgical strategies in the treatment of ICC can significantly increase the survival of ICC patients[14,80,81]. Yamamoto *et al*[82] and Ohashi *et al*[83] suggested that anatomic and extensive hepatectomy is the rational procedure for mass-forming ICC, while hepatectomy with extrahepatic duct excision, and hilar lymph nodal resection is the rational procedure for infiltrating ICC. The 3- and 5-year survival rates of ICC patients after curative resection (n = 56, 53% and 50%, respectively) were significantly higher than those of patients after non-curative resection (n = 67, 7% and 2% respectively, $P < 0.0001$). In 54 patients followed-up after curative resection, the rate of recurrence after surgery was 46%. The rate of recurrence was significantly higher in patients with various mass-forming ICC tumors ($P = 0.039$) than in those with other types of tumors or tumors > 3 cm in diameter than in those with tumors > 3 cm or < less 3 cm ($P = 0.006$)[84]. Kim *et al*[85] reported that the median survival time after non-curative resection is 3.0 mo. Chu *et al*[22] showed the the median survival time is 1.8 mo and 2.9 mo, respectively, after conservative management and palliative operations. Only a curative resection can prolong survival. ICC has no characteristic symptoms at its early stage and is often at its advanced stage when it is diagnosed. Consequently, the resectability rate is usually low, extended hepatectomy possibly in combination with resection of other structures (e.g. extrahepatic bile duct, portal vein and inferior vena cava) is generally required. Wu *et al*[86] described a case of initially unresectable, locally advanced intrahepatic cholangiocarcinoma that showed a remarkable regression after transcatheter arterial chemoembolization with degradable starch microspheres, allowing for subsequent successful curative resection. In a retrospectively study, Yamamoto *et al*[40] allocated 83 patients who had undergone resection to a standard surgery group (n = 56), in which the patients underwent hepatectomy alone or hepatectomy with bile duct resection, and an extended surgery group (n = 27), in which the patients underwent the standard operation combined with vessel resection and/or pancreatectomy. The 5-year survival rate was significantly higher in the standard surgery group (30%) than in the extended surgery group (10%, $P = 0.0061$). So they concluded that extended surgery does not improve the curative resection rate or the surgical outcome of ICC[40].

Lymphadenectomy

ICC frequently demonstrates lymphatic spread. Lymph node metastasis is a significant prognostic factor for IHCC. Whether lymph nodes are dissected, and what is the extent of dissection remain the two important questions to be solved. No consensus has been reached concerning the indications and value of lymph node dissection for ICC. Hepatectomy with extensive lymph node dissection is the standard operation for intrahepatic cholangiocarcinoma in Japan. However, lymph node dissection may not always be effective in reducing tumor recurrence. Chu *et al*[22] and Shimada *et al*[47] that lymph node dissection alone is not likely to improve the prognosis without further control of liver metastases. However, there are reported cases of long-term survival after extended surgical resection of intrahepatic cholangiocarcinoma with extensive lymph node metastasis[56,87].

Transplantation

Pichlmayr *et al*[88] reported that the median survival time of 18 patients with IHCC after liver transplantation was 5.0 mo, and the 1-year survival rate was 13.9%. Casavilla *et al*[89] performed liver transplantation for patients with unresectable tumor (n = 12) or advanced cirrhosis (n = 8) and found that the mortality within 30 d was 7.4%. Overall, the tumor-free survival rates were 64% and 57%, respectively at 1 year, 34% and 34%, respectively at 3 years, and 26% and 27%, respectively at 5 years after operation. About 59.3% patients experienced tumor recurrence. When patients with positive margins, multiple tumors, and lymph node involvement were excluded, the patient survival rate was 74%, 64% and 62%, at 1, 3, and 5 years, respectively after operation. A Mayo Clinic group[90] used preoperative irradiation and chemotherapy for patients with unresectable cholangiocarcinoma above the cystic duct without intrahepatic or extrahepatic metastases. Patients initially received external-beam irradiation plus bolus fluorouracil (5-FU), followed by brachytherapy with iridium and concomitant protracted venous infusion of 5-FU. 5-FU was then administered continuously through an ambulatory infusion pump until OLT. After irradiation, patients underwent an exploratory laparotomy to exclude metastatic disease. The patients have a median follow-up time of 44 mo (range 17-83 mo, 7 of 9 patients > 36 mo). Only 1 patient developed tumor relapse. The group concluded that OLT in combination with preoperative irradiation and chemotherapy is associated with prolonged disease-free, and overall survival in highly selected patients with early-stage cholangiocarcinoma[90]. A comparison of recent series is shown in Table 3.

Table 3 Comparison of recent series

Authors	Yr	Countries	Procedure (patients)	Prognosis (%)				Tumor recurrence (%)
				Median (mo)	1 yr	3 yr	5 yr	
Pichlmayr *et al*[88]	1995	Germany	Hepatic resection (32)	12.8				
			Liver transplantation (18)	5.0				
Casavilla *et al*[89]	1997	America	Hepatic resection (34)		60	37	31	56
			Liver transplantation (20)		70	29	18	55
Chu *et al*[22]	1997	Hong Kong, China	Conservative management (15)	1.8				
			Palliative operation (23)	2.9				
			Hepatic resection (39)	12.2	57.3	23.9	15.9	
Madariaga *et al*[98]	1998	Japan	Hepatic resection (34)	19	67	40	35	
Meyer *et al*[99]	2000	America	Liver transplantation (207)		72	48 (2 yr)	23	51
Kawarada *et al*[100]	2001	Japan	hepatic resection (37)	31.5	54.1	34	23.9	
Fu *et al*[101]	2004	China	Palliative or curative operation (79)	11.9	49.4	17.3	9.6	
Robles *et al*[102]	2004	Spain	Liver transplantation (23)		77	65	42	35
Lang *et al*[80]	2005	Germany	R0-resection (complete tumor removal) (16)	46	94	82		37.5
			R1-resection (microscopic tumor at the cutting margin) (11)	5	22	0		
Ghali *et al*[103]	2005	Canada	Liver transplantation (10)		90	80	20	80
Urahashi *et al*[104]	2007	Japan	HPD (hepatectomy with pancreatoduodenectomy) (12)		42	33	33	75
De Oliveira *et al*[105]	2007	America	Hepatic resection (R0-resection)	80			63	
			Hepatic resection (overall)	28			40	
Becker *et al*[106]	2008	America	Liver transplantation (280)		74		38	

Adjunctive therapy

Recurrence of ICC is due to failure in surgery, warranting consideration of adjuvant treatments. Neither adjuvant nor neoadjuvant therapy, however, has been shown to improve survival. Roayaie *et al*[91] performed chemo-radiation therapy for postoperative patients with positive resection margins or nodal invasion and did not find any difference in the actuarial disease-free survival between the patients with or without adjuvant chemo-radiation. Sanz-Altamira *et al*[92] used 5-fluorouracil, leucovorin, and carboplatin in patients with unresectable biliary tree carcinoma and found that 21% of the patients had significant responses. Ando *et al*[93] treated an IHCC patient with postoperative recurrence of multiple liver metastases, and a complete response was noted 1 year after the patient underwent 4 courses of hepatic arterial infusion therapy *via* a subcutaneously implanted injection port and received cisplatin. The research of Furuse *et al*[94] showed that, of the twenty-four patients not amenable to surgery, three had a response rate of 12.5%, thirteen had a stable disease, seven had a progressive disease, and one was not evaluated. Lee *et al*[95] treated 24 patients immunohistochemically proven cholangiocarcinoma patients with gemcitabine and cisplatin. Of these 24 patients, 5 had a partial response, 12 had a stable disease, and 7 had a progressive disease during treatment. These patients had a median survival time of 9.30 mo. In a study by Feisthammel *et al*[96], the response rate was 10% for patients with inoperable intrahepatic cholangiocarcinoma (n = 17) or gallbladder cancer (n = 13) after treatment with irinotecan followed by folinic acid and 5-FU, and an additional 10% of patients had a stable disease. The median overall survival time of was 166 d and 273 d, respectively and median progression-free survival time of intrahepatic cholangiocarcinoma and gallbladder cancer patients was 166-273 d, and 84-159 d, respectively. These results suggest that the present therapy is a useful option for advanced IHCC. Rai *et al*[97] reported a 59-year old lady who underwent orthotopic liver transplantation (OLT) for intrahepatic cholangiocarcinoma recurrence 13 mo after transplantation in spite of adjuvant chemotherapy. She survived 18 mo after her recurrent tumor was treated with radiofrequency ablation, suggesting that radiofrequency ablation can be used in treatment of recurrent tumor after liver transplantation.

REFERENCES

1 **Patel T**. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001; **33**: 1353-1357

2 **Patel T**. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer* 2002; **2**: 10

3 **Shaib Y**, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 115-125

4 **Shaib YH**, Davila JA, McGlynn K, El-Serag HB. Rising incidence of intrahepatic cholangiocarcinoma in the United States: a true increase? *J Hepatol* 2004; **40**: 472-477

5 **Khan SA**, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 2002; **37**: 806-813

6 **Taylor-Robinson SD**, Toledano MB, Arora S, Keegan TJ, Hargreaves S, Beck A, Khan SA, Elliott P, Thomas HC. Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968-1998. *Gut* 2001; **48**: 816-820

7 **Okuda K**, Nakanuma Y, Miyazaki M. Cholangiocarcinoma: recent progress. Part 1: epidemiology and etiology. *J Gastroenterol Hepatol* 2002; **17**: 1049-1055

8 **Mouzas IA**, Dimoulios P, Vlachonikolis IG, Skordilis P, Zoras O, Kouroumalis E. Increasing incidence of cholangiocarcinoma in Crete 1992-2000. *Anticancer Res* 2002; **22**: 3637-3641

9 **Wood R**, Brewster DH, Fraser LA, Brown H, Hayes PC, Garden OJ. Do increases in mortality from intrahepatic cholangiocarcinoma reflect a genuine increase in risk? Insights from cancer registry data in Scotland. *Eur J Cancer* 2003; **39**: 2087-2092

10 **Kato I**, Kuroishi T, Tominaga S. Descriptive epidemiology of subsites of cancers of the liver, biliary tract and pancreas in Japan. *Jpn J Clin Oncol* 1990; **20**: 232-237

11 **Sorensen HT**, Friis S, Olsen JH, Thulstrup AM, Mellemkjaer L, Linet M, Trichopoulos D, Vilstrup H, Olsen J. Risk of liver and other types of cancer in patients with cirrhosis: a nationwide cohort study in Denmark. *Hepatology* 1998; **28**: 921-925

12 **Welzel TM**, Graubard BI, El-Serag HB, Shaib YH, Hsing AW, Davila JA, McGlynn KA. Risk factors for intrahepatic and extrahepatic cholangiocarcinoma in the United States: a population-based case-control study. *Clin Gastroenterol Hepatol* 2007; **5**: 1221-1228

13 **Lazaridis KN**, Gores GJ. Cholangiocarcinoma. *Gastroenterology* 2005; **128**: 1655-1667

14 **Khan SA**, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. *Lancet* 2005; **366**: 1303-1314

15 **Oh SW**, Yoon YS, Shin SA. Effects of excess weight on cancer incidences depending on cancer sites and histologic findings among men: Korea National Health Insurance Corporation Study. *J Clin Oncol* 2005; **23**: 4742-4754

16 **Yamamoto S**, Kubo S, Hai S, Uenishi T, Yamamoto T, Shuto T, Takemura S, Tanaka H, Yamazaki O, Hirohashi K, Tanaka T. Hepatitis C virus infection as a likely etiology of intrahepatic cholangiocarcinoma. *Cancer Sci* 2004; **95**: 592-595

17 **Shin HR**, Lee CU, Park HJ, Seol SY, Chung JM, Choi HC, Ahn YO, Shigemastu T. Hepatitis B and C virus, Clonorchis sinensis for the risk of liver cancer: a case-control study in Pusan, Korea. *Int J Epidemiol* 1996; **25**: 933-940

18 **Jepsen P**, Vilstrup H, Tarone RE, Friis S, Sorensen HT. Incidence rates of intra- and extrahepatic cholangiocarcinomas in Denmark from 1978 through 2002. *J Natl Cancer Inst* 2007; **99**: 895-897

19 **McLean L**, Patel T. Racial and ethnic variations in the epidemiology of intrahepatic cholangiocarcinoma in the United States. *Liver Int* 2006; **26**: 1047-1053

20 **Lieser MJ**, Barry MK, Rowland C, Ilstrup DM, Nagorney DM. Surgical management of intrahepatic cholangiocarcinoma: a 31-year experience. *J Hepatobiliary Pancreat Surg* 1998; **5**: 41-47

21 **Valverde A**, Bonhomme N, Farges O, Sauvanet A, Flejou JF, Belghiti J. Resection of intrahepatic cholangiocarcinoma: a Western experience. *J Hepatobiliary Pancreat Surg* 1999; **6**: 122-127

22 **Chu KM**, Fan ST. Intrahepatic cholangiocarcinoma in Hong Kong. *J Hepatobiliary Pancreat Surg* 1999; **6**: 149-153

23 **Chu KM**, Lai EC, Al-Hadeedi S, Arcilla CE Jr, Lo CM, Liu CL, Fan ST, Wong J. Intrahepatic cholangiocarcinoma. *World J Surg* 1997; **21**: 301-305; discussion 305-306

24 **Poneros JM**, Tearney GJ, Shiskov M, Kelsey PB, Lauwers GY, Nishioka NS, Bouma BE. Optical coherence tomography of the biliary tree during ERCP. *Gastrointest Endosc* 2002; **55**: 84-88

25 **Slattery JM**, Sahani DV. What is the current state-of-the-art imaging for detection and staging of cholangiocarcinoma? *Oncologist* 2006; **11**: 913-922

26 **Bardales RH**, Stelow EB, Mallery S, Lai R, Stanley MW. Review of endoscopic ultrasound-guided fine-needle aspiration cytology. *Diagn Cytopathol* 2006; **34**: 140-175

27 **Crowe DR**, Eloubeidi MA, Chhieng DC, Jhala NC, Jhala D, Eltoum IA. Fine-needle aspiration biopsy of hepatic lesions: computerized tomographic-guided versus endoscopic ultrasound-guided FNA. *Cancer* 2006; **108**: 180-185

28 **Patel T**, Singh P. Cholangiocarcinoma: emerging approaches to a challenging cancer. *Curr Opin Gastroenterol* 2007; **23**: 317-323

29 **Liver Cancer Study Group of Japan**. Intrahepatic cholangiocarcinoma, macroscopic typing. In: Okamoto E (eds) Classification of primary liver cancer. Tokyo: Kanehara, 1997: 6-7

30 **Aishima S**, Kuroda Y, Nishihara Y, Iguchi T, Taguchi K, Taketomi A, Maehara Y, Tsuneyoshi M. Proposal of progression model for intrahepatic cholangiocarcinoma: clinicopathologic differences between hilar type and peripheral type. *Am J Surg Pathol* 2007; **31**: 1059-1067

31 **Isaji S**, Kawarada Y, Taoka H, Tabata M, Suzuki H, Yokoi H. Clinicopathological features and outcome of hepatic resection for intrahepatic cholangiocarcinoma in Japan. *J Hepatobiliary Pancreat Surg* 1999; **6**: 108-116

32 **Kajiyama K**, Maeda T, Takenaka K, Sugimachi K, Tsuneyoshi M. The significance of stromal desmoplasia in intrahepatic cholangiocarcinoma: a special reference of 'scirrhous-type' and 'nonscirrhous-type' growth. *Am J Surg Pathol* 1999; **23**: 892-902

33 **Sobin LH**, Wittekin C. UICC TNM classification of malignant tumors, 5th ed. New York: Wiley-Liss, 1997

34 **The Liver Cancer Study Group of Japan**. General rules for the clinical and pathological study of primary liver cancer. 2nd ed. Tokyo: Kanehara, 2003

35 **Uenishi T**, Yamazaki O, Yamamoto T, Hirohashi K, Tanaka H, Tanaka S, Hai S, Kubo S. Serosal invasion in TNM staging of mass-forming intrahepatic cholangiocarcinoma. *J Hepatobiliary Pancreat Surg* 2005; **12**: 479-483

36 **Okabayashi T**, Yamamoto J, Kosuge T, Shimada K, Yamasaki S, Takayama T, Makuuchi M. A new staging system for mass-forming intrahepatic cholangiocarcinoma: analysis of preoperative and postoperative variables. *Cancer* 2001; **92**: 2374-2383

37 **Nakajima T**, Kondo Y, Miyazaki M, Okui K. A histopathologic study of 102 cases of intrahepatic cholangiocarcinoma: histologic classification and modes of spreading. *Hum Pathol* 1988; **19**: 1228-1234

38 **Weinbren K**, Mutum SS. Pathological aspects of cholangiocarcinoma. *J Pathol* 1983; **139**: 217-238

39 **Sasaki A**, Aramaki M, Kawano K, Morii Y, Nakashima K, Yoshida T, Kitano S. Intrahepatic peripheral cholangiocarcinoma: mode of spread and choice of surgical treatment. *Br J Surg* 1998; **85**: 1206-1209

40 **Yamamoto M**, Takasaki K, Yoshikawa T. Extended resection for intrahepatic cholangiocarcinoma in Japan. *J Hepatobiliary Pancreat Surg* 1999; **6**: 117-121

41 **Washburn WK**, Lewis WD, Jenkins RL. Aggressive surgical resection for cholangiocarcinoma. *Arch Surg* 1995; **130**: 270-276

42 **Chou FF**, Sheen-Chen SM, Chen CL, Chen YS, Chen MC. Prognostic factors of resectable intrahepatic cholangiocarcinoma. *J Surg Oncol* 1995; **59**: 40-44

43 **Nakagawa T**, Kamiyama T, Kurauchi N, Matsushita M, Nakanishi K, Kamachi H, Kudo T, Todo S. Number of lymph node metastases is a significant prognostic factor in intrahepatic cholangiocarcinoma. *World J Surg* 2005; **29**: 728-733

44 **Yamamoto M**, Takasaki K, Yoshikawa T. Lymph node metastasis in intrahepatic cholangiocarcinoma. *Jpn J Clin Oncol* 1999; **29**: 147-150

45 **Uenishi T**, Hirohashi K, Kubo S, Yamamoto T, Yamazaki O, Kinoshita H. Clinicopathological factors predicting outcome after resection of mass-forming intrahepatic cholangiocarcinoma. *Br J Surg* 2001; **88**: 969-974

46 **Inoue K**, Makuuchi M, Takayama T, Torzilli G, Yamamoto J, Shimada K, Kosuge T, Yamasaki S, Konishi M, Kinoshita T, Miyagawa S, Kawasaki S. Long-term survival and prognostic factors in the surgical treatment of mass-forming type cholangiocarcinoma. *Surgery* 2000; **127**: 498-505

47 **Shimada M**, Yamashita Y, Aishima S, Shirabe K, Takenaka K, Sugimachi K. Value of lymph node dissection during resection of intrahepatic cholangiocarcinoma. *Br J Surg* 2001; **88**: 1463-1466

48 **Ohtsuka M**, Ito H, Kimura F, Shimizu H, Togawa A, Yoshidome H, Shimamura F, Shimizu Y, Miyazaki M. Extended hepatic resection and outcomes in intrahepatic cholangiocarcinoma. *J Hepatobiliary Pancreat Surg* 2003; **10**: 259-264

49 **Murakami Y**, Yokoyama T, Takesue Y, Hiyama E, Yokoyama Y, Kanehiro T, Uemura K, Matsuura Y. Long-term survival of peripheral intrahepatic cholangiocarcinoma with metastasis to the para-aortic lymph nodes. *Surgery* 2000; **127**: 105-106

50 **Yamamoto M**, Takasaki K, Imaizumi T, Ariizumi S, Matsumura N, Nakano M. A long-term survivor of intrahepatic cholangiocarcinoma with lymph node metastasis: a case report. *Jpn J Clin Oncol* 2002; **32**: 206-209

51 **Weber SM**, Jarnagin WR, Klimstra D, DeMatteo RP, Fong Y, Blumgart LH. Intrahepatic cholangiocarcinoma: resectability, recurrence pattern, and outcomes. *J Am Coll Surg* 2001; **193**: 384-391

52 **Nakeeb A**, Pitt HA, Sohn TA, Coleman J, Abrams RA, Piantadosi S, Hruban RH, Lillemoe KD, Yeo CJ, Cameron JL. Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. *Ann Surg* 1996; **224**: 463-473; discussion 473-475

53 **Liver Cancer Study Group of Japan**. Clinical and surgical findings. In: Liver Cancer Study Group of Japan, editors. Classification of Primary Liver Cancer. First English Edition. Tokyo: Kanehara Shuppan, 1997: 1-22

54 **Tsuji T**, Hiraoka T, Kanemitsu K, Takamori H, Tanabe D, Tashiro S. Lymphatic spreading pattern of intrahepatic cholangiocarcinoma. *Surgery* 2001; **129**: 401-407

55 **Okami J**, Dono K, Sakon M, Tsujie M, Hayashi N, Fujiwara Y, Nagano H, Umeshita K, Nakamori S, Monden M. Patterns of regional lymph node involvement in intrahepatic cholangiocarcinoma of the left lobe. *J Gastrointest Surg* 2003; **7**: 850-856

56 **Shirabe K**, Shimada M, Harimoto N, Sugimachi K, Yamashita Y, Tsujita E, Aishima S. Intrahepatic cholangiocarcinoma: its mode of spreading and therapeutic modalities. *Surgery* 2002; **131**: S159-S164

57 **Nozaki Y**, Yamamoto M, Ikai I, Yamamoto Y, Ozaki N, Fujii H, Nagahori K, Matsumoto Y, Yamaoka Y. Reconsideration of the lymph node metastasis pattern (N factor) from intrahepatic cholangiocarcinoma using the International Union Against Cancer TNM staging system for primary liver carcinoma. *Cancer* 1998; **83**: 1923-1929

58 **Isa T**, Kusano T, Shimoji H, Takeshima Y, Muto Y, Furukawa M. Predictive factors for long-term survival in patients with intrahepatic cholangiocarcinoma. *Am J Surg* 2001; **181**: 507-511

59 **Nakagohri T**, Konishi M, Inoue K, Oda T, Kinoshita T. Extended right hepatic lobectomy with resection of inferior vena cava and portal vein for intrahepatic cholangiocarcinoma. *J Hepatobiliary Pancreat Surg* 2000; **7**: 599-602

60 **Shimada K**, Sano T, Sakamoto Y, Esaki M, Kosuge T, Ojima H. Surgical outcomes of the mass-forming plus periductal infiltrating types of intrahepatic cholangiocarcinoma: a comparative study with the typical mass-forming type of intrahepatic cholangiocarcinoma. *World J Surg* 2007; **31**: 2016-2022

61 **Zhang Y**, Uchida M, Abe T, Nishimura H, Hayabuchi N, Nakashima Y. Intrahepatic peripheral cholangiocarcinoma: comparison of dynamic CT and dynamic MRI. *J Comput Assist Tomogr* 1999; **23**: 670-677

62 **Bhuiya MR**, Nimura Y, Kamiya J, Kondo S, Fukata S, Hayakawa N, Shionoya S. Clinicopathologic studies on perineural invasion of bile duct carcinoma. *Ann Surg* 1992; **215**: 344-349

63 **Braga HJ**, Imam K, Bluemke DA. MR imaging of intrahepatic cholangiocarcinoma: use of ferumoxides for lesion localization and extension. *AJR Am J Roentgenol* 2001; **177**: 111-114

64 **Harisinghani MG**, Barentsz J, Hahn PF, Deserno WM, Tabatabaei S, van de Kaa CH, de la Rosette J, Weissleder R. Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. *N Engl J Med* 2003; **348**: 2491-2499

65 **Peterson MS**, Murakami T, Baron RL. MR imaging patterns of gadolinium retention within liver neoplasms. *Abdom Imaging* 1998; **23**: 592-599

66 **Yeh TS**, Jan YY, Tseng JH, Chiu CT, Chen TC, Hwang TL, Chen MF. Malignant perihilar biliary obstruction: magnetic resonance cholangiopancreatographic findings. *Am J Gastroenterol* 2000; **95**: 432-440

67 **Manfredi R**, Barbaro B, Masselli G, Vecchioli A, Marano P. Magnetic resonance imaging of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 155-164

68 **Manfredi R**, Brizi MG, Masselli G, Vecchioli A, Marano P. [Malignant biliary hilar stenosis: MR cholangiography compared with direct cholangiography] *Radiol Med* 2001; **102**: 48-54

69 **Zidi SH**, Prat F, Le Guen O, Rondeau Y, Pelletier G. Performance characteristics of magnetic resonance cholangiography in the staging of malignant hilar strictures. *Gut* 2000; **46**: 103-106

70 **Anderson CD**, Rice MH, Pinson CW, Chapman WC, Chari RS, Delbeke D. Fluorodeoxyglucose PET imaging in the evaluation of gallbladder carcinoma and cholangiocarcinoma. *J Gastrointest Surg* 2004; **8**: 90-97

71 **Kim YJ**, Yun M, Lee WJ, Kim KS, Lee JD. Usefulness of 18F-FDG PET in intrahepatic cholangiocarcinoma. *Eur J Nucl Med Mol Imaging* 2003; **30**: 1467-1472

72 **Grobmyer SR**, Wang L, Gonen M, Fong Y, Klimstra D, D'Angelica M, DeMatteo RP, Schwartz L, Blumgart LH, Jarnagin WR. Perihepatic lymph node assessment in patients undergoing partial hepatectomy for malignancy. *Ann Surg* 2006; **244**: 260-264

73 **Kipp BR**, Stadheim LM, Halling SA, Pochron NL, Harmsen S, Nagorney DM, Sebo TJ, Therneau TM, Gores GJ, de Groen PC, Baron TH, Levy MJ, Halling KC, Roberts LR. A comparison of routine cytology and fluorescence in situ hybridization for the detection of malignant bile duct strictures. *Am J Gastroenterol* 2004; **99**: 1675-1681

74 **Baron TH**, Harewood GC, Rumalla A, Pochron NL, Stadheim LM, Gores GJ, Therneau TM, De Groen PC, Sebo TJ, Salomao DR, Kipp BR. A prospective comparison of digital image analysis and routine cytology for the identification of malignancy in biliary tract strictures. *Clin Gastroenterol Hepatol* 2004; **2**: 214-219

75 **Endo I**, Shimada H, Takeda K, Fujii Y, Yoshida K, Morioka D, Sadatoshi S, Togo S, Bourquain H, Peitgen HO. Successful duct-to-duct biliary reconstruction after right hemihepatectomy. Operative planning using virtual 3D reconstructed images. *J Gastrointest Surg* 2007; **11**: 666-670

76 **Abu-Hamda EM**, Baron TH. Endoscopic management of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 165-175

77 **Fritscher-Ravens A**, Broering DC, Sriram PV, Topalidis T, Jaeckle S, Thonke F, Soehendra N. EUS-guided fine-needle aspiration cytodiagnosis of hilar cholangiocarcinoma: a case series. *Gastrointest Endosc* 2000; **52**: 534-540

78 **Corvera CU**, Weber SM, Jarnagin WR. Role of laparoscopy in the evaluation of biliary tract cancer. *Surg Oncol Clin N Am* 2002; **11**: 877-891

79 **Yeh CN**, Jan YY, Yeh TS, Hwang TL, Chen MF. Hepatic resection of the intraductal papillary type of peripheral cholangiocarcinoma. *Ann Surg Oncol* 2004; **11**: 606-611

80 **Lang H**, Sotiropoulos GC, Frühauf NR, Dömland M, Paul A, Kind EM, Malagó M, Broelsch CE. Extended hepatectomy for intrahepatic cholangiocellular carcinoma (ICC): when is it worthwhile? Single center experience with 27 resections in 50 patients over a 5-year period. *Ann Surg* 2005; **241**: 134-143

81 **Neuhaus P**, Jonas S, Settmacher U, Thelen A, Benckert C, Lopez-Hänninen E, Hintze RE. Surgical management of proximal bile duct cancer: extended right lobe resection

increases resectability and radicality. *Langenbecks Arch Surg* 2003; **388**: 194-200
82 **Yamamoto J**, Kosuge T, Takayama T, Shimada K, Makuuchi M, Yoshida J, Sakamoto M, Hirohashi S, Yamasaki S, Hasegawa H. Surgical treatment of intrahepatic cholangiocarcinoma: four patients surviving more than five years. *Surgery* 1992; **111**: 617-622
83 **Ohashi K**, Nakajima Y, Tsutsumi M, Kanehiro H, Fukuoka T, Hisanaga M, Taki J, Nakae D, Konishi Y, Nakano H. Clinical characteristics and proliferating activity of intrahepatic cholangiocarcinoma. *J Gastroenterol Hepatol* 1994; **9**: 442-446
84 **Yamamoto M**, Takasaki K, Otsubo T, Katsuragawa H, Katagiri S. Recurrence after surgical resection of intrahepatic cholangiocarcinoma. *J Hepatobiliary Pancreat Surg* 2001; **8**: 154-157
85 **Kim HJ**, Yun SS, Jung KH, Kwun WH, Choi JH. Intrahepatic cholangiocarcinoma in Korea. *J Hepatobiliary Pancreat Surg* 1999; **6**: 142-148
86 **Wu Y**, Saiura A, Yamamoto J, Koga R, Asahara S, Kamei A, Takano K, Ikari T, Seki M, Yamaguchi T, Muto T. Locally advanced intrahepatic cholangiocarcinoma successfully resected after transcatheter arterial chemoembolization with degradable starch microspheres: report of a case. *Hepatogastroenterology* 2007; **54**: 1345-1347
87 **Asakura H**, Ohtsuka M, Ito H, Kimura F, Ambiru S, Shimizu H, Togawa A, Yoshidome H, Kato A, Miyazaki M. Long-term survival after extended surgical resection of intrahepatic cholangiocarcinoma with extensive lymph node metastasis. *Hepatogastroenterology* 2005; **52**: 722-724
88 **Pichlmayr R**, Lamesch P, Weimann A, Tusch G, Ringe B. Surgical treatment of cholangiocellular carcinoma. *World J Surg* 1995; **19**: 83-88
89 **Casavilla FA**, Marsh JW, Iwatsuki S, Todo S, Lee RG, Madariaga JR, Pinna A, Dvorchik I, Fung JJ, Starzl TE. Hepatic resection and transplantation for peripheral cholangiocarcinoma. *J Am Coll Surg* 1997; **185**: 429-436
90 **De Vreede I**, Steers JL, Burch PA, Rosen CB, Gunderson LL, Haddock MG, Burgart L, Gores GJ. Prolonged disease-free survival after orthotopic liver transplantation plus adjuvant chemoirradiation for cholangiocarcinoma. *Liver Transpl* 2000; **6**: 309-316
91 **Roayaie S**, Guarrera JV, Ye MQ, Thung SN, Emre S, Fishbein TM, Guy SR, Sheiner PA, Miller CM, Schwartz ME. Aggressive surgical treatment of intrahepatic cholangiocarcinoma: predictors of outcomes. *J Am Coll Surg* 1998; **187**: 365-372
92 **Sanz-Altamira PM**, Ferrante K, Jenkins RL, Lewis WD, Huberman MS, Stuart KE. A phase II trial of 5-fluorouracil, leucovorin, and carboplatin in patients with unresectable biliary tree carcinoma. *Cancer* 1998; **82**: 2321-2325
93 **Ando E**, Tanaka M, Yamashita F, Fukumori K, Sumie S, Yano Y, Sata M. Chemotherapy for hepatocellular carcinoma with portal hypertension due to tumor thrombus. *J Clin Gastroenterol* 2000; **31**: 247-249
94 **Furuse J**, Okusaka T, Funakoshi A, Yamao K, Nagase M, Ishii H, Nakachi K, Ueno H, Ikeda M, Morizane C, Horikawa Y, Mizuno N. Early phase II study of uracil-tegafur plus doxorubicin in patients with unresectable advanced biliary tract cancer. *Jpn J Clin Oncol* 2006; **36**: 552-556
95 **Lee GW**, Kang JH, Kim HG, Lee JS, Lee JS, Jang JS. Combination chemotherapy with gemcitabine and cisplatin as first-line treatment for immunohistochemically proven cholangiocarcinoma. *Am J Clin Oncol* 2006; **29**: 127-131
96 **Feisthammel J**, Schoppmeyer K, Mossner J, Schulze M, Caca K, Wiedmann M. Irinotecan with 5-FU/FA in advanced biliary tract adenocarcinomas: a multicenter phase II trial. *Am J Clin Oncol* 2007; **30**: 319-324
97 **Rai R**, Manas D, Rose J. Radiofrequency ablation of recurrent cholangiocarcinoma after orthotopic liver transplantation - a case report. *World J Gastroenterol* 2005; **11**: 612-613
98 **Madariaga JR**, Iwatsuki S, Todo S, Lee RG, Irish W, Starzl TE. Liver resection for hilar and peripheral cholangiocarcinomas: a study of 62 cases. *Ann Surg* 1998; **227**: 70-79
99 **Meyer CG**, Penn I, James L. Liver transplantation for cholangiocarcinoma: results in 207 patients. *Transplantation* 2000; **69**: 1633-1637
100 **Kawarada Y**, Yamagiwa K, Das BC. Analysis of the relationships between clinicopathologic factors and survival time in intrahepatic cholangiocarcinoma. *Am J Surg* 2002; **183**: 679-685
101 **Fu XH**, Tang ZH, Zong M, Yang GS, Yao XP, Wu MC. Clinicopathologic features, diagnosis and surgical treatment of intrahepatic cholangiocarcinoma in 104 patients. *Hepatobiliary Pancreat Dis Int* 2004; **3**: 279-283
102 **Robles R**, Figueras J, Turrion VS, Margarit C, Moya A, Varo E, Calleja J, Valdivieso A, Valdecasas JC, Lopez P, Gomez M, de Vicente E, Loinaz C, Santoyo J, Fleitas M, Bernardos A, Llado L, Ramirez P, Bueno FS, Jaurrieta E, Parrilla P. Spanish experience in liver transplantation for hilar and peripheral cholangiocarcinoma. *Ann Surg* 2004; **239**: 265-271
103 **Ghali P**, Marotta PJ, Yoshida EM, Bain VG, Marleau D, Peltekian K, Metrakos P, Deschenes M. Liver transplantation for incidental cholangiocarcinoma: analysis of the Canadian experience. *Liver Transpl* 2005; **11**: 1412-1416
104 **Urahashi T**, Yamamoto M, Ohtsubo T, Katsuragawa H, Katagiri S, Takasaki K. Hepatopancreatoduodenectomy could be allowed for patients with advanced intrahepatic cholangiocarcinoma. *Hepatogastroenterology* 2007; **54**: 346-349
105 **De Oliveira ML**, Cunningham SC, Cameron JL, Kamangar F, Winter JM, Lillemoe KD, Choti MA, Yeo CJ, Schulick RD. Cholangiocarcinoma: thirty-one-year experience with 564 patients at a single institution. *Ann Surg* 2007; **245**: 755-762
106 **Becker NS**, Rodriguez JA, Barshes NR, O'Mahony CA, Goss JA, Aloia TA. Outcomes analysis for 280 patients with cholangiocarcinoma treated with liver transplantation over an 18-year period. *J Gastrointest Surg* 2008; **12**: 117-122

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TOPIC HIGHLIGHT

Akio Inui, MD, PhD, Professor, *Series Editor*

What's new about ghrelin in 2008?

Akio Inui

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9th NPY Meeting was held for the first time in Japan in March 2008, which was organized by Akio Inui, Professor and Chairman, Department of Psychosomatic Internal Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

This TOPIC HIGHLIGHT is the "ghrelin version" of the proceedings of 9th NPY Meeting and presents examples of the critical interplay in ghrelin-NPY pathway in response to environmental, pharmacological and genetic challenges in the stomach and hypothalamus. Other major topics in connection with NPY and related peptides are published in the special issue of Nutrition, thus together representing a comprehensive review of state of the art knowledge of the operation in regulating multiple physiological functions in the periphery and CNS.

These papers are written by leaders in their respective scientific fields by the use of various approaches and models to examine the secretion and action of ghrelin, including manometric and force transducer methods to measure physiological and ghrelin peptides-induced GI motility, electrophysiology to measure neuronal activity in NPY neurons, NPY receptor knockout and other animal models to examine appetite, secretion of stomach ghrelin and leptin or gastric acid, and human studies in relation to *Helicobacter pylori*.

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TOPIC HIGHLIGHT

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Effects of ghrelin on interdigestive contractions of the rat gastrointestinal tract

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Abstract

Ghrelin causes interdigestive contractions of the stomach in rats. However, it remains unknown whether ghrelin causes interdigestive contractions in the small intestine. Four strain gauge transducers were implanted on the antrum, duodenum, proximal and distal jejunum. After an overnight fast, gastrointestinal (GI) contractions were recorded in freely moving conscious rats. Spontaneous phase Ⅲ-like contractions were observed at every 13-16 min in rat GI tract. The fasted motor patterns were replaced by the fed motor pattern immediately after food intake. Two minutes after finishing the spontaneous phase Ⅲ-like contractions in the antrum, acyl ghrelin (0.8, 2.4 and 8.0 μg/kg per min) was continuously infused for 30 min. Three-five minutes after the starting ghrelin infusion, augmented phase Ⅲ-like contractions were observed at the antrum, duodenum, and jejunum. Ghrelin infusion (0.8, 2.4 and 8.0 μg/kg per min) significantly increased motility index of phase Ⅲ-like contractions at the antrum and jejunum in a dose dependent manner, compared to that of saline injection. Thus, it is likely that exogenously administered ghrelin causes phase Ⅲ-like contraction at the antrum, which migrates to the duodenum and jejunum. The possible role of 5-HT, in addition to ghrelin, in mediating intestinal migrating motor complex (MMC), is discussed.

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Key words: Phase Ⅲ-like contractions; Strain gage transducers; Motility index

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GHRELIN AND INTERDIGESTIVE GASTRIC MOTILITY

In the interdigestive state, the stomach and small intestine show a remarkable motor pattern, known as the migrating motor complex (MMC)[1]. MMC consists of three phases; phase I (period of motor quiescence), phase Ⅱ (period of irregular low amplitude contractions) and phase Ⅲ (period of regular high amplitude contractions). In humans and dogs, MMC is usually observed every 90-120 min in the interdigestive state. In contrast, in rats, MMC cycle is less than 20 min and not so regular, compared to humans and dogs[2,3]. Exogenously administered motilin does not induce phase Ⅲ-like contractions in rats. Motilin or its receptors are not found in rats[4].

Ghrelin, a 28-amino acid peptide, was discovered as the endogenous ligand for growth hormone secretagogue receptor (GHS-R) from the rat stomach[5]. Because of a structural resemblance to motilin, ghrelin is known as the motilin-related peptide[6,7]. Ghrelin administration causes phase Ⅲ-like contraction at the antrum and duodenum in conscious rats[3,8]. We recently showed that gastric spontaneous phase Ⅲ-like contractions were abolished by ghrelin receptor antagonists[9]. This suggests that endogenous ghrelin regulates spontaneous phase Ⅲ-like contractions of the rat stomach.

However, it still remains unknown whether ghrelin regulates intestinal phase Ⅲ-like contractions in rats. In the current study, we investigated whether exogenously administered ghrelin stimulates phase Ⅲ-like contractions of gastrointestinal (GI) tract in conscious rats.

EFFECTS OF GHRELIN ON THE INTERDIGESTIVE GI CONTRACTIONS

Male Sprague-Dawley rats weighing 280-340 g were kept

in-group cages under conditions of controlled temperature (22-24℃), humidity and light (12 h light cycle starting at 7:00 am) with free access to laboratory chow and water. Protocols describing the use of rats were approved by the Institutional Animal Care and Use Committee of Zablocki VA Medical Center (Milwaukee) and carried out in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals". All efforts were made to minimize animal suffering and to reduce the number of animal in experiments.

After overnight fasting, the rats were anesthetized with intraperitoneal injection of pentobarbital sodium (45 mg/kg). Through a midline laparotomy, strain gauge transducers were implanted on the serosal surface of the antrum, duodenum and jejunum. Duodenal transducers were implanted at 5 cm distal from the pylorus. Jejunal transducers were implanted at 15 cm (the proximal jejunum: J-1) and 25 cm (the distal jejunum: J-2) distal from the pylorus, respectively. The wires from transducer were exteriorized through abdominal wall, ran under skin toward the back. Intravenous catheter was inserted into right jugular vein, and similarly exteriorized to the back, as previously reported[2,9]. The catheter was filled with heparinized saline (100 U/mL) to prevent coagulation. Wires and a catheter were protected by a protective jacket (Star Medical, Tokyo, Japan). After the surgery, rats were housed individual and were allowed to recover for one week before the experiments.

After the implantation of transducers, rats were given food once daily at 12:00 pm-16:00 pm, as previously reported[2]. Experiments of GI motility recording were started at 9:00 am every day. The wires from the transducer were connected to the recording system (PowerLab model 8SP, ADI instruments, Colorado Springs, CO). GI contractions were measured with free access to water in freely moving conscious rats. Spontaneous phase Ⅲ-like contractions were observed for 2-3 h. Phase Ⅲ-like contractions were defined as clustered potent contractions with amplitude of more than 4 g, as previously reported[10].

Fujino *et al*[3] reported that bolus injection of acyl ghrelin (1 g/rat; iv) induced phase Ⅲ-like contraction in conscious rats. In general, bolus injection of certain peptides abruptly increased its plasma level. In our previous study, acyl ghrelin (0.8 μg/kg per min) was continuously infused for 5 min and potent phase Ⅲ-like contractions were observed in the antrum in rats[9]. In our current study, acyl ghrelin (0.8, 2.4 and 8.0 μg/kg per min) was continuously infused for 30 min. Acyl ghrelin was purchased from Tocris Cookson (Ellisville, MO).

Motility index (MI), area under the curve, was calculated using a computer-assisted system (PowerLab, ADInstruments, Colorado Springs, CO). MI in GI tract was compared thirty minutes before and during the infusion of acyl ghrelin. Saline infused rats served as controls.

Results were shown as mean ± SE. ANOVA followed by student's *t*-test was used to assess the difference among groups. A P value < 0.05 was considered to be statistically significant.

It has been showed that spontaneous phase Ⅲ-like contractions are observed at 12-15 min intervals of the stomach[3,9,10] in conscious rats. In our current study, cyclic changes of contractions were detected in the antrum, duodenum, J-1 and J-2 including a quiescence period (phase Ⅰ-like contractions) followed by a grouping of strong contractions (phase Ⅲ-like contractions). Spontaneous phase Ⅲ-like contractions were observed at every 13-16 min in rat GI tract. The fasted motor patterns were replaced by the fed motor pattern immediately after food intake[11].

Two minutes after finishing the spontaneous phase Ⅲ-like contractions in the antrum, acyl ghrelin (0.8, 2.4 and 8.0 μg/kg per min) was continuously infused for 30 min. Three-five minutes after the starting ghrelin infusion, augmented phase Ⅲ-like contractions were observed at the antrum, duodenum, J-1 and J-2 (Figure 1).

Ghrelin infusion (0.8, 2.4 and 8.0 μg/kg per min) significantly increased MI of phase Ⅲ-like contractions at the antrum and jejunum compared to that of saline injection, in a dose-dependent manner (Figure 2).

It is well established that exogenously administered ghrelin causes phase Ⅲ-like contractions in the interdigestive state at the antrum and duodenum in rats[3]. However, it is not clear whether intestinal phase Ⅲ-like contractions are affected by ghrelin administration.

We evaluated the effects of peripherally infused ghrelin on gastrointestinal phase Ⅲ-like contractions in freely moving conscious rats. We demonstrated that ghrelin infusion induced phase Ⅲ-like contractions in the antrum, duodenum, proximal and distal jejunum in a dose-dependent manner (0.8-80 μg/kg per min). This suggests that gastric phase Ⅲ-like contractions induced by exogenously administered ghrelin migrate distally to the small intestine.

We have previously shown that GHS-R antagonists significantly inhibited spontaneous phase Ⅲ-like contractions in conscious rats[9], suggesting that endogenously released ghrelin regulates spontaneous phase Ⅲ-like contractions. We also showed the correlation between the plasma ghrelin levels and occurrence of gastric phase Ⅰ- and Ⅲ-like contractions of the antrum[9]. However, it is not clear whether intestinal phase Ⅲ-like contractions are regulated by endogenously released ghrelin. Previous report showed that ghrelin stimulates motility in the rat small intestine and that the stimulatory effect of ghrelin is mediated *via* cholinergic neurons of the myenteric plexus[12].

Our recent study showed that GHS-R antagonists inhibited phase Ⅲ-like contractions at the antrum, but not the duodenum and the jejunum[11]. Previous studies also showed that GHS-R antagonists did not affect phase Ⅲ-like contractions in the duodenum[8].

It is likely that exogenously administered ghrelin (a pharmacological dose of ghrelin) causes phase-Ⅲ like contraction at the antrum, which migrates to the jejunum. In contrast, endogenously released ghrelin causes spontaneous phase Ⅲ-like contractions at the antrum, which do not migrate to the small intestine.

It has been demonstrated that 5-HT is involved in mediating interdigestive contractions of the small intes-

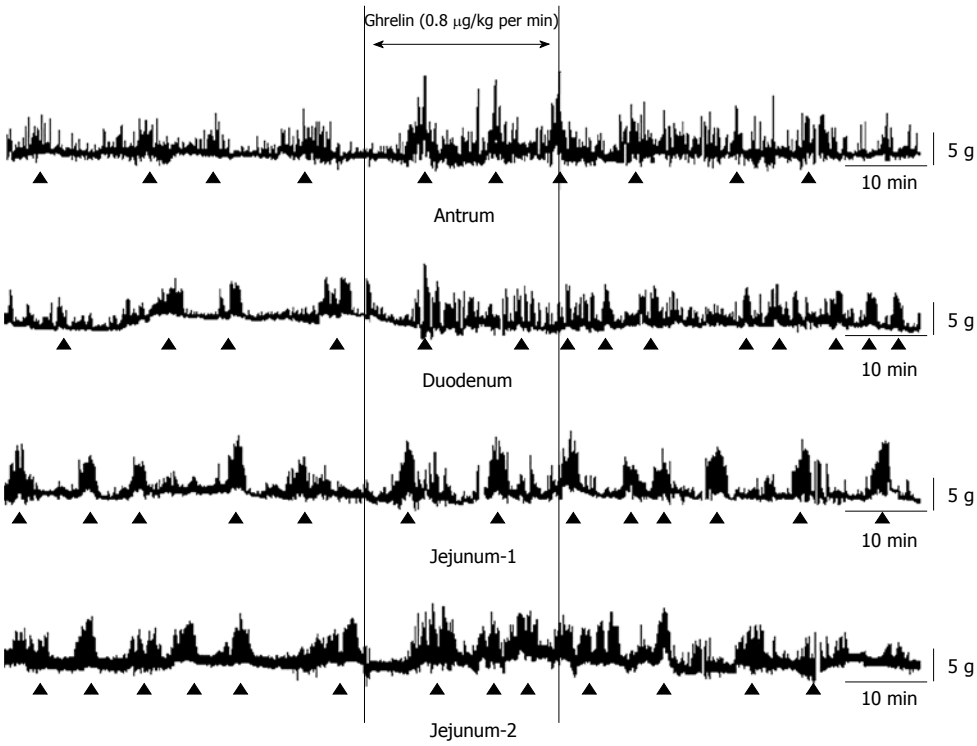

Figure 1 Effect of ghrelin infusion (0.8 μg/kg per min) on spontaneous phase Ⅲ-like contractions in conscious rats. Two minutes after finishing the spontaneous phase Ⅲ-like contractions in the antrum, acyl ghrelin (0.8 μg/kg per min) was continuously infused for 30 min. Three-five minutes after starting the ghrelin infusion, phase Ⅲ-like contractions were observed in response to ghrelin infusion at the antrum, duodenum, jejunum-1 and jejunum-2 (▲ indicates phase Ⅲ-like contractions).

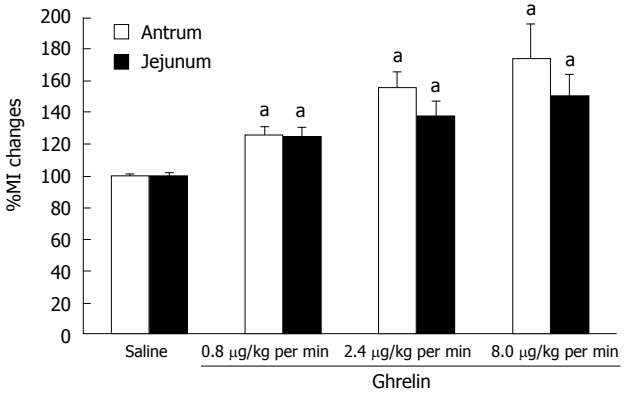

Figure 2 Effect of ghrelin on % changes of MI of phase Ⅲ-like contractions of GI tract ($^aP < 0.05$ *vs* saline, $n = 5$). Ghrelin infusion (0.8, 2.4 and 8.0 μg/kg) dose-dependently increased MI of phase Ⅲ-like contractions of the antrum and jejunum ($^aP < 0.05$ *vs* saline, $n = 5$).

tine in rats[13]. Subcutaneous or intravenous administration of 5-HT can induce intestinal migrating myoelectrical activity in rats[14,15]. Intestinal migrating myoelectrical activity was reduced by a 5-HT_3 antagonist, but not by a 5-HT_4 antagonist in conscious rats[15]. However, others showed that intestinal migrating myoelectrical activity was reduced by a 5-HT_4 antagonist, as well as a 5-HT_3 antagonist[16].

Our recent study showed that phase Ⅲ-like contractions at the jejunum, not the antrum and duodenum, were significantly attenuated by 5-HT_4 antagonists. In contrast, 5-HT_3 antagonists did not affect phase Ⅲ-like contractions in all of upper GI tract[11]. These suggest that spontaneous phase Ⅲ-like contractions at the jejunum is mediated *via* 5-HT_4 receptors, but not 5-HT_3 receptors.

5-HT_3 receptors[17] and 5-HT_4 receptors[18] are located on the cholinergic neurons of the myenteric plexus as well as sensory neurons of the intestinal mucosa[19]. Nerve endings of sensory neurons may well be the targets for the 5-HT released from enterochromaffin (EC) cells[20]. It is generally accepted that 5-HT stimulates intrinsic nerve fibers *via* 5-HT_4 receptors[21], while 5-HT stimulates extrinsic nerve fibers *via* 5-HT_3 receptors[19,22] in rats.

5-HT_3 receptors are located on the nerve terminal of vagal afferent of the duodenal mucosa in rats[23]. 5-HT released by mucosal stimuli initiates peristalsis by activating 5-HT_4 receptors on sensory CGRP neurons of the rat colon *in vitro*[24]. These suggest that 5-HT_4 receptors play a major role in mediating an intrinsic neural reflex. It is conceivable that luminally released 5-HT from duodenal EC cells initially stimulates duodenal phase Ⅲ-like contractions *via* 5-HT_4 receptors located on intrinsic primary afferent neurons (IPAN).

It has been shown that ghrelin receptors are synthesized in vagal afferent neurons and transported to the afferent terminal. This is the major pathway conveying ghrelin signals for starvation and growth hormone secretion to the brain[25]. Blockade of the gastric vagal neuron by vagotomy or perivagal application of capsaicin abolished ghrelin-induced feeding, GH secretion, and activation of NPY-producing and GHRH-producing neurons[25]. Ghrelin-induced acid secretion is also abolished by bilateral vagotomy[26].

Our recent study showed that spontaneous phase Ⅲ-like contractions were completely disappeared in vagotomized rats[11]. These results suggest that ghrelin-induced spontaneous phase-Ⅲ like is mediated *via* vagal pathways.

Spontaneous phase Ⅲ-like contractions are mainly regulated by ghrelin in the antrum, while spontaneous phase Ⅲ-like contractions are regulated by 5-HT in the jejunum. Released ghrelin from the gastric mucosa initi-

ates gastric phase Ⅲ-like contractions *via* vagal dependent pathways. Released 5-HT from intestinal EC cells induces intestinal phase Ⅲ-like contractions *via* IPAN in rats.

REFERENCES

1 **Szurszewski JH**. A migrating electric complex of canine small intestine. *Am J Physiol* 1969; **217**: 1757-1763
2 **Ariga H**, Imai K, Chen C, Mantyh C, Pappas TN, Takahashi T. Fixed feeding potentiates interdigestive gastric motor activity in rats: importance of eating habits for maintaining interdigestive MMC. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G655-G659
3 **Fujino K**, Inui A, Asakawa A, Kihara N, Fujimura M, Fujimiya M. Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *J Physiol* 2003; **550**: 227-240
4 **Depoortere I**, De Winter B, Thijs T, De Man J, Pelckmans P, Peeters T. Comparison of the gastroprokinetic effects of ghrelin, GHRP-6 and motilin in rats in vivo and in vitro. *Eur J Pharmacol* 2005; **515**: 160-168
5 **Kojima M**, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660
6 **Tomasetto C**, Karam SM, Ribieras S, Masson R, Lefebvre O, Staub A, Alexander G, Chenard MP, Rio MC. Identification and characterization of a novel gastric peptide hormone: the motilin-related peptide. *Gastroenterology* 2000; **119**: 395-405
7 **Asakawa A**, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 2001; **120**: 337-345
8 **Wang Y**, Dong L, Cheng Y, Zhao P. Effects of ghrelin on feeding regulation and interdigestive migrating complex in rats. *Scand J Gastroenterol* 2007; **42**: 447-453
9 **Ariga H**, Tsukamoto K, Chen C, Mantyh C, Pappas TN, Takahashi T. Endogenous acyl ghrelin is involved in mediating spontaneous phase III-like contractions of the rat stomach. *Neurogastroenterol Motil* 2007; **19**: 675-680
10 **Tatewaki M**, Harris M, Uemura K, Ueno T, Hoshino E, Shiotani A, Pappas TN, Takahashi T. Dual effects of acupuncture on gastric motility in conscious rats. *Am J Physiol Regul Integr Comp Physiol* 2003; **285**: R862-R872
11 **Taniguchi H**, Ariga H, Zheng J, Ludwig K, Mantyh C, Pappas TN, Takahashi T. Endogenous ghrelin and 5-HT regulate interdigestive gastrointestinal contractions in conscious rats. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G403-G411
12 **Edholm T**, Levin F, Hellstrom PM, Schmidt PT. Ghrelin stimulates motility in the small intestine of rats through intrinsic cholinergic neurons. *Regul Pept* 2004; **121**: 25-30
13 **Pineiro-Carrero VM**, Clench MH, Davis RH, Andres JM, Franzini DA, Mathias JR. Intestinal motility changes in rats after enteric serotonergic neuron destruction. *Am J Physiol* 1991; **260**: G232-G239
14 **Sagrada A**, Brancaccio N, Schiavone A. 5-Hydroxytryptamine affects rat migrating myoelectric complexes through different receptor subtypes: evidence from 5-hydroxytryptophan administration. *Life Sci* 1990; **46**: 1207-1216
15 **Lordal M**, Hellstrom PM. Serotonin stimulates migrating myoelectric complex via 5-HT3-receptors dependent on cholinergic pathways in rat small intestine. *Neurogastroenterol Motil* 1999; **11**: 1-10
16 **Axelsson LG**, Wallin B, Gillberg PG, Sjoberg B, Soderberg C, Hellstrom PM. Regulatory role of 5-HT and muscarinic receptor antagonists on the migrating myoelectric complex in rats. *Eur J Pharmacol* 2003; **467**: 211-218
17 **Miyata K**, Kamato T, Nishida A, Ito H, Yuki H, Yamano M, Tsutsumi R, Katsuyama Y, Honda K. Role of the serotonin3 receptor in stress-induced defecation. *J Pharmacol Exp Ther* 1992; **261**: 297-303
18 **Talley NJ**. Serotoninergic neuroenteric modulators. *Lancet* 2001; **358**: 2061-2068
19 **Gershon MD**. Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. *Aliment Pharmacol Ther* 1999; **13** Suppl 2: 15-30
20 **Berthoud HR**, Kressel M, Raybould HE, Neuhuber WL. Vagal sensors in the rat duodenal mucosa: distribution and structure as revealed by in vivo DiI-tracing. *Anat Embryol* (Berl) 1995; **191**: 203-212
21 **Foxx-Orenstein AE**, Kuemmerle JF, Grider JR. Distinct 5-HT receptors mediate the peristaltic reflex induced by mucosal stimuli in human and guinea pig intestine. *Gastroenterology* 1996; **111**: 1281-1290
22 **Blackshaw LA**, Grundy D. Effects of 5-hydroxytryptamine (5-HT) on the discharge of vagal mechanoreceptors and motility in the upper gastrointestinal tract of the ferret. *J Auton Nerv Syst* 1993; **45**: 51-59
23 **Glatzle J**, Sternini C, Robin C, Zittel TT, Wong H, Reeve JR Jr, Raybould HE. Expression of 5-HT3 receptors in the rat gastrointestinal tract. *Gastroenterology* 2002; **123**: 217-226
24 **Grider JR**, Kuemmerle JF, Jin JG. 5-HT released by mucosal stimuli initiates peristalsis by activating 5-HT4/5-HT1p receptors on sensory CGRP neurons. *Am J Physiol* 1996; **270**: G778-G782
25 **Date Y**, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K, Nakazato M. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 2002; **123**: 1120-1128
26 **Yakabi K**, Ro S, Onouhi T, Tanaka T, Ohno S, Miura S, Johno Y, Takayama K. Histamine mediates the stimulatory action of ghrelin on acid secretion in rat stomach. *Dig Dis Sci* 2006; **51**: 1313-1321

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TOPIC HIGHLIGHT

Akio Inui, MD, PhD, Professor, *Series Editor*

Growth hormone releasing peptide 2 reverses anorexia associated with chemotherapy with 5-fluorouracil in colon cancer cell-bearing mice

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Abstract

The cancer-associated anorexia-cachexia syndrome is observed in 80% of patients with advanced-stage cancer, and is one of the major obstacles in chemotherapy. Ghrelin is a orexigenic hormone that has been proposed to prevent anorexia. Aim of the study was to determine whether the addition of the ghrelin agonist growth hormone releasing peptide 2 (GHRP-2) to cytotoxic therapy with 5-fluorouracil (5-FU) prevents the anorexia associated with chemotherapy in cancer cachectic mice. Thirty-three BALB/c female tumour-bearing mice were randomized to receive a solution containing: (a) placebo; (b) GHRP-2; (c) 5-FU; or (d) 5-FU + GHRP-2. Ten BALB/c no tumour-bearing mice received placebo solution. Food intake and survival were checked. Six hours after the drug injection the cumulative food intake was significantly increased in mice treated with the combination of 5-FU + GHRP-2 *versus* the 5-FU alone (P = 0.0096). On day 3, the cumulative food intake of mice treated with GHRP-2, 5-FU and 5-FU + GHRP-2 significantly increased compared with naive and vehicle groups (P = 0.0007, P = 0.0038 and P = 0.0166, respectively). The median survival time was longer in 5-FU + GHRP-2 treated mice than in those with 5-FU, although it was not significant (18 d *versus* 15.5 d, P = 0.7). For the first time, we demonstrated that the addition of GHRP-2 to cytotoxic therapy with 5-FU improved appetite in tumour-bearing mice with anorexia/cachexia syndrome in early stage. These data suggest that GHRP-2 may improve the efficacy of therapy and the quality of life of cancer patients thank to the amelioration of their nutritional state.

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Key words: GHS; Ghrelin; Cancer anorexia-cachexia syndrome; Food intake; Chemotherapy; Colon cancer cell line; Murine model

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CHEMOTHERAPY-INDUCED ANOREXIA

Chemotherapy is the most effective treatment for most cancer patients because of its systemic distribution. Despite the recent advances in the treatment, many patients do not respond to therapy, and die of their diseases. The cancer-associated anorexia-cachexia syndrome is observed in 80% of patients with advanced-stage cancer, and it is a very powerful prognostic indicator of poor outcome and poor quality of life. This syndrome is also one of the major obstacles in cancer chemotherapy[1].

Ghrelin is the natural ligand of growth hormone secretagogue receptor. Growth hormone releasing peptide 2 (GHRP-2) is a synthetic compound that acts as a potent agonist of GHS receptor[2]. Increasing evidences support that GHRP-2, like ghrelin[3], exerts orexigenic

proprieties. Administration of GHRP-2 has shown to increase food intake and body weight in rodents[4] and in healthy men[5]. Since ghrelin has a short half-life, the more stable GHRP-2 was preferred in this experiment.

Aim of the study was to determine whether the addition of GHRP-2 to cytotoxic therapy with 5-fluorouracil (5-FU) prevents anorexia associated with chemotherapy in cancer cachectic mice[6]. Secondary aims were to examine the chronic effects of GHRP-2 on reduced appetite and survival in this animal model.

MURINE MODEL OF CANCER

Six-weeks-old female BALB/c mice (19-24 g) were housed individually in equal plastic cages and received *ad libitum* standard diet and tap water in a regulated environment. The murine colon cancer cell line (colon 26) was supplied by Dr. Hayashi (Kitazato University, Kanagawa, Japan). Colon 26 cancer cells (5×10^5) were dissolved in a 100 μL volume of PBS containing 0.02% EDTA and then implanted in the abdominal cavity of female BALB/c mice by intraperitoneal administration. Seven days later, when mice manifested the first symptoms of anorexia and cachexia, the tumour-bearing mice were randomized in 4 groups, each of them composed by: (a) 11 mice receiving 5% glucoside solution + PBS (vehicle); (b) 12 mice receiving GHRP-2 + 5% glucoside solution; (c) 12 mice receiving 5FU + PBS; and (d) 15 mice receiving 5-FU + GHRP-2. Ten BALB/c no tumour-bearing mice received 5% glucoside solution + PBS (naïve). The day of randomisation was considered as day 0 of the experiment. GHRP-2 (DalaDβNalAlaTrpDPheLysNH2) was supplied by Prof. Bowers (Tulane University, New Orleans, USA). It was dissolved in PBS and was subcutaneously administered at dose of 10 μg/mouse daily. 5-fluorouracil (Kyowa Hakko Kogyo Co. Ldt, Tokyo, Japan) was dissolved in 5% glucoside solution and then intraperitoneally administered at dose of 100 mg/kg weekly. In the acute experiment, food intake and body weight were measured at 0, 1, 2, 4 and 6 h after the first injection of the drugs and then daily. Food intake was evaluated by subtracting uneaten food from initially pre-measured food and checking for food spillage. All the experiments were approved by the animal care committees at the Kobe University and the Kagoshima University (Japan). Results are expressed as mean ± SE. Analysis of variance (ANOVA) followed by Bonferroni's *t* test was used to assess the differences among groups. *P*-values < 0.05 were considered significant. The survival curves after tumour implantation were analyzed by Kaplan-Meier survival test.

GHRELIN AGONIST GHRP-2 REVERSES CHEMOTHERAPY-INDUCED ANOREXIA

The median survival time was longer in the group treated with 5-FU + GHRP-2 than in the group treated with 5-FU, although it was not significant (18 d *versus* 15.5 d, *P* = 0.7) as shown in Figure 1. At day 0, 6 h after the drug injection, the cumulative food intake was significantly

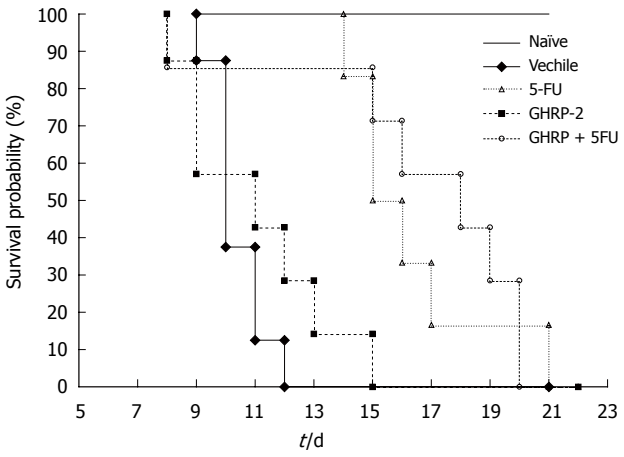

Figure 1 Kaplan-Meier curve of overall survival in colon 26-bearing mice (Naïve: 10 mice; Vehicle: 11 mice; GHRP-2: 12 mice; 5-FU: 12 mice; 5-FU + GHRP-2: 15 mice).

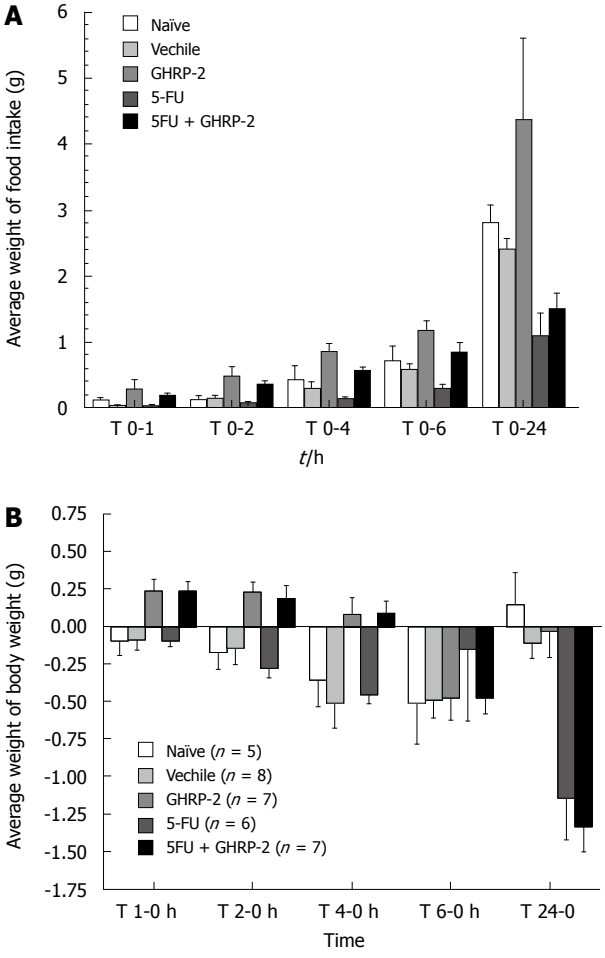

Figure 2 GHRP-2 reverses reduced food intake and body weight by 5-FU administration in colon 26-bearing mice-acute experiments. A: Food intake; B: Body weight.

increased in mice treated with the combination of 5-FU + GHRP-2 *versus* the 5-FU alone (*P* = 0.0096, Figure 2A). At day 0, 4 h after the drug injection, the cumulative body weight of the group treated with 5-FU + GHRP-2 showed a reduced loss of body weight compared with the group treated with 5-FU (*P* = 0.0074, Figure 2B). At day

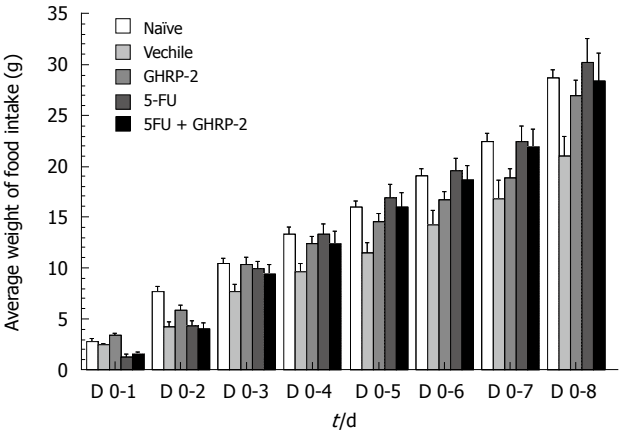

Figure 3 GHRP-2 increases cumulative food intake compared to vehicle controls in colon 26-bearing mice-chronic experiments.

2, the groups treated with 5-FU and 5-FU + GHRP-2 showed a higher loss of cumulative body weight compared with the group treated with GHRP-2 alone (P = 0.0003 and $P < 0.0001$, respectively, Figure 3). However, from day 3 to 5, the cumulative food intake of mice treated with 5-FU and 5-FU + GHRP-2 significantly increased compared with naive and vehicle groups (P = 0.0038 and P = 0.0166, respectively, Figure 3). The cumulative food intake was significantly increased in mice treated with GHRP-2 respect to naïve and vehicle groups throughout the observation period (P = 0.0007 and P = 0.0004, respectively, Figure 3). We could not follow the body weight changes since the tumour-bearing mice developed ascites after the inoculation of cancer cells into the abdominal cavity.

THERAPEUTIC ROLE OF GHRP-2 IN CANCER ANOREXIA-CACHEXIA

Up to 50% of cancer patients report changes in eating behavior at time of diagnosis, leading to weight loss[3]. This study suggests that the addition of GHRP-2 to cytotoxic therapy with 5-FU improved appetite in tumour-bearing mice with anorexia/cachexia syndrome in early stages, although a statistically significant improvement in survival was not achieved compared with mice treated with 5-FU. It is likely that GHRP-2 may overcome any resistance to the appetite-stimulating effects of ghrelin in cachectic animals and cancer patients with appetite loss. In our experiment GHRP-2 has shown to be a short-lasting, acute potent agent, which mimics the orexigenic effects of ghrelin[4,5]. In this study, we demonstrated for the first time that GHRP-2 reversed loss of food intake and body weight in tumour-bearing mice treated with chemotherapy. It is likely that preventing loss of appetite and weight associated with chemotherapy helps mice remain in a relatively balanced condition of electrolytes and hydration, thereby decreasing the side effect and increasing the efficacy of 5-FU. The characteristic of a long acting drug which allows to increased interval of time between two consecutive administrations of the drug, may be a further improve the quality of life in cancer patients.

This study has some limitations. The first is the difficulty to determinate if the survival and the weight loss experienced by tumour-bearing mice was due to tumour burden or to the efficacy or side effect of the 5-FU administration. Secondary, although GHRP-2 did increase the cumulative food intake in tumour-bearing mice, there was a lack of significant difference among groups in survival analysis which should be examined under various chemotherapy conditions and tumour models.

Due to the amelioration of the nutritional state, and of the side effects of chemotherapy, GHRP-2 may offer an interesting treatment for cachexia associated with cancer in order to improve the efficacy of therapy and the quality of life of cancer patients.

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REFERENCES

1 **Inui A**. Cancer anorexia-cachexia syndrome: current issues in research and management. *CA Cancer J Clin* 2002; **52**: 72-91
2 **Bowers CY**, Momany FA, Reynolds GA, Hong A. On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology* 1984; **114**: 1537-1545
3 **Neary NM**, Small CJ, Wren AM, Lee JL, Druce MR, Palmieri C, Frost GS, Ghatei MA, Coombes RC, Bloom SR. Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. *J Clin Endocrinol Metab* 2004; **89**: 2832-2836
4 **Tschop M**, Statnick MA, Suter TM, Heiman ML. GH-releasing peptide-2 increases fat mass in mice lacking NPY: indication for a crucial mediating role of hypothalamic agouti-related protein. *Endocrinology* 2002; **143**: 558-568
5 **Laferrere B**, Abraham C, Russell CD, Bowers CY. Growth hormone releasing peptide-2 (GHRP-2), like ghrelin, increases food intake in healthy men. *J Clin Endocrinol Metab* 2005; **90**: 611-614
6 **Seeliger H**, Guba M, Koehl GE, Doenecke A, Steinbauer M, Bruns CJ, Wagner C, Frank E, Jauch KW, Geissler EK. Blockage of 2-deoxy-D-ribose-induced angiogenesis with rapamycin counteracts a thymidine phosphorylase-based escape mechanism available for colon cancer under 5-fluorouracil therapy. *Clin Cancer Res* 2004; **10**: 1843-1852

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TOPIC HIGHLIGHT

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Characteristic features of ghrelin cells in the gastrointestinal tract and the regulation of stomach ghrelin expression and production

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Abstract

Ghrelin was isolated as an endogenous ligand for the GH secretagogue receptor from the rat stomach. Although physiological effects of ghrelin have been revealed by numerous studies, the regulation of stomach ghrelin remains obscure, and the factor that directly regulates ghrelin expression and production has not been identified. Here, we show some data regarding the characteristic features of ghrelin cells and the regulation of stomach ghrelin. In the gastrointestinal tract, ghrelin cells were identified as opened- and closed-type cells, and it was found that the number of ghrelin cells decreased from the stomach to the colon. The postnatal change in number of ghrelin cells in the stomach showed a sexually dimorphic pattern, indicating a role of estrogen in the regulation of stomach ghrelin. *In vitro* studies revealed that estrogen stimulated both ghrelin expression and production and that treatment with formestane, an aromatase (estrogen synthetase) inhibitor, decreased ghrelin expression level. On the other hand, leptin was found to inhibit both basal and estrogen-stimulated ghrelin expression. Moreover, both aromatase mRNA-expressing cells and leptin cells were found to be located close to ghrelin cells in the gastric mucosa. Furthermore, we found an inverse relationship between gastric ghrelin and leptin levels in a fasting state, and we revealed relative changes in expression of gastric ghrelin, estrogen and leptin in the postnatal rats. We propose that gastric estrogen and leptin directly regulate stomach ghrelin and that the balance control through gastric estrogen and leptin contributes to the altered ghrelin expression level in some physiological states.

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Key words: Stomach; Estrogen; Leptin; Regulate; Ghrelin; Expression; Physiological state

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INTRODUCTION

Ghrelin, a 28-amino-acid peptide with an essential n-octanoyl modification on the third amino acid, was purified as an endogenous ligand for the growth hormone (GH) secretagogue receptor from rat stomach in 1999[1]. In initial studies, ghrelin was shown to stimulate GH release from the pituitary both *in vivo* and *in vitro*[1,2], and ghrelin was later found to be also deeply involved in the regulation of feeding behavior and energy homeostasis[3-7].

Ghrelin is predominantly produced in the stomach[1]. Date *et al*[8] further revealed that X/A-like cells in the stomach were responsible for ghrelin production. They also found that in the gastrointestinal tract, the greatest amount of ghrelin was in the gastric mucosa, and smaller amounts were in the small and large intestines[8]. However, the detailed distribution and morphological characteristics of ghrelin cells in the whole gastrointestinal tract have not been elucidated.

On the other hand, it has been reported that both the expression and secretion of ghrelin are mainly influenced by changes in energy balance, *i.e.* increased by fasting and decreased after refeeding[3,9-11]. In addition, many other factors such as leptin, insulin, somatostatin and vagal activity have also been shown to be involved in the regulation of stomach ghrelin[9,12-14]. However, the factor that directly regulates ghrelin expression or production remains unclear, and little is known about the regulation of gastric ghrelin expression in various physiological states.

Therefore, in this review, we summarize the data obtained by our group regarding the distribution and morphological characteristics of gastrointestinal ghrelin cells, postnatal changes in stomach ghrelin, and the regulation of stomach ghrelin in some physiological states.

DISTRIBUTION OF GHRELIN CELLS IN THE RAT GASTROINTESTINAL TRACT

It was found that plasma ghrelin-like immunoreactivity levels were reduced by 65% in totally gastrectomized patients, suggesting that the stomach is the major source of circulating ghrelin[15]. To investigate the morphological characteristics and distribution of ghrelin cells, we used anti-acylated rat ghrelin antiserum to detect ghrelin-immunopositive (ip) cells in the rat gastrointestinal tract in a previous study[16]. We found that ghrelin cells were present throughout the whole gastrointestinal mucosa from the stomach to the colon, and that they could be classified into two types, *i.e.* closed-type cells (Figure 1A) and lumen-contacted opened-type cells (Figure 1B). Interestingly, in ghrelin cells, des-acylated ghrelin was found to be mainly localized to the perinucleus, while acylated ghrelin was found to be distributed in the periphery of the cytoplasm. These findings suggest that des-acylated ghrelin, which is from the Golgi complex, undergoes acylation in secretory granules in the periphery of the cytoplasm.

Further morphometric analysis revealed that the largest number of ghrelin cells was in the stomach and the next-largest number was in the duodenum, and very small numbers of ghrelin cells were observed in the ileum, cecum and colon[16]. These findings are in agreement with the results of another study regarding gastrointestinal ghrelin content[8]. On the other hand, in the stomach, very few opened-type ghrelin cells were observed; but it was found that the percentages of opened-type ghrelin cells in all ghrelin cells in various regions of the gastrointestine gradually increased in the direction from the stomach to the lower intestine, being particularly high in the ileum, cecum and colon[16]. It is generally accepted that opened-type endocrine cells in the gastrointestinal tract are mainly regulated by luminal signals, whereas closed-type cells in the gastrointestinal tract receive modulation from hormones, neuronal stimulation or mechanical distension[17]. Therefore, the distinct distributions of opened- and closed-type ghrelin cells in the gastrointestine suggest that the ghrelin cells may be modulated by different stimulators and may play different physiological roles in various regions of the gastrointestinal tract.

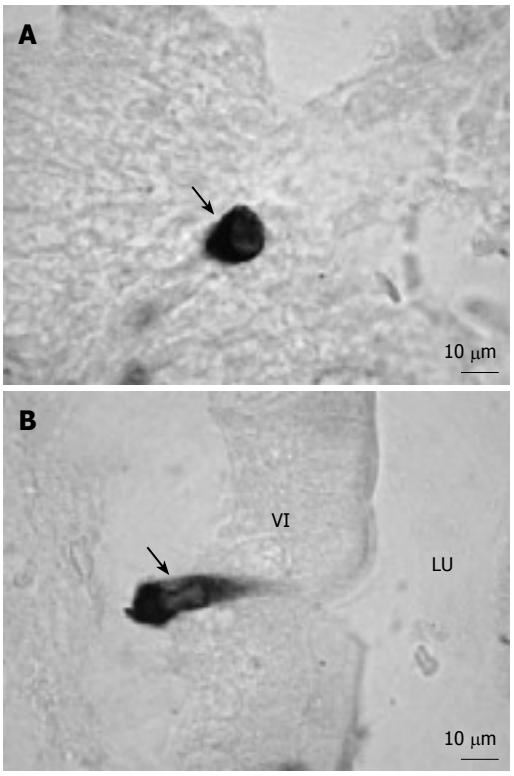

Figure 1 Representative microphotographs of ghrelin-ip cells in the rat gastrointestinal tract[16]. A: Closed-type ghrelin cells (arrow) were found in the crypt of the duodenum; B: Opened-type ghrelin cells (arrow) in contact with the lumen were found in the villi of the duodenum. Bar = 10 μm. VI: Villi; LU: Lumen.

POSTNATAL CHANGES IN STOMACH GHRELIN

In a recent study, the mRNA expression level of gastric ghrelin was shown to be elevated progressively during the second and third postnatal weeks[18]. Gualillo *et al*[19] also reported a gradual increase in the expression level of the gastric ghrelin gene after birth in the rat (up to 90 days). In agreement with these results, we found that gastric ghrelin expression was detectable just after birth in both male and female rats, and that expression levels of gastric ghrelin gradually increased during postnatal development (up to 8 wk of age) (Figure 2A and B).

Accordingly, ghrelin-ip and ghrelin mRNA-expressing cells were also observed just after birth[20]. At 1 wk of age, these ghrelin cells were mainly localized in the glandular base of the fundic gland, and then the distribution of ghrelin cells gradually extended from the glandular base to the glandular neck with increasing age in both sexes[20]. An interesting finding in that study was that two kinds of stained ghrelin cells, weakly stained and strongly stained cells, were found in female rats at the early stage of development (1 and 3 wk of age), whereas staining in most of the ghrelin cells was strong in male rats and 7-wk-old female rats[20]. With increasing age, weakly stained cells were replaced by

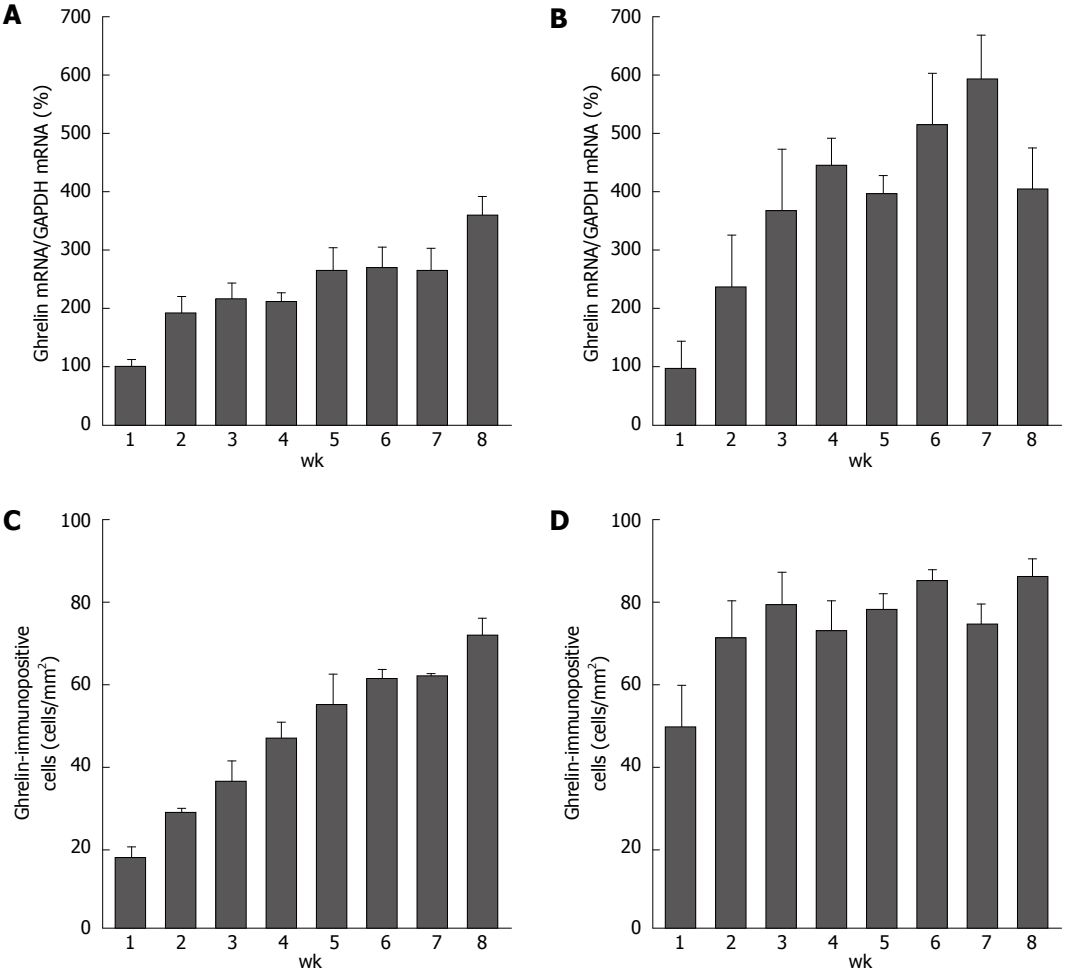

Figure 2 Changes in ghrelin mRNA levels and the densities of ghrelin-ip cells during the postneonatal period in the rat stomach[20]. The data in (A) and (B) are shown as the % of 1-wk-old (1 wk) rats (100%) and each bar represents the mean ± SE (n = 3). A: Ghrelin mRNA levels in the male stomach; B: Ghrelin mRNA levels in the female stomach; C: The densities of ghrelin-ip cells (cells/mm^2) in the male stomach; D: The densities of ghrelin-ip cells (cells/mm^2) in the female stomach.

strongly stained cells, resulting in almost no change in the density of ghrelin cells in female rats throughout the whole period of postnatal development (Figure 2D), in contrast to the increase in ghrelin mRNA expression level. On the other hand, in male rats, ghrelin cell density showed an age-dependent increase after birth (Figure 2C), and the increase was in concert with the increase in ghrelin expression level. The sexual dimorphism of ghrelin cell density suggests that ghrelin cells in female rats differentiate at an earlier stage of development than they do in male rats, and that sex steroids such as estrogen may be involved in the regulation of stomach ghrelin.

REGULATION OF STOMACH GHRELIN BY GASTRIC ESTROGEN AND LEPTIN IN SOME PHYSIOLOGICAL STATES

Regulatory role of gastric estrogen

A role of estrogen in the regulation of stomach ghrelin has also been suggested by several studies. In a previous study, the levels of gastric ghrelin mRNA and plasma ghrelin and the number of ghrelin cells were found to be transiently increased by ovariectomy in female rats[21]. In addition, it was found that ghrelin cells express estrogen receptor α (ERα)[21]. On the other hand, Ueyama *et al*[22] recently demonstrated that aromatase, an estrogen synthetase, is expressed in parietal cells of the rat stomach and that gastric parietal cells are capable of producing and secreting a substantial amount of estrogen. These findings indicate that gastric estrogen plays a role in the regulation of stomach ghrelin.

Therefore, in a previous study, using isolated stomach cells, which are rich in ghrelin cells, we determined the direct effect of estrogen on stomach ghrelin expression and production[23]. In that study, we found that estrogen treatment significantly stimulated ghrelin mRNA expression (Figure 3A) and production in a dose-dependent manner and that treatment of minced stomach tissue with formestane, an aromatase inhibitor, decreased ghrelin expression level[23]. Given that a significant increase in estrogen concentration in the portal vein compared with that in the artery was observed in intact rats, but not in gastrectomized rats[22], the concentration of gastric estrogen must be higher than that of plasma estrogen. Moreover, neither the gastric ghrelin expression nor the plasma

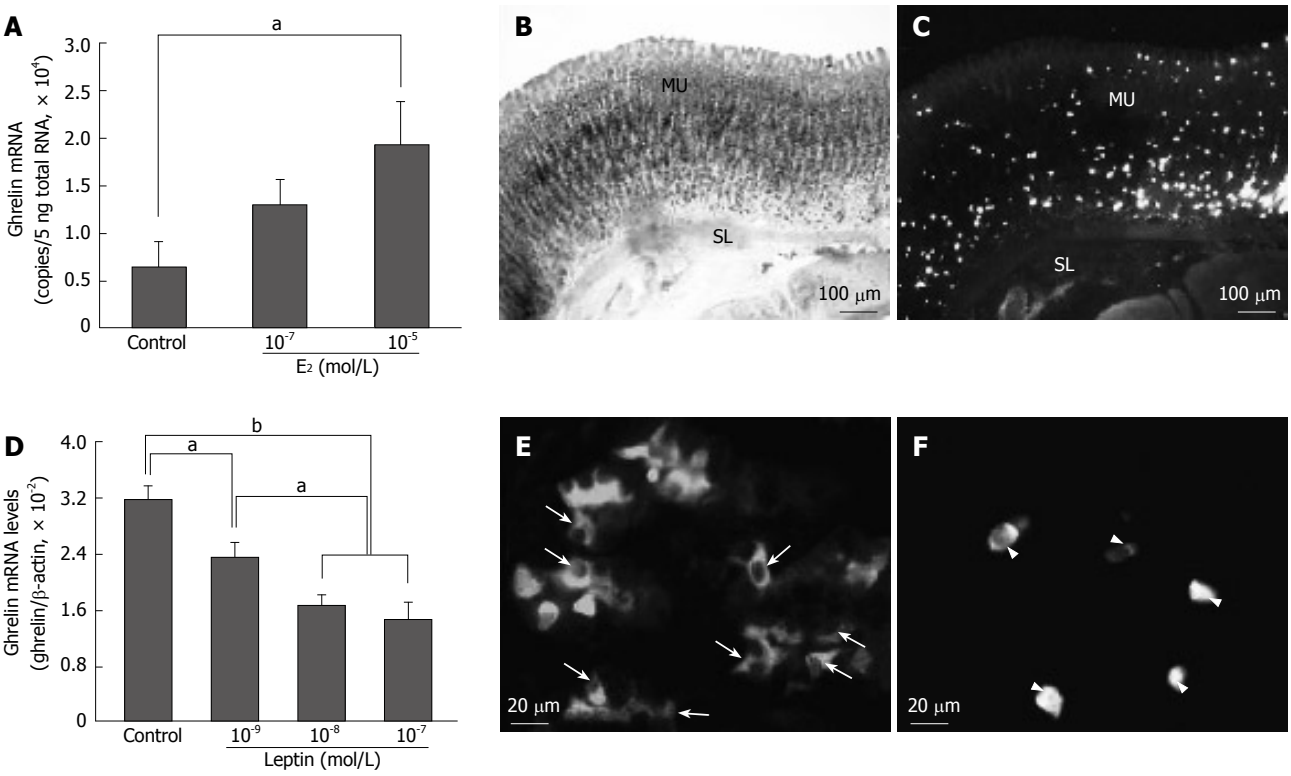

Figure 3 ***In vitro*** **effects of estrogen and leptin on ghrelin mRNA expression and distributions of aromatase mRNA-expressing, leptin-ip and ghrelin-ip cells in the gastric mucosa[23,27].** A: Changes in ghrelin mRNA expression after estrogen treatment; B: Aromatase mRNA-expressing cells were found in the glandular body of the fundic gland; C: Ghrelin-ip cells were found sporadically throughout the gastric mucosa; D: Changes in ghrelin mRNA expression after leptin treatment. Ghrelin mRNA level is expressed relative to β-actin mRNA level; E: Leptin-ip cells (arrow) were found in the lower half of the fundic gland; F: Ghrelin-ip cells (arrowhead) were found in the gastric mucosa. (A, D) Data are presented as mean ± SE. *n* = 3-4/group. $^{a}P < 0.05$; $^{b}P < 0.01$. Bar = 100 μm (B, C) and 20 μm (E, F) respectively. MU: Mucosa; SL: Smooth muscle layer.

ghrelin concentration was altered by gonadectomy[23]. Furthermore, ghrelin cells and aromatase mRNA-expressing cells were found to be located close together in the gastric mucosa (Figure 3B and C), suggesting that ghrelin cells are exposed to gastric estrogen. All of these results strongly suggest that estrogen produced in the stomach directly stimulates ghrelin expression and production in the rat stomach.

Regulatory role of gastric leptin

Reciprocal circadian rhythms in circulating ghrelin and leptin levels and antagonistic hypothalamus-mediated control of appetite by ghrelin and leptin have been revealed by several studies[3,6,24]; however, results of studies on the modulation of ghrelin expression by leptin are inconsistent. One group showed that leptin administration to rats for five days stimulated gastric ghrelin mRNA expression[11], whereas another group reported the opposite results[9]. Due to these conflicting results, the role of leptin in regulation of ghrelin expression remains unclear. On the other hand, leptin was initially thought to be adipocyte-derived[25], and it has also been identified in various tissues, including the stomach[26]. The fact that the release of gastric leptin is rapidly stimulated by food intake or CCK treatment suggests that gastric leptin is involved in the short-term control of energy balance[26], although adipocyte leptin is known to be a long-term regulator of energy balance.

In contrast to the stimulatory effect of estrogen, in another study, we found that leptin inhibits both basal (Figure 3D) and estrogen-stimulated ghrelin expression *in vitro*[27]. The fact that both long and short forms of the leptin receptor have been identified in the human and rat gastric mucosa[28,29] strongly suggests that the gastric epithelium could be a direct target of gastric leptin. Consistent with results of these studies, mRNAs of both OB-Ra and OB-Rb were found to be expressed in the rat gastric fundus, and no inhibitory effect of leptin on ghrelin expression was observed in leptin receptor-defective Zucker fatty (fa/fa) rats, indicating that leptin inhibits ghrelin expression *via* the leptin receptor[27]. Furthermore, it was revealed that leptin cells were mainly located in the lower half of the gastric mucosa, where most of the ghrelin cells were tightly surrounded by leptin cells (Figure 3E and F), suggesting that gastric leptin has a paracrine role in regulation of ghrelin cells, and that ghrelin cells may be exposed to a higher concentration of gastric leptin than that of plasma leptin since leptin infusion at 0.1 nmol/L, which can mimic the plasma leptin concentration under basal conditions in rats, has been shown to be incapable of suppressing ghrelin release from the isolated rat stomach[30].

Relative changes in gastric ghrelin, estrogen and leptin levels in some physiological states

Direct regulation of stomach ghrelin by gastric estrogen

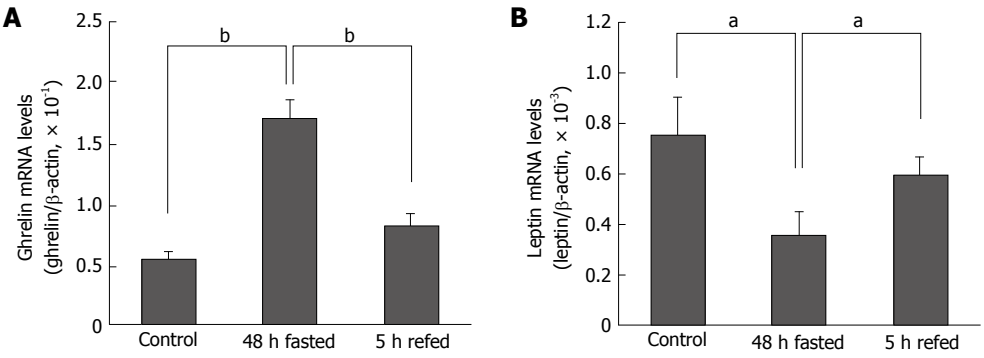

Figure 4 mRNA expression levels of gastric ghrelin and leptin under different feeding conditions[27]. (A) Effects of 48 h of fasting and 5 h of refeeding on gastric ghrelin expression; (B) Effects of 48 h of fasting and 5 h of refeeding on gastric leptin expression. Ghrelin and leptin mRNA levels are expressed relative to β-actin mRNA levels. Data are presented as mean ± SE. n = 3-4/group. $^{a}P < 0.05$; $^{b}P < 0.01$.

and leptin raises the possibility that gastric estrogen and leptin contribute to the altered ghrelin level in some physiological states. It is generally accepted that the most important physiological state for the regulation of stomach ghrelin is fasting. Results of numerous studies have shown that levels of both ghrelin expression and secretion are increased by fasting and decreased after refeeding[3,9-11]. Therefore, in a recent study, we determined the changes in gastric estrogen and leptin levels in relation to gastric ghrelin expression level under a fasting condition[27]. In that study, however, neither the expression level of gastric aromatase nor the concentration of estrogen in the portal vein was altered by fasting[27], although the expression level of gastric ghrelin was significantly elevated as predicted (Figure 4A). In contrast, both gastric leptin expression (Figure 4B) and concentrations were found to be significantly decreased by fasting[27]. In addition, refeeding of fasted animals induced an increase in gastric leptin expression level (Figure 4B), which was also opposite to the decreased gastric ghrelin expression level after food intake (Figure 4A).

In another study, relative changes in expressions of gastric ghrelin, aromatase and leptin in postnatal rats were investigated. We found that gastric ghrelin expression levels significantly increased from 2 wk of age to 4 wk of age. Similarly, gastric aromatase expression level was also significantly elevated in 4-wk-old rats compared with the level in 2-wk-old rats. In contrast, gastric leptin expression level decreased from 2 wk of age to 4 wk of age.

Although the mechanism underlying the fluctuations in gastric estrogen and leptin levels in a certain physiological state remains to be elucidated, these fluctuations in gastric estrogen and leptin levels together with the fact that estrogen and leptin produced in the stomach directly regulate gastric ghrelin expression led us to propose a regulatory model of stomach ghrelin expression in some physiological states. Under a basal (fed) condition, the expression of gastric ghrelin is maintained at a certain level due to a balance between positive regulation from gastric estrogen and negative regulation from gastric leptin. In postnatal development, gastric estrogen level increases and gastric leptin level decreases with increasing age, resulting in gradual elevation of ghrelin expression level during postnatal development. Similarly, under a fasting condition, when fasting reduces gastric leptin level with no change in gastric estrogen level, this balance is also broken by attenuated negative regulation due to decreased gastric leptin level and finally results in increased ghrelin expression. These two models do not, however, exclude the possibility of involvement of other factors such as neural control through the vagal nerve system or other hormones inside and outside the stomach such as gastric somatostatin and insulin. But, we believe that the balance control through gastric estrogen and leptin at least partially contributes to the altered ghrelin expression level in some physiological states.

CONCLUSION

In the gastrointestinal tract, ghrelin cells were identified as opened- and closed-type cells. The greatest number of ghrelin cells was found in the stomach, and it was found that the number of opened-type cells gradually increases in the direction from the stomach to the lower intestine. Stomach ghrelin cells in female rats differentiate at an earlier stage of development than they do in male rats. Gastric estrogen and leptin directly regulate ghrelin expression and production in the stomach, and the balance control through positive regulation from gastric estrogen and negative regulation from gastric leptin contributes to the altered ghrelin expression level in some physiological states. These findings provide new insights into the physiological regulation of stomach ghrelin, and may be important for the development of methods for controlling high ghrelin expression levels in some negative energy balance states, which directly contribute to increased food intake and adiposity. However, the regulatory mechanism of ghrelin is thought to be more complicated, and may involve other factors such as vagal activity and hormones outside the stomach. Further studies are necessary to elucidate the roles of these factors.

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REFERENCES

1 **Kojima M**, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660
2 **Yamazaki M**, Nakamura K, Kobayashi H, Matsubara M, Hayashi Y, Kangawa K, Sakai T. Regulational effect of ghrelin on growth hormone secretion from perifused rat anterior pituitary cells. *J Neuroendocrinol* 2002; **14**: 156-162
3 **Tschop M**, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000; **407**: 908-913
4 **Tschop M**, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML. Circulating ghrelin levels are decreased in human obesity. *Diabetes* 2001; **50**: 707-709
5 **Wren AM**, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 2000; **141**: 4325-4328
6 **Nakazato M**, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature* 2001; **409**: 194-198
7 **Shintani M**, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* 2001; **50**: 227-232
8 **Date Y**, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000; **141**: 4255-4261
9 **Asakawa A**, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 2001; **120**: 337-345
10 **Cummings DE**, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001; **50**: 1714-1719
11 **Toshinai K**, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, Kangawa K, Matsukura S. Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. *Biochem Biophys Res Commun* 2001; **281**: 1220-1225
12 **Lucidi P**, Murdolo G, Di Loreto C, De Cicco A, Parlanti N, Fanelli C, Santeusanio F, Bolli GB, De Feo P. Ghrelin is not necessary for adequate hormonal counterregulation of insulin-induced hypoglycemia. *Diabetes* 2002; **51**: 2911-2914
13 **Shimada M**, Date Y, Mondal MS, Toshinai K, Shimbara T, Fukunaga K, Murakami N, Miyazato M, Kangawa K, Yoshimatsu H, Matsuo H, Nakazato M. Somatostatin suppresses ghrelin secretion from the rat stomach. *Biochem Biophys Res Commun* 2003; **302**: 520-525
14 **Williams DL**, Grill HJ, Cummings DE, Kaplan JM. Vagotomy dissociates short- and long-term controls of circulating ghrelin. *Endocrinology* 2003; **144**: 5184-5187
15 **Ariyasu H**, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab* 2001; **86**: 4753-4758
16 **Sakata I**, Nakamura K, Yamazaki M, Matsubara M, Hayashi Y, Kangawa K, Sakai T. Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. *Peptides* 2002; **23**: 531-536
17 **Solcia E**, Rindi G, Buffa R, Fiocca R, Capella C. Gastric endocrine cells: types, function and growth. *Regul Pept* 2000; **93**: 31-35
18 **Lee HM**, Wang G, Englander EW, Kojima M, Greeley GH Jr. Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. *Endocrinology* 2002; **143**: 185-190
19 **Gualillo O**, Caminos JE, Kojima M, Kangawa K, Arvat E, Ghigo E, Casanueva FF, Dieguez C. Gender and gonadal influences on ghrelin mRNA levels in rat stomach. *Eur J Endocrinol* 2001; **144**: 687-690
20 **Sakata I**, Tanaka T, Matsubara M, Yamazaki M, Tani S, Hayashi Y, Kangawa K, Sakai T. Postnatal changes in ghrelin mRNA expression and in ghrelin-producing cells in the rat stomach. *J Endocrinol* 2002; **174**: 463-471
21 **Matsubara M**, Sakata I, Wada R, Yamazaki M, Inoue K, Sakai T. Estrogen modulates ghrelin expression in the female rat stomach. *Peptides* 2004; **25**: 289-297
22 **Ueyama T**, Shirasawa N, Numazawa M, Yamada K, Shelangouski M, Ito T, Tsuruo Y. Gastric parietal cells: potent endocrine role in secreting estrogen as a possible regulator of gastro-hepatic axis. *Endocrinology* 2002; **143**: 3162-3170
23 **Sakata I**, Tanaka T, Yamazaki M, Tanizaki T, Zheng Z, Sakai T. Gastric estrogen directly induces ghrelin expression and production in the rat stomach. *J Endocrinol* 2006; **190**: 749-757
24 **Friedman JM**, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998; **395**: 763-770
25 **Zhang Y**, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425-432
26 **Bado A**, Levasseur S, Attoub S, Kermorgant S, Laigneau JP, Bortoluzzi MN, Moizo L, Lehy T, Guerre-Millo M, Le Marchand-Brustel Y, Lewin MJ. The stomach is a source of leptin. *Nature* 1998; **394**: 790-793
27 **Zhao Z**, Sakata I, Okubo Y, Koike K, Kangawa K, Sakai T. Gastric leptin, but not estrogen and somatostatin, contributes to the elevation of ghrelin mRNA expression level in fasted rats. *J Endocrinol* 2008; **196**: 529-538
28 **Sobhani I**, Bado A, Vissuzaine C, Buyse M, Kermorgant S, Laigneau JP, Attoub S, Lehy T, Henin D, Mignon M, Lewin MJ. Leptin secretion and leptin receptor in the human stomach. *Gut* 2000; **47**: 178-183
29 **Wang MY**, Zhou YT, Newgard CB, Unger RH. A novel leptin receptor isoform in rat. *FEBS Lett* 1996; **392**: 87-90
30 **Kamegai J**, Tamura H, Shimizu T, Ishii S, Sugihara H, Oikawa S. Effects of insulin, leptin, and glucagon on ghrelin secretion from isolated perfused rat stomach. *Regul Pept* 2004; **119**: 77-81

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TOPIC HIGHLIGHT

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Feeding behavior and gene expression of appetite-related neuropeptides in mice lacking for neuropeptide Y Y5 receptor subclass

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Abstract

Neuropeptide Y (NPY) is a potent neurotransmitter for feeding. Besides NPY, orexigenic neuropeptides such as agouti-related protein (AgRP), and anorexigenic neuropeptides such as α-melatonin stimulating hormone (MSH) and cocaine-amphetamine-regulated transcript (CART) are also involved in central feeding regulation. During fasting, NPY and AgRP gene expressions are up-regulated and POMC and CART gene expressions are down-regulated in hypothalamus. Based on the network of peptidergic neurons, the former are involved in positive feeding regulation, and the latter are involved in negative feeding, which exert these feeding-regulated peptides especially in paraventricular nucleus (PVN). To clarify the compensatory mechanism of knock-out of NPY system on feeding, change in gene expressions of appetite-related neuropeptides and the feeding behavior was studied in NPY Y5-KO mice. Food intake was increased in Y5-KO mice. Fasting increased the amounts of food and water intake in the KO mice more profoundly. These data indicated the compensatory phenomenon of feeding behavior in Y5-KO mice. RT-PCR and ISH suggested that the compensation of feeding is due to change in gene expressions of AgRP, CART and POMC in hypothalamus. Thus, these findings indicated that the compensatory mechanism involves change in POMC/CART gene expression in arcuate nucleus (ARC). The POMC/CART gene expression is important for central compensatory regulation in feeding behavior.

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Key words: Neuropeptide Y; Y5 receptor; Feeding; Arcuate nucleus; Knockout mice

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INTRODUCTION

Feeding behavior is a complicated process to regulate the body weight. The body weight is determined by balance between calorie intake mainly by feeding and energy consumption including exercise, body temperature and metabolism. Obesity derived from excess of food intake results in the metabolic syndrome, diabetes mellitus and associated cardiovascular diseases. Since hyperphagia contributes onset and progression of the metabolic syndrome and so on, central feeding regulation is an important issue to be clarified[1,2].

Feeding behavior is regulated strictly by more than 20 appetite-related neuropeptides which are expressed in feeding center in central hypothalamus[1-4]. The main regulation to control feeding are the opposing controls between the orexigenic NPY (neuropeptide Y)/AgRP (agouti-related protein) neurons and the anorexigenic POMC (proopiomelanocortin)/CART (cocaine-amphetamine-regulated transcript) neurons, which originate from the arcuate nucleus (ARC) to the paraventricular nucleus (PVN)[3-6]. The main pathway of two opposite neuron groups obtain and integrate nutritional informations, and communicate information regarding nutrient status and energy stores to the second-order neurons of feeding regulation in PVN[3,5,6]. Among these appetite-related neuropeptides, NPY is the endogenous, strongest orexigenic neuropeptide and fasting induces the most re-

markable increase in NPY gene expression in hypothalamus, indicating its principal and physiological relevance in feeding regulation[3-7].

We have been studying the regulatory mechanism of NPY gene expression in hypothalamus, so as to elucidate the mechanism of feeding-related regulation of NPY gene expression[8-11]. Leptin is an anti-obesity hormone derived from adipose tissue and reduces the NPY gene expression in hypothalamus. This inhibition of NPY gene expression by leptin is shown to be due to activation of SOCS3 in the NPY neurons in arcuate nucleus[11].

In contrast the NPY receptors have been classified into at least 6 subclasses (Y1-y6). Y1, Y2, Y4, Y5, and y6 receptors have been cloned and Y1, Y2 and Y5 receptors are mainly involved in central feeding regulation in hypothalamic ARC and PVN[12-19]. NPY-induced marked induction of feeding behavior intracerebroventricular (icv) is mediated through mainly Y1 and Y5 receptors in mammalians[13,15,16]. Interestingly, although activation of Y1 and Y5 receptors is involved in NPY-induced hyperphagia, the Y1-KO (knockout) and Y5-KO mice develop the late-onset obesity with increase in food intake and adiposity, while NPY-KO mice did not change the feeding behavior or body weight[17,20]. This implies a compensatory mechanism in feeding behavior in these KO mice, which is important for understanding the long-term treatment with the current Y1 and Y5 receptor antagonists for anti-obesity drugs. In addition the compensation against the orexigenic NPY system has not been elucidated to date yet. Therefore, first we chose the Y5-KO mice to investigate the central compensatory mechanism for knockout of NPY system, since the expression of Y5 receptor is much restricted in the brain[16,18,21]. Then we characterized change in the feeding behavior and gene expressions of various appetite-related neuropeptides in the hypothalamus of the NPY Y5-KO mice.

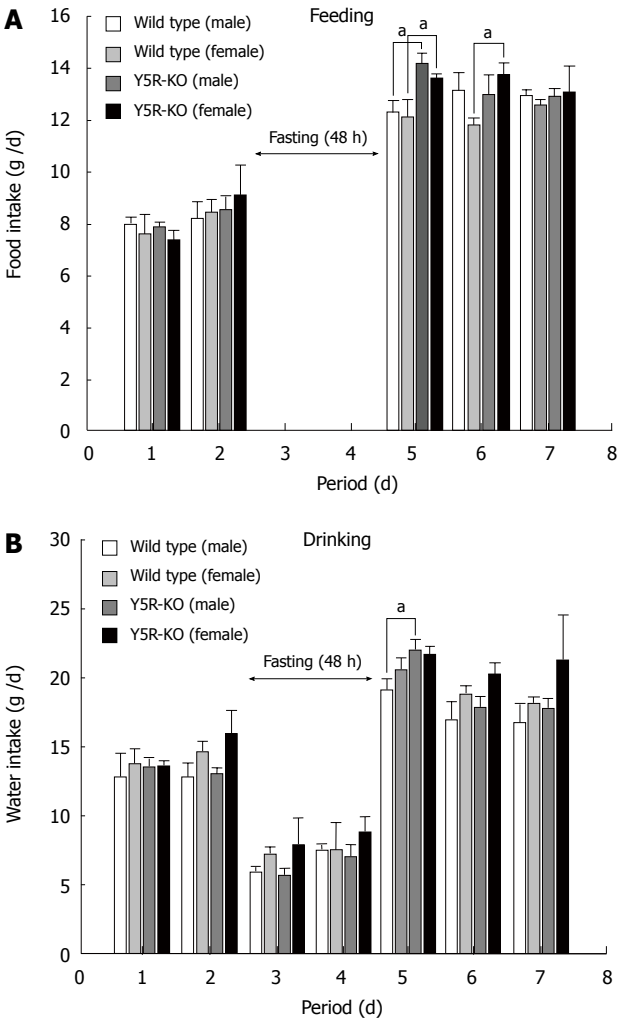

Figure 1 Fasting (48 h)-induced change in food intake (A) and water intake (B) in NPY Y5-KO mice. Fasting (48 h)-induced change in daily amounts of food intake and water intake in Y5-KO mice was measured and compared with those in wild-type (C57BL/6N) mice at 10 wk old. $^{a}P < 0.05$ (unpaired Student's *t* test).

FEEDING BEHAVIOR IN WILD-TYPE C57BL/6N MICE

NPY is the endogenous neuropeptide with most orexigenic potency in hypothalamus, and its injection icv or direct injection into PVN in hypothalamus produces marked increase in feeding behavior in rodents[13-16]. Fasting or central glucoprivation evoked by 2-deoxyglucose (2DG) induces eating with simultaneous increase in orexigenic NPY and AgRP gene expressions in ARC, followed by augmented NPY peptide release[7,8]. Since this fasting-induced food intake is significantly inhibited by selective Y1 or Y5 antagonists, the induction is mediated through Y1 and Y5 receptors (apparently, mainly through Y1 receptors in mice)[13,16]. This NPY/AgRP neurons are essential for feeding in adult mice[22].

In our experiments with male 10-wk-old wild-type mice, fasting for 48 h increased daily food intake and daily water intake by 53% and 50%, respectively (Figure 1). The increase in food intake followed by the concomitant increase in the orexigenic NPY and AgRP gene expressions and decrease in the anorexigenic POMC gene expression in ARC[4]. RT-PCR indicated that NPY and AgRP gene expression is increased remarkably and galanin gene expression is increased moderately, while orexin, MCH, CART or POMC mRNA did not change significantly. These findings suggested that fasting-induced food intake is involved markedly in NPY/AgRP gene expression and moderately in galanin gene expression in ARC in wild-type mice. NPY and AgRP coexist in the same neurons in ARC, and NPY and AgRP gene expressions were increased simultaneously and remarkably by fasting[4,7,8].

In contrast to the concept that the NPY system plays a pivotal role in central feeding regulation, the following studies have performed that in the NPY-KO and NPY Y1-KO mice their body weights or feeding behaviors did not change or that in NPY Y5-KO mice the body weight and food intake increased conversely[17,18,20]. This suggested the existence of a compensatory mechanism other than the NPY system. The existence of multiple

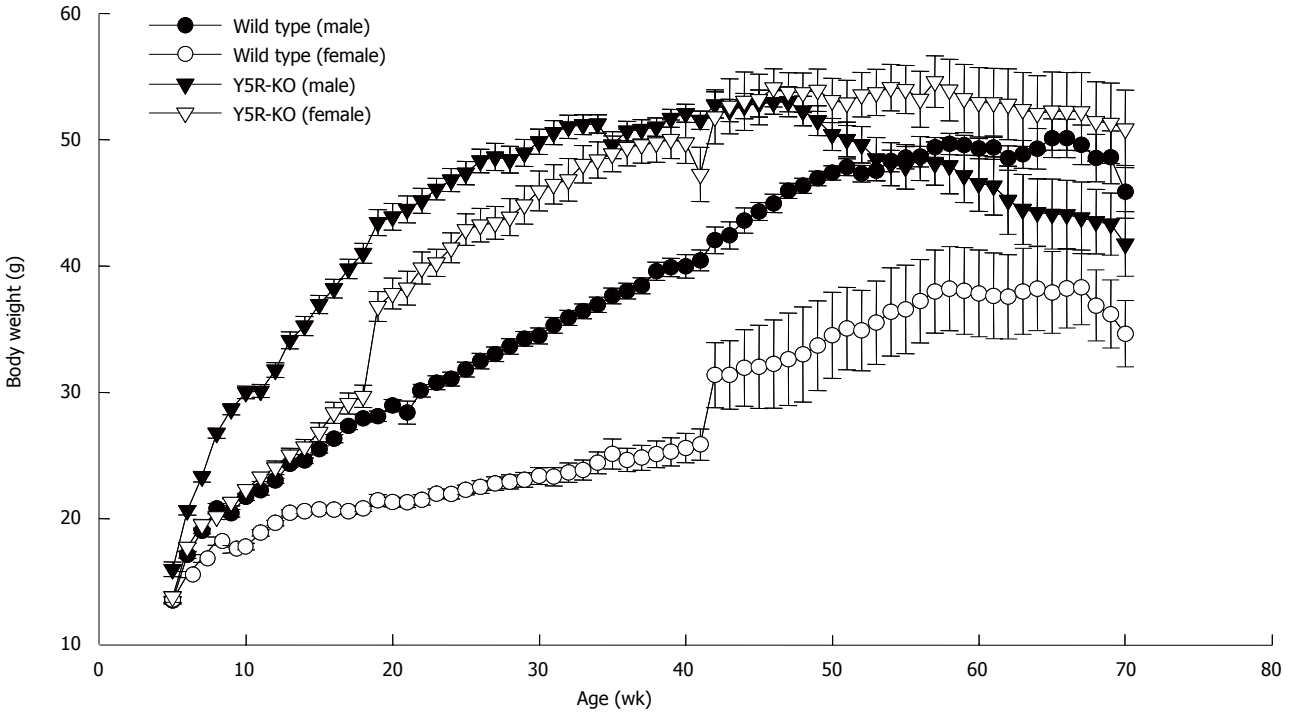

Figure 2 Growth curves of wild-type (C57BL/6N) and NPY Y5-KO mice from 4 to 69 wk old. Data are the mean ± SE. From 4 to 40 wk old, the body weight of Y5-KO mice was twice as much as that of wild-type mice.

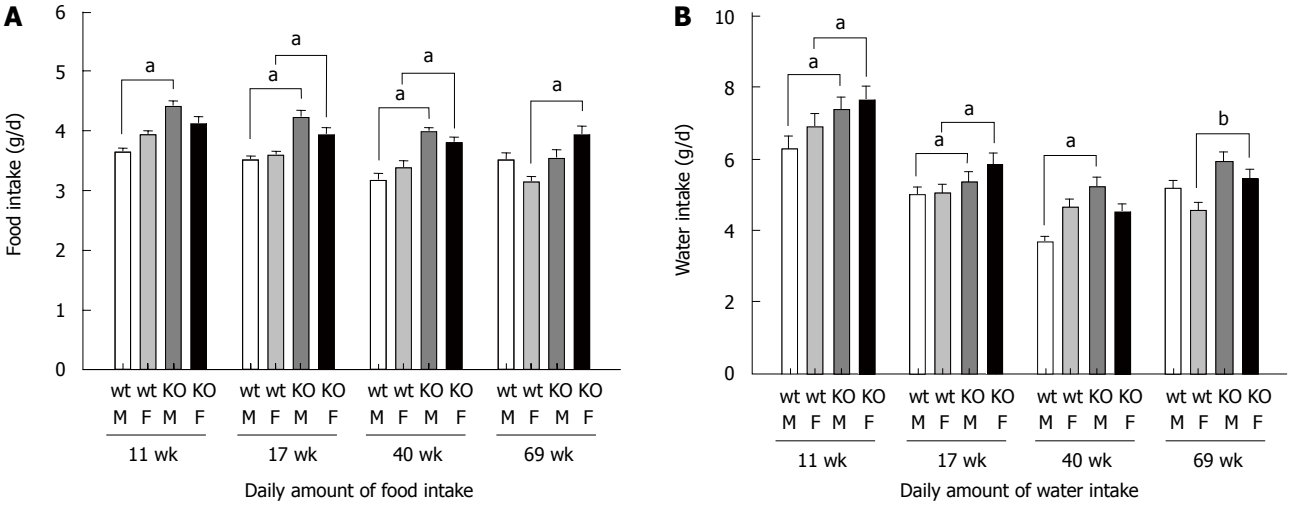

Figure 3 Change in feeding and drinking in NPY Y5-KO mice. Daily amounts of food intake (A) and water intake (B) in Y5-KO mice were measured and compared with those in wild-type (C57BL/6N) mice at the same age (from 11 to 69 wk old). $^{a}P < 0.05$, $^{b}P < 0.01$ (unpaired Student's *t* test). M: Male; F: Female.

feeding-regulatory systems is very important for the homeostasis of body weight. Therefore, we tried to elucidate the compensatory system except for NPY by using NPY Y5-KO mice.

COMPENSATORY FEEDING BEHAVIOR IN NPY Y5-KO MICE

As shown in Figure 2, growth curve of wild-type and NPY Y5-KO mice from 4 to 69 wk old. Although NPY is an orexigenic peptide, obviously Y5-KO mice have obese phenotype. From 4 to 40 wk old, the body weight of Y5-KO mice was twice as much as that of wild-type mice. The increase was observed in both male and female gender but more remarkably in female gender. Because NPY is related with regulation of release of female gonadotropins from pituitary glands, and because female in human being tends to be fatty after menopause, activation through Y5 receptors may be involved in suppression of onset and progression of obesity in female.

As shown in Figure 3, when Y5-KO mice were freely fed, their daily food intake and daily water intake were significantly higher than those of wild-type mice at any age. This suggested that overeating in Y5-KO mice produced obesity of the mice. Next, we measured the fasting (48 h)-induced change in food intake in Y5-KO mice

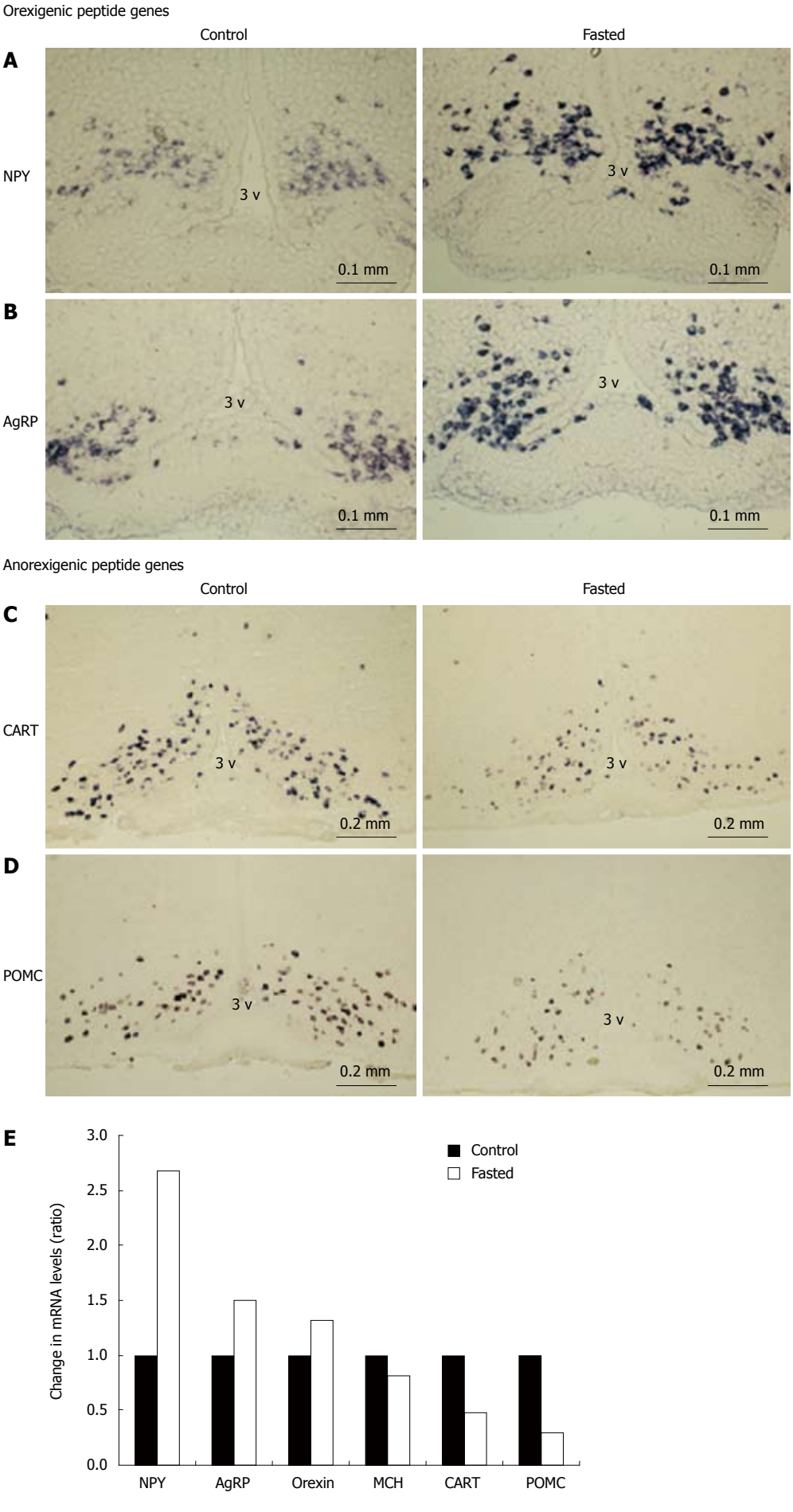

Figure 4 Effect of fasting on appetite-related gene expression in hypothalamus. Fasting (48 h)-induced change in gene expression of appetite-related genes (A: NPY; B: AgRP; C: CART; D: POMC) in Y5-KO mice was measured in the hypothalamus and change in signal intensity was quantitated in panel E at 10 wk old. 3v: Third ventricle; NPY: neuropeptide Y; AgRP: agouti-related protein; MCH: melanin-concentrating hormone; CART: cocaine-amphetamine-regulated transcript; POMC: proopiomelanocortin.

(Figure 1A). Compared with fasting-induced food intake in wild-type mice, obviously fasting-induced feeding was augmented by about 2 times. The fasting-induced water intake in Y5-KO mice was significantly increased following increase in food intake (Figure 1B). Thus, obesity in NPY Y5-KO mice is probably due to overeating *ad libitum* and on fasting.

To deny the possibility that obesity might be due to decrease in energy consumption, the decrease rate in body weight was studied during fasting (48 h) in Y5-KO mice. The body weght loss in 2 d of Y5-KO mice was almost the same to that in wild-type mice (data not shown). This indicated that the energy consumption rate is not changed in Y5-KO mice, but the obesity in Y5-KO mice is simply due to increased food intake *ad libitum* and on fasting.

CHANGE IN GENE EXPRESSION OF APPETITE-RELATED PEPTIDES IN HYPOTHALAMUS

The compensatory feeding behavior might be due to change in gene expressions of appetite-related neuropeptides other than NPY. Therefore, the change in gene expression of feeding-regulating peptides in hypothalamus was investigated, first, when NPY Y5-KO mice were fed freely. Under ordinary conditions in NPY Y5-KO mice the NPY and AgRP gene expressions were diminished probably due to disuse[4]. The decrease in NPY and AgRP gene expression which expresses in the same neuron group in ARC may be due to increased leptin level following obesity in Y5-KO mice. The gene expression of orexin, MCH, or CART was not changed in hypothalamus; but the POMC gene expression was significantly decreased by RT-PCR and ISH, suggesting that the synthesis of anorexigenic POMC-derived peptides such as -MSH is decreased. This decrease in POMC gene expression appears to be the principal cause of the compensatory overeating.

AUGMENTATION OF FASTING-INDUCED CHANGE IN GENE EXPRESSION IN NPY Y5-KO MICE

Fasting for 48 h produces augmented the fasting-induced food intake, with the concomitant increase in the orexigenic NPY and AgRP gene expressions and decrease in the anorexigenic POMC gene expression in ARC in wild-type mice[4]. Obviously the fasting-induced feeding behavior is augmented in Y5-KO mice (Figure 1). Next we investigated whether changes in gene expressions of appetite-regulated neuropeptides in hypothalamus are involved in 48 h-fasting-induced induced feeding behavior (Figure 4). NPY and AgRP gene expressions were induced by 48 h-fasting more profoundly in NPY Y5-KO mice than those in wild-type mice (Figure 4A and B). In contrast, CART and POMC gene expression were conversely decreased more markedly by fasting in Y5-KO mice. In contrast orexin and MCH (melanin-concentrating hormone) gene expressions were not changed by fasting in the mouse hypothalamus (Figure 4E). Thus, the augmentation of fasting-induced feeding behavior in NPY Y5-KO mice was accompanied by the concomitant fasting-induced changes in NPY/AgRP (both increase) and POMC/CART (both decrease) gene expression. Because the NPY system probably dysfunctions in NPY Y5-KO mice, the concomitant increase in AgRP gene expression and decrease in POMC and CART gene expression are the cause of fasting-induced augmentation of feeding behavior in NPY Y5-KO mice. The compensatory mechanism of feeding is probably due to overfunction of compensation by POMC and partly CART gene expressions which results in the late-onset obesity in Y5-KO mice.

CONCLUSION

Feeding behavior and energy balance are regulated in complicated manner by networks of neurons with classical neurotransmitters and appetite-related peptides. In this article, we showed that the compensatory feeding behavior occurs in NPY Y5-KO mice when the NPY system is probably inhibited, so that the late-onset obesity has appeared. This is probably due to the compensatory change in POMC gene expression. At present the mechanism of compensatory change in POMC/AgRP gene expression is still unknown, and remains to be clarified. This compensatory mechanism is not dependent on the technical procedure of knockout mice. Questions regarding when the compensatory mechanism was been completed, and whether the compensation might occur essential only in adult brain[22,23].

The investigation about the regulation of NPY, AgRP and POMC gene expression in hypothalamic nuclei is useful for elucidation of central feeding regulation and also for development of novel anti-obesity drugs in future.

REFERENCES

1 **Schwartz MW**, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000; **404**: 661-671
2 **Morton GJ**, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. *Nature* 2006; **443**: 289-295
3 **Murphy KG**, Bloom SR. Gut hormones and the regulation of energy homeostasis. *Nature* 2006; **444**: 854-859
4 **Higuchi H**, Yamaguchi T, Niki T. [Regulation of hypothalamic neuropeptide expression and feeding behavior in NPY-Y5 knockout (KO) mice] *Nippon Yakurigaku Zasshi* 2006; **127**: 92-96
5 **Kalra SP**, Kalra PS. Neuropeptide Y: a physiological orexigen modulated by the feedback action of ghrelin and leptin. *Endocrine* 2003; **22**: 49-56
6 **O'Rahilly S**, Yeo GS, Farooqi IS. Melanocortin receptors weigh in. *Nat Med* 2004; **10**: 351-352
7 **Bertile F**, Oudart H, Criscuolo F, Maho YL, Raclot T. Hypothalamic gene expression in long-term fasted rats: relationship with body fat. *Biochem Biophys Res Commun* 2003; **303**: 1106-1113

8 **Minami S**, Kamegai J, Sugihara H, Suzuki N, Higuchi H, Wakabayashi I. Central glucoprivation evoked by administration of 2-deoxy-D-glucose induces expression of the c-fos gene in a subpopulation of neuropeptide Y neurons in the rat hypothalamus. *Brain Res Mol Brain Res* 1995; **33**: 305-310
9 **Higuchi H**, Nakano K, Kim CH, Li BS, Kuo CH, Taira E, Miki N. Ca2+/calmodulin-dependent transcriptional activation of neuropeptide Y gene induced by membrane depolarization: determination of Ca(2+)- and cyclic AMP/phorbol 12-myristate 13-acetate-responsive elements. *J Neurochem* 1996; **66**: 1802-1809
10 **Muraoka O**, Xu B, Tsurumaki T, Akira S, Yamaguchi T, Higuchi H. Leptin-induced transactivation of NPY gene promoter mediated by JAK1, JAK2 and STAT3 in the neural cell lines. *Neurochem Int* 2003; **42**: 591-601
11 **Higuchi H**, Hasegawa A, Yamaguchi T. Transcriptional regulation of neuronal genes and its effect on neural functions: transcriptional regulation of neuropeptide Y gene by leptin and its effect on feeding. *J Pharmacol Sci* 2005; **98**: 225-231
12 **Woldbye DP**, Larsen PJ. The how and Y of eating. *Nat Med* 1998; **4**: 671-672
13 **Iyengar S**, Li DL, Simmons RM. Characterization of neuropeptide Y-induced feeding in mice: do Y1-Y6 receptor subtypes mediate feeding? *J Pharmacol Exp Ther* 1999; **289**: 1031-1040
14 **Yokosuka M**, Dube MG, Kalra PS, Kalra SP. The mPVN mediates blockade of NPY-induced feeding by a Y5 receptor antagonist: a c-FOS analysis. *Peptides* 2001; **22**: 507-514
15 **Gerald C**, Walker MW, Criscione L, Gustafson EL, Batzl-Hartmann C, Smith KE, Vaysse P, Durkin MM, Laz TM, Linemeyer DL, Schaffhauser AO, Whitebread S, Hofbauer KG, Taber RI, Branchek TA, Weinshank RL. A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature* 1996; **382**: 168-171
16 **Kanatani A**, Mashiko S, Murai N, Sugimoto N, Ito J, Fukuroda T, Fukami T, Morin N, MacNeil DJ, Van der Ploeg LH, Saga Y, Nishimura S, Ihara M. Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice. *Endocrinology* 2000; **141**: 1011-1016
17 **Marsh DJ**, Hollopeter G, Kafer KE, Palmiter RD. Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nat Med* 1998; **4**: 718-721
18 **Pedrazzini T**, Seydoux J, Kunstner P, Aubert JF, Grouzmann E, Beermann F, Brunner HR. Cardiovascular response, feeding behavior and locomotor activity in mice lacking the NPY Y1 receptor. *Nat Med* 1998; **4**: 722-726
19 **Naveilhan P**, Hassani H, Canals JM, Ekstrand AJ, Larefalk A, Chhajlani V, Arenas E, Gedda K, Svensson L, Thoren P, Ernfors P. Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor. *Nat Med* 1999; **5**: 1188-1193
20 **Erickson JC**, Clegg KE, Palmiter RD. Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature* 1996; **381**: 415-421
21 **Huang XF**, Han M, Storlien LH. The level of NPY receptor mRNA expression in diet-induced obese and resistant mice. *Brain Res Mol Brain Res* 2003; **115**: 21-28
22 **Luquet S**, Perez FA, Hnasko TS, Palmiter RD. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 2005; **310**: 683-685
23 **Melnick I**, Pronchuk N, Cowley MA, Grove KL, Colmers WF. Developmental switch in neuropeptide Y and melanocortin effects in the paraventricular nucleus of the hypothalamus. *Neuron* 2007; **56**: 1103-1115

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TOPIC HIGHLIGHT

Akio Inui, MD, PhD, Professor, *Series Editor*

Different effects of ghrelin, des-acyl ghrelin and obestatin on gastroduodenal motility in conscious rats

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Abstract

Three peptides, ghrelin, des-acyl ghrelin and obestatin are derived from a common prohormone, preproghrelin by posttranslational processing, originating from endocrine cells in the stomach. To examine the effects of these peptides, we applied the manometric measurement of gastrointestinal motility in freely moving conscious rat models. Ghrelin exerts stimulatory effects on the motility of antrum and duodenum in both fed and fasted state of animals. Des-acyl ghrelin exerts inhibitory effects on the motility of antrum, but not on the motility of duodenum in the fasted state of animals. Obestatin exerts inhibitory effects on the motility of antrum and duodenum in the fed state, but not in the fasted state of animals. NPY Y2 or Y4 receptors in the brain may mediate the action of ghrelin, CRF type 2 receptors in the brain mediate the action of des-acyl ghrelin, whereas CRF type 1 and type 2 receptors in the brain mediate the action of obestatin. Vagal afferent pathways might be involved in the action of ghrelin, but not involved in the action of des-acyl ghrelin, whereas vagal afferent pathways might be partially involved in the action of obestatin.

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Key words: Ghrelin; Des-acyl ghrelin; Obestatin; Gastrointestinal motility; Hypothalamus

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INTRODUCTION

Ghrelin, des-acyl ghrelin and obestatin are derived from a prohormone, preproghrelin by posttranslational processing. Ghrelin was first identified as endogenous ligand for growth hormone secretagogue receptors (GHS-R) with O-n-octanoyl acid modification at serine 3 position[1]. On the other hand, des-acyl ghrelin has no O-n-octanoyl acid modification[1]. Obestatin was predicted to be formed from preproghelin by a bioinformatic approach[2]. Obestatin was initially reported to be endogenous ligand for orphan G protein-coupled receptor GPR39[2]; however, recent studies have found no specific binding of obestatin to various types of GPR39-expressing cells[3-5]. Ghrelin is a potent stimulator of food intake and gastrointestinal motility[6], while des-acyl ghrelin exerts opposite effects on food intake and gastrointestinal motility[7]. The effects of obestatin on food intake and gastrointestinal motility have been controversial[8-13]. Very recently we have reported that obestatin exerts inhibitory action on gastroduodenal motility in the fed state of conscious rats[14]. Previous studies have shown that food intake and gastroduodenal motility are tightly related. For example, feeding stimulatory peptides such as NPY and ghrelin stimulate gastroduodenal motility[15,16], while feeding inhibitory peptides such as CRF and urocortin inhibit the gastroduodenal motility[17]. Here, we overview different effects of ghrelin, des-acyl ghrelin and obestatin on gastroduodenal motility by using freely moving conscious rat models.

MANOMETRIC MEASUREMENT OF GASTROINTESTINAL MOTILITY IN CONSCIOUS RATS

We developed freely moving conscious rat model to

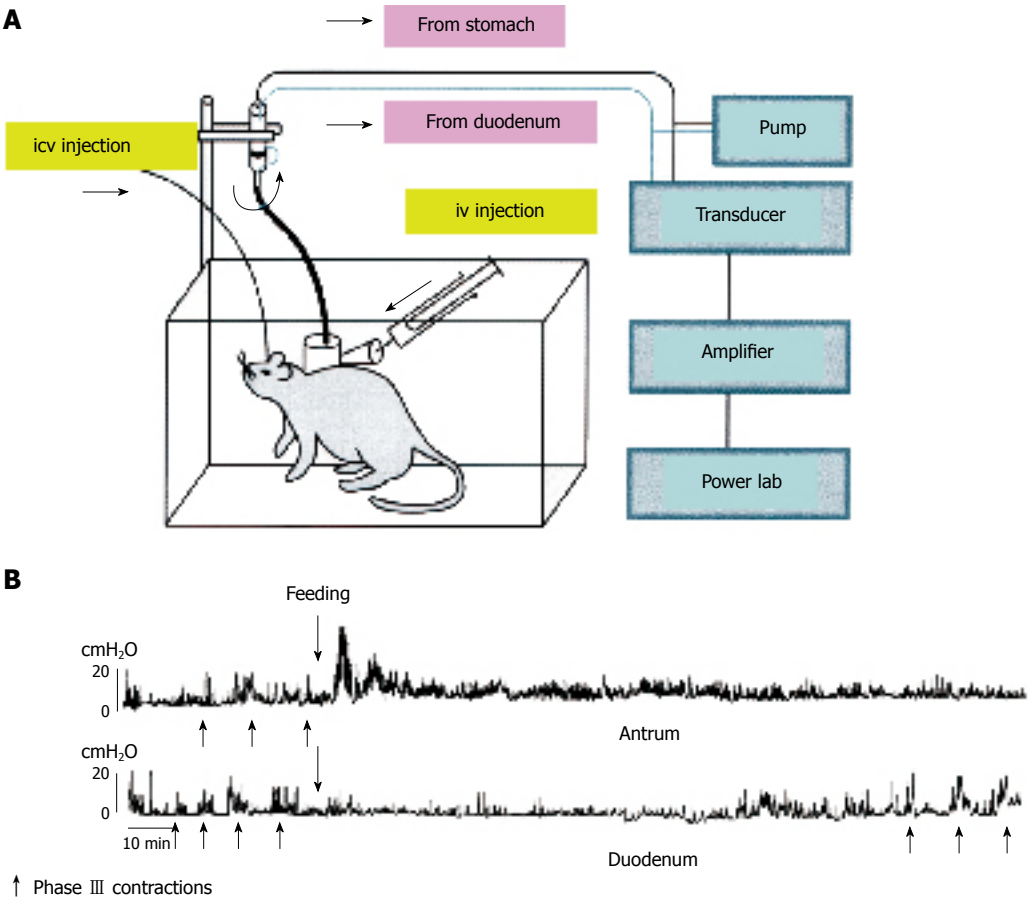


Figure 1 Measurement of gastrointestinal motility. A: Manometric measurement of gastroduo-denal motility in freely moving conscious rats; B: Fasted and fed motor activities in the antrum and duodenum. Phase Ⅲ-like contractions are indicated by arrows.

measure the gastrointestinal motility[15] (Figure 1A). This model permits the measurement of gastrointestinal motility in animals in the physiological fed and fasted states by a manometric method[15]. In the fasted state, the cyclic change of pressure waves were detected in both antrum and duodenum, including the quiescence period during which relatively low amplitude contractions occur (phase I-like contractions), followed by a grouping of strong contractions (phase Ⅲ-like contractions) (Figure 1B). The frequency of the onset of phase Ⅲ-like contractions was 5.3 ± 0.5/h (n = 6) in the antrum and 5.6 ± 0.8/h (n = 6) in the duodenum[16]. After food intake, such fasted motor pattern was disrupted and replaced by a fed motor pattern, which consisted of irregular contractions of high frequency (Figure 1B). The fed pattern continued for 85.7 ± 6.8 min (n = 5) in the duodenum and for more than 240 min in the antrum when rats were given 3 g of chow, and then replaced by the fasted motor pattern[14].

GHRELIN AND GASTRODUODENAL MOTILITY

Intracerebroventricular (icv) and intravenous (iv) injection of ghrelin stimulated the % motor index (%MI) in the antrum and induced the fasted motor activity in the duodenum when given in the fed state of animals[16] (Figure 2A). Icv and iv injection of ghrelin increased the frequency of phase Ⅲ-like contractions in both antrum and duodenum when given in the fasted state of animals[16]. The effects of iv injection of ghrelin on gastroduodenal motility were blocked by iv injection of GHS-R antagonist, but not by icv injection of GHS-R antagonist[16] (Figure 2B). In vagotomized animals, iv injection of ghrelin-induced the fasted motility in both antrum and duodenum when given in the fed state, iv injection of GHS-R antagonist completely blocked phase Ⅲ-like contractions in both antrum and duodenum[16]. Immunoneutralization of NPY in the brain blocked the stimulatory effects of ghrelin on the gastroduodenal motility[16] (Figure 2C). These results indicate that ghrelin released from the stomach may act on the ghrelin receptor on vagal afferent nerve terminals and NPY neurons in the brain may mediate the action of ghrelin on the gastroduodenal motility. *C-Fos* expression in the arcuate nucleus (ARC) in the hypothalamus and in the nucleus tractus solitarius (NTS) induced by intraperitoneal (ip) injection of ghrelin confirmed this effect (Figure 2D). Our previous study showed that immunoneutralization of NPY in the brain completely blocked the phase Ⅲ-like contractions in the duodenum of normal rats, and Y2 and Y4 receptor agonists induced the phase Ⅲ-like contractions in the duodenum when given in the fed state of animals[15]. Combined together, in normal animals ghrelin may stimulate gastroduodenal motility by activating the GHS-R on vagal afferent nerve terminals and affect NPY neurons in the hypothalamus, Y2 and/or Y4 receptors in the brain may mediate the action of ghrelin (Figure 3). Once the brain mechanism is eliminated by truncal vagotomy, ghrelin might be primarily involved in the regulation of fasted molility through GHS-R on the stomach and duodenum.

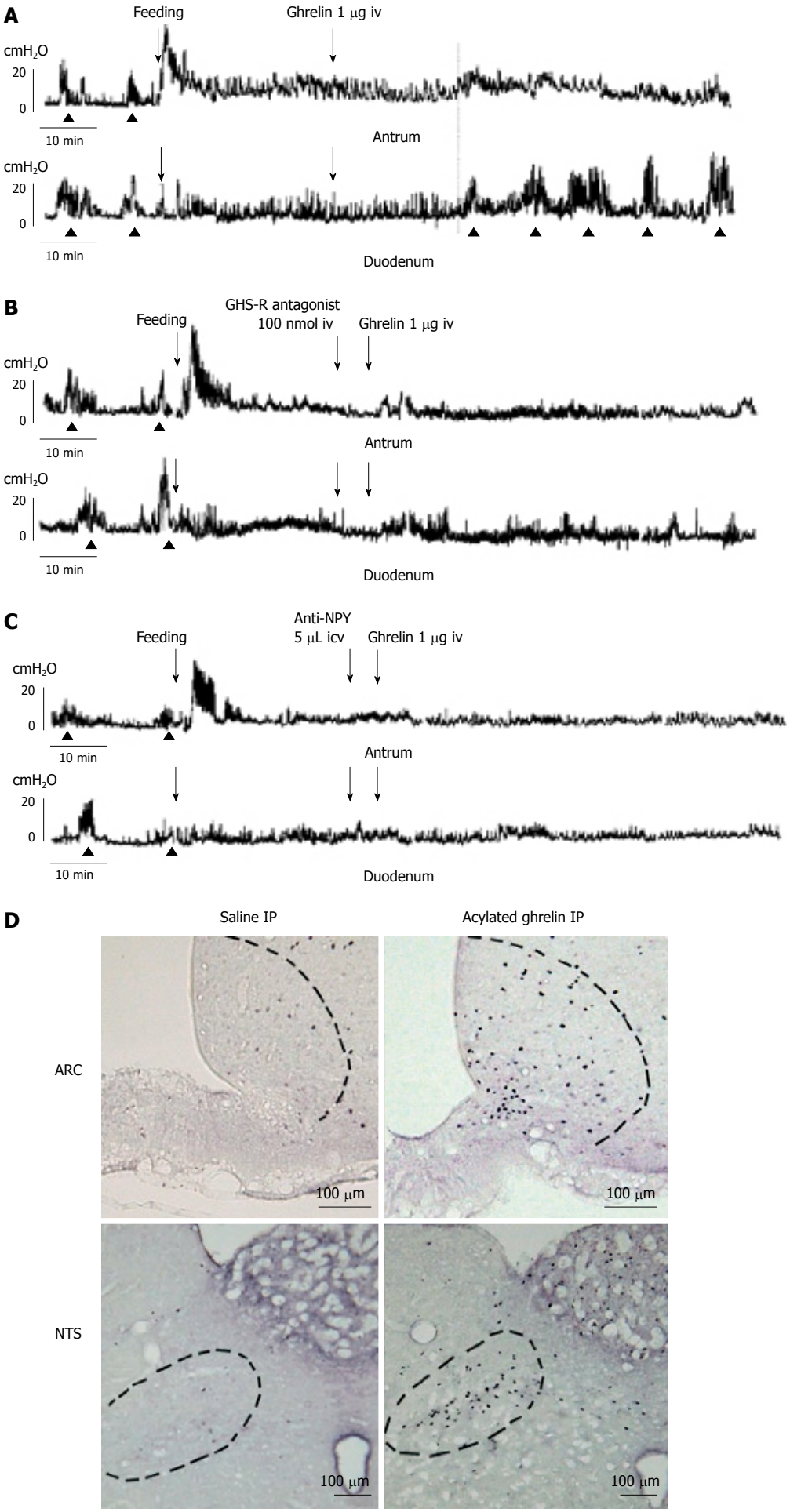
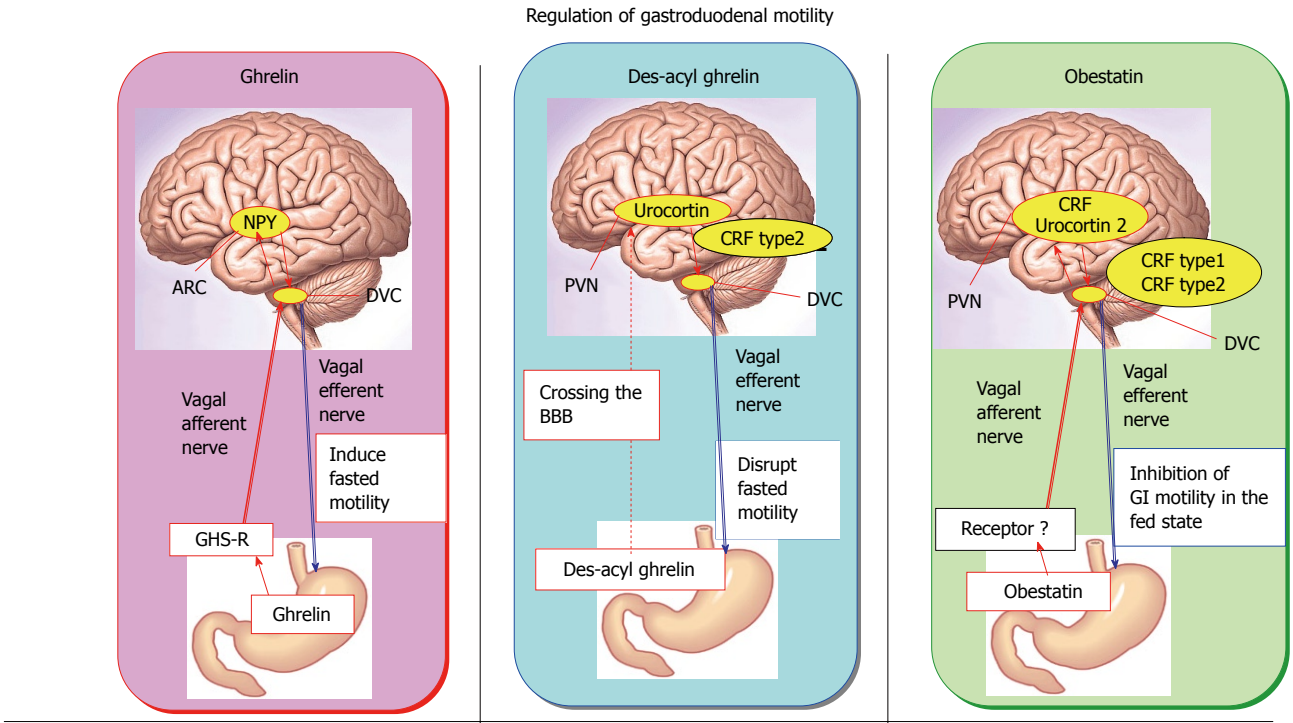


Figure 2 Ghrelin and gastroduodenal motility. A: Effects of iv injection of ghrelin on the fed motor activity of the antrum and duodenum. Iv injection of ghrelin induces the fasted pattern in the duodenum and increases the motor activity in the antrum; B: Iv injection of GHS-R antagonist completely blocks the effect of iv injection of ghrelin; C: Icv injection of NPY antiserum completely blocks the effect of iv injection of ghrelin; D: The density of c-Fos-positive cells in the arcuate nucleus (ARC) and nucleus tractus solitarius (NTS) increases with ip injection of ghrelin compared to saline-injected control.



	Ghrelin		Des-acyl ghrelin		Obestatin	
	Fasted state	Fed state	Fasted state	Fed state	Fasted state	Fed state
Stomach	↑	↑	↓	—	—	↓
Duodenum	↑	↑	↓	—	—	↓
Hypothalamus neuron	NPY		Urocortin		CRF, urocortin	
Brain receptor	Y2, Y4		CRF type 2		CRF type 1, type 2	
Vagal afferent	+		-		+	

Figure 3 Summary diagram of different effects of ghrelin, des-acyl ghrelin and obestatin on the gastroduodenal motility and brain mechanisms mediating the action of these peptide.

Human ghrelin has a structural resemblance to human motilin, and human ghrelin receptors exhibit a 50% identity with human motilin receptors[18]. Therefore, the role of ghrelin in the gastrointestinal motility is comparable with that of motilin[19,20]. Motilin originates from the endocrine cells in the duodenum[19], while ghrelin originates from the endocrine cells in the stomach[21], both of them are involved in the regulation of phase Ⅲ contractions in the gastrointestinal tracts. Motilin induces fasted motility in the stomach and duodenum when it is given peripherally, but not when given centrally[20,22], while ghrelin induces fasted motility in the duodenum when it is given both peripherally and centrally[16]. Since it is known that gastric acidification modulates the action of motilin[23], we examined the relationship between the effects of ghrelin on gastroduodenal motility and intragastric pH. The results showed that within 30 min after feeding, low intragastric pH (pH 2.5 ± 0.2) inhibited the effects iv injected ghrelin on gastroduodenal motility, and that this effect was reversed by an increase of intragastric pH (pH 5.4 ± 0.6) within 60 min after feeding, or by pretreatment of famotidine (intragastric pH 6.0-6.7)[16]. These results suggest that the sensitivity of the GHS-R in the gastrointestinal tract might be inhibited by low intragastric pH.

DES-ACYL GHRELIN AND GASTRODUODENAL MOTILITY

Central and peripheral administration of des-acyl ghrelin has been shown to significantly decrease food intake in food-deprived mice and decrease gastric emptying[6]. Transgenic mice with overexpression of the des-acyl ghrelin gene exhibited a decrease in body weight, food intake and fat mass weight accompanied by moderately decreased linear growth compared with their nontransgenic littermates[6]. In rats, des-acyl ghrelin injected intraperitoneally (ip) effectively decreased food intake in food-deprived rats, and decreased the dark-phase food intake in free-feeding rats, but failed to decrease the light-phase food intake in free-feeding rats[7].

Icv and iv injections of des-acyl ghrelin disrupted fasted motility in the antrum, but not in the duodenum[7] (Figire 4A). The frequencies of fasted motility in the antrum were decreased to 58.9% and 54.5% by des-acyl ghrelin injcted icv and iv, respectively[7]. However icv and iv injections of des-acyl ghrelin did not alter fed motor activity in both the antrum and duodenum[7] (Figure 4A). These data indicate that the dominant role of exogenous des-acyl ghrelin affects fasted motility in the antrum, but not in the duodenum. The results showed that capsaicin

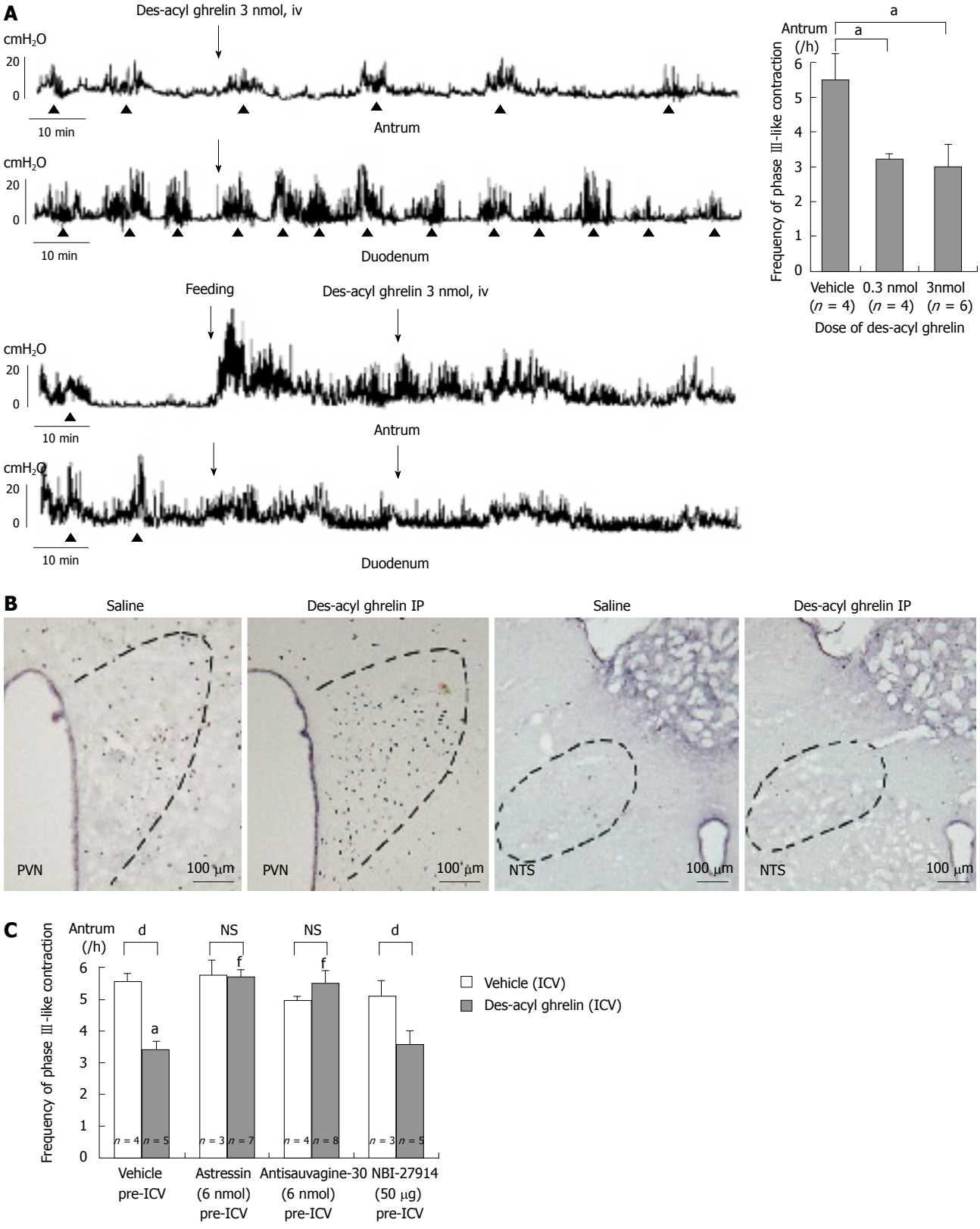


Figure 4 Des-acyl ghrelin and gastroduodenal motility. A: Effects of iv injection of des-acyl ghrelin on the fasted and fed motor activities of the antrum and duodenum. Iv injection of des-acyl ghrelin decreases the frequency of phase Ⅲ-like contractions in the antrum, but not in the duodenum. Iv injection of des-acyl ghrelin does not affect fed motor activity in both antrum and duodenum. [a]$P < 0.05$; B: The density of c-Fos-positive cells in the paraventricular nucleus (PVN) is increased by ip injection of des-acyl ghrelin compared to saline-injected control, whereas that in the NTS is not altered; C: The decreased frequency of phase Ⅲ-like contractions induced by iv injection of des-acyl ghrelin is restored to normal in pretreatment of nonselective CRF receptor antagonist astressin and the selective CRF type 2 receptor antagonist antisauvagine-30, but not CRF type 1 receptor antagonist NBI-27914. [d]$P < 0.01$, [f]$P < 0.001$ compared with a.

treatment did not alter the disruptive effect of iv injection of des-acyl ghrelin on fasted motility in the antrum[7]. These results were consistent with electrophysiological studies, which showed that peripheral administration of

ghrelin suppressed firing of the vagal afferent pathways, whereas des-acyl ghrelin had no effect on vagal afferent pathways[24]. Difference in the involvement of vagal afferent pathways in the action of ghrelin and des-acy ghrelin was confirmed by c-*Fos* expression in the NTS. Ip injection of ghrelin significantly increased the density of c-*Fos*-positive cells in the NTS (Figure 2D), while ip injection of des-acyl ghrelin induced no change in the density of c-*Fos*-positive cells in the NTS compared with vehicle-injected controls[7] (Figure 4B). Taken together, these results suggest that peripherally administered des-acyl ghrelin may cross the blood-brain barrier (BBB) and act directly on the brain receptor and disrupt the fasted motility in the antrum (Figure 3).

The results showed that the centrally administered CRF type 2 receptor antagonist, but not the CRF type 1 receptor antagonist, blocked the effects of centrally and peripherally administered des-acyl ghrelin on gastric motility[7] (Figure 4C). Among two CRF receptor subtypes, CRF type 1 receptor is highly involved in anxiety-related behavior and CRF type 2 receptor is involved in regulating food intake and peripheral functions such as gastric acid secretion or gastric emptying. CRF is a relatively selective ligand for CRF type 1 receptor, whereas urocortin is a ligand more selective for CRF type 2 receptor[25,26]. The density of c-*Fos*-positive cells in the paraventricular nucleus (PVN) was significantly increased by ip injection of des-acyl ghrelin compared to vehicle-injected controls[7] (Figure 4B). These data suggest that peripherally administered des-acyl ghrelin may activate neurons in the PVN by crossing the BBB, and exert inhibitory effects on the antral motility *via* CRF type 2 receptor in the brain (Figure 3).

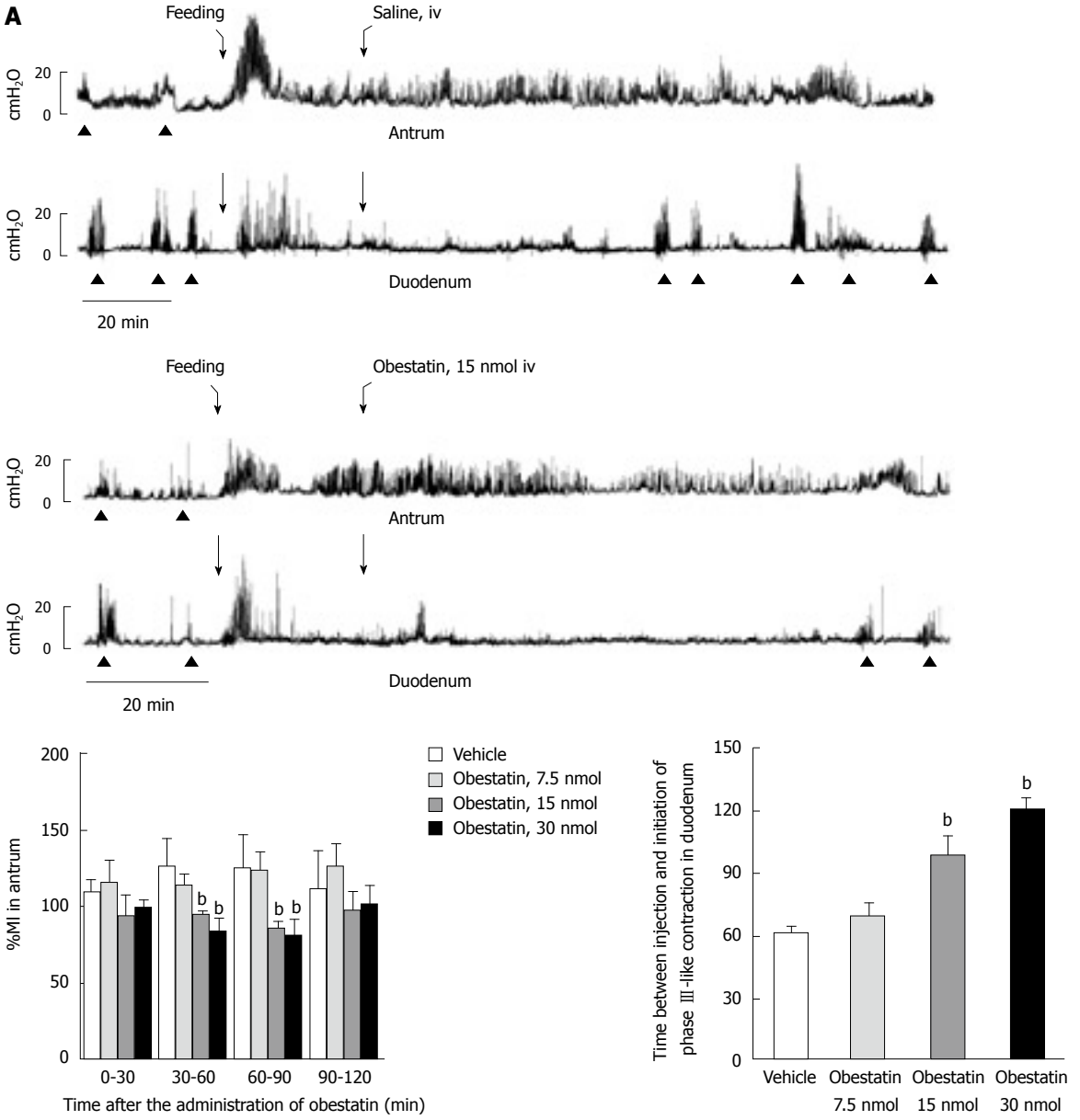
OBESTATIN AND GASTRODUODENAL MOTILITY

Zhang *et al*[2] first reported that ip injection of obestatin suppressed cumulative food intake, decreased body weight gain, and inhibited gastric emptying and jejunal muscle contraction in mice. Since then, however, the inhibitory effects of obestatin on food intake and gastrointestinal motility have remained controversial[8-13]. Most of the previous studies which showed the negative effects of obestatin on the gastrointestinal motility have only measured the gastric emptying or MMC cycle time as indices for motor activity. In our previous study, for more precise analysis, motor activity in both fed and fasted states was quantified by the %MI, and we measured the time taken to the initiation of phase Ⅲ-like contractions in the antrum and duodenum of conscious rats[14].

Results showed that motor activity in the antrum and duodenum was inhibited when obestatin was given iv to conscious rats in the fed state, but not when it was given in the fasted state[14]. Iv injection of obestatin decreased the %MI of fed motility in the antrum and prolonged the time before the return of fasted motility in the duodenum[14] (Figure 5A). Such inhibitory actions were the opposite of those obtained with ghrelin[16]. The results showed that the inhibitory action of obestatin appeared 30-90 min after iv injection[14] (Figure 5A), which is consistent with the timing of the effects of iv injection of ghrelin (approximately 30 min) on gastroduodenal motility[16] (Figure 2A). Iv injection of obestatin induced a significant increase in the number of c-*Fos*-positive cells in the PVN compared to saline-injected controls[14] (Figure 5B). Immunofluorescence overlap staining showed that the PVN neurons activated by iv injection of obestatin contain CRF or urocortin 2[14] (Figure 5B). The involvement of CRF type 1 and type 2 receptors in the action of obestatin on the gastroduodenal motility was examined[14]. Results showed that the inhibitory action of iv injection of obestatin on the motor activities in the antrum and duodenum were blocked by icv injection of CRF type 1 and type 2 receptor antagonists, suggesting that both types of CRF receptors in the brain may mediate the action of peripherally injected obestatin on gastroduodenal motility[14] (Figure 5C). The results showed that vagal afferent nerve blockade by capsaicin reverses the inhibitory effects of obestatin on duodenal motility, but does not alter the inhibitory effects of obestatin on antral motility[14]. These results suggest that vagal afferent pathways might be involved partially, but not entirely, in the action of obestatin. Involvement of vagal afferent pathways was confirmed by the finding that the number of c-*Fos*-positive neurons in the NTS was increased by iv injection of obestatin[14]. In addition to vagal afferent pathways, it is possible that circulating obestatin acts on brain targets directly by crossing the BBB, because a previous study has shown that there is a rapid influx of iv-injected ^{125}I-labeled obestatin from the blood to the brain[27]. Therefore, the lack of effects of obestatin on antral motility during capsaicin treatment might be explained by direct action of peripherally injected obestatin on brain targets by crossing the BBB, similar to what has been observed for des-acyl ghrelin. We further examined whether obestatin can antagonize the stimulatory effects of ghrelin on gastroduodenal motility[14]. We found that obestatin failed to antagonize the ability of ghrelin either to stimulate the %MI in the antrum or to accelerate the initiation of fasted motility in the duodenum when administrated in the fed state[14]. These results were consistent with previous studies in which obestatin failed to antagonize the ability of ghrelin to stimulate gastric emptying or to shorten the MMC cycle time[8].

GPR39 was initially proposed as the receptor for obestatin[2], and GPR39 expression has been detected in peripheral organs such as the duodenum and kidney, but not in the pituitary or hypothalamus[4]. However recent publications indicate that obestatin is unlikely to be the endogenous ligand for GPR39 on the basis of a lack of specific binding of obestatin to GPR39 receptor-expressing cells[2,4,5,28]. Nevertheless, although binding of obestatin to the receptor GPR39 remains controversial, the functional effect of obestatin on gastrointestinal motility has been clearly demonstrated in our study.

In conclusion, our study indicates that obestatin inhibits gastroduodenal motility in the fed state, but not in


A
Feeding
Saline, iv
cmH2O
20
0
Antrum
Duodenum
20 min
Feeding
Obestatin, 15 nmol iv
Antrum
Duodenum
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%MI in antrum
200
150
100
50
0
Vehicle
Obestatin, 7.5 nmol
Obestatin, 15 nmol
Obestatin, 30 nmol
b
0-30
30-60
60-90
90-120
Time after the administration of obestatin (min)
Time between injection and initiation of phase Ⅲ-like contraction in duodenum
150
120
90
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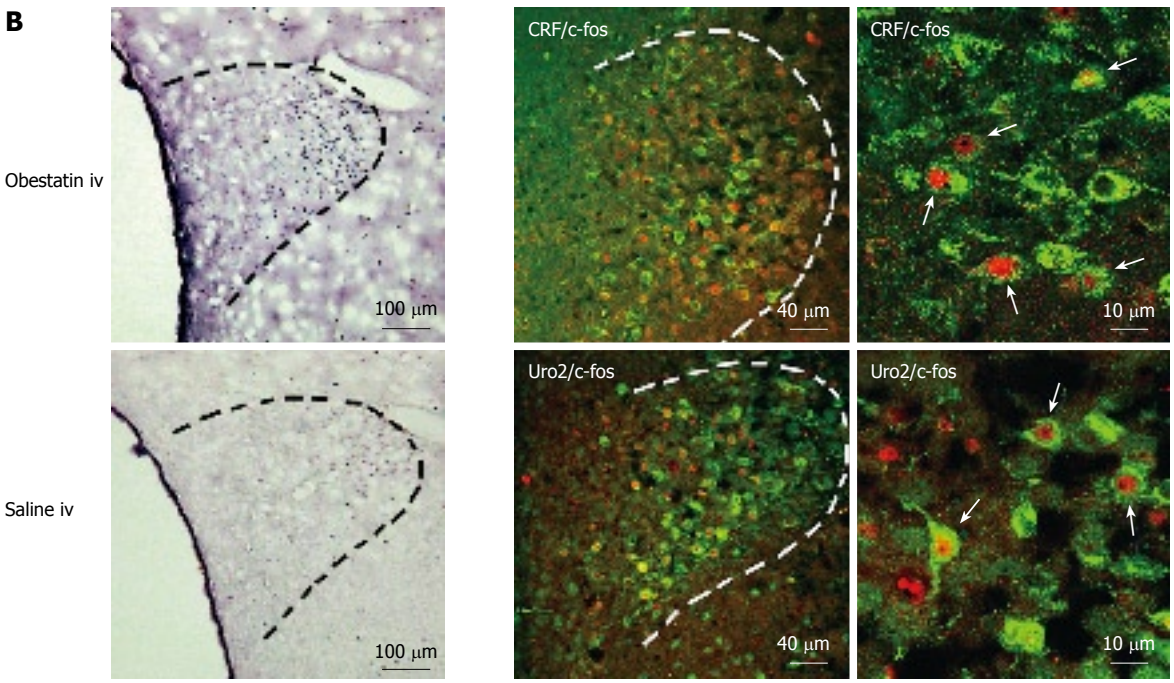
30
0
Vehicle
Obestatin 7.5 nmol
Obestatin 15 nmol
Obestatin 30 nmol


B
Obestatin iv
Saline iv
100 μm
CRF/c-fos
40 μm
10 μm
Uro2/c-fos

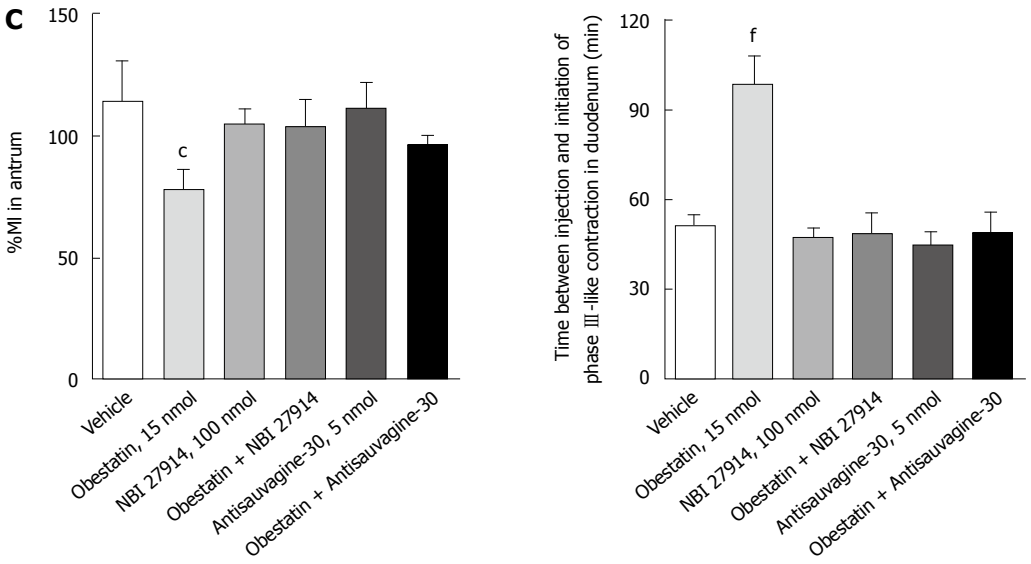


Figure 5 Obestatin and gastroduodenal motility. A: Effects of iv injection of obestatin on the fed motor activity of the antrum and duodenum. Iv injection of obestatin dose dependently decreases the %MI during the 30-90-min period after injection of obestatin in the antrum, and prolongs the time between the initiation of phase Ⅲ-like contractions and injection of obestatin in the duodenum. $^{b}P < 0.01$, compared with vehicle-injected controls; B: The density of c-Fos-positive cells in the PVN is increased by iv injection of obestatin compared to saline-injected control. CRF-positive or urocortin 2-positive neurons are overlapped with c-Fos-positive neurons in the PVN; C: The decrease in %MI that is observed 30-60 min after iv injection of obestatin is reversed by icv injection of the CRF type 1 antagonist NBI-27914 and the CRF type 2 receptor antagonist antisauvagine-30. The elongation of the time between injection of obestatin and initiation of phase Ⅲ-like contractions in the duodenum induced by iv injection of obestatin is also reversed by icv injection of NBI-27914 and antisauvagine-30. $^{c}P < 0.05$, $^{f}P < 0.01$, compared with vehicle-injected controls.

the fasted state of conscious rats. In the brain, CRF- and urocortin 2-containing neurons might be activated by iv injection of obestatin, and at the level, CRF type1 and type 2 receptors might be involved in the inhibitory action of obestatin on antral and duodenal motility (Figure 3). Vagal afferent pathways might be involved partially, but not entirely, in these actions of obestatin (Figure 3).

REFERENCES

1 **Kojima M**, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660
2 **Zhang JV**, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 2005; **310**: 996-999
3 **Chartrel N**, Alvear-Perez R, Leprince J, Iturrioz X, Reaux-Le Goazigo A, Audinot V, Chomarat P, Coge F, Nosjean O, Rodriguez M, Galizzi JP, Boutin JA, Vaudry H, Llorens-Cortes C. Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". *Science* 2007; **315**: 766; author reply 766
4 **Holst B**, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, Storjohann L, Stidsen CE, Jones R, Beck-Sickinger AG, Schwartz TW. GPR39 signaling is stimulated by zinc ions but not by obestatin. *Endocrinology* 2007; **148**: 13-20
5 **Tremblay F**, Perreault M, Klaman LD, Tobin JF, Smith E, Gimeno RE. Normal food intake and body weight in mice lacking the G protein-coupled receptor GPR39. *Endocrinology* 2007; **148**: 501-506
6 **Asakawa A**, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, Kasuga M. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. *Gut* 2005; **54**: 18-24
7 **Chen CY**, Inui A, Asakawa A, Fujino K, Kato I, Chen CC, Ueno N, Fujimiya M. Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in conscious rats. *Gastroenterology* 2005; **129**: 8-25
8 **Bassil AK**, Haglund Y, Brown J, Rudholm T, Hellstrom PM, Naslund E, Lee K, Sanger GJ. Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract. *Br J Pharmacol* 2007; **150**: 58-64
9 **Bresciani E**, Rapetti D, Dona F, Bulgarelli I, Tamiazzo L, Locatelli V, Torsello A. Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. *J Endocrinol Invest* 2006; **29**: RC16-RC18
10 **De Smet B**, Thijs T, Peeters TL, Depoortere I. Effect of peripheral obestatin on gastric emptying and intestinal contractility in rodents. *Neurogastroenterol Motil* 2007; **19**: 211-217
11 **Gourcerol G**, Million M, Adelson DW, Wang Y, Wang L, Rivier J, St-Pierre DH, Tache Y. Lack of interaction between peripheral injection of CCK and obestatin in the regulation of gastric satiety signaling in rodents. *Peptides* 2006; **27**: 2811-2819
12 **Lagaud GJ**, Young A, Acena A, Morton MF, Barrett TD, Shankley NP. Obestatin reduces food intake and suppresses body weight gain in rodents. *Biochem Biophys Res Commun* 2007; **357**: 264-269
13 **Nogueiras R**, Pfluger P, Tovar S, Arnold M, Mitchell S, Morris A, Perez-Tilve D, Vazquez MJ, Wiedmer P, Castaneda TR, DiMarchi R, Tschop M, Schurmann A, Joost HG, Williams LM, Langhans W, Dieguez C. Effects of obestatin on energy balance and growth hormone secretion in rodents. *Endocrinology* 2007; **148**: 21-26
14 **Ataka K**, Inui A, Asakawa A, Kato I, Fujimiya M. Obestatin inhibits motor activity in the antrum and duodenum in the fed state of conscious rats. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G1210-G1218
15 **Fujimiya M**, Itoh E, Kihara N, Yamamoto I, Fujimura M, Inui A. Neuropeptide Y induces fasted pattern of duodenal motility via Y(2) receptors in conscious fed rats. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G32-G38
16 **Fujino K**, Inui A, Asakawa A, Kihara N, Fujimura M, Fujimiya M. Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *J Physiol* 2003; **550**: 227-240
17 **Kihara N**, Fujimura M, Yamamoto I, Itoh E, Inui A, Fujimiya M. Effects of central and peripheral urocortin on fed and

fasted gastroduodenal motor activity in conscious rats. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G406-G419

18 **Asakawa A**, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 2001; **120**: 337-345

19 **Itoh Z**. Motilin and clinical application. *Peptides* 1997; **18**: 593-608

20 **Sarna SK**, Gonzalez A, Ryan RP. Enteric locus of action of prokinetics: ABT-229, motilin, and erythromycin. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G744-G752

21 **Date Y**, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000; **141**: 4255-4261

22 **Hashmonai M**, Go VL, Yaksh T, Szurszewski JH. Effect of central administration of motilin on migrating complexes in the dog. *Am J Physiol* 1987; **252**: G195-G199

23 **Yamamoto O**, Matsunaga Y, Haga N, Mizumoto A, Itoh Z. Inhibition of phase III activity by acidifying stomach in vagally denervated and innervated dogs with gastric pouches. *Gastroenterology* 1994; **106**: 1533-1541

24 **Date Y**, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K, Nakazato M. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 2002; **123**: 1120-1128

25 **Chang CP**, Pearse RV 2nd, O'Connell S, Rosenfeld MG. Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. *Neuron* 1993; **11**: 1187-1195

26 **Coskun T**, Bozkurt A, Alican I, Ozkutlu U, Kurtel H, Yegen BC. Pathways mediating CRF-induced inhibition of gastric emptying in rats. *Regul Pept* 1997; **69**: 113-120

27 **Pan W**, Tu H, Kastin AJ. Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin. *Peptides* 2006; **27**: 911-916

28 **Zhang JV**, Klein C, Ren PG, Kass S, Donck LV, Moechars D, Hsueh AJ. Response to Comment on "Obestatin, a Peptide Encoded by the Ghrelin Gene, Opposes Ghrelin's Effects on Food Intake". *Science* 2007; **315**: 766

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TOPIC HIGHLIGHT

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Ghrelin and *Helicobacter pylori* infection

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Abstract

Ghrelin is primarily secreted from the stomach and has been implicated in the coordination of eating behavior and weight regulation. Ghrelin also plays an essential role in the mechanism of gastric mucosal defense. Thus, it is important to clarify which diseases primarily influence changes in plasma ghrelin concentrations. *Helicobacter pylori* (*H pylori*) infection is involved in the pathogenesis of gastritis, gastric and duodenal ulcer, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma. *H pylori* eradication is related to body weight change. Compared, *H pylori* infected and negative subjects with normal body mass index, plasma ghrelin concentration, gastric ghrelin mRNA, and the number of ghrelin producing cells in gastric mucosa are significantly lower in *H pylori* infected subjects than in *H pylori*-negative controls. Plasma ghrelin concentration decreases with the progression of gastric atrophy. Impaired gastric ghrelin production in association with atrophic gastritis induced by *H pylori* infection accounts for the decrease in plasma ghrelin concentration. However, the ratio of plasma acylated ghrelin to total ghrelin levels is higher in patients with chronic atrophic gastritis than in healthy subjects. This may result from the compensatory increase in plasma active ghrelin concentration in response to gastric atrophy. After *H pylori* eradication, gastric preproghrelin mRNA expression is increased nearly 4-fold in most cases. However, changes in plasma ghrelin concentrations before and after *H pylori* cure are not associated with the gastric ghrelin production. Plasma ghrelin changes are inversely correlated with both body weight change and initial plasma ghrelin levels.

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Key words: Ghrelin; *Helicobacter pylori*; Eradication; Body weight; Leptin

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INTRODUCTION

Ghrelin, a 28 amino acid peptide isolated from rat and human stomach, possesses strong growth hormone-releasing activity and plays central as well as peripheral roles in food intake, gastric motility, and acid secretion[1-3]. Ghrelin has been shown to evoke weight gain by actions in the hypothalamus[3]. Plasma ghrelin concentrations rise before meals and fall after meals. This peptide also contributes to the regulation of both somatic growth and adipose tissue mass, and is therefore, a short-term, meal-related orexigen as well as a long-term regulator of body weight[4,5]. Circulating ghrelin concentrations in newborns are not associated with gender, body weight, or hormonal parameter[6]. In children and adults, however, plasma ghrelin concentrations are lower in obese subjects compared with those with normal body weight and lean subjects[7]. The decrease of plasma ghrelin concentrations appears to compensate for the positive energy balance in obese individuals[7].

SOURCES OF GHRELIN AND *HELICOBACTER PYLORI* (*H PYLORI*) INFECTION

Ghrelin is predominantly produced by the stomach[8], whereas substantially lower amounts are derived from bowel[9], pituitary, kidney, placenta, hypothalamus[8], lung, kidney, and A-cells of the pancreatic islet. Thus, it is important to clarify which organ primarily influences changes in plasma ghrelin concentrations in various diseases. Although the majority of circulating ghrelin is produced in the stomach, other sources may increase or decrease ghrelin secretion in a compensatory manner. After gastrectomy, for example, plasma ghrelin level is

surprisingly reduced only by 65%[5].

The gastric ghrelin is produced in X/A like-cells of enteroendocrine cells/oxyntic glands in the mammalian gastric mucosa[9]. Thus, there exists the possibility that chronic persistent damage of the gastric mucosa, such as chronic gastritis, might affect ghrelin production, leading to changes in food intake and body weight. *H pylori* is a gram-negative bacterium that colonizes the stomach. *H pylori* infection is involved in the pathogenesis of gastritis, gastric and duodenal ulcer, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma[10-12]. More than 50% of the adult population is infected with *H pylori* worldwide. *H pylori* infection first leads to atrophic gastritis and intestinal metaplasia, which may further lead to dysplasia and gastric carcinoma. Thus, it is an intriguing question whether *H pylori* infection affects gastric ghrelin production and consequently alters plasma ghrelin concentration.

RELATIONSHIP BETWEEN PLASMA GHRELIN LEVELS AND BODY MASS INDEX IN *H PYLORI* INFECTED PATIENTS

In determining whether ghrelin is involved in long-term energy homeostasis, several studies have found that circulating ghrelin is elevated in individuals with anorexia nervosa[7], reduced in obesity[7,13,14] and normalized with weight gain[13] or weight loss. Circulating ghrelin levels are negatively correlated with the percentage of body fat, fat mass, body mass index (BMI), body weight, insulin, leptin, and T3 in cross-sectional and longitudinal studies examining anorexia nervosa and obesity. In *H pylori*-infected subjects, however, the correlation between BMI and circulating ghrelin levels was weak[15,16]. This suggested that *H pylori* infection could affect plasma ghrelin levels strongly.

EFFECT OF *H PYLORI* INFECTION ON PLASMA GHRELIN LEVELS

Several investigators reported the relationship between plasma ghrelin levels and *H pylori* infection. Nwokolo *et al*[17] reported that plasma ghrelin concentrations increased after the eradication of *H pylori*. On the contrary, Gokcel *et al*[18] reported that *H pylori* infection has no effect on plasma ghrelin levels. Although the relationship between *H pylori* infection and plasma ghrelin concentrations had been still controversial in Western countries, Japanese investigators revealed the effects of *H pylori* infection on plasma ghrelin concentrations[17,18]. The direct relationship between *H pylori* infection and gastric ghrelin production, which could influence plasma ghrelin concentrations, have been demonstrated. Osawa *et al*[19] and Tatsuguchi *et al*[16] investigated the association of *H pylori* infection with gastric ghrelin production in the human stomach concomitantly examining plasma ghrelin concentrations.

PLASMA GHRELIN CONCENTRATIONS ARE LOWER IN *H PYLORI*-POSITIVE SUBJECTS

Several investigators clarified the effect of *H pylori* infection on plasma ghrelin levels. Plasma ghrelin concentrations were significantly lower in *H pylori*-positive patients than in *H pylori*-negative controls[15,16,19]. Its level is obviously independent of sex and BMI and varied among *H pylori* infected subjects even with same BMI[15]. Mean plasma ghrelin levels in *H pylori*-positive subjects remain two-third of those of *H pylori*-negative subjects[19]. In addition to several clinical factors including BMI, food intake, and serum insulin levels[7,20], *H pylori* infection is also another determinant of plasma ghrelin levels as well as body mass index.

EXPRESSION LEVELS OF GASTRIC GHRELIN ARE LOWER IN *H PYLORI*-POSITIVE SUBJECTS

It is important to focus on the gastric mucosa in order to better understand the effects of *H pylori* infection on the alteration of ghrelin expression. Gastric ghrelin mRNA levels were much lower in *H pylori*-positive patients than in *H pylori*-negative controls using real-time quantitative RT-PCR[16,19]. The median of gastric ghrelin mRNA expression levels in *H pylori*-positive subjects was less than one 45th of that in *H pylori*-negative controls[19]. Moreover, plasma ghrelin concentrations were in parallel with the gastric ghrelin mRNA expression levels in *H pylori*-positive patients. Therefore, the attenuation of the ghrelin production in the gastric mucosa accounts for the decrease in the plasma ghrelin concentrations in *H pylori*-positive individuals[19].

GHRELIN-PRODUCING CELLS IN THE GASTRIC MUCOSA ARE FEWER IN *H PYLORI*-POSITIVE SUBJECTS

Ghrelin immuno-reactive cells are seen in the lower half of fundic epithelial glands[9]. Immunoreactivity is concentrated in the basal cytoplasm of the positive cells. The number of ghrelin-positive cells in the gastric mucosa of *H pylori*-positive individuals was significantly lower than those of *H pylori*-negative individuals[19]. Furthermore, the numbers of ghrelin-positive cells in the gastric mucosa fell significantly in accompaniment to the decrease in plasma ghrelin concentrations in *H pylori*-positive subjects[16,19]. These results reinforce the fact that the attenuation of the gastric ghrelin production caused by *H pylori* infection accounts for the decrease in the plasma ghrelin concentrations in *H pylori*-positive individuals.

PLASMA GHRELIN CONCENTRATIONS ARE ASSOCIATED WITH THE DEGREE OF GASTRIC ATROPHY IN *H PYLORI*-POSITIVE SUBJECTS

Since *H pylori* infection first induces gastric atrophy in its pathological course, it is important to clarify the association between plasma ghrelin concentration and degree of gastric atrophy in *H pylori*-positive patients. Several reports revealed that groups of *H pylori*-positive subjects with higher degrees of gastric atrophy tended to have lower plasma ghrelin concentrations, leading to a negative association between plasma ghrelin concentration and gastric atrophy grade[15,16,19]. Moreover, activity and topography of gastritis affects circulating ghrelin levels[21]. Histological severity of mononuclear cell infiltration and glandular atrophy of the corpus significantly influenced the expression levels of ghrelin mRNA, its peptide contents and the density of immunoreactive cells, indicating that gastric ghrelin biosynthesis seems to be affected by chronic mucosal inflammation and/or atrophy in association with *H pylori* infection. In addition, plasma ghrelin concentrations in *H pylori*-positive patients correlated with serum pepsinogen I concentration as well as pepsinogen I / Ⅱ ratio. Pepsinogen I and pepsinogen Ⅱ differ in their location in the stomach. Both are located in the chief and mucous neck cells of the oxyntic gland mucosa in the gastric corpus but only pepsinogen Ⅱ is present in the gastric antrum. A pepsinogen I / Ⅱ ratio < 3 is considered to be a reliable marker for severe atrophic gastritis[22]. Serum levels of pepsinogen I as well as the ratio of pepsinogen I / Ⅱ fell significantly as plasma ghrelin concentrations decreased, indicating the positive association between plasma ghrelin and pepsinogen I concentrations as well as pepsinogen I / Ⅱ ratios in *H pylori*-positive patients[19]. Collectively, these results reveal that plasma ghrelin concentrations are associated with the progression of gastric atrophy. Although geographical differences in the prevalence of atrophic gastritis in Asians and Westerners would require additional consideration, these findings strongly suggest that the reduction of ghrelin-producing cells in the gastric mucosa by *H pylori* infection results in the lower plasma ghrelin concentration in *H pylori*-positive patients.

Checchi *et al*[23] reported that serum ghrelin levels are negatively affected by autoimmune gastritis as well as by *H pylori* associated gastritis, and represent the most sensitive and specific noninvasive markers for selecting those patients at high risk for having gastric damage. Of particular interest is the fact that the measurement of serum ghrelin levels is superior to that of pepsinogen I / Ⅱ ratio and serum gastrin to predict gastric damage.

STOMACH REGULATES ENERGY BALANCE *VIA* ACYLATED GHRELIN AND DESACYLATED GHRELIN

It is known that ghrelin circulates in two different forms: the so-called acylated ghrelin, octanoylated, in serine 3, and the so-called desacylated, without the octanoyl group[24]. This latter form is dramatically less potent on the GHS-receptor than the acylated form[25]. Acylated ghrelin is involved in the regulation of GH secretion, energy balance, gastrointestinal motility, cardiac performance, and anxiety[8]. Administered acylated ghrelin induces body weight gain and adiposity by promoting food intake and decreasing fat use or energy expenditure[26]. In contrast to acylated ghrelin, desacylated ghrelin induces a negative energy balance by decreasing food intake and delaying gastric emptying[27]. The effect is mediated *via* the hypothalamus. Although derived from the same precursor, the inverse effects of these two peptides suggest that the stomach might be involved as an endocrine organ in the regulation of the energy balance.

PLASMA ACYLATED GHRELIN LEVELS ARE HIGHER IN PATIENTS WITH CHRONIC ATROPHIC GASTRITIS

Total plasma ghrelin concentrations decrease in patients with gastric atrophy secondary to *H pylori* infection, and the levels are related to the degree of atrophy. These finding might be explained by the loss of ghrelin-producing cells caused by inflammatory and/or atrophic changes. Campana *et al*[28] reported that plasma acylated ghrelin levels were higher in patients with chronic atrophic gastritis than in healthy subjects. This opposite tendency compared to total plasma ghrelin concentration may result from the compensatory increase in plasma active ghrelin concentration in response to gastric atrophy. This hypothesis seems to be supported by a recent report showing that a significant decrease in gastric pH was found after injection of exogenous ghrelin. Gastric atrophy causes an increase gastric pH, leading to an increase in serum gastrin levels. Both the increase in acylated ghrelin and gastrin could represent a compensatory mechanism to stimulate gastric acid production.

GHRELIN HAS A PROTECTIVE EFFECT AGAINST MUCOSAL INJURY OF STOMACH

H pylori infection induces gastric mucosal damage including gastric ulcer and chronic gastritis. It is an intriguing question whether lower plasma ghrelin level in *H pylori*-infected patients affects gastric mucosa. Sibilia *et al*[29] reported that ghrelin protects against ethanol-induced gastric ulcers in rats. Similarly, Konturek *et al*[30] reported that ghrelin expression of gastric mucosa is enhanced after exposure to ethanol, and ghrelin exhibits a strong gastroprotection due to its anti-inflammatory action mediated by prostaglandins. The gastroprotective effect of ghrelin is accompanied by a significant rise in the gastric blood flow, which is known to play an essential role in the mechanism of gastric mucosal defense. This ghrelin-induced hyperemia could be prob-

ably attributed to the direct vasodilatory effect of this peptide.

PLASMA GHRELIN LEVEL AND BODY WEIGHT

Eradication of *H pylori* has been a standard therapy for peptic ulcer disease[10,11] and improves gastritis[10]. Much attention has recently been directed to the relationship between obesity and *H pylori* infection. Several studies showed that *H pylori* infection is inversely related to obesity. For example, Wu *et al*[31] reported that the seropositivity of *H pylori* infection was significantly lower in morbid obesity patients. Furuta *et al*[32] showed the body weight gain after *H pylori* cure. As ghrelin is mainly synthesized and secreted by gastric mucosa, it has been assumed that the inverse effect of *H pylori* infection on body weight may attribute to the difference of plasma ghrelin concentrations in patients with or without *H pylori* infection[33]. This hypothesis states that an increase of gastric ghrelin production after *H pylori* cure may elevate plasma ghrelin concentration resulting in body weight gain.

PLASMA GHRELIN LEVELS AND BODY WEIGHT GAIN AFTER *H PYLORI* ERADICATION

Do plasma ghrelin levels affect body weight gain after *H pylori* eradication, or body weight gain affect plasma ghrelin levels? Gastric ghrelin production is decreased by *H pylori* infection and increased by eradication therapy[19]. As ghrelin is a body weight regulating peptide, much attention has been paid to the nutritional status and the dynamics of gastric and plasma ghrelin in response to *H pylori* infection[31,32]. In this respect, Nwokolo *et al*[17] reported that plasma ghrelin levels increased at 6 wk after *H pylori* cure in 10 patients in UK. Because plasma ghrelin levels increased significantly by 75%, they proposed that increased ghrelin following *H pylori* eradication may play a role in obesity. This could lead to increased appetite and weight gain, and contribute to the increasing obesity seen in Western populations where *H pylori* prevalence is low. Also, Czesnikiewicz-Guzik *et al*[34] reported that plasma ghrelin levels increased significantly to two-fold levels at 4 wk after *H pylori* cure in 41 patients in Poland. After Nwokolo's report, it has been believed that plasma ghrelin concentrations will increase after *H pylori* cure due to the increase of gastric ghrelin production, leading to body weight gain[16]. However, their reports suggested that plasma ghrelin levels increased, but did not reveal a changes of plasma ghrelin levels in patients with body weight gain after *H pylori* cure. Plasma ghrelin levels decrease as a compensatory effect in obesity patients who has positive energy balance. Therefore, it is questionable whether plasma ghrelin levels increase in a condition of body weight gain after eradication. Another study found that plasma ghrelin levels were unaffected[35]. In fact, plasma ghrelin concentration is not simply regulated by the levels of gastric ghrelin production. Even in healthy humans, plasma ghrelin concentration is tightly correlated with body weight[7]. Therefore, it has been proposed[36] that it should be re-examined whether the rise in plasma ghrelin following *H pylori* eradication exists and whether it can be an important determinant of body weight increase. It is possible that only a subpopulation of infected patients may show a rise in ghrelin following eradication.

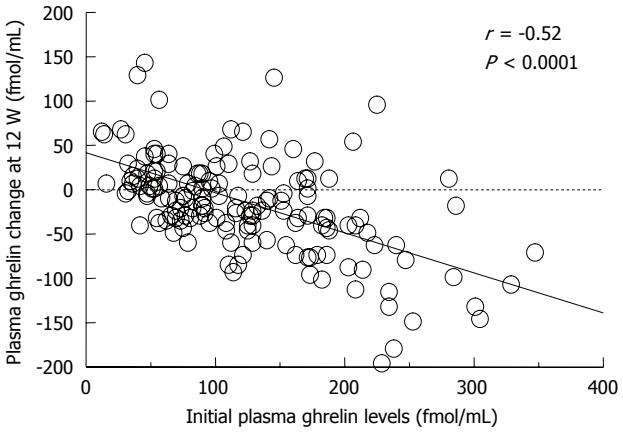

Figure 1 The relationship between the initial plasma ghrelin levels and the change in plasma ghrelin levels at 12 wk after *H pylori* cure. The change was obtained by subtracting the levels before the treatment from the levels at 12 wk after. The change in 12 wk correlated inversely with initial plasma ghrelin levels. This figure is cited from *J Gastroenterol* 2006; 41: 954.

DISPARATE CHANGES IN PLASMA GHRELIN AFTER *H PYLORI* CURE

The effect of *H pylori* eradication on plasma ghrelin concentration was reported in 134 Japanese patients[37]. Interestingly, mean plasma ghrelin concentrations decreased significantly from 120 fmol/mL before *H pylori* eradication to 103 fmol/mL at 12 wk after *H pylori* eradication. However, its levels after treatment changed diversely among enrolled patients. In fact, levels increased in 50 patients and decreased in 84 patients. There are some potential mechanisms leading to disparate changes in plasma ghrelin levels after *H pylori* eradication. The relationship between the initial plasma ghrelin levels and their changes were analyzed after *H pylori* cure. Figure 1 shows the relationship between the initial plasma ghrelin levels, and the changes in plasma ghrelin concentration at 12 wk after *H pylori* cure. Interestingly, higher initial plasma ghrelin levels decreased after the cure, but lower initial plasma ghrelin levels did not change significantly. The change of plasma ghrelin concentration after 12 wk was inversely correlated with the initial plasma ghrelin levels.

EXPRESSION LEVELS OF GASTRIC GHRELIN INCREASES AFTER *H PYLORI* CURE

The effect of *H pylori* eradication on the ghrelin pro-

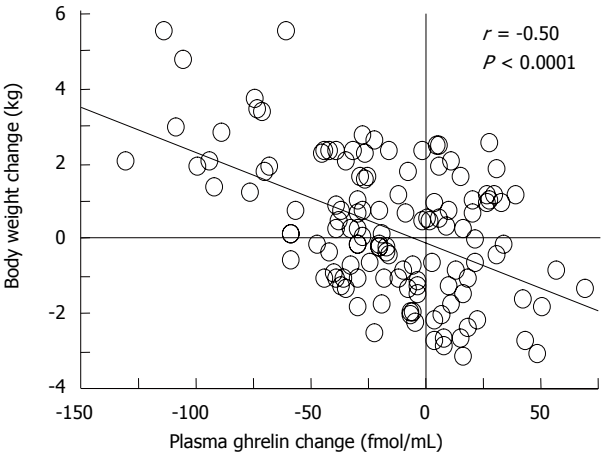

Figure 2 The relationship between the plasma ghrelin change and body weight change at 12 wk after *H pylori* cure. The alteration of plasma ghrelin levels correlated inversely with body weight change after *H pylori* cure. This figure is cited from *J Gastroenterol* 2006; 41: 954.

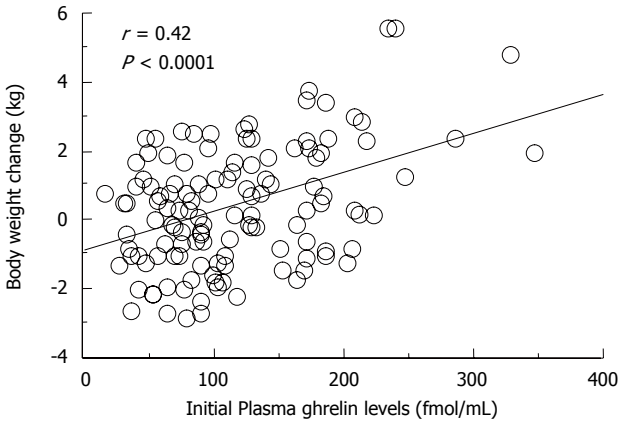

Figure 3 The relationship between the initial plasma ghrelin levels and body weight change at 12 wk after *H pylori* cure. Initial plasma ghrelin levels correlated positively with body weight changes. This figure is cited from *J Gastroenterol* 2006; 41: 954.

duction in the gastric mucosa was reported in several studies. Tatsuguchi *et al*[16] reported that ghrelin immunoreactive cells increase in the gastric mucosa after *H pylori* eradication irrespective of the recovery of glandular atrophy. Osawa *et al*[37] also reported that the number of ghrelin positive cells per oxyntic gland was increased in 77 patients and was unchanged in 57 patients after *H pylori* eradication. The number of ghrelin producing cells tend to increase despite the change of plasma ghrelin levels before and after *H pylori* cure. In recent reports, gastric glandular atrophy recovers gradually over the long term after *H pylori* eradication. Arkkila *et al*[38] reported that atrophy can diminish or even disappear, especially in the antrum, during a 1-year follow-up after eradication of infection. If glandular atrophy recovers by *H pylori* cure, the number of ghrelin producing cell may increase more and more. Osawa *et al*[37] compared gastric preproghrelin mRNA expression levels before and 12 wk after treatment using the corpus mucosa. Median preproghrelin mRNA expression was increased nearly 4-fold after *H pylori* cure. Preproghrelin mRNA expression was also increased in the antral mucosa. No correlation was observed between the changes in plasma ghrelin and those of gastric preproghrelin mRNA or ghrelin positive cells after *H pylori* cure. Similarly, Isomoto *et al*[21] reported that preproghrelin mRNA expression was increased in the corpus mucosa at 4 wk after *H pylori* cure. Therefore, gastric ghrelin production is enhanced after *H pylori* eradication even in patients with decreased plasma ghrelin concentrations.

BODY WEIGHT CHANGES CORRELATE INVERSELY WITH CHANGES IN PLASMA GHRELIN CONCENTRATION

Body weight gain is a well-known effect of *H pylori* eradication and plasma ghrelin concentration is influenced by body weight change[38,39]. The question as to whether ghrelin is involved in weight gain after *H pylori* cure has been discussed[19]. Figure 2 showed clearly that the change in plasma ghrelin is inversely correlated with body weight change after *H pylori* cure[37]. Plasma ghrelin decreased in 23 of 28 patients (82%) with more than 2 kg of weight gain, and in all 7 patients with more than 3 kg of weight gain. These data suggest that plasma ghrelin concentration after *H pylori* cure is more strongly influenced by body weight change than the increase of gastric preproghrelin mRNA and ghrelin producing cells.

In contrast, patients with less than 2 kg of body weight gain or with body weight loss had minor changes of plasma ghrelin levels. Increased plasma ghrelin levels after the cure in European studies can be associated with patients having minor change of body weight. The racial difference of enrolled subjects may account for the discrepancy. In this respect, Asians including Japanese are more prone to central adiposity than are white individuals[40]. As body fat storage is closely associated with plasma ghrelin levels, the racial difference of body fat distribution may account for the discrepancy.

INITIAL PLASMA GHRELIN LEVELS CAN BE A PREDICTIVE FACTOR OF BODY WEIGHT GAIN AFTER *H PYLORI* ERADICATION

Initial plasma ghrelin levels before eradication therapy were significantly higher in those whose plasma ghrelin decreased after treatment[37]. In addition, these subjects had a significantly greater increase in body weight than those with increased plasma ghrelin after treatment. Figure 3 shows the positive correlation between the initial plasma ghrelin levels and body weight changes. In particular, 12 of 14 patients (86%) with more than 200 fmol/mL of initial ghrelin levels had an increase in body weight, suggesting high levels of initial plasma ghrelin can be a predictive factor of body weight gain after *H pylori* eradication. The correlation between initial

plasma ghrelin levels and weight changes suggests the participation of ghrelin in the weight gain after *H pylori* eradication. The weight gain after *H pylori* eradication does not simply result from an increase in plasma ghrelin by the recovery of gastric ghrelin production.

However, additional research is needed to clarify the relationship between the body weight gain and the plasma ghrelin levels after *H pylori* cure in Western population.

CONCLUSION

Plasma ghrelin concentrations are influenced by the presence of chronic gastritis in association with *H pylori* infection. The decrease in gastric ghrelin production accounts for lower concentrations of plasma ghrelin in *H pylori*-positive individuals. Gastric ghrelin production increases after *H pylori* cure. Plasma ghrelin concentrations decrease in subjects with body weight gain after *H pylori* cure. Initial plasma ghrelin levels before eradication can be a predictive factor for body weight gain after *H pylori* cure.

REFERENCES

1 **Date Y**, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochem Biophys Res Commun* 2001; **280**: 904-907

2 **Masuda Y**, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K. Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun* 2000; **276**: 905-908

3 **Nakazato M**, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature* 2001; **409**: 194-198

4 **Asakawa A**, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 2001; **120**: 337-345

5 **Cummings DE**, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, Purnell JQ. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *N Engl J Med* 2002; **346**: 1623-1630

6 **Bellone S**, Rapa A, Vivenza D, Vercellotti A, Petri A, Radetti G, Bellone J, Broglio F, Ghigo E, Bona G. Circulating ghrelin levels in newborns are not associated to gender, body weight and hormonal parameters but depend on the type of delivery. *J Endocrinol Invest* 2003; **26**: RC9-RC11

7 **Shiiya T**, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukura S. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *J Clin Endocrinol Metab* 2002; **87**: 240-244

8 **Kojima M**, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660

9 **Date Y**, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000; **141**: 4255-4261

10 **Marshall BJ**, Goodwin CS, Warren JR, Murray R, Blincow ED, Blackbourn SJ, Phillips M, Waters TE, Sanderson CR. Prospective double-blind trial of duodenal ulcer relapse after eradication of Campylobacter pylori. *Lancet* 1988; **2**: 1437-1442

11 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789

12 **Wotherspoon AC**, Doglioni C, Diss TC, Pan L, Moschini A, de Boni M, Isaacson PG. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori. *Lancet* 1993; **342**: 575-577

13 **Tolle V**, Kadem M, Bluet-Pajot MT, Frere D, Foulon C, Bossu C, Dardennes R, Mounier C, Zizzari P, Lang F, Epelbaum J, Estour B. Balance in ghrelin and leptin plasma levels in anorexia nervosa patients and constitutionally thin women. *J Clin Endocrinol Metab* 2003; **88**: 109-116

14 **Otto B**, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, Heiman ML, Lehnert P, Fichter M, Tschop M. Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. *Eur J Endocrinol* 2001; **145**: 669-673

15 **Shiotani A**, Miyanishi T, Uedo N, Iishi H. Helicobacter pylori infection is associated with reduced circulating ghrelin levels independent of body mass index. *Helicobacter* 2005; **10**: 373-378

16 **Tatsuguchi A**, Miyake K, Gudis K, Futagami S, Tsukui T, Wada K, Kishida T, Fukuda Y, Sugisaki Y, Sakamoto C. Effect of Helicobacter pylori infection on ghrelin expression in human gastric mucosa. *Am J Gastroenterol* 2004; **99**: 2121-2127

17 **Nwokolo CU**, Freshwater DA, O'Hare P, Randeva HS. Plasma ghrelin following cure of Helicobacter pylori. *Gut* 2003; **52**: 637-640

18 **Gokcel A**, Gumurdulu Y, Kayaselcuk F, Serin E, Ozer B, Ozsahin AK, Guvener N. Helicobacter pylori has no effect on plasma ghrelin levels. *Eur J Endocrinol* 2003; **148**: 423-426

19 **Osawa H**, Nakazato M, Date Y, Kita H, Ohnishi H, Ueno H, Shiiya T, Satoh K, Ishino Y, Sugano K. Impaired production of gastric ghrelin in chronic gastritis associated with Helicobacter pylori. *J Clin Endocrinol Metab* 2005; **90**: 10-16

20 **Date Y**, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes* 2002; **51**: 124-129

21 **Isomoto H**, Nishi Y, Ohnita K, Mizuta Y, Kohno S, Ueno H, Nakazato M. The Relationship between Plasma and Gastric Ghrelin Levels and Strain Diversity in Helicobacter pylori Virulence. *Am J Gastroenterol* 2005; **100**: 1425-1427

22 **Samloff IM**, Varis K, Ihamaki T, Siurala M, Rotter JI. Relationships among serum pepsinogen I, serum pepsinogen II, and gastric mucosal histology. A study in relatives of patients with pernicious anemia. *Gastroenterology* 1982; **83**: 204-209

23 **Checchi S**, Montanaro A, Pasqui L, Ciuoli C, Cevenini G, Sestini F, Fioravanti C, Pacini F. Serum ghrelin as a marker of atrophic body gastritis in patients with parietal cell antibodies. *J Clin Endocrinol Metab* 2007; **92**: 4346-4351

24 **Hosoda H**, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem Biophys Res Commun* 2000; **279**: 909-913

25 **Thompson NM**, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC, Wells T. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* 2004; **145**: 234-242

26 **Inui A**. Ghrelin: an orexigenic and somatotrophic signal from the stomach. *Nat Rev Neurosci* 2001; **2**: 551-560

27 **Asakawa A**, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, Kasuga M. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. *Gut* 2005;

54: 18-24
28 **Campana D**, Nori F, Pagotto U, De Iasio R, Morselli-Labate AM, Pasquali R, Corinaldesi R, Tomassetti P. Plasma acylated ghrelin levels are higher in patients with chronic atrophic gastritis. *Clin Endocrinol* (Oxf) 2007; **67**: 761-766
29 **Sibilia V**, Rindi G, Pagani F, Rapetti D, Locatelli V, Torsello A, Campanini N, Deghenghi R, Netti C. Ghrelin protects against ethanol-induced gastric ulcers in rats: studies on the mechanisms of action. *Endocrinology* 2003; **144**: 353-359
30 **Konturek PC**, Brzozowski T, Pajdo R, Nikiforuk A, Kwiecien S, Harsch I, Drozdowicz D, Hahn EG, Konturek SJ. Ghrelin-a new gastroprotective factor in gastric mucosa. *J Physiol Pharmacol* 2004; **55**: 325-336
31 **Wu MS**, Lee WJ, Wang HH, Huang SP, Lin JT. A case-control study of association of Helicobacter pylori infection with morbid obesity in Taiwan. *Arch Intern Med* 2005; **165**: 1552-1555
32 **Furuta T**, Shirai N, Xiao F, Takashima M, Hanai H. Effect of Helicobacter pylori infection and its eradication on nutrition. *Aliment Pharmacol Ther* 2002; **16**: 799-806
33 **Blaser MJ**, Atherton JC. Helicobacter pylori persistence: biology and disease. *J Clin Invest* 2004; **113**: 321-333
34 **Czesnikiewicz-Guzik M**, Loster B, Bielanski W, Guzik TJ, Konturek PC, Zapala J, Konturek SJ. Implications of oral Helicobacter pylori for the outcome of its gastric eradication therapy. *J Clin Gastroenterol* 2007; **41**: 145-151
35 **Isomoto H**, Nakazato M, Ueno H, Date Y, Nishi Y, Mukae H, Mizuta Y, Ohtsuru A, Yamashita S, Kohno S. Low plasma ghrelin levels in patients with Helicobacter pylori-associated gastritis. *Am J Med* 2004; **117**: 429-432
36 **Peeters TL**. Ghrelin: a new player in the control of gastrointestinal functions. *Gut* 2005; **54**: 1638-1649
37 **Osawa H**, Kita H, Ohnishi H, Nakazato M, Date Y, Bowlus CL, Ishino Y, Watanabe E, Shiiya T, Ueno H, Hoshino H, Satoh K, Sugano K. Changes in plasma ghrelin levels, gastric ghrelin production, and body weight after Helicobacter pylori cure. *J Gastroenterol* 2006; **41**: 954-961
38 **Arkkila PE**, Seppala K, Farkkila MA, Veijola L, Sipponen P. Helicobacter pylori eradication in the healing of atrophic gastritis: a one-year prospective study. *Scand J Gastroenterol* 2006; **41**: 782-790
39 **Leidy HJ**, Gardner JK, Frye BR, Snook ML, Schuchert MK, Richard EL, Williams NI. Circulating ghrelin is sensitive to changes in body weight during a diet and exercise program in normal-weight young women. *J Clin Endocrinol Metab* 2004; **89**: 2659-2664
40 **McNeely MJ**, Boyko EJ, Shofer JB, Newell-Morris L, Leonetti DL, Fujimoto WY. Standard definitions of overweight and central adiposity for determining diabetes risk in Japanese Americans. *Am J Clin Nutr* 2001; **74**: 101-107

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TOPIC HIGHLIGHT

Akio Inui, MD, PhD, Professor, *Series Editor*

Ghrelin and gastric acid secretion

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Abstract

Ghrelin, a novel growth hormone-releasing peptide, was originally isolated from rat and human stomach. Ghrelin has been known to increase the secretion of growth hormone (GH), food intake, and body weight gain when administered peripherally or centrally. Ghrelin is also known to stimulate the gastric motility and the secretion of gastric acid. In the previous studies, the action of ghrelin on acid secretion was shown to be as strong as that of histamine and gastrin in *in-vivo* experiment. In the studies, the mechanism for the action of ghrelin was also investigated. It was shown that vagotomy completely inhibited the action of ghrelin on the secretion of gastric acid suggesting that vagal nerve is involved in the mechanism for the action of ghrelin on acid secretion. As famotidine did not inhibit ghrelin-induced acid secretion in the study by Masuda *et al*, they concluded that histamine was not involved in the action of ghrelin on acid secretion. However, we have shown that famotidine completely inhibited ghrelin-induced acid secretion and histidine decarboxylase (HDC) mRNA was increased in gastric mucosa by ghrelin injection which is inhibited by vagotomy Our results indicate that histamine is involved in the action of ghrelin on acid secretion. Furthermore synergistic action of gastrin and ghrelin on gastric acid secretion was shown. Although gastrin has important roles in postprandial secretion of gastric acid, ghrelin may be related to acid secretion during fasting period or at night. However, further studies are needed to elucidate the physiological role of ghrelin in acid secretion.

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Key words: Ghrelin; Acid secretion; Vagal nerve; Vagotomy; Histamine; Histidine decarboxylase

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GHRELIN AND THE REGULATION OF GASTRIC ACID SECRETION

Ghrelin, a novel growth-hormone-releasing peptide, was originally isolated from rat and human stomachs[1] and was demonstrated to localize in the endocrine cells of the stomach and hypothalamus[1,2]. Ghrelin has been known as a multifunctional hormone. Ghrelin increases secretion of growth hormone (GH), food intake, and body weight gain when administered peripherally or centrally[1,3-9]. Ghrelin has also positive cardiovascular effects. In humans, infusion of ghrelin decreases systemic vascular resistance and increases cardiac output in patients with heart failure. On the functions of stomach, ghrelin was also known to stimulate gastric motility and the secretion of gastric acid when administered peripherally or centrally[10,11]. As ghrelin has such multiple actions on many organs, physiological roles of ghrelin *in vivo* might be important even if many of them are not elucidated.

The mechanism for the action of ghrelin on feeding, growth hormone secretion and the secretion of gastric acid was studied and demonstrated that the vagal nerve was involved in the action of ghrelin[10-12]. When ghrelin was administered peripherally, ghrelin induced c-fos expression in the neurons of the arcuate nucleus of rats[12]. However, ghrelin did not induce c-fos expression in the neuron in both capsaicin-treated rats and vagotomized rats[12]. The effects of ghrelin on feeding were also abolished with capsaisin treatment and vagotomy[12]. These results suggest that gastric vagal afferent is the major pathway conveying ghrelin's signals for starvation and GH secretion to the brain. On the action of ghrelin on the secretion of gastric acid, vagal nerve was indicated to be involved in the action of ghrelin[10]. Masuda and coworkers indicated that the action of ghrelin on the secretion of gastric acid was abolished by the pretreatment

with atropine or bilateral cervical vagotomy[10]. Date and co-workers demonstrated that intracerebroventricular (ICV) administration of ghrelin induced the increase in the secretion of gastric acid and vagotomy and the pretreatment with atropine abolished the action of ghrelin[11]. They also demonstrated that ICV administration of ghrelin induced c-fos expression in the neurons of the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus nerve (DMX)[11]. Taken together, these results suggest that vagal nerve plays important roles in the action of ghrelin on the secretion of gastric acid.

GHRELIN AND HISTAMINE RELEASE

As dictated above, ghrelin stimulates the secretion of gastric acid[10,11]. The mechanism for the action of ghrelin on acid secretion was also demonstrated that the vagal nerve was involved in the action of ghrelin[10,11]. However, the details of the mechanism after the activation of vagal nerve by ghrelin administration remains to be elucidated.

In the mechanism for acid secretion, vagal nerve has been known to play an important role, especially in the regulation by central nervous system[13] and in the stimulation by the distenstion of stomach[14]. Vagal nerve has stimulatory and inhibitory actions on acid secretion[15,16] and it contains and releases several neurotransmitters such as acetylcholine[17], gastrin-releaseing peptide (GRP)[18], substance P[19]. Calcitonin gene-related peptide[19] and pituitary adenylate cyclase activating peptide (PACAP)[20]. Among these, GRP has a stimulatory action on gastrin release from G cells[21]. Gastrin released by GRP stimulation is the most important physiological secretagogue and it has a primary role in postprandial acid secretion[22]. Gastrin stimulates acid secretion *via* enterochromaffin-like cells (ECL cell)[23]. Another transmitter, PACA, was also found to increase histamine release from ECL cell[24]. PACAP-immunoreactive nerve fibers are abundant in the gastric mucosa of both rat and humans[25] and gastric ECL cells possesses PACAP receptors(PAC1)[26]. Gastrin is now known as major stimulant of histamine release from gastric ECL cells[27]. Histamine released from ECL cells acts on parietal cells through H_2 receptor[28]. It was indicated that the activation of vagal nerve induced an increase in histamine release from gastric mucosa[29]. Accordingly it is plausible that histamine release may be involved in the increased acid secretion induced by administration of ghrelin.

In a previous report, Masuda *et al*[10] demonstrated that vagal nerve is involved in the action of ghrelin on acid secretion. However, they concluded that histamine was not involved in the action of ghrelin on acid secretion, as famotidine did not inhibited ghrelin-induced acid secretion[10]. Tthe mechanism that was demonstrated by Masuda seemed to require further investigation**,** because the vagal stimulation[29], as well as transmitter PACAP, is known to increase release of histamine from ECL cells[24]. Therefore, if ghrelin administration stimulates the vagal nerve, an increase in histamine release would consequently occur.

Previously we attempted to clarify whether histamine

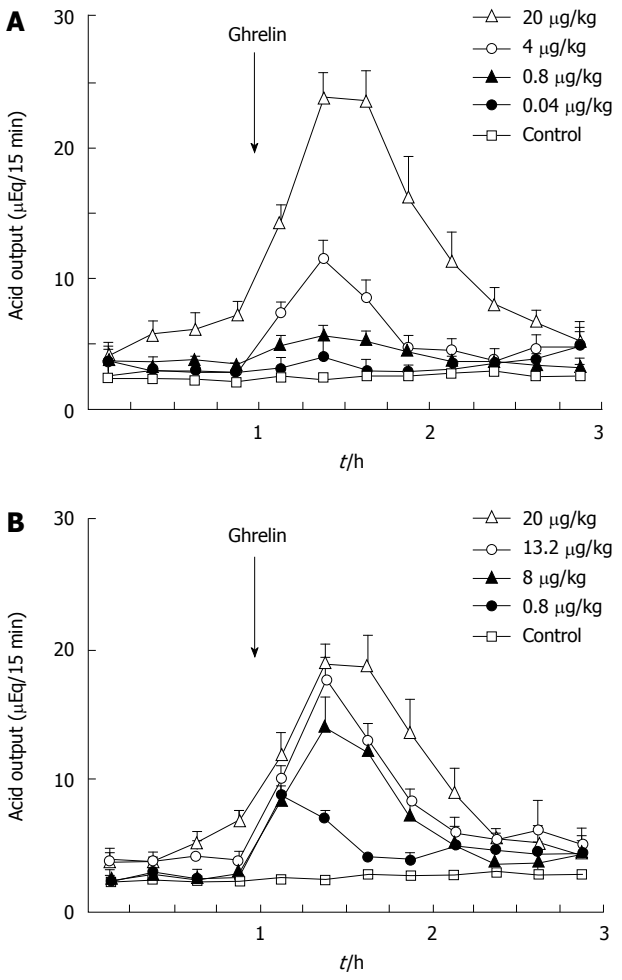

Figure 1 Effects of rat ghrelin (A) and gastrin-17 (B) on acid output in gastric lumen-perfused rats. A: Time course of acid output by Ⅳ administration of ghrelin (0.04, 0.8, 4, 20 μg/kg) in gastric lumen perfused rats; B: Time course of acid output by Ⅳ administration of gastrin (0.8, 8, 13.2 and 20 μg/kg) in gastric lumen perfused rats. Saline was administered in control rats. Each value represents the mean ± SE of acid output at 15 min intervals. *n* = 4, 5, 6, 7. Reprinted with permission[30].

is involved in the action of ghrelin[30]. In our study, ghrelin (0.8-20 μg/kg) dose-dependently increased gastric acid secretion and the action of ghrelin (20 μg/kg, 6×10^{-9} mol/kg) on acid secretion was almost as efficient as that of gastrin (20 μg/kg, 9.2×10^{-9} mol/kg) (Figure 1) [30]. The study demonstrated that vagotomy abolished the increase in acid secretion by ghrelin administration (Figure 2)[30] and also that famotidine completely inhibited the stimulatory action of ghrelin [30]. Furthermore, administration of ghrelin significantly increased HDC mRNA concentration of gastric mucosa (Figure 3) [30]. Vagotomy also abolished the increase in HDC mRNA by ghrelin administration (Figure 4) [30]. Furthermore, in the study, isolated vascularly-perfused stomachs that lacked vagal innervation were prepared in order to examine the effect of ghrelin on histamine release. Although the infusion of gastrin (2.1 μg/10 min) increased histamine release, ghrelin (5 μg/10 min) did not induce histamine release from isolated rat stomachs. Taken together, the results demonstrate the mechanism of action of ghrelin involves the vagal nerve as well as increases in release and synthesis of histamine,

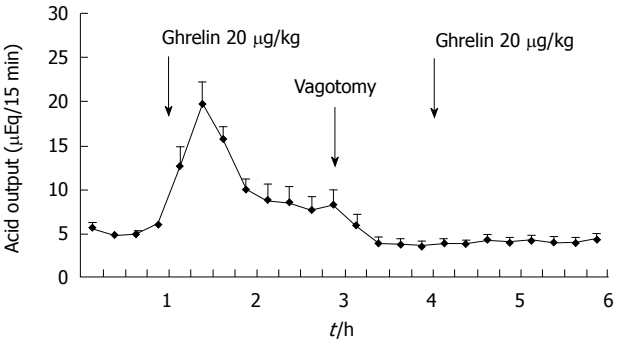

Figure 2 Effect of famotidine on ghrelin-stimulated acid output in gastric lumen-perfused rats. Time course of acid output by the Ⅳ administration of famotidine (0.33 mg/kg) and ghrelin (20 μg/kg) in gastric lumen-perfused rats. Famotidine was administered intravenously 15 min before ghrelin injection. Each value represents the mean ± SE of acid output at 15 min intervals. n = 4. Reprinted with permission[30].

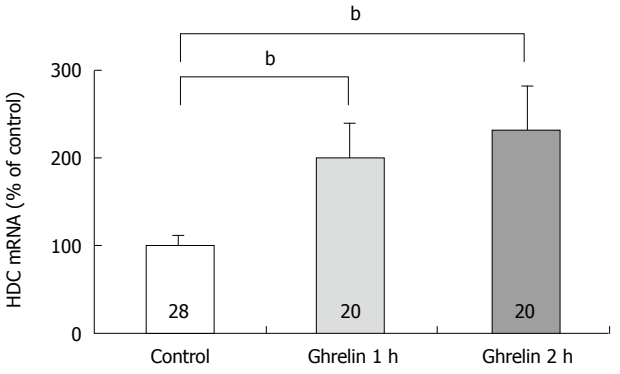

Figure 3 Effect of ghrelin on the concentration of HDC mRNA in rat gatsric mucosa. Ghrelin (20 μg/kg) was administered intravenously. The concentration of HDC mRNA was measured by real time RT-PCR using LightCycler. Control: The concentration of HDC mRNA in gastric mucosa of rats with saline administration. 1 h; the concentration of HDC mRNA in gastric mucosa of rats with ghrelin administration, the stomachs were excised 1 h after the administration. 2 h; the concentration of HDC mRNA in gastric mucosa of rats with ghrelin administration, the stomachs were excised 2 h after the administration. Each value represents the mean ± SE of HDC mRNA demonstrated as % of control. n = 28, 20, 20. ^bP < 0.01. Reprinted with permission[30].

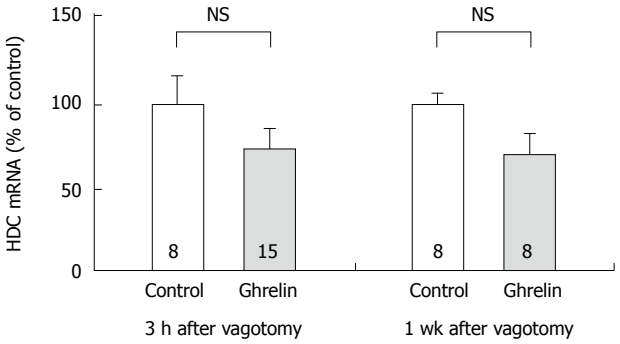

Figure 4 Effect of vagotomy on the concentration of HDC mRNA in rat gastric mucosa stimulated by ghrelin administration. Subdiaphragmatic vagotomy was performed in rats 3 h or 7 d before the experiments. Ghrelin (20 μg/kg) was administered Ⅳ. The concentration of HDC mRNA was measured by real time RT-PCR using Light Cycler. Control: The concentration of HDC mRNA in gastric mucosa of rats with saline administration. Ghrelin: The concentration of HDC mRNA in gastric mucosa of rats with ghrelin administration, the stomachs were excised 2 h after the administration. 3 h after vagotomy, the experiments were performed 3 h after vagotomy. 1 wk after vagotomy, the experiments were performed 7 d after vagotomy. Each value represents the mean ± SE of HDC mRNA demonstrated as % of control. n = 8 or 15. NS: Not significant. Reprinted with permission[30].

consequently induces the stimulation of parietal cells.

The reason that Masuda *et al* did not observe the inhibitory effect of famotidine on the action of ghrelin is unclear. However, there is a difference between the administration methods in the study by Matsuda *et al* and those used in our study. We administered ghrelin intravenously, while Matsuda *et al* administered ghrelin subcutaneously. The difference in the results may thus be due to the difference in the administration methods.

GHRELIN AND GASTRIN IN THE MECHANISM FOR THE REGULATION OF GASTRIC ACID SECRETION

Gastrin is known to be released into the circulation in response to food[31], and to play an important roles in increasing postprandial acid secretion[22], and to exert activity on ECL cells[23]. It was shown that vagotomy did not affect the maximal response of acid secretion to gastrin administration thus indicating that the action of gastrin was not primarily dependent on vagal nerve[32]. On the other hand, ghrelin is released during fasting period, and food intake suppresses its release[33]. As vagotomy completely abolished ghrelin-induced acid secretion and HDC mRNA production, ghrelin is thought to induce histamine release *via* vagal nerve activation, consequently resulting in increased acid secretion by parietal cells. Accordingly the mechanism for the secretion of ghrelin appears to differ from that of gastrin.

Recently, however, synergistic action of ghrelin and ghrelin on gastric acid secretion was reported from two groups[34,35]. Fukumoto *et al* have shown that Ⅳ administration of gastrin induced transient increases of ghrelin levels within 10 minutes and that simultaneous administration of both gastrin and ghrelin resulted in a synergistic increase of gastric acid secretion in rat[35]. They supposed that gastrin may directly stimulate ghrelin release and both hormones may increase gastric acid secretion synergistically[35]. We also presented the data that shows synergistic effects of gastrin and ghrelin on gastric acid secretion and histamine production by gastric mucosa which involves the vagal nerve[34]. Circulating ghrelin decreases soon after the initiation of feeding, gastrin oppositely increase with food intake. Generally simultaneous increases of gastrin and ghrelin do not occur. Therefore physiological role of this synergistic action of these hormones remain to be clarified. We are supposing this synergistic action may occurs in subjects with Hp infection that induces hypergastrinemia in fasting when ghrelin level rises.

CENTRAL REGULATION OF GASTRIC ACID SECRETION BY GHRELIN

The peripheral vagal nerve and the nuclei of central nerves are thought to be involved in the mechanism of

action of ghrelin[6,12]. Ghrelin administered Ⅳ induced Fos expression only in the neurons of the arcuate nucleus of the hypothalamus in rats[12]. On the other hand, ICV injection induced Fos expression in the neurons of NTS and DMX of the medulla oblongata and other nuclei such as several hypothalamic nuclei, the dentate gyrus, the hippocampus, and the cerebral cortex[6,11]. The hypothalamus is known to be the center for hunger and satiety[36]. In particular, the arcuate nucleus of the hypothalamus is activated by ghrelin administration and this region is known to have important role in controlling food intake relating to the action of leptin[37]. Date *et al*[12] indicated that Ⅳ injection of ghrelin induces Fos expression in neuropeptide Y (NPY)-producing and growth hormone-releasing hormone (GHRH)-producing neurons in the arcuate neucleus. It is known that injection of NPY into cerebral ventricles or directly into the hypothalamus of rats potently stimulates food intake[38]. Therefore, the activation of NPY neuron in the arcuate nucleus of the hypothalamus by ghrelin is thought to relate to the stimulatory effect of ghrelin on food intake. However, it is still unclear whether activation of the arcuate nucleus induces increase in the secretion of gastric acid. It has been shown that ghrelin receptors are synthesized in vagal afferent neurons and transported to the afferent terminals[12]. Date *et al*[12] suggested that the gastric vagal afferent nerve, which is capsaicin sensitive, is the major pathway conveying ghrelin signals for starvation and growth hormone secretion to the brain. It is possible that the same pathway mediates the mechanism for the action on the secretion of gastric acid when ghrelin is administered Ⅳ. On the other hand, as described above, DMX, NTS and several nuclei of the brain are apparently activated by ICV administration of ghrelin[6,11]. As DMX has been demonstrated to relate to the secretion of gastric acid, the activation of DMX may be related to the central nervous system mechanism for the action of ghrelin on acid secretion. Furthermore, vagotomy also abolished the stimulatory effect of ICV administration of ghrelin on acid secretion, thus the vagal efferent may also be involved in the action of cerebral ghrelin[11].

The neuronal pathway mediating the peripheral ghrelin appears to be different from that mediating central ghrelin. Although the vagal afferent nerve was demonstrated to be involved in the action of peripheral ghrelin[10,12], the role of vagal efferent nerve remained to be elucidated. However, as the action of cerebral ghrelin is thought to involve vagal nerve, possibly vagal efferent nerve, and the results of our previous study demonstrated that the actions of ghrelin on acid secretion and HDC mRNA production require the vagal nerve to be intact, it is possible that vagal efferent nerve is also involved in the action of peripheral ghrelin. A likely hypothesis based on the results of our study is that peripheral ghrelin stimulation of gastric acid secretion initiates the activation of central regulatory system that induces the activation of vagal nerve, resulting in possibly release of neurotransmitters and ECL cells stimulation.

Recently, as to the action of ghrelin, involvement of NO synthesis has been indicated[40]. Bilgin *et al* reported that ghrelin stimulates the secretion of gastric acid through NO as a mediator, because acid secretion induced by ghrelin administration was inhibited by applying L-NAME in rats. Previously the gastroprotective effect of ghrelin was reported and this can be attenuated by pretreatment of L-NAME suggesting involvement of NO pathways in the effect of ghrelin[40]. As NO increases mucosal blood flow, the effect of ghrelin on the secretion of gastric acid may partially dependent of an increase of mucosal blood flow.

THE PHYSIOLOGICAL ROLE OF GHRELIN IN THE REGULATION OF ACID SECRETION

The physiological role of ghrelin in the regulation of acid secretion still remains unclear. Comparing the secretion of ghrelin with that of gastrin, the secretion of gastrin is induced by food intake[26], while the secretion of ghrelin is increased during fasting period[33]. As described above, there are many differences between gastrin and ghrelin in the actions, their roles may be different. Gastrin is known to play important roles in the postprandial secretion of gastric acid, while ghrelin may be related to acid secretion during fasting period or at night. To elucidate the physiological role of ghrelin in acid secretion, further studies are required.

REFERENCES

1 **Kojima M**, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660

2 **Date Y**, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000; **141**: 4255-4261

3 **Tschop M**, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000; **407**: 908-913

4 **Wren AM**, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 2000; **141**: 4325-4328

5 **Kamegai J**, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I. Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. *Endocrinology* 2000; **141**: 4797-4800

6 **Nakazato M**, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature* 2001; **409**: 194-198

7 **Asakawa A**, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 2001; **120**: 337-345

8 **Shintani M**, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway.

Diabetes 2001; **50**: 227-232
9 **Wren AM**, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR. Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 2001; **86**: 5992
10 **Masuda Y**, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K. Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun* 2000; **276**: 905-908
11 **Date Y**, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochem Biophys Res Commun* 2001; **280**: 904-907
12 **Date Y**, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K, Nakazato M. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 2002; **123**: 1120-1128
13 **Tache Y**, Goto Y, Hamel D, Pekary A, Novin D. Mechanisms underlying intracisternal TRH-induced stimulation of gastric acid secretion in rats. *Regul Pept* 1985; **13**: 21-30
14 **Grossman MI**. Secretion of acid and pepsin in response to distention of vagally innervated fundic gland area in dogs. *Gastroenterology* 1962; **42**: 718-721
15 **Antia F**, Rosiere CE, Robertson C, Grossman MI. Effect of vagotomy on gastric secretion and emptying time in dogs. *Am J Physiol* 1951; **166**: 470-479
16 **Debas HT**, Konturek SJ, Walsh JH, Grossman MI. Proof of a pyloro-oxyntic reflex for stimulation of acid secretion. *Gastroenterology* 1974; **66**: 526-532
17 **Ternaux JP**, Falempin M, Palouzier B, Chamoin MC, Portalier P. Presence of cholinergic neurons in the vagal afferent system: biochemical and immunohistochemical approaches. *J Auton Nerv Syst* 1989; **28**: 233-242
18 **Dockray GJ**, Vaillant C, Walsh JH. The neuronal origin of bombesin-like immunoreactivity in the rat gastrointestinal tract. *Neuroscience* 1979; **4**: 1561-1568
19 **Sternini C**. Vagal afferent innervation of the enteric nervous system. In: Ritter S, Ritter RC, Barnes CD. editors. Neuroanatomy and physiology of abdominal vagal afferents. Boca Raton, FL: CRC Press, 1992: 135-156
20 **Tornoe K**, Hannibal J, Georg B, Schmidt PT, Hilsted L, Fahrenkrug J, Holst JJ. PACAP 1-38 as neurotransmitter in the porcine antrum. *Regul Pept* 2001; **101**: 109-121
21 **Sugano K**, Park J, Soll AH, Yamada T. Stimulation of gastrin release by bombesin and canine gastrin-releasing peptides. Studies with isolated canine G cells in primary culture. *J Clin Invest* 1987; **79**: 935-942
22 **Richardson CT**, Walsh JH, Hicks MI, Fordtran JS. Studies on the mechanisms of food-stimulated gastric acid secretion in normal human subjects. *J Clin Invest* 1976; **58**: 623-631
23 **Chen D**, Monstein HJ, Nylander AG, Zhao CM, Sundler F, Hakanson R. Acute responses of rat stomach enterochromaffinlike cells to gastrin: secretory activation and adaptation. *Gastroenterology* 1994; **107**: 18-27
24 **Lindstrom E**, Bjorkqvist M, Boketoft A, Chen D, Zhao CM, Kimura K, Hakanson R. Neurohormonal regulation of histamine and pancreastatin secretion from isolated rat stomach ECL cells. *Regul Pept* 1997; **71**: 73-86
25 **Kivipelto L**, Absood A, Arimura A, Sundler F, Hakanson R, Panula P. The distribution of pituitary adenylate cyclase-activating polypeptide-like immunoreactivity is distinct from helodermin- and helospectin-like immunoreactivities in the rat brain. *J Chem Neuroanat* 1992; **5**: 85-94
26 **Zeng N**, Kang T, Lyu RM, Wong H, Wen Y, Walsh JH, Sachs G, Pisegna JR. The pituitary adenylate cyclase activating polypeptide type 1 receptor (PAC1-R) is expressed on gastric ECL cells: evidence by immunocytochemistry and RT-PCR. *Ann N Y Acad Sci* 1998; **865**: 147-156
27 **Prinz C**, Zanner R, Gratzl M. Physiology of gastric enterochromaffin-like cells. *Annu Rev Physiol* 2003; **65**: 371-382
28 **Chew CS**, Hersey SJ, Sachs G, Berglindh T. Histamine responsiveness of isolated gastric glands. *Am J Physiol* 1980; **238**: G312-G320
29 Uvnas B. The part played by the pyloric region in the cephalic phase of gastric secretion. *Acta Physiol Scan* 1942; **4** (Suppl 13): 1-7
30 **Yakabi K**, Ro S, Onouhi T, Tanaka T, Ohno S, Miura S, Johno Y, Takayama K. Histamine mediates the stimulatory action of ghrelin on acid secretion in rat stomach. *Dig Dis Sci* 2006; **51**: 1313-1321
31 **Feldman M**, Walsh JH, Wong HC, Richardson CT. Role of gastrin heptadecapeptide in the acid secretory response to amino acids in man. *J Clin Invest* 1978; **61**: 308-313
32 **Emas S**, Grossman MI. Effect of truncal vagotomy on acid and pepsin responses to histamine and gastrin in dogs. *Am J Physiol* 1967; **212**: 1007-1012
33 **Cummings DE**, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001; **50**: 1714-1719
34 **Ro S**, Tanaka T, Ochiai M, Yakabi K. The interaction between gastrin and ghrelin on acid secfretion in rat stomsch. *Gastroentwrology* 2004; **26**: A147
35 **Fukumoto K**, Nakahara K, Katayama T, Miyazatao M, Kangawa K, Murakami N. Synergistic action of gastrin and ghrelin on gastric acid secretion in rats. *Biochem Biophys Res Commun* 2008; **374**: 60-63
36 **Bray GA**, Fisler J, York DA: Neuroendocrine control of the development of obesity: understanding gained from studies of experimental animal models. *Front Neuroendocrinol* 1990; **11**: 128-181
37 **Satoh N**, Ogawa Y, Katsuura G, Hayase M, Tsuji T, Imagawa K, Yoshimasa Y, Nishi S, Hosoda K, Nakao K. The arcuate nucleus as a primary site of satiety effect of leptin in rats. *Neurosci Lett* 1997; **224**: 149-152
38 **Stanley BG**, Kyrkouli SE, Lampert S, Leibowitz SF. Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides* 1986; **7**: 1189-1192
39 **Wyrwicka W**, Garcia R. Effect of electrical stimulation of the dorsal nucleus of the vagus nerve on gastric acid secretion in cats. *Exp Neurol* 1979; **65**: 315-325
40 **Bilgin HM**, Tumer C, Diken H, Kelle M, Sermet A. Role of ghrelin in the regulation of gastric acid secretion involving nitrergic mechanisms in rats. *Physiol Res* 2008; **57**: 563-568

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c-Fos overexpression increases the proliferation of human hepatocytes by stabilizing nuclear Cyclin D1

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Abstract

AIM: To investigate the effect of stable c-Fos overexpression on immortalized human hepatocyte (IHH) proliferation.

METHODS: IHHs stably transfected with c-Fos (IHH-Fos) or an empty vector (IHH-C) were grown in medium supplemented with 1% serum or stimulated with 10% serum. Cell proliferation was assessed by cell counts, 3H-thymidine uptake and flow cytometry analyses. The levels of cell cycle regulatory proteins (Cyclin D1, E, A) cyclin dependent kinases (cdk) cdk2, cdk4, cdk6, and their inhibitors p15, p16, p21, p27, total and phosphorylated GSK-3β and epidermal growth factor receptor (EGF-R) were assayed by Western blotting. Analysis of *Cyclin D1* mRNA levels was performed by reverse transcription-polymerase chain reaction and real-time polymerase chain reaction (PCR) analysis. Stability of Cyclin D1 was studied by cycloheximide blockade experiments.

RESULTS: Stable c-Fos overexpression increased cell proliferation under low serum conditions and resulted in a two-fold increase in [^{3}H]-thymidine incorporation following serum addition. Cell cycle analysis by flow cytometry showed that c-Fos accelerated the cell cycle kinetics. Following serum stimulation, Cyclin D1 was more abundantly expressed in c-Fos overexpressing cells. Cyclin D1 accumulation did not result from increased transcriptional activation, but from nuclear stabilization. Overexpression of c-Fos correlated with higher nuclear levels of inactive phosphorylated GSK-3β, a kinase involved in Cyclin D1 degradation and higher levels of *EGF-R* mRNA, and EGF-R protein compared to IHH-C both in serum starved, and in serum stimulated cells. Abrogation of EGF-R signalling in IHH-Fos by treatment with AG1478, a specific EGF-R tyrosine kinase inhibitor, prevented the phosphorylation of GSK-3β induced by serum stimulation and decreased Cyclin D1 stability in the nucleus.

CONCLUSION: Our results clearly indicate a positive role for c-Fos in cell cycle regulation in hepatocytes. Importantly, we delineate a new mechanism by which c-Fos could contribute to hepatocarcinogenesis through stabilization of Cyclin D1 within the nucleus, evoking a new feature to c-Fos implication in hepatocellular carcinoma.

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Key words: c-Fos; Cyclin D1; GSK-3; Cell growth; Cell cycle; Hepatoma; Epidermal growth factor

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, with an increasing number of new cases emerging each year. Etiologically it is linked to chronic viral infections (hepatitis B and C viruses), alcohol-related cirrhosis or aflatoxin B1 exposure, which

all cause disruptions in signal transduction cascades leading to abnormalities in gene expression.

The proto-oncogene *c-fos* is an important member of the activating protein 1 (AP-1) transcription factor involved in major cellular functions such as transformation, proliferation, differentiation and apoptosis[1,2]. Such a large variety of functions is achieved by the combination of different Jun (c-Jun, JunB or JunD) and Fos (c-Fos, FosB, ΔfosB, Fra-1, Fra-2) family members forming various AP-1 homo and heterodimers. *c-fos* is an immediate early gene whose expression is rapidly and transiently induced after mitogenic stimulation[3]. The role of c-Fos in cell proliferation and transformation remains controversial. c-Fos is required during all phases of the cell cycle in exponentially growing cells and is a potent inducer of cell proliferation[4]. However, some studies have suggested that c-Fos poorly contributes to proliferation[5], was totally dispensable for[6], or even down-regulated, cell growth[7,8]. Overexpression of c-Fos leads to morphological transformation of fibroblasts[9,10], and to osteosarcoma formation in transgenic mice[11,12]. Apart from one study describing a negative role for c-Fos in hepatocellular tumorigenesis[8], several reports rather support a potential positive role for c-Fos in this process. High expression levels of c-Fos were determined in tumour tissue compared to the adjacent non-tumour liver in human HCC[13-15], as well as in several models of HCC in rodents[16-18]. A recent study in humans identified a subtype of HCC sharing gene expression patterns with foetal hepatoblasts which can be distinguished from another HCC subtype closer to adult hepatocytes[19]. Interestingly, c-Fos, but not c-Jun expression was higher in the foetal subtype which displayed a poorer prognosis and a greater tendency to invasion than the adult subtype. In addition, the expression of DNA 5-methylcytosine transferase, a c-Fos target gene involved in DNA methylation[20] is increased in human tumour cells and in HCCs[21]. Despite these studies showing that c-Fos overexpression might be an important step towards the development of liver cancer, its precise role in hepatocarcinogenesis remains ill-defined.

In order to clarify c-Fos implication in hepatocarcinogenesis, we examined the effect on proliferation of stable c-Fos overexpression in immortalized human hepatocytes (IHH). We show, for the first time, that a positive role for c-Fos on hepatocyte proliferation can be attained by stabilization of Cyclin D1 in the nuclear compartment, a mechanism which has not been described as a c-Fos related process in any cell type to date.

MATERIALS AND METHODS

Cell culture and reagents

IHH were cultured in Williams' medium E (Invitrogen, Cergy Pontoise, France) supplemented with 100 mL/L fetal calf serum (FCS) (Biochrom AG, Cambridge, UK), 1% penicillin-streptomycin, 1% Glutamax and 1% DMEM sodium pyruvate (Invitrogen). Specific reagents were AG1478 (Calbiochem, San Diego, CA) and cycloheximide (CHX) (Euromedex, Souffelweyersheim, France).

Generation of stably transfected cells

The human *c-fos* cDNA was inserted into the cytomegalovirus driven pCIneo expression vector (Promega, Charbonnières, France) containing a neomycin resistance gene to obtain the pCIneo-*cfos* vector. Cells were stably transfected by electroporation (230V, 960 μF) in PBS-Hepes Buffer 10 mmol/L, pH 7.4 with the empty vector (pCIneo) or with pCIneo-*cfos*. Two days post-transfection, stable clones were selected in media containing 500 μg/mL of G418 (Invitrogen). The resistant clones were pooled after 3 wk of selection, and maintained with G418. c-Fos overexpression was verified by Western blot analysis as shown in Figure 1A.

Growth curve analysis

Cells were plated in triplicate at a density of 1.0×10^5 per well in six-well plates, and cultured in low serum (1% FCS) conditions for 5 d. Triplicate cultures were trypsinized and diluted in an equal volume of trypan blue solution (Invitrogen). Viable cells were counted daily in a haemocytometer counting chamber.

[^{3}H]-thymidine incorporation

DNA synthesis was determined by measuring [^{3}H]-thymidine incorporation. Cells were plated onto 24-well plates at a density of 1.0×10^5 cells/well in quadruplets. Cells were serum deprived for 24 h, and serum stimulated in culture media containing 1.5 μCi/mL tritiated thymidine ([^{3}H]dT) (specific activity of 740 GBq/mmol) (Perkin-Elmer, Waltham, Ma) for 4 h. Cells were fixed and washed in ice-cold 10% trichloroacetic acid. DNA was solubilized in 0.1 mol/L NaOH for 1 h at 37℃. [^{3}H]dT incorporated into the DNA was measured using liquid scintillation counting.

Flow cytometry

DNA cell cycle analysis was measured by 5-bromodeoxyuridine (BrdU) incorporation and propidium iodide staining of the nuclei by flow cytometry (FACScalibur, BD Biosciences, Mansfield, MA) and analyzed with the ProCellQuest software provided by the manufacturer. Cell cycle progression was measured by pulse/chase experiments. Cells plated at a density of 5×10^5 per 6-cm dish were serum starved for 24 h, serum stimulated for 12 h and stained with BrdU (30 μg/mL) for 1 h. Cells were then chased with BrdU free medium for 0, 3, 6, 9, 12 h, stained with propidium iodide and harvested in 70% ethanol. Cells were then treated with 2 N HCl and pepsin (0.2 mg/mL) for 30 min. BrdU content was analyzed using a FITC-labeled monoclonal antibody to BrdU (BD Pharmingen, Le-Pont-de-Claix, France). Labeled cells were washed and resuspended in PBS containing propidium iodide (10 μg/mL) for 30 min prior to flow cytometric analysis.

Western blot analysis

Nuclear proteins were extracted as described[22]. Total proteins were extracted with lysis buffer [1% (v/v) SDS, 1 mmol/L Na_3VO_4, 10 mmol/L Tris pH 7.4, 1% benzonase) for 10 min at room temperature and heated for

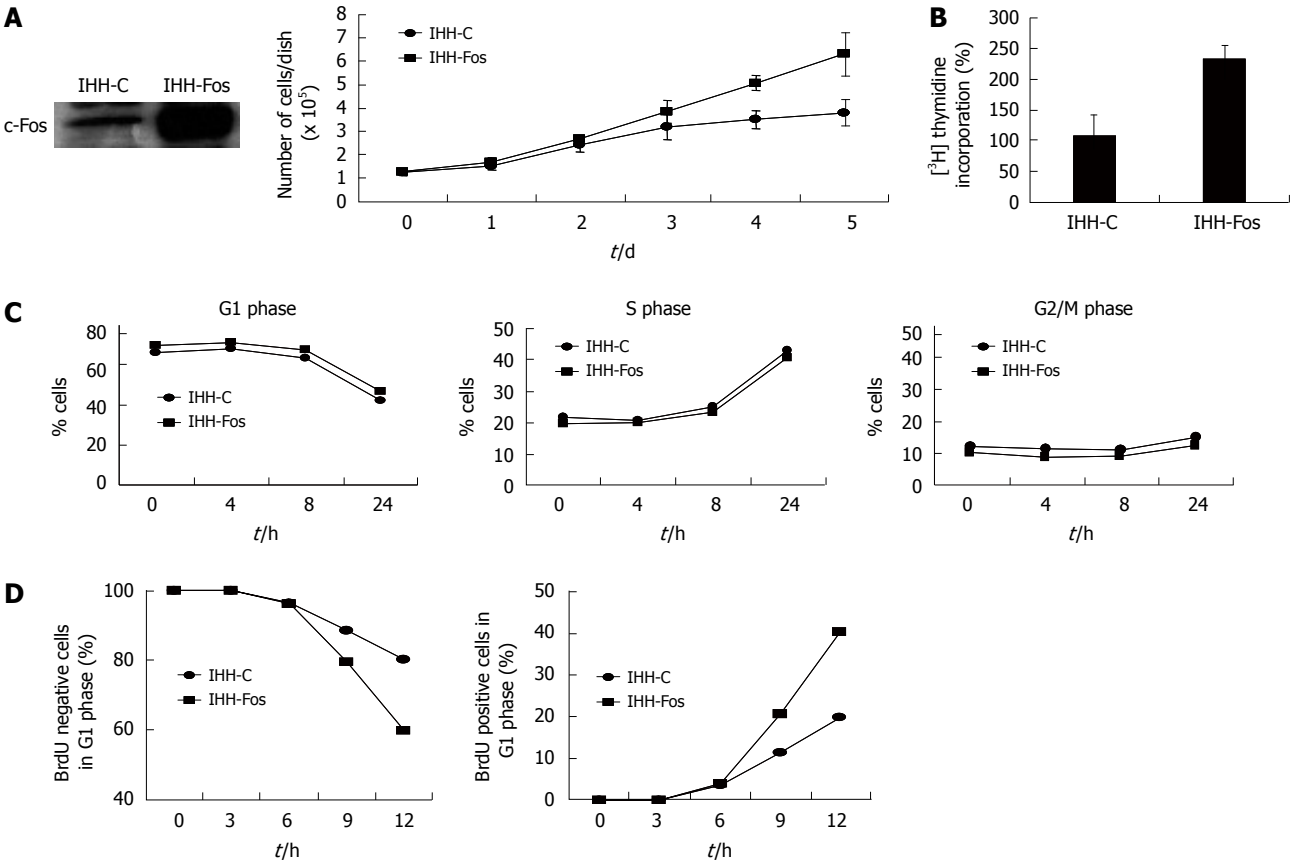

Figure 1 Overexpression of c-Fos accelerates the cell cycle. A: IHH-C and IHH-Fos were grown in 1% FCS, cultured for 5 d and counted daily. Cell growth was determined by counting the number of attached cells every day. Results are the mean ± SE of three independent experiments; B: [^{3}H] thymidine incorporation into DNA. Non-synchronized IHH-C or IHH-Fos serum starved for 24 h then serum stimulated for 4 h were incubated with [^{3}H] thymidine for 4 h. DNA was extracted as described in materials and methods, and [^{3}H] thymidine incorporation into DNA was assessed by scintillation counting. Results are expressed as percentage of increase of [^{3}H] thymidine incorporation in serum-stimulated cells over that of quiescent cells for each cell population. Results are the mean ± SE of six independent experiments; C: Flow cytometry analysis for quantification of cell cycle phase distribution and progression through cell cycle. IHH-C or IHH-Fos serum starved for 24 h were incubated with BrdU for 1 h and stained with propidium iodide 0, 4, 8 and 24 h after serum stimulation. The percentage of cells in each phase is plotted against time. Results of a representative experiment are shown (out of 3); D: IHH-C or IHH-Fos serum starved for 24 h were serum stimulated for 12 h, BrdU pulsed for 1 h, chased with fresh medium for 0, 3, 6, 9, 12 h, and then stained with propidium iodide. The percentage of BrdU-negative cells in the G1 phase (G1 exit) (left panel) and of the BrdU-positive cells in the G1 phase (G1 entry) (right panel) of the cell cycle is plotted against time. Results are representative of four independent experiments.

5 min at 100℃. Equal quantities of nuclear proteins were fractionated on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes by electroblotting. The antibodies used in this study were as follows: c-Fos, Cyclin D1, Cyclin E, Cyclin A, cdk2, cdk4, cdk6, p15, p16, p21, p27 and EGF-R (Santa Cruz Biotechnology, Santa Cruz, CA), GSK3β (Affinity BioReagents, Golden, CO), Phospho-GSK3β (Serine9) (Abcam, Paris, France). Immunoreactive bands were visualized using the ECL kit (Amersham BioSciences, Saclay, France) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR analysis

mRNA was isolated from cells by Nucleospin RNA II kit (Macherey-Nagel, Hoerdt, France) following the manufacturer's instructions. RNA (1 μg) was reverse transcribed using ThermoScript™ RT-PCR System (Invitrogen, Cergy-Pontoise, France). Real-time quantitative PCR was performed using the following primers: *Cyclin D1*: forward 5'-GCATGTTCGTGGCCTCTAAGA-3'; reverse 5'-CGGTGTAGATGCACAGCTTCTC-3', *EGF-R*: forward 5'-GCGTCTCTTGCCGGAATGT-3' and reverse 5'-GGCTCACCCTCCAGAAGGTT-3'. Real-time quantitative PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA)) using SYBRGreen PCR core reagents (Applied Biosystems). Fold changes in mRNA were calculated by the $\Delta\Delta C_t$ method using *cyclophilin A* (forward: 5'-CAAATGCTGGACCCAACACA-3'; reverse: 5'-TGCCATCCAACCACTCAGTCT-3') as a standard. All PCR reactions were done in triplicate.

Statistical analysis

Data were expressed as means ± SE. Student's *t*-test was performed and statistical significance was considered as $P < 0.05$.

RESULTS

Growth rate and cell cycle regulation by c-Fos overexpression

To determine whether c-Fos could modulate hepatocyte growth, we carried out growth curve assays. While

cell growth was similar in the presence of 10% FCS in IHH-C and IHH-Fos (data not shown), we observed that the growth pattern of the two cell lines differed in low serum conditions (1% FCS). While the number of IHH-Fos increased exponentially over 5 d in culture, IHH-C number increased slowly during the first 3 d of culture, and then reached a plateau, due to the induction of cell death by serum deprivation (Figure 1A and data not shown). Thus, c-Fos overexpression correlated with a more rapid growth in low serum conditions. The effect of c-Fos overexpression on cell proliferation was further established by measuring [^{3}H]dT incorporation following 4 h serum stimulation of cells deprived of serum for 24 h. The increase of [^{3}H]dT incorporation induced by serum was 2.2 times higher in IHH-Fos than in IHH-C (231% and 107%, respectively), and the difference was statistically significant ($P < 0.001$) (Figure 1B). To further analyze the role of c-Fos on the cell cycle, cell cycle phase distribution and cell cycle kinetics were analyzed by flow cytometry. Following serum stimulation, the percentage of cells in the G1 phase decreased, while the percentage of cells in the S-phase increased 24 h after serum stimulation. However, the percentage of cells in the different stages of the cell cycle was comparable in IHH-Fos and IHH-C (Figure 1C). Cell cycle progression was measured by BrdU pulse/chase experiments. The rate at which BrdU positive cells progress into G1 indicates the rate of transit through S, G2 and M phases. Similarly, the rate at which BrdU negative cells become depleted from the G1 pool indicates the transit rate through G1. We show that IHH-Fos quit (Figure 1D, left panel) and enter (Figure 1D, right panel) G1 faster than IHH-C, which reflects a global increase in cell cycle kinetics. The fact that the cell cycle profile was not altered by c-Fos indicates that the acceleration is proportional in all phases of the cycle. These data taken together indicate that c-Fos overexpression increases the growth of exponentially growing cells cultured in low serum medium as well as the proliferation response induced by serum refeeding.

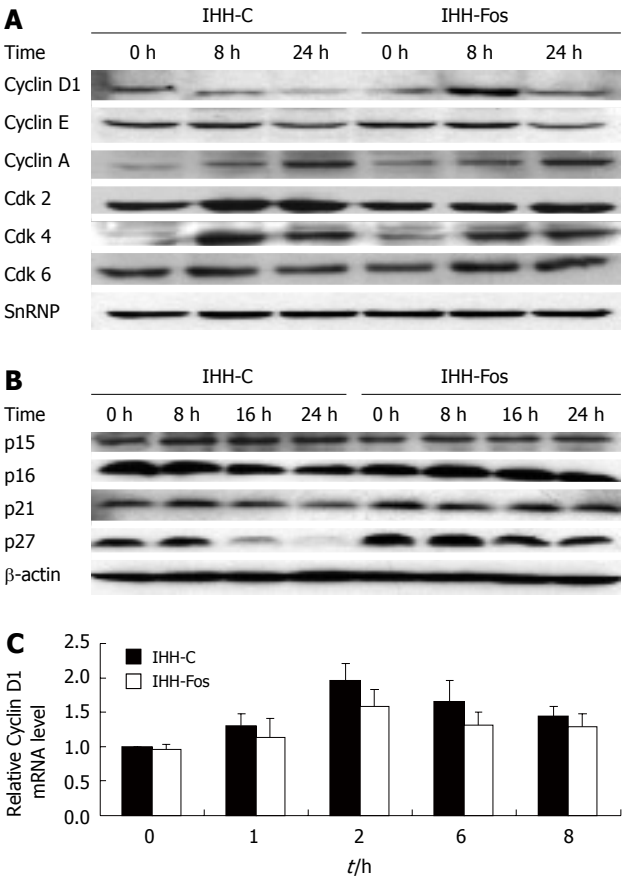

Figure 2 Induction of cell cycle regulatory proteins after serum refeeding. IHH-C or IHH-Fos were serum starved for 24 h. Nuclear (A) or total (B) extracts prepared before or after serum stimulation for 8 h or 24 h were immunoblotted with antibodies, as indicated. Loading of nuclear or total extracts was normalized using a SnRNP or a β-actin antibody, respectively. Results of a representative experiment are shown (out of 3); C: Quantitative real time PCR of Cyclin D1 mRNA levels in quiescent IHH-C or IHH-Fos serum stimulated for the indicated times. Bars indicate mean ± SE of three independent experiments each performed in triplicate.

Induction of cell cycle regulatory proteins by c-Fos

The levels of various cell cycle regulatory proteins before and after serum stimulation of IHH-C and IHH-Fos were analyzed by Western blotting experiments. In both cell lines, serum addition induced an increase in the nuclear levels of Cyclin A, cdk2 and cdk4, but no change in Cyclin E. Of interest, the nuclear levels of Cyclin D1 were increased after 8 h of stimulation in IHH-Fos, but not in IHH-C (Figure 2A). In addition, the levels of p27 were higher in the absence of serum stimulation or following serum stimulation in IHH-Fos than in IHH-C (Figure 2B).

Quantitative RT-PCR analysis was performed to determine whether the increase of Cyclin D1 at 8 h of serum stimulation in IHH-Fos was controlled transcriptionally. Interestingly, a similar 2-fold increase in *Cyclin D1* mRNA 2 h following serum stimulation was observed in IHH-Fos and IHH-C, without any significant differences at any of the time points (Figure 2C), indicating that the higher levels of Cyclin D1 in the nucleus in IHH-Fos are not due to transcriptional mechanisms.

Cyclin D1 stabilization in the nucleus

We next determined whether post-translational regulations could explain the increase of nuclear Cyclin D1 in serum-stimulated IHH-Fos. CHX, a translational inhibitor, was used to block protein synthesis. While in IHH-C, nuclear Cyclin D1 protein levels started to decline as from 1 h, and decreased by 85% after 2 h of CHX treatment, Cyclin D1 nuclear levels were decreased by only 20% upon 2 h of CHX treatment in IHH-Fos (Figure 3), indicating that c-Fos overexpression correlates with increased stability of nuclear Cyclin D1.

Inactivation of GSK-3β in IHH-Fos contributes to Cyclin D1 stabilization

Previous studies have indicated that Cyclin D1 degradation is triggered by GSK-3β-induced phosphorylation on a single threonine residue (Thr-286)[23]. Of note, phosphorylated GSK-3β is the inactive form of the protein[24]. We, therefore, compared the level of

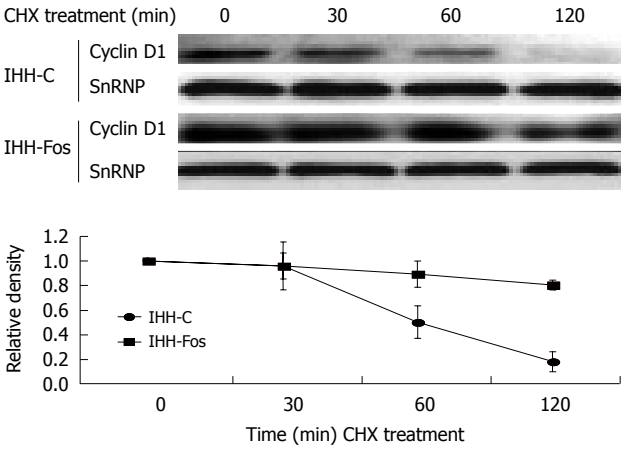

Figure 3 Nuclear Cyclin D1 stability is increased by c-Fos overexpression. IHH-C or IHH-Fos serum starved for 24 h were serum stimulated for 6 h and then treated with CHX (30 μg/mL) for 30 min, 1 h or 2 h. Nuclear extracts were immunoblotted with an antibody against Cyclin D1, and normalized with a SnRNP antibody, as indicated (Upper panels). The Cyclin D1 over SnRNP ratios were quantified by densitometric analysis of the immunoreactive bands (Lower panel). The results are the mean ± SE of three independent experiments.

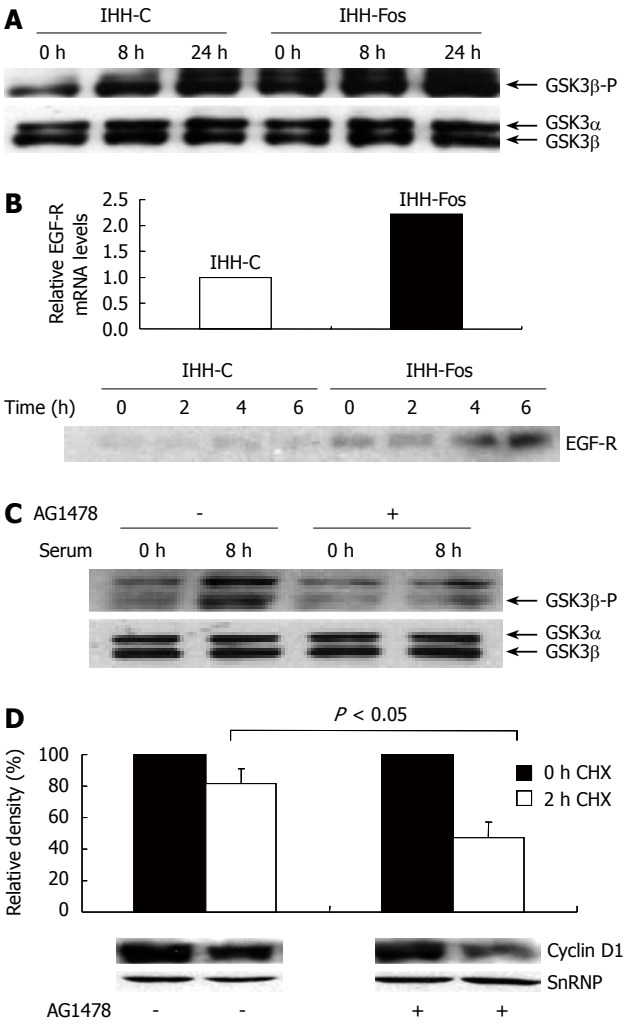

Figure 4 Stimulation of EGF-R signaling by c-Fos overexpression. A: Total and phosphorylated levels of nuclear GSK-β. IHH-C or IHH-Fos were serum starved for 24 h. Nuclear extracts prepared from unstimulated (0 h), 8 h or 24 h serum stimulated IHH-C or IHH-Fos, were immunoblotted with an antibody against phosphorylated or total GSK3β. B: Upper panel, detection of EGF-R mRNA in IHH-C and IHH-Fos by quantitative real time PCR analysis of mRNA isolated from cells grown in the presence of serum. Lower panel, Western blot analysis of EGF-R in total cell extracts from IHH-C and IHH-Fos cells serum starved for 24 h (0) or stimulated with serum for the indicated times; C: Serum deprived IHH-Fos were pre-treated (+) or not (-) with AG1478 (10 μmol/L) for 1 h. Nuclear proteins were prepared from non stimulated and 8 h serum-stimulated cells. Phosphorylated and total GSK3β levels were detected by Western blot; D: Serum-deprived IHH-Fos were pretreated or not with AG1478 (10 μmol/L) for 1 h, then serum-stimulated for 6 h. Nuclear proteins were extracted before (0 h, filled columns) or after 2 h (empty columns) of CHX treatment (30 μg/mL). Cyclin D1 levels were quantified by Western blotting. The immunoreactive bands were quantified by densitometric analysis after loading normalization of the blot using a SnRNP antibody. The results are expressed as the % of Cyclin D1/SnRNP expression and are the mean ± SE of 3 independent experiments. The lower panel illustrates one representative experiment.

phosphorylated GSK-3β between the two cell lines by Western blot analysis. As shown in Figure 4A, the levels of GSK3β phosphorylation were much higher in the nucleus of IHH-Fos than in IHH-C, both in unstimulated and in serum stimulated conditions, indicating higher basal, and induced levels of inactive GSK-3β in IHH-Fos (Figure 4A). Therefore, a decrease in active GSK-3β in IHH-Fos could be responsible for the increased stability of nuclear Cyclin D1 after serum stimulation.

Several signaling pathways are able to induce GSK-3β phosphorylation, including the two main cascades targeted by tyrosine kinase receptors: the phosphatidyl inositol 3-kinase (PI3K) and the Ras/mitogen activated protein kinase pathways[24]. Since the epidermal growth factor receptor (EGF-R) is a known transcriptional target of AP-1[25-27], we tested the hypothesis that overexpression of EGF-R might contribute to high levels of GSK-3β phosphorylation in IHH-Fos. Quantitative RT-PCR analysis revealed a 2.2-fold increase in the basal level of *EGF-R* mRNA in IHH-Fos compared to IHH-C (Figure 4B). Higher levels of EGF-R protein were also observed in serum starved and serum-stimulated IHH-Fos compared to IHH-C (Figure 4B), strongly indicative of increased EGF-R signaling in c-Fos-overexpressing cells. Altogether, these data suggest that increased EGF-R signaling might contribute, at least partly, to increased levels of GSK-3β phosphorylation in IHH-Fos cells.

To demonstrate the implication of EGF-R in GSK-3β-mediated Cyclin D1 stabilization, IHH-Fos cells were treated with AG1478, a specific inhibitor of the EGF-R tyrosine kinase before serum stimulation. Western blot analysis indicated that AG1478 treatment did block the phosphorylation of GSK-3β induced by serum (Figure 4C). Interestingly, the decrease in the nuclear level of Cyclin D1 protein observed after a 2 h-CHX treatment of IHH-Fos cells stimulated by serum was more important in cells treated with AG1478 (50% decrease) than in untreated cells (10% decrease), and the difference was statistically significant ($P < 0.05$, Figure 4D), confirming that blockade of EGF-R induced signaling in IHH-Fos leads to a more rapid nuclear Cyclin D1 degradation.

DISCUSSION

Our results indicate that c-Fos overexpression accelerates

cell growth under reduced serum concentration suggesting that hepatocytes overexpressing c-Fos become relatively independent of the presence of growth factors. We show that c-Fos enhances DNA synthesis after serum stimulation, and accelerates hepatocyte cell cycle progression without altering the overall distribution of cells in each phase due to a proportional acceleration of cell cycle kinetics in all phases.

Our results are at variance with those obtained in immortalized murine hepatocytes[8]. In this model, c-Fos conditional expression for 48 h was shown to decrease cell growth and [^{3}H]dT incorporation of cells grown in serum-supplemented medium. Besides species differences (murine *vs* human cells), the discrepancy in results can be explained by the use of very different cellular models which cannot be compared. The human hepatocytes used in our study were immortalized by SV40 T antigen while murine hepatocytes were immortalized using truncated c-Met[28]. Overexpression of c-Fos in our model was permanently established as the result of stable transfection, while in Mikula's study a c-Fos-estrogen receptor fusion protein was expressed for a limited period (1-3 d) following estradiol treatment of the cultures[8]. Furthermore, the function of the conditionally expressed c-Fos protein may have been modified, since gene fusion has been shown to alter the function of Fos family proteins[29].

We aimed to determine whether the positive role of c-Fos on hepatocyte proliferation depicted in our study was mediated through changes in cell cycle regulation. Different studies have reported an effect of *c-fos* gene deletion or c-Fos protein overexpression on Cyclin D1[9,30,31], Cyclin E[31] or Cyclin A[32] expression, depending on the cell type studied. In our study, while the levels of Cyclin E and A and their associated kinases varied with a similar pattern in both cell types following serum stimulation, nuclear Cyclin D1 levels were higher in IHH-Fos compared to IHH-C 8 h after serum re-feeding. Contrary to previous reports describing c-Fos as a transcriptional activator of Cyclin D1[30], the higher levels of nuclear Cyclin D1 in IHH-Fos than in IHH-C were not due to differences in transcriptional regulation, but to increased protein stability in the nucleus. A similar lack of correlation between Cyclin D1 mRNA and protein expression has been previously described in an *in vivo* experimental model of HCC[33]. Our results strongly suggest a mechanism whereby c-Fos induces nuclear accumulation of Cyclin D1 without affecting the total cellular amount of the protein.

The Cyclin D1 protein is quite unstable, with a half-life of less than 30 min[34]. It accumulates in the nucleus during the G1 phase and exits into the cytoplasm during the S phase. Nuclear export of Cyclin D1, and its subsequent ubiquitination and proteolysis, are dependent on phosphorylation on a single threonine residue (Thr-286) performed mainly by GSK-3β[23], a protein kinase active only when dephosphorylated. In contrast to Cyclin D1, GSK-3β is predominantly cytoplasmic during G1 phase, but a considerable amount becomes nuclear during S phase[23]. We show herein that phosphorylated levels of nuclear GSK-3β are higher in IHH-Fos than in IHH-C. Lower levels of active GSK-3β would consequently lead to a decrease in Cyclin D1 phosphorylation, resulting in its nuclear accumulation in IHH-Fos. Since EGF-R is a known transcriptional target of AP-1[25-27], we tested the possibility that c-Fos overexpression increases the activation of the pathways downstream to EGF signaling. EGF-R activates both the PI3K and the mitogen-activated protein kinase cascades[35], two upstream activators of GSK-3β phosphorylation[24,36]. In support of an involvement of EGF-R signaling in GSK-3β inactivation and nuclear cylin D1 stabilization, we show that IHH-Fos display increased levels of expression of *EGF-R* mRNA and protein than IHH-C. Furthermore, blocking the activation of the EGF-R tyrosine kinase significantly accelerates the rate of Cyclin D1 degradation assessed in CHX experiments. Upregulated expression of EGF-R is a frequent finding in HCC[37-39], and increased EGF-R signaling has been associated with a poorer prognosis[40]. c-Fos is also frequently overexpressed in HCC tumoral tissues[13-15,41]. Our data, therefore, suggest that a causal relationship could exist between c-Fos and EGF-R overexpression in HCC.

Our finding of high levels of nuclear Cyclin D1 associated with c-Fos overexpression adds further support for a contributing effect of c-Fos on HCC development. Indeed, Cyclin D1 exit from the nucleus during S phase is essential for regulated cell division, and its retention in the nucleus is a cancer promoting or predisposing event[42]. Thus, expression of a Cyclin D1 mutant that cannot be phosphorylated by GSK-3β, and remains nuclear throughout the cell cycle is highly transforming and induces tumour growth in nude mice[43].

In accordance with previous reports[32,44], we also found that p27 protein levels were higher in c-Fos overexpressing cells. It is now well recognized that the family of p21/p27 proteins plays a dual role in cell cycle regulation. On one hand, they bind to cdk2 complexes and inhibit their kinase activities. On the other hand, they are able to promote the activation of Cyclin D1/cdk4-6 by complex stabilization, and by facilitating the nuclear import of these complexes, without inhibiting Cyclin D-associated kinase activity[45-48]. In our study, higher levels of p27 in IHH-Fos could, therefore, represent another mechanism contributing to the increase in nuclear levels of Cyclin D1, although the precise mechanisms linking c-Fos and p27 overexpression are currently unknown. Nevertheless, the mechanism is not at the level of transcription, as indicated by our quantitative PCR analysis (data not shown).

To conclude, our results clearly indicate a positive role for c-Fos in cell cycle regulation in hepatocytes. Importantly, we delineate a new mechanism by which c-Fos could contribute to hepatocarcinogenesis through stabilization of Cyclin D1 within the nucleus, evoking a new feature to c-Fos implication in HCC.

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COMMENTS

Background

Human hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. Among the numerous genes potentially implicated in hepatocarcinogenesis, the proto-oncogene c-Fos, a member of activating protein 1 (AP-1) transcription factor is a good candidate. Apart from one study reporting a negative role for c-Fos in hepatocellular tumorigenesis, several papers rather support a positive role in this process. High expression levels of c-Fos were determined in tumor tissue compared to the adjacent non-tumor liver in human HCC. However, in different cell types or tissues, the role of c-Fos in cell proliferation and/or transformation remains controversial. This study was designed to determine whether c-Fos could contribute to hepatocarcinogenesis by increasing cell proliferation.

Research frontiers

The role of c-Fos on hepatocyte proliferation has never been studied in human cells, but only in murine hepatocytes. These cells had been immortalized and stably transfected by c-Fos using different techniques than those reported in the present study. The authors showed that c-Fos overexpression led to decreased hepatocyte proliferation. However, these results did not appear consistent with most studies suggesting a positive role for c-Fos in hepatocarcinogenesis.

Innovations and breakthroughs

This study shows for the first time that c-Fos deregulates hepatocyte proliferation by stabilizing Cyclin D1 in the nucleus which is a cancer promoting or predisposing event.

Applications

Strategies designed to suppress c-Fos expression in HCC could contribute reducing hepatocyte proliferation and thereby cancer development.

Terminology

Human immortalized hepatocytes are hepatocytes which have been transfected by SV40 T antigen, allowing them to proliferate when cultured contrary to normal hepatocytes. However these immortalized cells are not tumorigenic *in vitro* and *in vivo*.

Peer review

This is an interesting study. Authors investigated the effect of stable c-Fos overexpression on IHH proliferation.

REFERENCES

1 **Shaulian E**, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* 2002; **4**: E131-E136
2 **Eferl R**, Wagner EF. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* 2003; **3**: 859-868
3 **Angel P**, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1991; **1072**: 129-157
4 **Pai SR**, Bird RC. c-fos expression is required during all phases of the cell cycle during exponential cell proliferation. *Anticancer Res* 1994; **14**: 985-994
5 **Kovary K**, Bravo R. The jun and fos protein families are both required for cell cycle progression in fibroblasts. *Mol Cell Biol* 1991; **11**: 4466-4472
6 **Brusselbach S**, Mohle-Steinlein U, Wang ZQ, Schreiber M, Lucibello FC, Muller R, Wagner EF. Cell proliferation and cell cycle progression are not impaired in fibroblasts and ES cells lacking c-Fos. *Oncogene* 1995; **10**: 79-86
7 **Balsalobre A**, Jolicoeur P. Fos proteins can act as negative regulators of cell growth independently of the fos transforming pathway. *Oncogene* 1995; **11**: 455-465
8 **Mikula M**, Gotzmann J, Fischer AN, Wolschek MF, Thallinger C, Schulte-Hermann R, Beug H, Mikulits W. The proto-oncoprotein c-Fos negatively regulates hepatocellular tumorigenesis. *Oncogene* 2003; **22**: 6725-6738
9 **Miao GG**, Curran T. Cell transformation by c-fos requires an extended period of expression and is independent of the cell cycle. *Mol Cell Biol* 1994; **14**: 4295-4310
10 **Hennigan RF**, Hawker KL, Ozanne BW. Fos-transformation activates genes associated with invasion. *Oncogene* 1994; **9**: 3591-3600
11 **Grigoriadis AE**, Schellander K, Wang ZQ, Wagner EF. Osteoblasts are target cells for transformation in c-fos transgenic mice. *J Cell Biol* 1993; **122**: 685-701
12 **Wang ZQ**, Grigoriadis AE, Mohle-Steinlein U, Wagner EF. A novel target cell for c-fos-induced oncogenesis: development of chondrogenic tumours in embryonic stem cell chimeras. *EMBO J* 1991; **10**: 2437-2450
13 **Feng DY**, Zheng H, Tan Y, Cheng RX. Effect of phosphorylation of MAPK and Stat3 and expression of c-fos and c-jun proteins on hepatocarcinogenesis and their clinical significance. *World J Gastroenterol* 2001; **7**: 33-36
14 **Yuen MF**, Wu PC, Lai VC, Lau JY, Lai CL. Expression of c-Myc, c-Fos, and c-jun in hepatocellular carcinoma. *Cancer* 2001; **91**: 106-112
15 **Tabor E**. Tumor suppressor genes, growth factor genes, and oncogenes in hepatitis B virus-associated hepatocellular carcinoma. *J Med Virol* 1994; **42**: 357-365
16 **Masui T**, Nakanishi H, Inada K, Imai T, Mizoguchi Y, Yada H, Futakuchi M, Shirai T, Tatematsu M. Highly metastatic hepatocellular carcinomas induced in male F344 rats treated with N-nitrosomorpholine in combination with other hepatocarcinogens show a high incidence of p53 gene mutations along with altered mRNA expression of tumor-related genes. *Cancer Lett* 1997; **112**: 33-45
17 **Yao X**, Hu JF, Daniels M, Yien H, Lu H, Sharan H, Zhou X, Zeng Z, Li T, Yang Y, Hoffman AR. A novel orthotopic tumor model to study growth factors and oncogenes in hepatocarcinogenesis. *Clin Cancer Res* 2003; **9**: 2719-2726
18 **Borlak J**, Meier T, Halter R, Spanel R, Spanel-Borowski K. Epidermal growth factor-induced hepatocellular carcinoma: gene expression profiles in precursor lesions, early stage and solitary tumours. *Oncogene* 2005; **24**: 1809-1819
19 **Lee JS**, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 2006; **12**: 410-416
20 **Bakin AV**, Curran T. Role of DNA 5-methylcytosine transferase in cell transformation by fos. *Science* 1999; **283**: 387-390
21 **Saito Y**, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H, Hirohashi S. Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. *Int J Cancer* 2003; **105**: 527-532
22 **Sadowski HB**, Gilman MZ. Cell-free activation of a DNA-binding protein by epidermal growth factor. *Nature* 1993; **362**: 79-83
23 **Diehl JA**, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998; **12**: 3499-3511
24 **Cohen P**, Frame S. The renaissance of GSK3. *Nat Rev Mol Cell Biol* 2001; **2**: 769-776
25 **Johnson AC**, Murphy BA, Matelis CM, Rubinstein Y, Piebenga EC, Akers LM, Neta G, Vinson C, Birrer M. Activator protein-1 mediates induced but not basal epidermal growth factor receptor gene expression. *Mol Med* 2000; **6**: 17-27
26 **Zenz R**, Scheuch H, Martin P, Frank C, Eferl R, Kenner L, Sibilia M, Wagner EF. c-Jun regulates eyelid closure and skin tumor development through EGFR signaling. *Dev Cell* 2003; **4**: 879-889
27 **Mialon A**, Sankinen M, Soderstrom H, Junttila TT, Holmstrom T, Koivusalo R, Papageorgiou AC, Johnson RS, Hietanen S, Elenius K, Westermarck J. DNA topoisomerase I

is a cofactor for c-Jun in the regulation of epidermal growth factor receptor expression and cancer cell proliferation. *Mol Cell Biol* 2005; **25**: 5040-5051

28 **Amicone L**, Spagnoli FM, Spath G, Giordano S, Tommasini C, Bernardini S, De Luca V, Della Rocca C, Weiss MC, Comoglio PM, Tripodi M. Transgenic expression in the liver of truncated Met blocks apoptosis and permits immortalization of hepatocytes. *EMBO J* 1997; **16**: 495-503

29 **Schuermann M**, Hennig G, Muller R. Transcriptional activation and transformation by chimaeric Fos-estrogen receptor proteins: altered properties as a consequence of gene fusion. *Oncogene* 1993; **8**: 2781-2790

30 **Brown JR**, Nigh E, Lee RJ, Ye H, Thompson MA, Saudou F, Pestell RG, Greenberg ME. Fos family members induce cell cycle entry by activating cyclin D1. *Mol Cell Biol* 1998; **18**: 5609-5619

31 **Sunters A**, McCluskey J, Grigoriadis AE. Control of cell cycle gene expression in bone development and during c-Fos-induced osteosarcoma formation. *Dev Genet* 1998; **22**: 386-397

32 **Sunters A**, Thomas DP, Yeudall WA, Grigoriadis AE. Accelerated cell cycle progression in osteoblasts overexpressing the c-fos proto-oncogene: induction of cyclin A and enhanced CDK2 activity. *J Biol Chem* 2004; **279**: 9882-9891

33 **Ramljak D**, Calvert RJ, Wiesenfeld PW, Diwan BA, Catipovic B, Marasas WF, Victor TC, Anderson LM, Gelderblom WC. A potential mechanism for fumonisin B(1)-mediated hepatocarcinogenesis: cyclin D1 stabilization associated with activation of Akt and inhibition of GSK-3beta activity. *Carcinogenesis* 2000; **21**: 1537-1546

34 **Diehl JA**, Zindy F, Sherr CJ. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev* 1997; **11**: 957-972

35 **Normanno N**, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene* 2006; **366**: 2-16

36 **Roux PP**, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, Sonenberg N, Blenis J. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J Biol Chem* 2007; **282**: 14056-14064

37 **Nicholson RI**, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer* 2001; **37** Suppl 4: S9-S15

38 **Ito Y**, Takeda T, Sakon M, Tsujimoto M, Higashiyama S, Noda K, Miyoshi E, Monden M, Matsuura N. Expression and clinical significance of erb-B receptor family in hepatocellular carcinoma. *Br J Cancer* 2001; **84**: 1377-1383

39 **Breuhahn K**, Longerich T, Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. *Oncogene* 2006; **25**: 3787-3800

40 **Daveau M**, Scotte M, Francois A, Coulouarn C, Ros G, Tallet Y, Hiron M, Hellot MF, Salier JP. Hepatocyte growth factor, transforming growth factor alpha, and their receptors as combined markers of prognosis in hepatocellular carcinoma. *Mol Carcinog* 2003; **36**: 130-141

41 **Moghaddam SJ**, Haghighi EN, Samiee S, Shahid N, Keramati AR, Dadgar S, Zali MR. Immunohistochemical analysis of p53, cyclinD1, RB1, c-fos and N-ras gene expression in hepatocellular carcinoma in Iran. *World J Gastroenterol* 2007; **13**: 588-593

42 **Gladden AB**, Diehl JA. Location, location, location: the role of cyclin D1 nuclear localization in cancer. *J Cell Biochem* 2005; **96**: 906-913

43 **Alt JR**, Cleveland JL, Hannink M, Diehl JA. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev* 2000; **14**: 3102-3114

44 **Kobayashi K**, Phuchareon J, Inada K, Tomita Y, Koizumi T, Hatano M, Miyatake S, Tokuhisa T. Overexpression of c-fos inhibits down-regulation of a cyclin-dependent kinase-2 inhibitor p27Kip1 in splenic B cells activated by surface Ig cross-linking. *J Immunol* 1997; **158**: 2050-2056

45 **LaBaer J**, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E. New functional activities for the p21 family of CDK inhibitors. *Genes Dev* 1997; **11**: 847-862

46 **Soos TJ**, Kiyokawa H, Yan JS, Rubin MS, Giordano A, DeBlasio A, Bottega S, Wong B, Mendelsohn J, Koff A. Formation of p27-CDK complexes during the human mitotic cell cycle. *Cell Growth Differ* 1996; **7**: 135-146

47 **Cheng M**, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM, Sherr CJ. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J* 1999; **18**: 1571-1583

48 **Sherr CJ**, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999; **13**: 1501-1512

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BASIC RESEARCH

Leptin transiently antagonizes ghrelin and long-lastingly orexin in regulation of Ca^{2+} signaling in neuropeptide Y neurons of the arcuate nucleus

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Abstract

AIM: To explore the mechanism for interactions of leptin with ghrelin and orexin in the arcuate nucleus (ARC) activating neuropeptide Y (NPY) neurons during physiological regulation of feeding.

METHODS: Single neurons from ARC of adult rats with matured feeding function were isolated. $[Ca^{2+}]_i$ was measured to monitore their activities. The time course of leptin effects on ghrelin-induced *versus* orexin-induced $[Ca^{2+}]_i$ increases in NPY neurons was studied.

RESULTS: Administration of ghrelin or orexin-A at 10^{-10} mol/L increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in NPY neurons isolated from the ARC of adult rats. Upon administration of leptin at 10^{-14}-10^{-12} mol/L, ghrelin-induced $[Ca^{2+}]_i$ increases were initially (< 10 min) inhibited but later restored, exhibiting a transient pattern of inhibition. In contrast, orexin-induced $[Ca^{2+}]_i$ increases were inhibited by leptin in a long-lasting manner. Furthermore, a prior administration of leptin inhibited orexin action but not ghrelin action to increase $[Ca^{2+}]_i$.

CONCLUSION: Leptin counteracted ghrelin effects transiently and orexin effects long-lastingly in NPY neurons. The transient property with which leptin counteracts ghrelin action in NPY neurons may allow the fasting-associated increase in ghrelin levels to activate NPY neurons in the presence of physiological leptin and to stimulate feeding.

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Key words: Leptin; Ghrelin; Orexin; Arcuate nucleus; Neuropeptide Y; Ca^{2+}; Feeding; Phosphatidylinositol 3-kinase; Phosphodiesterase 3; Signal transducer and activator of transcription 3

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INTRODUCTION

Food intake is controlled by the feeding regulatory centers, in which the arcuate nucleus (ARC) in the hypothalamus is considered the first order center that senses and integrates a variety of central and peripheral factors[1]. In the ARC, neuropeptide Y (NPY) neurons that coexpress agouti-related peptide (AgRP) are mandatory for feeding[2,3], while proopiomelanocortin (POMC) neurons are essential for satiety[1]. Orexin-A and -B (hypocretin-1 and -2) are orexigenic peptides[4] localized in neurons in the lateral hypothalamus (LH)[5], an area implicated in feeding behavior[6]. Fasting and lowering glucose concentrations increase prepro-orexin mRNA level[4] and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in orexin neurons[7], suggesting a possible physiological role of orexins in feeding. Ghrelin is an orexigenic peptide released predominantly from the stomach[8-10] and also from the intestine and pancreas[11-13]. Ghrelin is the only one orexigenic peptide of the peripheral origin known. The plasma concentration of ghrelin increases before meals and rapidly declines upon food intake[14]. Orexin levels might also change during a day,

since this peptide is implicated in regulation of sleep/wakefulness[15]. These rhythmic changes in ghrelin and orexin levels may alter their inputs on the feeding center and thereby regulate feeding. Both ghrelin[9,16-17] and orexin[18] stimulate food intake primarily by activating NPY neurons in the ARC.

Leptin, a powerful anorectic hormone produced and released from the adipocytes, is present constantly in the plasma at the nanomolar range[14] and is considered to enter the brain through the blood-brain barrier[19]. Therefore, leptin is likely to act continuously on the feeding center and thereby modulate the efficacy of orexigenic substances. The primary action of leptin in the feeding center is inhibition of NPY neurons as well as activation of POMC neurons in the ARC. The obesity syndrome in ob/ob mice resulting from lack of functional leptin is attenuated by the loss of neuropeptide Y[20]. Therefore, elucidation of the interaction of leptin with ghrelin and with orexin in the ARC NPY neurons may provide a clue to understand the neuronal mechanisms for physiological regulation of feeding. It has recently been shown that leptin counteracts ghrelin action to increase cytosolic free $[Ca^{2+}]_i$ in NPY neurons in the ARC, and that the PI3-kinase (phosphatidylinositol 3-kinase)-PDE3 (phosphodiesterase 3) signaling plays a key role in the leptin action[17]. In the present study, we isolated single neurons from ARC of adult rats with matured feeding function and monitored their activities by measuring $[Ca^{2+}]_i$. We studied the time course of leptin effects on ghrelin-induced *vs* orexin-induced $[Ca^{2+}]_i$ increases in NPY neurons.

MATERIALS AND METHODS

Animals and preparation of single neurons from the ARC of adult rats

Adult male Sprague-Dawley (SD) rats were maintained on a 12-h light/dark cycle and given conventional food and water ad libitum. The ARC was isolated from the brain of 5-8-wk-old SD rats and single neurons were prepared according to the procedures reported previously[16,21] with slight modifications. Briefly, rats were anaesthetized with an intraperitoneal injection of carbamic acid ethyl ester (900 mg/kg) and decapitated, and their brains were removed. Brain slices containing the ARC were prepared and the whole ARC of the left and right sides was cut out. The dissected tissues were washed with 10 mmol/L HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) containing 10 mmol/L glucose. Then they were incubated in HKRB supplemented with 20 U/mL papain (Sigma Chemical Co., St. Louis, MO), 0.015 mg/mL deoxyribonuclease, 0.75 mg/mL bovine serum albumin and 1mmol/L cysteine for 15 min at 36℃ in a shaking water bath, followed by gentle mechanical trituration for 5-10 min. After trituration, the cell suspension was centrifuged at 100 × *g* for 5 min. The pellet was resuspended in HKRB and distributed on coverslips. The cells were kept at 20℃ in moisture-saturated dishes for up to 10 h. The animal protocols were approved by the Jichi Medical School Institute of Animal Care and Use Committee.

Measurements of $[Ca^{2+}]_i$ in single neurons of the ARC

At 2 to 10 h after cell preparation, $[Ca^{2+}]_i$ was measured by ratiometric fura-2 microfluorometry in combination with digital imaging as previously reported[16,21]. Briefly, following incubating with 2 μmol/L fura-2-AM for 30 min at room temperature, the cells were mounted in a chamber and superfused with HKRB at 1 mL/min at 34℃. Fluorescence images due to excitation at 340 nm and 380 nm were detected every 8.0 s with an intensified charge-coupled device (ICCD) camera, and the ratio image was produced by an Argus-50 system (Hamamatsu Photonics Co., Hamamatsu, Japan). Ratio values were converted to $[Ca^{2+}]_i$ according to calibration curves. Data were taken from the cells identified as neurons by the procedures reported previously[16,21].

Post-$[Ca^{2+}]_i$ imaging immunocytochemistry and identification of NPY neurons

Neurochemical identification of the neurons that exhibited $[Ca^{2+}]_i$ responses were performed according to the original method[22] with slight modification[16]. Briefly, the cells were fixed with 4% paraformaldehyde over night. They were blocked in 10% normal goat serum (NGS) and in 0.1 mol/L PBS for 1 h at room temperature. Primary antiserum against NPY (DiaSorin, Stillwater, MN) was diluted 1:1000 in PBS containing 1.5% NGS and was incubated 24 h at 4℃. The antiserum were then rinsed and incubated with biotinylated secondary antibody raised against rabbit IgG (Vector Laboratories Inc., Burlingame, CA; diluted at 1:400) for 1 h at room temperature. The secondary antibody was rinsed, and the sections were labeled with avidin-peroxidase complex (ABC kit, Vector) for 1 h and color-developed with 3,3'-diaminobenzidine (DAB). Control experiments were carried out by omitting the primary antiserum.

To correlate $[Ca^{2+}]_i$ and immunocytochemical data, photographs of all the cells in the microscopic field subjected to $[Ca^{2+}]_i$ measurements were taken at the end of $[Ca^{2+}]_i$ imaging. Based on these photographs, the cells in which $[Ca^{2+}]_i$ was recorded were correlated with their corresponding immunocytochemical results.

Criteria for $[Ca^{2+}]_i$ responses and determination of response amplitude

Ghrelin, orexin-A and leptin were administered to the superfusion solution. Amplitudes of $[Ca^{2+}]_i$ increases in response to agents were calculated by subtracting pre-stimulatory basal $[Ca^{2+}]_i$ levels from peak $[Ca^{2+}]_i$ levels. When increases in $[Ca^{2+}]_i$ took place within 5 min after addition of agents and their amplitudes were 150 nmol/L or larger, they were considered responses. Suppression by leptin was judged by the following criteria. In Figures 1 and 2, when the peak amplitude of ghrelin-induced $[Ca^{2+}]_i$ increase was decreased to a level of 40%

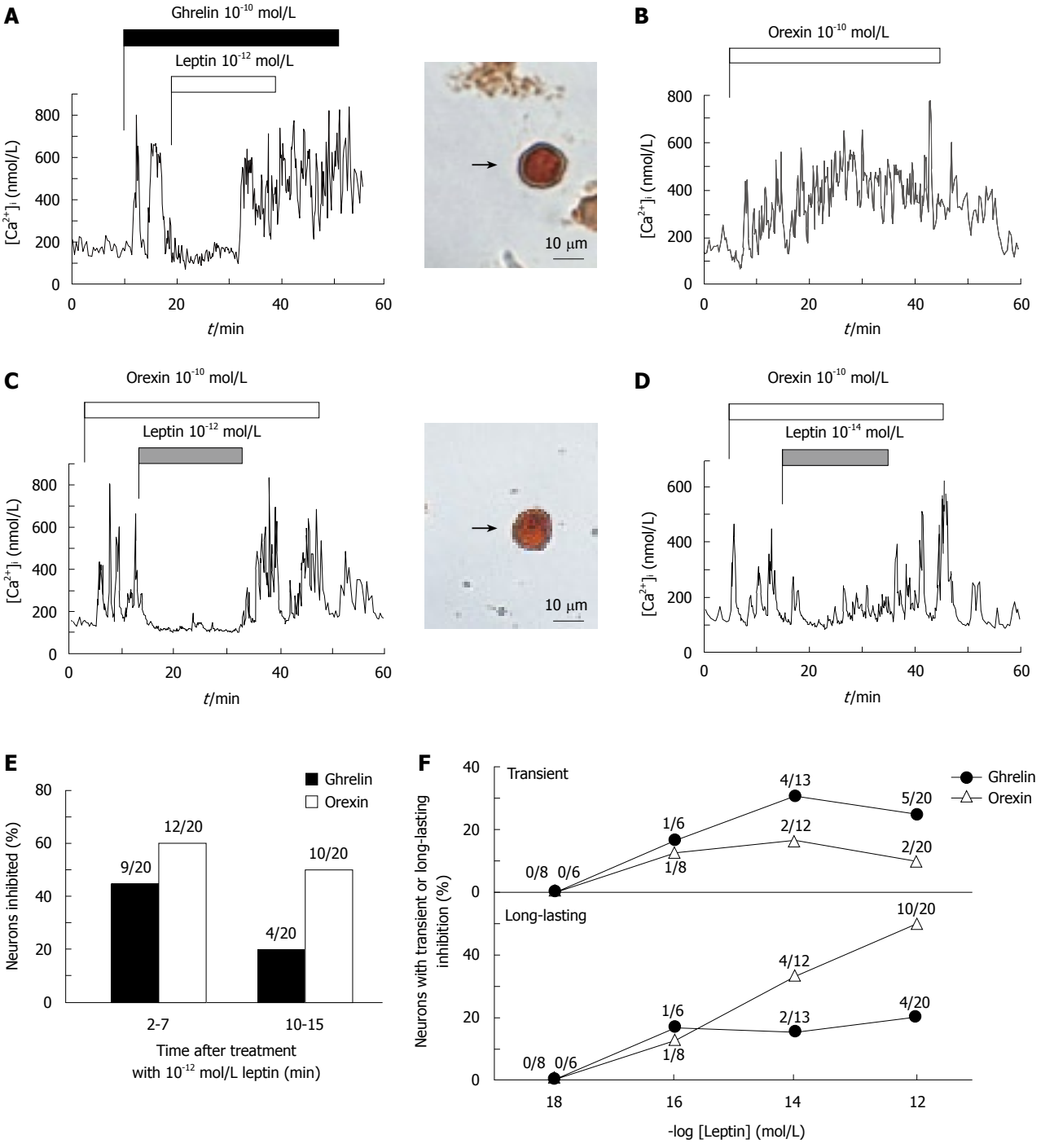

Figure 1 Ghrelin- and orexin-induced $[Ca^{2+}]_i$ increases were suppressed by leptin in a transient or long-lasting manner in NPY neurons. A-D: Ghrelin and orexin-A at 10^{-10} mol/L increased $[Ca^{2+}]_i$ in single neurons isolated from the ARC, and these $[Ca^{2+}]_i$ increases were suppressed by administration of leptin at 10^{-12} mol/L (A, C) and 10^{-14} mol/L (D). Following $[Ca^{2+}]_i$ measurements, the neurons in (A) and (C) were proved to contain NPY by immunocytochemistry using anti-NPY antibody (A, C; right panels). The bars indicate 10 μm. E: Leptin inhibited ghrelin-induced $[Ca^{2+}]_i$ increases in a greater number of neurons in 2-7 min after administration than in 10-15 min. In contrast, leptin inhibited orexin-A-induced $[Ca^{2+}]_i$ increases in the majority of neurons in 2-7 min and 10-15 min after administration. The numbers above the bars indicate the number of neurons inhibited by leptin over that responded to ghrelin or orexin-A. F: The number of neurons whose $[Ca^{2+}]_i$ responses to ghrelin or orexin-A were suppressed by leptin in either a transient or long-lasting manner is expressed by percentage. The numbers above the points indicate the number of neurons inhibited by leptin in the specified manner over that responded to ghrelin or orexin-A.

or smaller for at least 5 min and the recovery of $[Ca^{2+}]_i$ increase was observed after washing out leptin, it was considered inhibition. In Figure 3, repetitive additions of ghrelin or orexin-A twice induced repeated $[Ca^{2+}]_i$ increases, and the second challenge to ghrelin or orexin-A was performed in the presence of leptin. When the amplitude of the $[Ca^{2+}]_i$ response to the second addition was less than 150 nmol/L, it was considered inhibition.

Solutions and chemicals

The measurements were carried out in HKRB solution composed of 129 mmol/L NaCl, 5.0 mmol/L $NaHCO_3$,

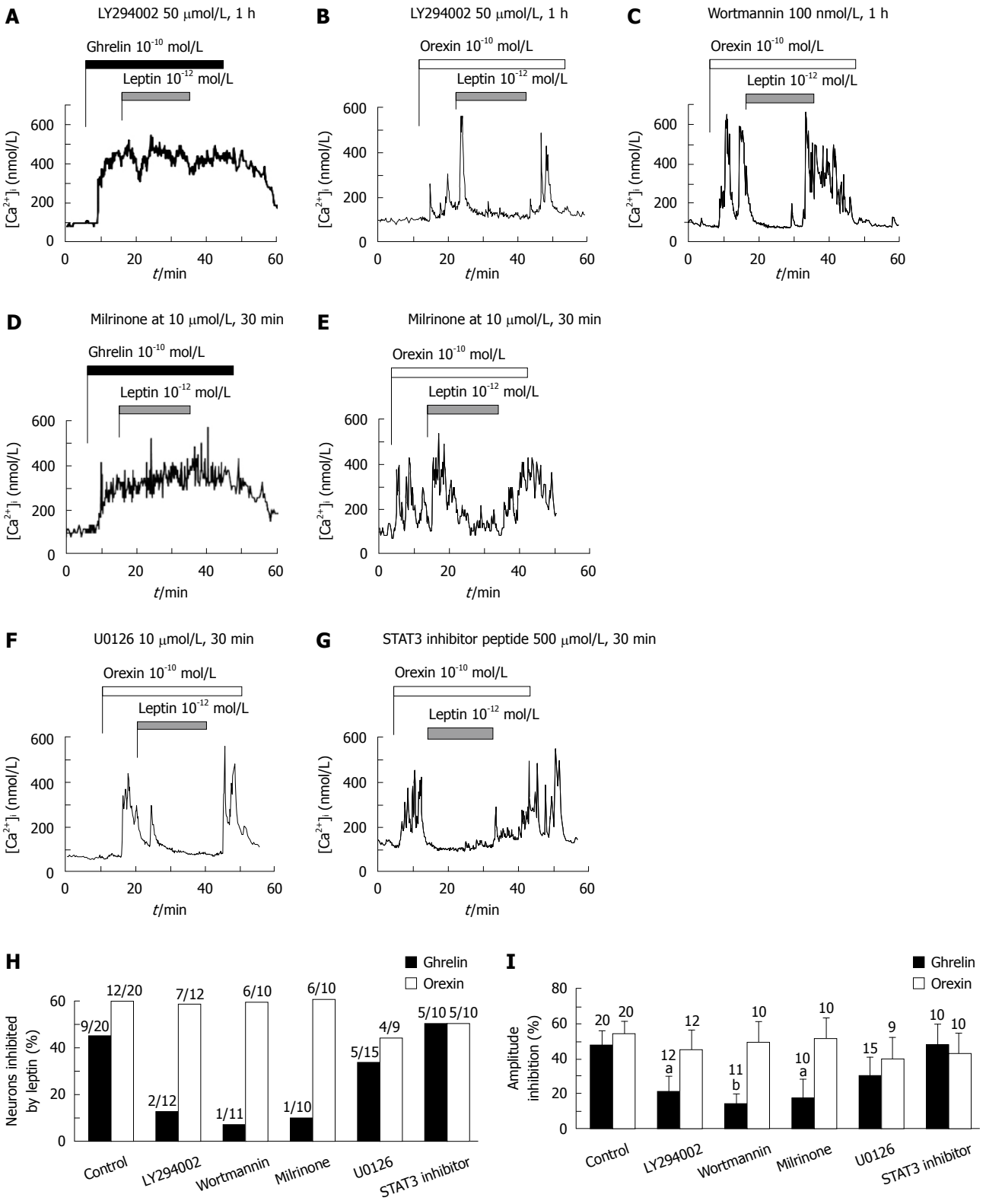

Figure 2 Leptin suppresses ghrelin-induced and orexin-induced $[Ca^{2+}]_i$ increases *via* different signaling pathways in NPY neurons. A-G: Effects of inhibitors for signaling molecules on the leptin action against ghrelin and orexin. Preincubation for 1 h with 50 μmol/L LY294002, an inhibitor for PI3-kinase, interfered with leptin action to suppress $[Ca^{2+}]_i$ responses to ghrelin (A), but not orexin (B). Preincubation for 1 h with 100 nmol/L wortmannin, another PI3-kinase inhibitor, little affected the leptin suppression of orexin-induced $[Ca^{2+}]_i$ increases (C). Preincubation for 30 min with 10 μmol/L milrinone, a PDE3 inhibitor, blocked the leptin action to suppress $[Ca^{2+}]_i$ responses to ghrelin (D), but not orexin (E). Preincubation for 30 min with 10 μmol/L U0126, an inhibitor for MAP-kinase, little affected the leptin action to suppress orexin-induced $[Ca^{2+}]_i$ increases (F). Preincubation for 30 min with a STAT3 inhibitor peptide at 500 μmol/L, did not significantly alter the leptin suppression of orexin-induced $[Ca^{2+}]_i$ increases (G). H: The number of neurons whose $[Ca^{2+}]_i$ responses to ghrelin or orexin were suppressed by leptin in the presence of inhibitors is expressed by percentage. The numbers above the bars indicate the number of neurons inhibited by leptin over that responded to ghrelin or orexin-A. I: Reduction of amplitudes of $[Ca^{2+}]_i$ responses to ghrelin or orexin-A by leptin is expressed by percentage. $^aP < 0.05$, $^bP < 0.01$ *vs* control. The data regarding ghrelin in H and I were in part taken from our previous paper[17].

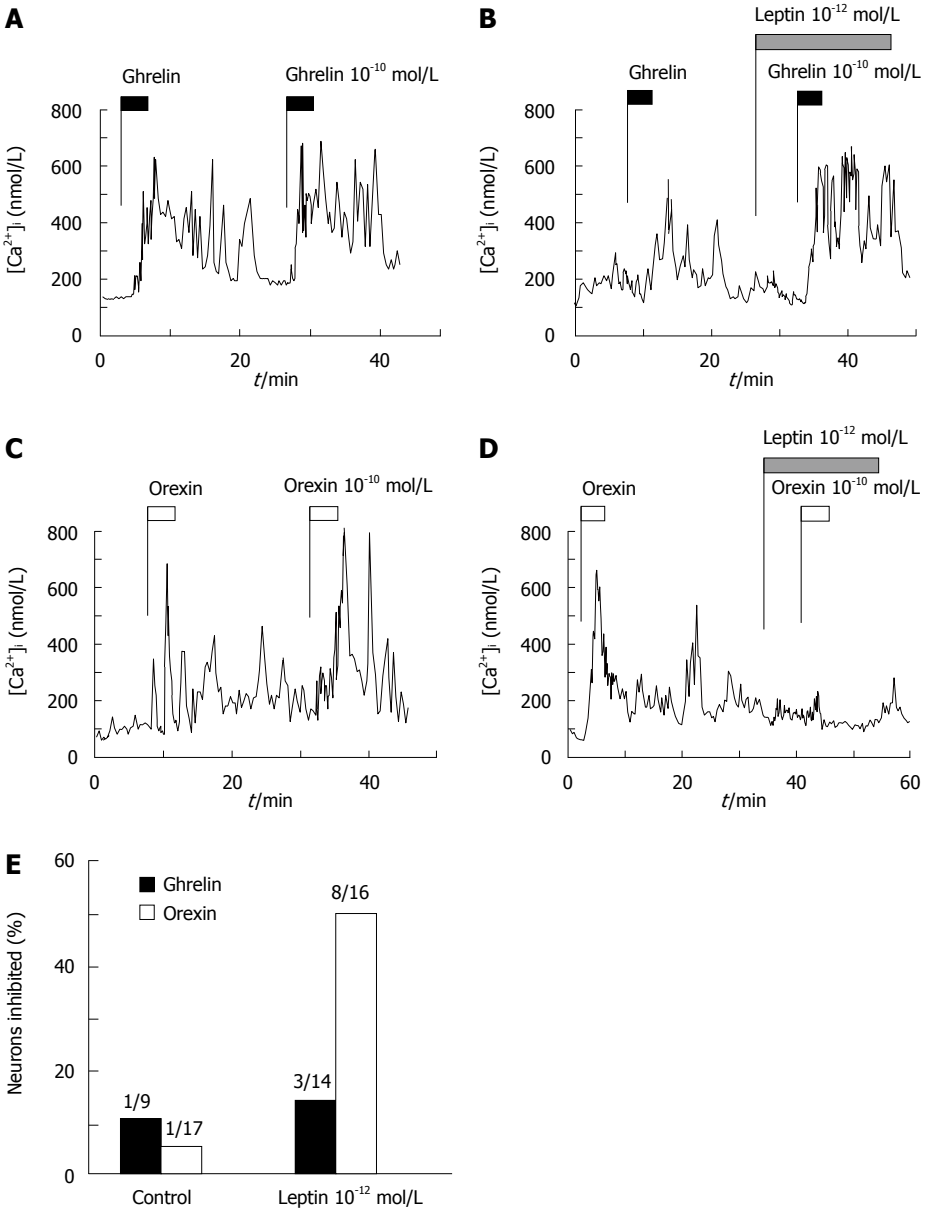

Figure 3 Prior treatment with leptin suppressed $[Ca^{2+}]_i$ responses to subsequent administration of orexin, but not ghrelin, in NPY neurons. A, C: Repeated administrations of ghrelin or orexin-A at 10^{-10} mol/L increased $[Ca^{2+}]_i$ twice in single ARC neurons; B, D: Prior administration of leptin failed to inhibit the effect of ghrelin added subsequently (B), but inhibited the effect of orexin-A (D); E: The number of neurons in which $[Ca^{2+}]_i$ responses to the second ghrelin or orexin addition were suppressed by prior administration of leptin or HKRB (Control) is expressed by percentage. The numbers above the bars indicate the number of neurons whose second responses were inhibited over that exhibited first responses to ghrelin or orexin-A.

4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.8 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, and 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH 7.4. Fura 2-acetoxymethylester was obtained from Dojin Chemical (Kumamoto, Japan). Ghrelin and orexin-A were obtained from Peptide Institute, Inc. (Osaka, Japan), Leptin was from R&D Systems (Mineapolis, MN).

Data presentation and statistical analysis

The data are presented as the mean ± SE (*n*: number of neurons). Each study was based on at least 7 neurons prepared from at least 3 rats. Student's paired or unpaired *t*-test was used to evaluate differences and values of $P < 0.05$ were considered to be significant.

RESULTS

Leptin inhibits ghrelin- and orexin-A-induced $[Ca^{2+}]_i$ increases in NPY neurons

Single neurons isolated from the ARC were superfused with HKRB and subjected to measurements of $[Ca^{2+}]_i$ with fura-2 fluorescence imaging. Administration of ghrelin or orexin-A at 10^{-10} mol/L for 40-50 min into superfusion solutions increased $[Ca^{2+}]_i$ in a continuous manner (Figure 1A and B) as reported previously[16-18]. The $[Ca^{2+}]_i$ increases in response to ghrelin and orexin took place in an oscillatory manner. The peaks of $[Ca^{2+}]_i$ responses to ghrelin [486 ± 49 nmol/L (n = 33)] and orexin-A [433 ± 47 nmol/L (n = 27)] were significantly ($P < 0.001$) higher than the corresponding basal $[Ca^{2+}]_i$ levels prior to administration of the peptides [107 ± 12 nmol/L (n = 33) for ghrelin; 110 ± 10 nmol/L (n = 27) for orexin].

Both ghrelin-induced (Figure 1A) and orexin-induced $[Ca^{2+}]_i$ increases (Figure 1C) were inhibited by administration of 10^{-12} mol/L leptin in the ARC neurons, which were subsequently proven to be immunoreactive to NPY (Figure 1A and C, right panels). The results that leptin counteracts ghrelin and orexin actions on $[Ca^{2+}]_i$ in the ARC NPY neurons confirm previous reports[16-18].

Leptin inhibits ghrelin-induced $[Ca^{2+}]_i$ increases transiently and orexin-A-induced $[Ca^{2+}]_i$ increases long-lastingly in NPY neurons

Typical results of the effects of leptin on $[Ca^{2+}]_i$ responses to ghrelin and orexin-A are shown in Figure 1; leptin at 10^{-12} mol/L inhibited ghrelin-induced $[Ca^{2+}]_i$ increases in a transient manner (Figure 1A) and orexin-induced $[Ca^{2+}]_i$ increases in a longer-lasting manner (Figure 1C) during the 20 min period of leptin administration. Among 20 neurons that exhibited $[Ca^{2+}]_i$ responses to ghrelin, administration of 10^{-12} mol/L leptin inhibited $[Ca^{2+}]_i$ increases in 9 neurons (45%) during the earlier 2-7 min of leptin treatment, but only in 4 neurons (20%) later in the 10-15 min period of treatment (Figure 1E), showing attenuation of the counteracting effect of leptin for ghrelin in the later period (Figure 1F). In contrast, among 20 neurons that exhibited $[Ca^{2+}]_i$ responses to orexin, administration of 10^{-12} mol/L leptin inhibited $[Ca^{2+}]_i$ increases in 12 neurons (60%) during the 2-7 min of leptin treatment, and in 10 neurons (50%) in the 10-15 min period of treatment (Figure 1E), showing a long-lasting counteracting effect of leptin (Figure 1F). The long-lasting effect was also evoked by leptin at a lower concentration of 10^{-14} mol/L (Figure 1D). Leptin at both 10^{-14} mol/L and 10^{-12} mol/L counteracted ghrelin-induced $[Ca^{2+}]_i$ increases predominantly in a transient manner and orexin-induced $[Ca^{2+}]_i$ increases mainly in a long-lasting manner (Figure 1F).

Pretreatment with leptin inhibited orexin-induced, but not ghrelin-induced, $[Ca^{2+}]_i$ increases in NPY neurons

The data that leptin inhibited ghrelin-induced $[Ca^{2+}]_i$ increases transiently prompted us to hypothesize that efficacy of leptin is attenuated by time. Therefore, we examined whether prior administration of leptin is less effective in counteracting ghrelin action. Repetitive additions of ghrelin or orexin-A twice induced repeated $[Ca^{2+}]_i$ increases twice in a similar manner (Figure 3A and C). Following infusion of leptin that had started 8 min in advance, the addition of ghrelin induced $[Ca^{2+}]_i$ increases with amplitudes comparable to those in the control without leptin (Figure 3B), and the similar result was observed in the majority of neurons (Figure 3E). This result indicates a marked attenuation of inhibitory ability of leptin by time. In contrast, infusion of leptin that started 8 min in advance inhibited $[Ca^{2+}]_i$ responses to the addition of orexin-A in 8 of 16 orexin-responsive neurons (50%) (Figure 3D and E). This incidence of the inhibition by leptin administered in prior to orexin was comparable to the inhibition by leptin administered after orexin (12 of 20 neurons, 60%) (Figure 1E). These data indicate that the ability of leptin to counteract orexin action is well preserved without appreciable attenuation.

We next examined whether the difference in the time dependence of leptin action on ghrelin-*vs* orexin-induced $[Ca^{2+}]_i$ increases could involve different leptin signaling mechanisms in NPY neurons. Leptin is linked to several signaling pathways, which include phosphatidylinositol 3 (PI3)-kinase and, its downstream effecter phosphodiesterase 3 (PDE3)[23], signal transducer and activator of transcription 3 (STAT3)[24], and mitogen-activated protein (MAP)-kinase[25]. We have previously shown that leptin suppresses ghrelin-induced $[Ca^{2+}]_i$ increases *via* PI3-kinase- and PDE3-, but not MAP-kinase- and STAT3-, mediated pathway[17]. Therefore, whether these signaling mechanisms could be involved in the leptin action to counteract orexin-induced $[Ca^{2+}]_i$ increases was examined. Pretreatment with inhibitors for PI3-kinase, LY294002 (Figure 2A) or wortmannin (data not shown), blocked the leptin action to suppress $[Ca^{2+}]_i$ responses to ghrelin in both the response incidence (Figure 2H) and response amplitude (Figure 2I). Likewise, pretreatment with an inhibitor for PDE3, milrinone, blocked the leptin action against ghrelin (Figure 2D, H and I). These results confirm previous report[17]. In contrast, LY294002 (Figure 2B), wortmannin (Figure 2C) and milrinone (Figure 2E) failed to significantly affect the leptin suppression of orexin-induced $[Ca^{2+}]_i$ increases in both the response incidence and amplitude (Figure 2H and I). Furthermore, pretreatment with a MAP kinase inhibitor U0126 (Figure 2F) or a STAT3 inhibitor peptide (Figure 2G) little altered the leptin ability to inhibit $[Ca^{2+}]_i$ responses to orexin in both the response incidence and amplitude (Figure 2H and I), the results similar to those reported for ghrelin-induced $[Ca^{2+}]_i$ increases[17].

DISCUSSION

The present data indicate that leptin inhibits ghrelin-induced $[Ca^{2+}]_i$ increases in a transient manner and orexin-induced $[Ca^{2+}]_i$ increases in a long-lasting manner. The transient action of leptin to inhibit ghrelin-induced $[Ca^{2+}]_i$ increases is not due to insufficient concentration of leptin, since leptin at a lower concentration of 10^{-14} mol/L is already maximal in counteracting the ghrelin effect[17] and more specifically in exhibiting the transient inhibitory property (Figure 1F). Furthermore, the transient property for leptin inhibition of ghrelin-induced $[Ca^{2+}]_i$ increases is neither due to excessive concentration of ghrelin, since the ghrelin concentration of 10^{-10} mol/L used in the present study is close to a maximal, but never super-maximal concentration in activating NPY neurons[16]. Therefore, the transient manner with which leptin counteracts ghrelin action reflects the intrinsic property of interaction between leptin and ghrelin.

The present study clearly indicated that the leptin signaling underlying the inhibition of $[Ca^{2+}]_i$ responses to orexin-A in NPY neurons is distinct from that to ghrelin. The transient action of leptin to inhibit ghrelin-induced $[Ca^{2+}]_i$ increases in NPY neurons may be mediated by the leptin signaling *via* PI3-kinase and PDE3, since the inhibitors for these enzymes block the leptin action (Figure 2A and D)[17]. The ghrelin signaling *via* the cAMP system could be the target for this leptin signaling[17]. On the other hand, the long-lasting action of leptin to counteract orexin-induced $[Ca^{2+}]_i$ increases

was not affected by inhibitors for PI3-kinase, PDE3, MAP-kinase and STAT3, well known leptin signaling molecules. This result suggests that the long-lasting counteracting action of leptin for orexin is mediated by a yet unidentified leptin signaling, which may long-lastingly inhibit the orexin-stimulated PLC-PKC-IP3 pathway reported previously[18], though further study is definitely needed to elucidate the signaling interaction between leptin and orexin.

The transient nature with which leptin counteracts the ghrelin action on NPY neurons may serve as the neuronal mechanism that allows fasting-associated increases in ghrelin levels to activate the ARC NPY neurons in the continuous presence of leptin[14] and thereby stimulate feeding.

Both ghrelin and orexin have been suggested to be concerned with obesity and type 2 diabetes[26,27]. Based on the present results, leptin resistance could alter the sensitivity to ghrelin and orexin in the ARC NPY neurons, which may alter the energy metabolism and thereby influence the pathogenesis of obesity and type 2 diabetes.

COMMENTS

Background

Ghrelin and orexins are potent orexigenic peptides working primarily via activating neuropeptide Y (NPY) neurons in the arcuate nucleus (ARC). NPY neurons in the ARC also serve as a major target for the anorexigenic leptin. Therefore, interactions of leptin with ghrelin and orexin in the ARC NPY neurons may play a key role in physiological regulation of feeding.

Research frontiers

The authors study the time course of leptin effects on ghrelin-induced *vs* orexin-induced $[Ca^{2+}]_i$ increases in NPY neurons.

Innovations and breakthroughs

The study clearly indicated that the leptin signaling underlying the inhibition of $[Ca^{2+}]_i$ responses to orexin-A in NPY neurons is distinct from that to ghrelin. The transient action of leptin to inhibit ghrelin-induced $[Ca^{2+}]_i$ increases in NPY neurons may be mediated by the leptin signaling *via* PI3-kinase and PDE3, The ghrelin signaling *via* the cAMP system could be the target for this leptin signaling.

Applications

The transient nature with which leptin counteracts the ghrelin action on NPY neurons may serve as the neuronal mechanism that allows fasting-associated increases in ghrelin levels to activate the ARC NPY neurons in the continuous presence of leptin[14] and thereby stimulate feeding.

Peer review

A well designed, in-depth paper about the time course of leptin effects on ghrelin-induced *vs* orexin-induced $[Ca^{2+}]_i$ increases in NPY neurons.

REFERENCES

1 **Schwartz MW**, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000; **404**: 661-671

2 **Gropp E**, Shanabrough M, Borok E, Xu AW, Janoschek R, Buch T, Plum L, Balthasar N, Hampel B, Waisman A, Barsh GS, Horvath TL, Bruning JC. Agouti-related peptide-expressing neurons are mandatory for feeding. *Nat Neurosci* 2005; **8**: 1289-1291

3 **Luquet S**, Perez FA, Hnasko TS, Palmiter RD. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 2005; **310**: 683-685

4 **Sakurai T**, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998; **92**: 573-585

5 **Date Y**, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. *Proc Natl Acad Sci USA* 1999; **96**: 748-753

6 **Oomura Y**, Ooyama H, Sugimori M, Nakamura T, Yamada Y. Glucose inhibition of the glucose-sensitive neurone in the rat lateral hypothalamus. *Nature* 1974; **247**: 284-286

7 **Muroya S**, Uramura K, Sakurai T, Takigawa M, Yada T. Lowering glucose concentrations increases cytosolic Ca2+ in orexin neurons of the rat lateral hypothalamus. *Neurosci Lett* 2001; **309**: 165-168

8 **Kojima M**, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660

9 **Nakazato M**, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature* 2001; **409**: 194-198

10 **Asakawa A**, Inui A, Kaga T, Katsuura G, Fujimiya M, Fujino MA, Kasuga M. Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut* 2003; **52**: 947-952

11 **Date Y**, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000; **141**: 4255-4261

12 **Date Y**, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes* 2002; **51**: 124-129

13 **Dezaki K**, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K, Yada T. Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca2+ signaling in beta-cells: implication in the glycemic control in rodents. *Diabetes* 2004; **53**: 3142-3151

14 **Cummings DE**, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001; **50**: 1714-1719

15 **Chemelli RM**, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 1999; **98**: 437-451

16 **Kohno D**, Gao HZ, Muroya S, Kikuyama S, Yada T. Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca2+ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* 2003; **52**: 948-956

17 **Kohno D**, Nakata M, Maekawa F, Fujiwara K, Maejima Y, Kuramochi M, Shimazaki T, Okano H, Onaka T, Yada T. Leptin suppresses ghrelin-induced activation of neuropeptide Y neurons in the arcuate nucleus via phosphatidylinositol 3-kinase- and phosphodiesterase 3-mediated pathway. *Endocrinology* 2007; **148**: 2251-2263

18 **Muroya S**, Funahashi H, Yamanaka A, Kohno D, Uramura K, Nambu T, Shibahara M, Kuramochi M, Takigawa M, Yanagisawa M, Sakurai T, Shioda S, Yada T. Orexins (hypocretins) directly interact with neuropeptide Y, POMC and glucose-responsive neurons to regulate Ca 2+ signaling in a reciprocal manner to leptin: orexigenic neuronal pathways in the mediobasal hypothalamus. *Eur J Neurosci* 2004; **19**: 1524-1534

19 **Banks WA**, Kastin AJ, Huang W, Jaspan JB, Maness LM. Leptin enters the brain by a saturable system independent

of insulin. *Peptides* 1996; **17**: 305-311
20 **Erickson JC**, Hollopeter G, Palmiter RD. Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. *Science* 1996; **274**: 1704-1707
21 **Muroya S**, Yada T, Shioda S, Takigawa M. Glucose-sensitive neurons in the rat arcuate nucleus contain neuropeptide Y. *Neurosci Lett* 1999; **264**: 113-116
22 **Yada T**, Vigh S, Arimura A. Pituitary adenylate cyclase activating polypeptide (PACAP) increases cytosolic-free calcium concentration in folliculo-stellate cells and somatotropes of rat pituitary. *Peptides* 1993; **14**: 235-239
23 **Zhao AZ**, Huan JN, Gupta S, Pal R, Sahu A. A phosphatidylinositol 3-kinase phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. *Nat Neurosci* 2002; **5**: 727-728
24 **Gao Q**, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI, Fu XY. Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. *Proc Natl Acad Sci USA* 2004; **101**: 4661-4666
25 **Benomar Y**, Roy AF, Aubourg A, Djiane J, Taouis M. Cross down-regulation of leptin and insulin receptor expression and signalling in a human neuronal cell line. *Biochem J* 2005; **388**: 929-939
26 **Yada T**, Dezaki K, Sone H, Koizumi M, Damdindorj B, Nakata M, Kakei M. Ghrelin regulates insulin release and glycemia: physiological role and therapeutic potential. *Curr Diabetes Rev* 2008; **4**: 18-23
27 **Tsuneki H**, Sugihara Y, Honda R, Wada T, Sasaoka T, Kimura I. Reduction of blood glucose level by orexins in fasting normal and streptozotocin-diabetic mice. *Eur J Pharmacol* 2002; **448**: 245-252

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BASIC RESEARCH

Metabolism for cyclosporin A during liver regeneration after partial hepatectomy in rats

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Abstract

AIM: To elucidate the metabolism and the effect of the cyclosporin A (CyA) as a representative immunosuppressive drug used in transplantation in a partially hepatectomized rat model.

METHODS: CyA was administered to rats that underwent a 70% hepatectomy. These rats were randomly assigned into three groups according to the dose of CyA administration as follows; (group 1) water, (group 2) 5 mg/kg CyA, (group 3) 10 mg/kg CyA. On postoperative days-1, 3, 7 and 14, the rats were killed to analyze the serum concentration of CyA, the liver regeneration ratio, biochemical or histological markers, and mRNA expression using reverse transcriptase-polymerase chain reaction method to determine albumin and cytochrome p450 expression.

RESULTS: The serum concentration of CyA in group 3 was significantly higher than group 2 during liver regeneration. CyA enhanced the liver regeneration in a dose dependent manner. The mRNA expression associated with CyA metabolism was significantly decreased on day 14, while preserving the albumin producing activity.

CONCLUSION: These data indicate that the p-450 activity required to metabolize the CyA may be reduced during regeneration of the remnant liver after a hepatectomy, which may, therefore, be linked to difficulty in controlling the optimal dose of CyA during early period of LDLT.

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Key words: Cyclosporin A; Liver regeneration; Partial hepatectomy; Rat

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INTRODUCTION

Orthotopic liver transplantation is an established treatment for patients with end-stage liver disease. However, donor organ shortages remain extremely problematic. To address this issue, living donor liver transplantation (LDLT) was developed[1]. During transplantation, the liver graft is subjected to a variety of potential hepatic injuries including ischemic injury associated with organ harvesting and the obligate storage before revascularization, reperfusion injury following revascularization, immunological attack caused by the immune system of the recipient, toxicity of certain drugs used during the post-transplant period, and certain infections[2,3]. After transplantation the liver graft goes into a regeneration process, which may be important for the overall success of the transplant procedures. Notably, the liver graft must be capable of normal growth, repair, and regeneration in the presence of immunosuppressive drugs such as calcineurin inhibitors. The aim of the present study was, therefore, to investigate the pharmacokinetics of cyclosporin A (CyA)[4] and its effect on liver regeneration and metabolic

activity to elucidate the mechanism of metabolic activity and serum concentration of cyclosporin A as an example of calcineurin inhibitors administered during liver regeneration in a rat model.

MATERIALS AND METHODS

Animals and treatments

Adult male Sprague Dawley rats, weighting 250-320 g (CRJ Charles River Japan, Kanagawa, Japan), were provided with water, and a standard laboratory diet ad libitum. All of the studies were performed according to the rules and regulations of the University of Nagasaki Research Animal Resources Guidelines.

Surgical procedures

A 70% hepatectomy was carried out according to the method described by Higgins and Anderson[5] under light ether anesthesia. Surgery was performed between 9:00 and 12:00 a.m. to avoid diurnal variation in the regenerative responses. The rats were randomly assigned to three groups, and treated daily by gavage beginning immediately after the hepatectomy. Group 1 animals were given water. Group 2 animals received 5 mg/kg CyA (Neoral®, Novartis Pharma, Basel) and group 3 animals received 5 or 10 mg/kg CyA. These CyA doses were selected based on the results reported by Morii *et al*[6].

In each group, five rats were killed before and at day 1, 3, 7 and 14 after the hepatectomy. Immediately before they were sacrifices, blood samples were obtained from the inferior vena cava. The remnant liver was removed to investigate hepatic restoration. The experimental protocol is demonstrated in Figure 1.

Serum concentration of CyA

The serum concentration of CyA was measured in the whole blood by fluorescence polarization according to the manufacturer's protocols (AxSYM® analyzer, Abbott, Tokyo)[7].

Regeneration ratio

The liver regeneration ratio in each experiment was defined as the ratio of the remaining liver weight to the initial body weight.

Serum ALT and T-Bil level

To evaluate liver toxicity of CyA administration, plasma concentrations of alanine aminotransferase (ALT) and total bilirubin (T-Bil) were examined using an automated analyzing system according to the manufacturer's protocol.

RT-PCR analysis

Total hepatic RNA was prepared by the method as described previously[8] and used for the determination of the expression levels of albumin (ALB) and cytochrome-P 3A2 (CYP3A2). In addition, the level of gene expression of glyceral-dehyde-3-phosphate-dehydrogenase (GAPDH) was measured as an internal control. Complementary DNA (c-DNA) was prepared from total RNA by the method described previously[8]. The primers used in the present study are listed in Table 1.

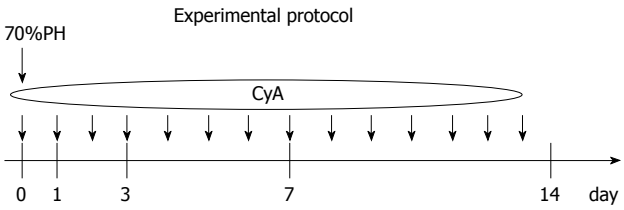

Figure 1 Administration schedule of CyA. Rats underwent a 70% hepatectomy immediately followed by the daily administration of CyA for up to 14 d per os. Blood samples were collected on post operative day 1, 3, 7 and 14.

Statistical analysis

The data are expressed as the mean ± SD (n = 5). Statistical analyses were performed by unpaired, two tailed Student's t-test. A P value less than 0.05 was considered to be significant.

RESULTS

Changes of serum concentration of CyA during liver regeneration

Figure 2 shows that the concentration of CyA reached a maximum during 3 to 7 d, and gradually declined thereafter. The levels of CyA in the PH group were significantly higher than that in control group.

The effect of CyA on liver regeneration ratio

As shown in Figure 3, the lower concentration of CyA (5 mg) did not affect the liver regeneration potential during the observation period; however, the rate of liver regeneration was significantly higher than that in the low CyA group on postoperative day 7.

Changes of hepatocyte specific gene expression during liver regeneration

Alb mRNA expression remained constant during liver regeneration, while hepatocyte specific p450 activity-CYP3A2 was significantly reduced on postoperative day 14 (Figure 4).

The effect of CyA on liver function

Rats were anesthetized and blood samples were collected through the tail vein at the indicated time points. ALT and T-Bil levels were measured as indicators of liver function. On day 1, plasma ALT concentrations increased during the first 24 h after the hepatectomy and then decreased gradually returning to the preoperative values at 72 h. There was no significant difference between the groups (Figure 5).

As shown in Figure 5, the ALT level in control animals were slightly increased, and thereafter gradually reduced. There was no statistically significant difference in any of the groups.

Table 1 Primers used in the present study. The hepatocyte specific gene expression levels were determined by using RT-PCR method. As hepatocyte specific parameters, albumin and CYP3A2 were selected. As an internal control, GAPDH gene expression was also examined. The primer sequences and optimal PCR conditions were summarized

Gene	Sequence (5'-3' sense/antisense)	Reaction condition			Product size	Cycles
		Denaturation	Annealing	Elongation		
GAPDH	TTCAACGGCACAGTCAAG CACACCCATCACAAACAT	95℃, 1 min	60℃, 1 min	72℃, 2 min	240 bp	26
CYP3A2	TACTACAAGGGCTTAGGGAG CTTGCCTGTCTCCGCCTCTT	94℃, 1 min	60℃, 1 min	72℃, 2 min	348 bp	27
ALB	ATACACCCAGAAAGCACCTC CAGAGTGGAAGGTGAAGGTC	94℃, 1 min	60℃, 1 min	72℃, 2 min	305 bp	27

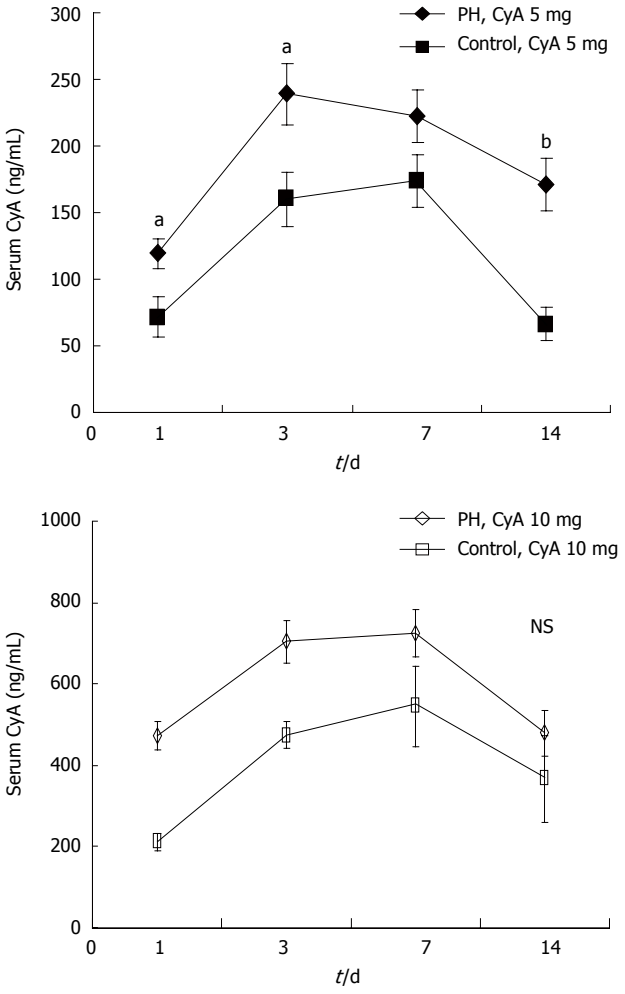

Figure 2 Changes in the serum concentration of CyA during liver regeneration. The values are expressed as the mean ± SD of 5 samples in each group. The concentration of CyA reached a maximum during 3 to 7 d, and gradually declined thereafter. The level of CyA in the PH group was significantly higher than that in control group. $^{a}P < 0.05$, $^{b}P < 0.01$.

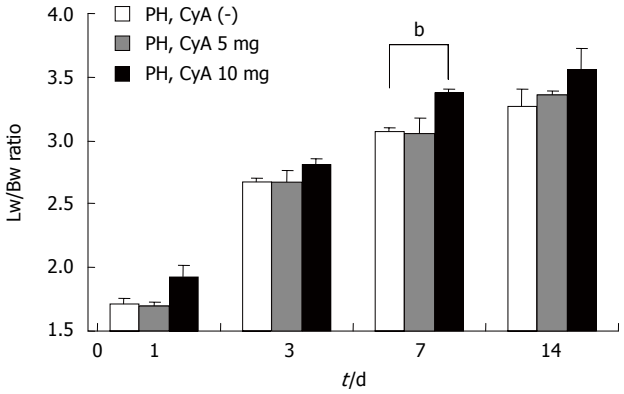

Figure 3 The effect of CyA on the liver regeneration ratio. The values are expressed as the mean ± SE of 5 samples in each group. The low concentration of CyA (5 mg) did not affect the liver regeneration potential during the observation period; however, the rate of liver regeneration was significantly higher than that in the-low CyA group on postoperative day 7. $^{b}P < 0.01$.

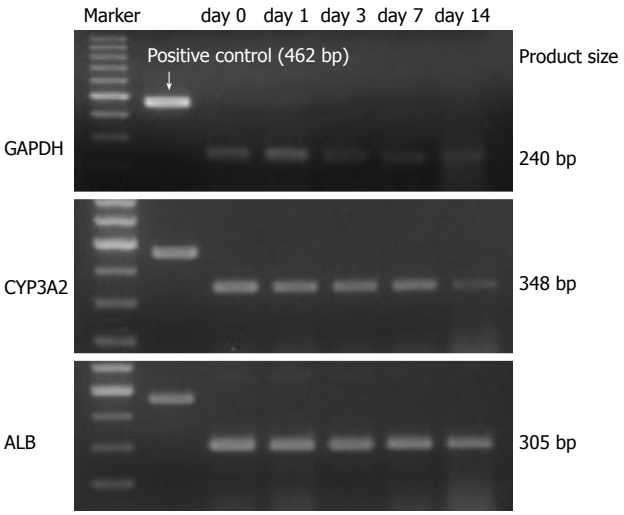

Figure 4 Changes of hepatocyte specific gene expression during liver regeneration. Alb mRNA expression remained constant during liver regeneration, while the hepatocyte specific p450 activity-CYP3A2 significantly decreased on postoperative day 14.

DISCUSSION

The present study, investigated the pharmacokinetics of the CyA in a rat two thirds hepatectomy model, for the first time. The results yielded important information concerning the interrelationship between the CyA and regenerating liver. (1) The metabolism is retarded in a regenerating liver, which is actually seen in clinical partial liver transplantation. (2) CyA has possible hepatotrophic effect on the regenerating liver in a CyA-dose dependent manner. (3) The p450 activity of the regenerating liver was down-regulated after CyA administration.

As expected, the serum concentrations of CyA after

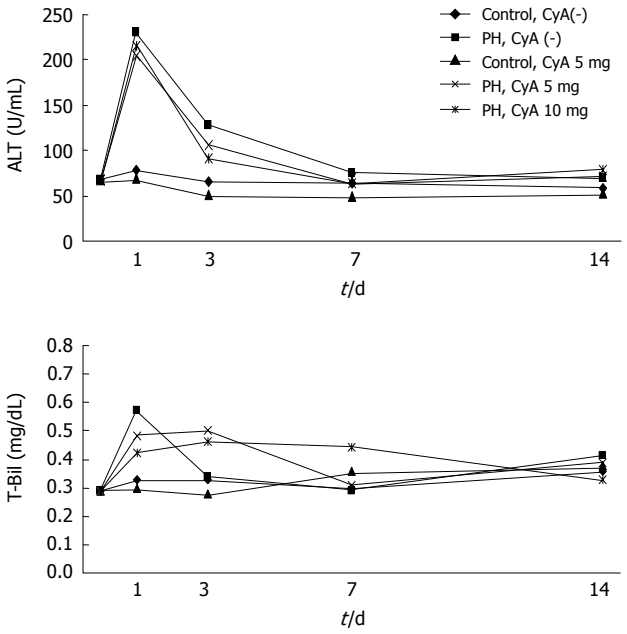

Figure 5 The effect of CyA on liver function. Rats were anesthetized and blood samples were collected through the tail vein at the indicated time points. ALT and T-Bil level were measured as indicators of liver function. On day 1, ALT level were significantly increased and thereafter gradually reduced. There was no statistically significant difference in each group ($P > 0.05$).

a hepatectomy were significantly higher than that seen in the sham operated group as previously reported in clinical settings. There are several possible explanations for this, including increased absorption, decreased volume of distribution, or decreased clearance.

However, an increased absorption is not likely. The CyA used in this study was the microemulsified type, and the absorption is bile independent. Therefore, absorption of the CyA was table in both groups[9]. The volume of distribution should be smaller in the partially hepatectomized rats. In other words, a smaller volume of distribution could increase the relative blood level of an immunosuppressant for a given dose.

Another possibility for the higher levels of CyA after the partial hepatectomy is reduced hepatic immunosuppressive clearance, which may be explained by two possible mechanisms. One is simply because of the reduced hepatic mass available to metabolize the drugs. Another possibility is that immediately after hepatectomy, the hepatic mass is reduced because of the surgical excision of hepatic tissue. As a result, the ability to clear substances through the liver is reduced. For instance, the indocyanine green half-life is increased four-fold after a 60% hepatectomy and by 33% after a 40% hepatectomy[10]. In the rats after a two thirds hepatectomy, the whole-organ reduced form of-cytochrome c reductase and cytochrome p-450 activity are reduced by half. After a 90% hepatectomy, galactose clearance in rats was reduced by 90% within 24 h after surgery. The genetic data regarding the cytochrome p-450 gene suggested that the metabolic activity of specific enzymes responsible for drug metabolism is reduced in the remaining hepatic tissue. Marie *et al* reported that the cytochrome p-450 activity decreased soon after a two-thirds hepatectomy in rats, returning to 90% of the initial activity 2 wk postoperatively. In the present study, the expression analysis of cytochrome p450 activity was performed using RT-PCR method. The results showed the cytochrome p-450 activity remained at the initial stage of liver regeneration, finally declined in the late stages. Although, no other liver specific enzymes were examined, a previous study demonstrated that the levels of mRNA for enzymes responsible for gluconeogenesis and the acute phase proteins are increased up to four fold after a hepatectomy[11]. Therefore, there are adaptive changes in the hepatic tissue after a partial hepatectomy. Collectively, the activity of enzymes that support hepatic regeneration is increased, whereas the activity of the enzymes responsible for drug metabolism is reduced.

The present data also suggest that cyclosporine enhances the hepatic regenerative response without affecting the individual hepatocellular function. Among immunosuppressive drugs currently in clinical use, azathioprine and steroids have been reported to exert an antiproliferative action on the regenerating liver. Azathioprine inhibits the DNA or RNA synthesis of hepatocytes, acting as an antimetabolite, whereas the action of steroids is more complex. Several investigators have suggested a functional linkage between lymphoid tissues and hepatocytes. Craddock *et al* reported that a partial hepatectomy induced proliferation of hepatocytes as well as lymphoid tissues. Another study recently suggested a very close and positive interrelationship between hepatocyte replication and lymphocyte activities[12]. The new potent immunosuppressor, cyclosporin A has been extensively compared with azathioprine and steroids. It primarily inhibits T-lymphocyte responses, and has no functional effects on other hematopoietic cells or phagocytic cells[13-16]. The present study on hepatectomized rats confirmed the antimitotic action of these immunosuppressants on hepatocytes, although the degree of suppression was less than that seen in previous reports.

Notably, there seems to be some discrepancy in these data showing that the statistical difference of the serum concentration of CyA between 5 mg treated and control animals was not observed in the groups treated with 10 mg of CyA treated as demonstrated in Figure 2. However, this is probably a reflection of-the fact that the liver regenerative effect of CyA at a higher dose may improve the impaired metabolic potential for CyA itself.

The limitation of this study is that this model potentially does not require immunosuppresion; therefore, further research will be needed to elucidate the underlining mechanism for these findings in a partial liver transplant model.

In conclusion, these results indicate that CyA levels in hepatectomized rats were significantly higher in control rats without a hepatectomy, probably because of the decreased volume of distribution, and/or decreased clearance by reduced metabolic activity. The possible hepatotrophic effect of CyA on the regenerating liver has also been confirmed.

REFERENCES

1 **Chen CL**, Fan ST, Lee SG, Makuuchi M, Tanaka K. Living-donor liver transplantation: 12 years of experience in Asia. *Transplantation* 2003; **75**: S6-S11

2 **Burton JR Jr**, Rosen HR. Diagnosis and management of allograft failure. *Clin Liver Dis* 2006; **10**: 407-435, x

3 **Said A**, Einstein M, Lucey MR. Liver transplantation: an update 2007. *Curr Opin Gastroenterol* 2007; **23**: 292-298

4 **Pichlmayr R**, Neuhaus P, Ringe B, Wonigeit K, Burdelski M, Verner L, Lauchart W, Schmidt FW. Developments in liver transplantation. *Jpn J Surg* 1985; **15**: 409-419

5 **Higgins G**, Anderson R. Experimental pathology of the liver. *Arch Pathol* 1931; **12**: 186-202

6 **Morii Y**, Kawano K, Kim YI, Aramaki M, Yoshida T, Kitano S. Augmentative effect of cyclosporin A on rat liver regeneration: influence on hepatocyte growth factor and transforming growth factor-beta(1). *Eur Surg Res* 1999; **31**: 399-405

7 **Sabate I**, Liron FJ, Gonzalez Alba JM, Ginard M, Virgili J, Baro S, Figueras J, Gonzalez Segura C, Jaurrieta E. Comparison of cyclosporine immunoassays (AxSYM and RIA) for assessing pharmacokinetic parameters in liver transplant patients. *Transplant Proc* 1999; **31**: 2421-2422

8 **Konno Y**, Sekimoto M, Nemoto K, Degawa M. Sex difference in induction of hepatic CYP2B and CYP3A subfamily enzymes by nicardipine and nifedipine in rats. *Toxicol Appl Pharmacol* 2004; **196**: 20-28

9 **Tredger JM**. Using cyclosporine Neoral immediately after liver transplantation. United Kingdom Neoral Pilot Study Group. *Ther Drug Monit* 1995; **17**: 638-641

10 **Prasse KW**, Bjorling DE, Holmes RA, Cornelius LM. Indocyanine green clearance and ammonia tolerance in partially hepatectomized and hepatic devascularized, anesthetized dogs. *Am J Vet Res* 1983; **44**: 2320-2323

11 **Tygstrup N**, Jensen SA, Krog B, Pietrangelo A, Shafritz DA. Expression of messenger RNA for liver functions following 70% and 90% hepatectomy. *J Hepatol* 1996; **25**: 72-78

12 **Sakai A**, Pfeffermann R, Kountz SL. Liver regeneration and lymphocyte activation. *Surg Gynecol Obstet* 1976; **143**: 914-918

13 **White DJ**, Calne RY, Plumb A. Mode of action of cyclosporin A: a new immunosuppressive agent. *Transplant Proc* 1979; **11**: 855-859

14 **Hellman A**, Goldman JM. Effects of cyclosporin A on human granulopoiesis in vitro. *Transplantation* 1980; **30**: 386-387

15 **White DJ**, Plumb AM, Pawelec G, Brons G. Cyclosporin A: an immunosuppressive agent preferentially active against proliferating T cells. *Transplantation* 1979; **27**: 55-58

16 **Larsson EL**. Cyclosporin A and dexamethasone suppress T cell responses by selectively acting at distinct sites of the triggering process. *J Immunol* 1980; **124**: 2828-2833

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RAPID COMMUNICATION

Protective effects of anti-ricin A-chain RNA aptamer against ricin toxicity

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Abstract

AIM: To investigate the therapeutic potential of an RNA ligand (aptamer) specific for the catalytic ricin A-chain (RTA), the protective effects of a 31-nucleotide RNA aptamer (31RA), which formed a high affinity complex with RTA, against ricin-induced toxicity in cell-based luciferase translation and cell cytotoxicity assays were evaluated.

METHODS: To test the therapeutic potential of anti-RTA aptamers in Chinese hamster ovary (CHO) AA8 cells stably transfected with a tetracycline regulatable promoter, ricin ribotoxicity was measured using luciferase and ricin-induced cytotoxicity was ascertained by MTS cell proliferation assay with tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium].

RESULTS: Inhibition of protein synthesis by ricin in CHO AA8 cells resulted in diminished luciferase activity and treatment with polyclonal antibody against deglycosylated RTA (dgA) neutralized the inhibitory effects of ricin on luciferase activity and protected against ricin-induced cytotoxicity as measured by MTS assay. The 31RA anti-RTA aptamer inhibited the translation of luciferase mRNA in cell-free reticulocyte translation assay. 31RA aptamer also partially neutralized the inhibitory effects of ricin on luciferase activity and partially protected against ricin-induced cytotoxicity in CHO AA8 cells.

CONCLUSION: We have shown that anti-RTA RNA aptamer can protect against ricin ribotoxicity in cell-based luciferase and cell cytotoxicity assays. Hence, RNA aptamer that inhibits RTA enzymatic activity represents a novel class of nucleic acid inhibitor that has the potential to be developed as a therapeutic agent for the treatment of ricin intoxication.

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Key words: Ricin inhibitor; RNA aptamer; Luciferase assay

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INTRODUCTION

Ricin, a lectin from the castor bean plant *R. communis* is considered one of the most potent plant toxins. Ricin poisoning can cause severe tissue damage and inflammation and can result in death. More than 750 cases of accidental or deliberate ricin poisoning have been described in humans. Most accidental exposures occur by ingestion of the seeds of castor beans whereby the toxin is released after the seed coat is damaged. The ingested toxin causes severe gastrointestinal damage with symptoms including nausea, vomiting, diarrhea, and abdominal pain and may progress to hypotension, liver failure, renal dysfunction, and death due to multiorgan failure or cardiovascular collapse[1]. Ricin administered

intragastrically to mice induced villus atrophy and epithelial damage in the proximal small intestine[2]. In experimental exposed animals, intravenous injection of ricin to mice induced severe inflammatory responses and clinical symptoms resembling hemolytic uremic syndrome (HUS), including thrombotic microangiopathy, hemolytic anemia, thrombocytopenia, and acute renal failure[3].

Ricin belongs to a group of toxins referred to as ribosome-inactivating proteins (RIPs)[4,5] and is composed of two glycoproteins, the A-chain (RTA) and the B-chain (RTB), linked by a disulfide bond[6]. RTB binds to galactose residues on cell surface receptors and cell binding is followed by endocytic uptake. A proportion of the ricin moves from early endosomes to the trans-Golgi network and to the endoplasmic reticulum (ER) lumen. In the ER lumen, RTA and RTB dissociate and RTA is retrograde transported across the ER membrane into the cytosol[7]. RTA is a ribotoxin that possesses RNA N-glycosidase activity that disables protein translation by depurinating a single adenine in the 28 S eukaryotic ribosomal RNA[8] which prevents the binding of elongation factor 2, thereby terminating protein synthesis[9,10]. The irreversible poisoning of the ribosome and inhibition of protein synthesis may lead to eventual cell death.

Since currently there is no antidote or specific therapy available for ricin poisoning, the discovery of antitoxins is a high priority. Ricin ribotoxicity can be counteracted by several different types of antitoxins including neutralizing anti-ricin antibodies, small molecule RTA inhibitors, polynucleotide active site inhibitors and polynucleotide substrate analogues[11,12]. *In vitro* selection had been used to generate RNA ligands (aptamers) specific for the catalytic ricin A-chain[13]. An initial 80-nucleotide RNA ligand was minimized to a 31-nucleotide RNA aptamer (31RA) that contained all sequences and structures necessary for forming high affinity complexes with RTA and blocking enzymatic activity of RTA *in vitro*. A transient cell-based luciferase assay had been utilized for quantifying protein synthesis inhibition by bacterial toxins[14]. In this report, we utilized a stable cell-based luciferase assay and showed that 31RA aptamer also neutralized the inhibitory effects of ricin on translation inhibition in cell-free and cell-based luciferase assays and ricin-induced cytotoxicity assay. The use of a stably transfected cell-based luciferase assay will facilitate the development of high throughput screening for inhibitors of ricin as potential antidotes for the treatment of ricin intoxication.

MATERIALS AND METHODS

Cell-free luciferase translation assay

Ricin (*Ricinus communis* agglutinin Ⅱ) and ricin A-chain (RTA) were purchased from Vector Laboratories, Inc. (Burlington, CA). Anti-deglycosylated ricin A-chain (anti-dgA) antibody was IgG purified by protein-A sepharose from pooled polyclonal antisera obtained from mice hyperimmunized with dgA (USAMRIID, Fort Detrick, MD). The anti-RTA RNA aptamer (31RA) (GGCGAAUUCAGGGGACGUAGCAAUGACUGCC)[13] was synthesized by Sigma-Genosys. Rabbit reticulocyte lysate (nuclease treated), amino acid (complete) mixture, luciferase control RNA, RNasin inhibitor, nuclease-free water, and luciferin substrate (CFT luciferase reporter buffer) were purchased from Promega (Madison, WI). 31RA aptamer was diluted and heated for 3 min at 65℃, cooled to 25℃ and incubated at 25℃ for 10 min. After incubation, aptamer and toxin were mixed together and incubated at 25℃ for an additional 10 min. As a standard control, ricin (1.6 to 200 ng/mL) and RTA (0.4 to 50 ng/mL) diluted in PBS buffer were added to a V-shaped 96-well microtiter plate. Rabbit reticulocyte lysate, RNAsin, amino acid complete mixture, nuclease-free deionized water, and luciferase mRNA were mixed together and kept on ice. Five μL of each standard and treatment group were added to microtiter plate, and then 25 μL of the lysate mixture was added to each well. The plate was wrapped in a damp paper towel, placed in a plastic bag and incubated for 90 min at 37℃. After incubation, 5 μL of reaction mixture was transferred to a black microtiter plate and 45 μL of the luciferin reaction buffer was added to each well. Luminescence was measured as counts per second (CPS) using a SpectraMax Luminometer (Molecular Devices). Data were presented as the % of control (PBS only or no treatment) [CPS experimental/CPS PBS control × 100] as previously described[15]. Statistical analyses were calculated using Microsoft Excel 7.0 and SigmaPlot™ V3.01. Three separate assays were performed for each experimental group.

Cell-based luciferase and cytotoxicity assays

Chinese Hamster Ovary AA8 (CHO AA8) cells offered a stably transfected luciferase reporter cell system, whereby expression of the luciferase gene was under the transcriptional control of a tetracycline-repressible promoter system (Tet-Off™ Expression System from Clontech/BD Biosciences). CHO AA8 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin and streptomycin and incubated at 37℃ in a humidified 5% CO_2 incubator. Isotype control mouse IgG, anisomycin and doxycycline were obtained from Sigma-Aldrich.

To suppress luciferase expression, CHO AA8 cells were cultured in DMEM containing 10% FBS and doxycycline (Dox) (1 μg/mL). For the kinetics of induction of luciferase activity, cells were trypsinized and seeded at 2×10^5 cells/well in 6-well plate without Dox and 3 h after cell plating, residual and cell-attached Dox was removed by several washes with PBS. Cells were subjected to the indicated treatment and incubated for different lengths of time before cell lysis. As control, cells were treated with anisomycin (10 μg/mL). Equal amounts of protein (20 μg) were assayed for luciferase activity using the Bright-Glo™ Luciferase Assay System (Promega) and luminescence was measured with EG&G Berthold microplate luminometer (MicroLumat Plus LB96V).

To evaluate the protective effects of anti-dgA Ab

and 31RA aptamer, 5000 CHO AA8 cells were plated in 96-well plates in the absence of Dox overnight. Ricin was co-incubated with anti-dgA IgG or control IgG at a ratio of 1 μg ricin to 10 μg antibody for 30 min at 37℃ in 5% CO_2 as previously described[16]. The ricin-antibody mixture was added to CHO AA8 cells in triplicate wells. Prior to use, the 31RA aptamer was heated at 65℃ for 3 min and cool at 25℃ for 10 min. Various dilutions of the 31RA aptamer were added to ricin and incubated at 37℃ for 30-40 min before incubation with cells. Cells were harvested 24 h after treatment for luciferase assay.

For ricin-induced cytotoxicity assay, CHO AA8 cells were seeded in 96-well flat-bottom plates at 5000 cells/well in 100 μL DMEM plus 10% FBS and incubated at 37℃ overnight. Ricin was pre-incubated with anti-ricin Ab or aptamer as described above and cell viability was assayed at 48 h post-treatment. Cytotoxicity of CHO AA8 cells induced by ricin was quantitated in triplicate wells using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega) and the plate was read using a 492 nm absorbance filter in a Perkin Elmer HTS700 BioAssay plate reader.

RESULTS

Hale ML[15] developed a cell-free *in vitro* translation assay for measuring the ribotoxicity of ribosome-inactivating toxins based on inhibition of protein translation of the luciferase mRNA in a rabbit reticulocyte assay. As shown in Figure 1 (upper panels), the amount of luciferase translated, as measured by luminescence, was inversely proportional to the concentration of RTA and ricin holoenzyme. The protective effects of 31RA aptamer were first evaluated using the cell-free translation assay. Preincubation of RTA (5 ng/mL) with 31RA aptamer at both 1 and 5 ng/mL protected equally against RTA-induced translation inhibition while dose-dependent protection was observed with a higher dose of RTA (10 ng/mL) (Figure 1A, lower panel). When preincubated with the ricin holoenzyme, 31RA aptamer partially neutralized protein synthesis inhibition by ricin (Figure 1B, lower panel).

To evaluate the protective effects of 31RA aptamer against ricin ribotoxicity in cells, we utilized a cell-based luciferase assay to complement the cell-free luciferase translation assay. For the cell-based luciferase assay, CHO AA8 cells stably transfected with a luciferase reporter gene under a tetracycline-repressible promoter were used to measure the ribotoxicity of ricin. In the presence of ATP, the luciferase enzyme catalyzes the oxidation of D-luciferin to produce light and the light output corresponds to the concentration of the luciferase enzyme and activity. The light output (Relative light unit, RLU) was used to measure the dose-dependent inhibition of luciferase activity by ricin compared to anisomycin, a small molecule protein synthesis inhibitor known to target ribosomal RNA (rRNA) at an adjacent site distinct from that targeted by ricin[10]. First, the kinetics of induction of expression of the luciferase reporter gene after removal of doxycycline

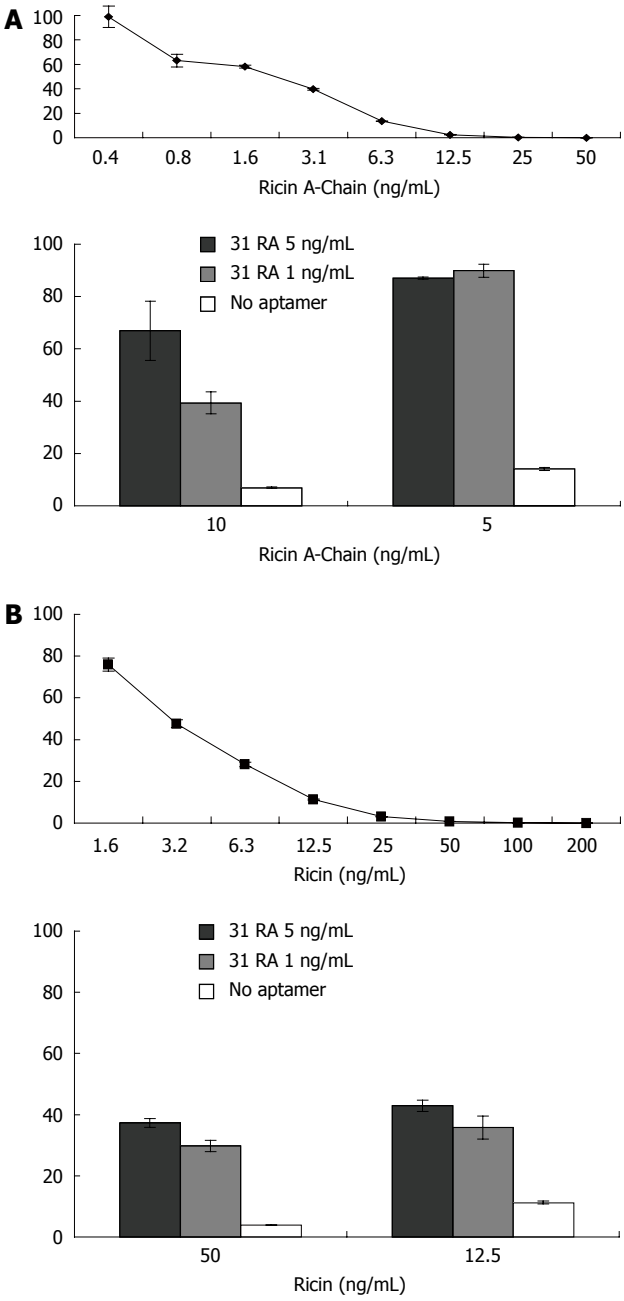

Figure 1 Cell-free luciferase translation assay for biological activity of ricin and protective effects of anti-RTA 31RA aptamer. A: Effects of increasing concentrations of RTA on luciferase activity in cell-free translation assay (upper panel) and neutralization by 31RA aptamer (lower panel); B: Effects of increasing concentrations of ricin holoenzyme on luciferase activity in cell-free translation assay (upper panel) and neutralization by 31RA aptamer (lower panel).

was determined by measuring luciferase activity over a 24-h period in the absence and presence of ricin or anisomycin (Figure 2A) The increase in luciferase activity was observed at 3 h after induction and ricin (1 μg/mL) and anisomycin (10 μg/mL) completely inhibited the increase in luciferase activity. Treatment of CHO AA8 cells with increasing concentrations of ricin resulted in increased inhibition of luciferase activity in a dose-dependent manner compared to untreated control and maximal inhibition was obtained with > 0.5 μg/mL ricin (Figure 2B).

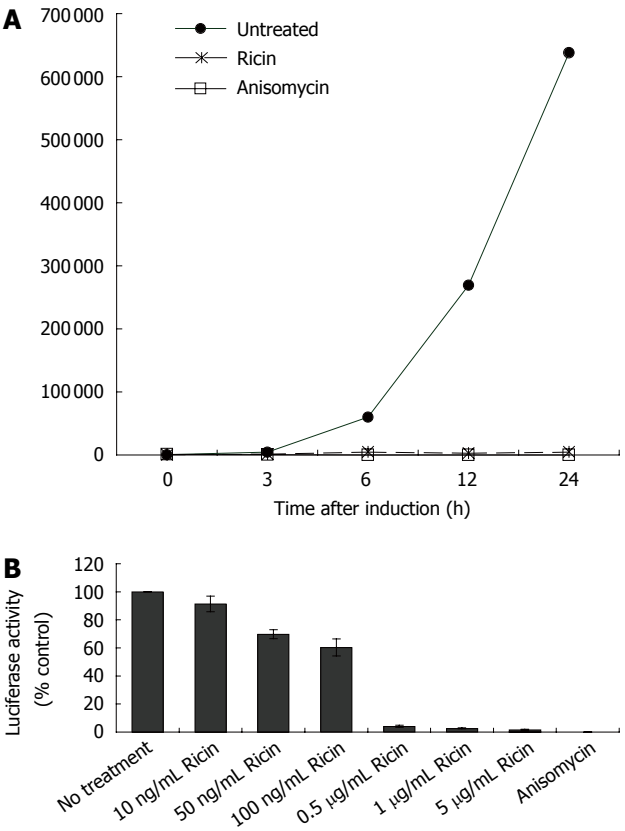

Figure 2 Cell-based luciferase assay for biological activity of ricin in CHO AA8 cells. A: Kinetics of induction of luciferase activity in CHO AA8 cells and inhibition by ricin and anisomycin. CHO AA8 cells were cultured in medium containing doxycycline (Dox) to suppress luciferase expression. For induction of luciferase expression, medium containing Dox was removed and replaced with medium (untreated) or medium containing ricin (1 μg/mL) or anisomycin (10 μg/mL). At various timepoints (0, 3, 6, 12, 24 h) after treatment, luciferase activity was measured. Light output is expressed as RLU; B: Dose-dependent inhibition of luciferase activity by increasing concentrations of ricin or anisomycin as control.

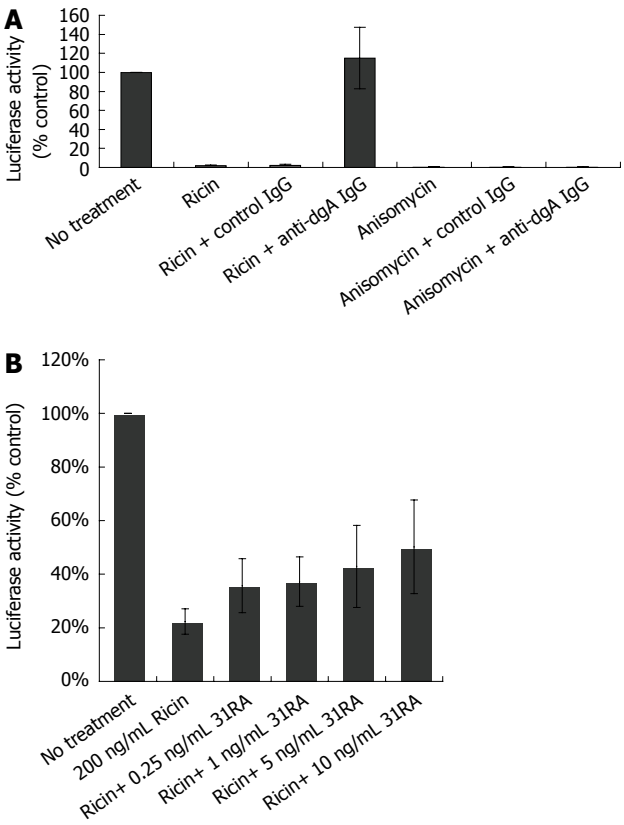

Figure 3 Protective effects of polyclonal anti-dgA IgG and anti-RTA 31RA aptamer on ricin-inhibited luciferase activity. A: Protective effects of anti-dgA IgG against ricin-inhibited luciferase activity, but not anisomycin-inhibited luciferase activity; B: Protective effects of various concentrations of 31RA aptamer against ricin-inhibited luciferase activity.

To determine the utility of this cell-based assay for testing specificity of antitoxins against ricin toxicity, the effects of polyclonal anti-deglycosylated ricin A chain (dgA) antibody in neutralizing the inhibitory effects of ricin on luciferase activity were determined. We have previously shown that anti-dgA Ab protected against ricin-induced cytotoxicity of RAW 264.7 mouse macrophage cells and ricin-induced lung injury and lethality[17]. CHO AA8 cells were treated with ricin or anisomycin in the presence of control IgG or IgG purified from the sera from dgA-immunized mice (anti-dgA IgG) and luciferase activity was measured 24 h later. As depicted in Figure 3A, anti-dgA IgG specifically neutralized the inhibitory effects of ricin on luciferase activity, but not that of anisomycin. Compared to anti-dgA IgG, 31RA aptamer partially protected against ricin-induced ribotoxicity as assessed by luciferase assay (Figure 3B).

To examine the effects of increasing doses of ricin on viability of CHO AA8 cells using the MTS assay. As shown in Figure 4A, treatment of CHO AA8 cells with increasing concentrations of ricin resulted in decreased cell survival. Interestingly, similar to the dose-dependent inhibition of luciferase activity (Figure 2B), ricin (100 ng/mL) induced ~50% cell death while > 0.5 μg/mL induced > 80% cell death (Figure 4A). Treatment with anti-dgA IgG, but not control IgG protected against cytotoxicity induced by ricin, and not anisomycin in CHO AA8 cells (Figure 4A). Pretreatment of ricin with 31RA aptamer also neutralized ricin-induced cytotoxicity in CHO AA8 cells (Figure 4B) and RAW264.7 mouse macrophage cells (data not shown).

DISCUSSION

In vitro selection is a powerful molecular tool for the generation of ligands for a wide variety of targets for therapeutic purposes. RNA aptamers that bind to human immunodeficiency virus type I Rev also inhibit viral replication[18]. Therefore, aptamers that recognize and inhibit ricin might be useful therapeutic agents. Interestingly, although the 31RA aptamer specific for the catalytic RTA bore no resemblance to the normal RTA substrate, i.e. the sarcin-ricin loop (SRL) and was not depurinated by RTA[13], it contained all sequences and structures necessary for interacting with RTA. This minimal 31-nucleotide RNA formed high affinity complexes with RTA (K_d = 7.3 nmol/L) which could compete with SRL for binding to RTA and inhibited RTA depurination of the SRL and could partially protect protein translation from RTA inhibition in *in vitro* translation assay. The IC_{50} of the aptamer for

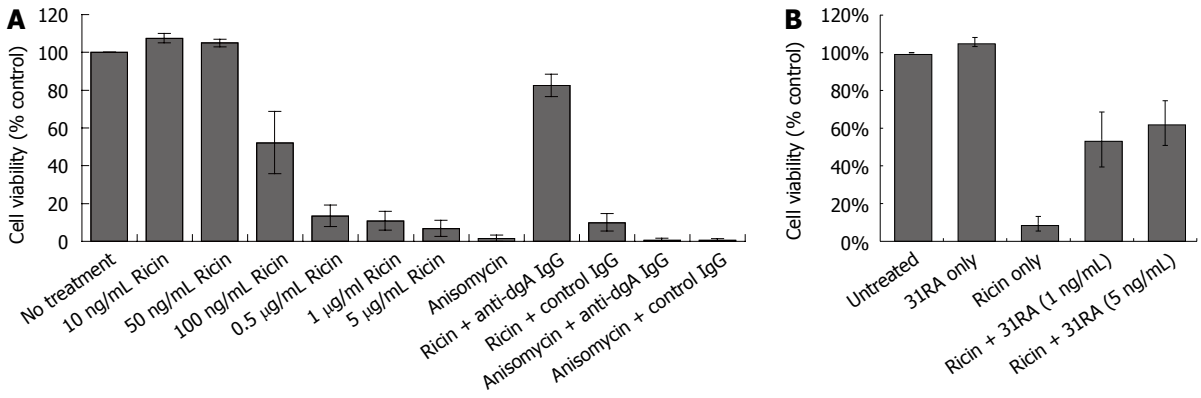

Figure 4 Protective effects of polyclonal anti-dgA IgG and anti-RTA 31RA aptamer on ricin-induced cell cytotoxicity as measured by MTS assay. A: Protective effects of anti-dgA IgG against ricin-induced cytotoxicity, but not anisomycin-induced cytotoxicity; B: Protective effects of 31RA aptamer against ricin-induced cytotoxicity.

RTA in the latter assay was 100 nmol/L, roughly 3 orders of magnitude lower than a small molecule inhibitor of ricin, pteroic acid[19]. Here we showed that 31RA aptamer can inhibit ricin ribotoxicity in cell-free and cell-based luciferase translation assays and cell cytotoxicity assay. It will also be interesting to determine if the 31RA aptamer will be effective against ricin after cell internalization which will be relevant for post-exposure treatment. We are currently testing various methods for intracellular delivery of the 31RA aptamer to determine if internalized aptamer can block toxicity of intracellular ricin A-chain. Hence, anti-RTA aptamers have the potential to be developed as ricin inhibitors and the therapeutic effects of aptamers *in vivo* in animal models of ricin intoxication remain to be determined.

Luciferase and luminescence assays, commonly used readouts for small molecule screens because of high sensitivity and linear signal response, can readily be adapted for high throughput screening of large libraries of compounds[20]. We have utilized a CHO Tet-Off luciferase system for measuring the biological activity of ricin based on protein synthesis inhibition and have shown that this assay can be used for measuring the protective effects of ricin inhibitors including anti-RTA neutralizing antibodies and an anti-RTA RNA aptamer. Compared to the transient luciferase-based assay described by Zhao *et al*[14] which utilized adenoviral transduction to deliver the luciferase gene, our cell-based assay is a stable cell system whereby the luciferase gene is stably integrated and its expression can be regulated by removal of tetracycline or doxycycline. Another advantage of using a stable cell system compared to adenoviral transduction is that the variability due to batch-to-batch variations of viral titers and infection efficiency are avoided. The conventional assays for protein synthesis utilize radioactive amino acids, but these assays suffer from significant sample-to-sample variability and insufficient sensitivity for high throughput assays. Hence, the adaptation of the stable luciferase-based assay in combination with cell cytotoxicity assay will be useful for high throughput screening of compounds for inhibitors of ricin and other related ribotoxins.

COMMENTS

Background

Ricin, a lectin from the castor bean plant *Ricinus communis* is considered one of the most potent plant toxins. Ricin poisoning can cause severe tissue damage and inflammation and can result in death. More than 750 cases of accidental or deliberate ricin poisoning have been described in humans. Most accidental exposures occur by ingestion of the seeds of castor beans whereby the toxin is released after the seed coat is damaged. The ingested toxin causes severe gastrointestinal damage with symptoms including nausea, vomiting, diarrhea, and abdominal pain and may progress to hypotension, liver failure, renal dysfunction, and death due to multiorgan failure or cardiovascular collapse.

Research frontiers

Since currently there is no antidote or specific therapy available for ricin poisoning, the discovery of antitoxins is a high priority. Ricin ribotoxicity can be counteracted by several different types of antitoxins including neutralizing anti-ricin antibodies, small molecule RTA inhibitors, polynucleotide active site inhibitors and polynucleotide substrate analogues. The development of specific inhibitors of RTA will offer novel insights into the development of effective therapeutics against ricin poisoning.

Innovations and breakthroughs

In vitro selection had been used to generate RNA ligands (aptamers) specific for the catalytic ricin A-chain. An initial 80-nucleotide RNA ligand was minimized to a 31-nucleotide RNA aptamer (31RA) that contained all sequences and structures necessary for forming high affinity complexes with RTA and blocking enzymatic activity of RTA *in vitro*. In this report, authors utilized a stable cell-based luciferase assay and showed that 31RA aptamer also neutralized the inhibitory effects of ricin on translation inhibition in cell-free and cell-based luciferase assays and ricin-induced cytotoxicity assay.

Applications

The use of a stably transfected cell-based luciferase assay will facilitate the development of high throughput screening for inhibitors of ricin as potential antidotes for the treatment of ricin intoxication.

Peer review

The manuscript deals with the inhibitory effects of a previously described aptamer against ricin toxicity. The authors employ an *in vitro* translation and a cell-based luciferase assay. This is an interesting paper.

REFERENCES

1 **Audi J**, Belson M, Patel M, Schier J, Osterloh J. Ricin poisoning: a comprehensive review. *JAMA* 2005; **294**: 2342-2351

2 **Yoder JM**, Aslam RU, Mantis NJ. Evidence for widespread epithelial damage and coincident production of monocyte chemotactic protein 1 in a murine model of intestinal ricin intoxication. *Infect Immun* 2007; **75**: 1745-1750

3 **Korcheva V**, Wong J, Corless C, Iordanov M, Magun B. Administration of ricin induces a severe inflammatory

response via nonredundant stimulation of ERK, JNK, and P38 MAPK and provides a mouse model of hemolytic uremic syndrome. *Am J Pathol* 2005; **166**: 323-339
4 **Olsnes S**, Refsnes K, Pihl A. Mechanism of action of the toxic lectins abrin and ricin. *Nature* 1974; **249**: 627-631
5 **Stirpe F**, Barbieri L. Ribosome-inactivating proteins up to date. *FEBS Lett* 1986; **195**: 1-8
6 **Lord JM**, Roberts LM, Robertus JD. Ricin: structure, mode of action, and some current applications. *FASEB J* 1994; **8**: 201-208
7 **Lord JM**, Deeks E, Marsden CJ, Moore K, Pateman C, Smith DC, Spooner RA, Watson P, Roberts LM. Retrograde transport of toxins across the endoplasmic reticulum membrane. *Biochem Soc Trans* 2003; **31**: 1260-1262
8 **Endo Y**, Tsurugi K. The RNA N-glycosidase activity of ricin A-chain. The characteristics of the enzymatic activity of ricin A-chain with ribosomes and with rRNA. *J Biol Chem* 1988; **263**: 8735-8739
9 **Olsnes S**, Pihl A. Different biological properties of the two constituent peptide chains of ricin, a toxic protein inhibiting protein synthesis. *Biochemistry* 1973; **12**: 3121-3126
10 **Wool IG**, Gluck A, Endo Y. Ribotoxin recognition of ribosomal RNA and a proposal for the mechanism of translocation. *Trends Biochem Sci* 1992; **17**: 266-269
11 **Rainey GJ**, Young JA. Antitoxins: novel strategies to target agents of bioterrorism. *Nat Rev Microbiol* 2004; **2**: 721-726
12 **Mantis NJ**. Vaccines against the category B toxins: Staphylococcal enterotoxin B, epsilon toxin and ricin. *Adv Drug Deliv Rev* 2005; **57**: 1424-1439
13 **Hesselberth JR**, Miller D, Robertus J, Ellington AD. In vitro selection of RNA molecules that inhibit the activity of ricin A-chain. *J Biol Chem* 2000; **275**: 4937-4942
14 **Zhao L**, Haslam DB. A quantitative and highly sensitive luciferase-based assay for bacterial toxins that inhibit protein synthesis. *J Med Microbiol* 2005; **54**: 1023-1030
15 **Hale ML**. Microtiter-based assay for evaluating the biological activity of ribosome-inactivating proteins. *Pharmacol Toxicol* 2001; **88**: 255-260
16 **Dertzbaugh MT**, Rossi CA, Paddle BM, Hale M, Poretski M, Alderton MR. Monoclonal antibodies to ricin: in vitro inhibition of toxicity and utility as diagnostic reagents. *Hybridoma* (Larchmt) 2005; **24**: 236-243
17 **Pratt TS**, Pincus SH, Hale ML, Moreira AL, Roy CJ, Tchou-Wong KM. Oropharyngeal aspiration of ricin as a lung challenge model for evaluation of the therapeutic index of antibodies against ricin A-chain for post-exposure treatment. *Exp Lung Res* 2007; **33**: 459-481
18 **Symensma TL**, Giver L, Zapp M, Takle GB, Ellington AD. RNA aptamers selected to bind human immunodeficiency virus type 1 Rev in vitro are Rev responsive in vivo. *J Virol* 1996; **70**: 179-187
19 **Miller DJ**, Ravikumar K, Shen H, Suh JK, Kerwin SM, Robertus JD. Structure-based design and characterization of novel platforms for ricin and shiga toxin inhibition. *J Med Chem* 2002; **45**: 90-98
20 **de Wet JR**, Wood KV, DeLuca M, Helinski DR, Subramani S. Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* 1987; **7**: 725-737

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RAPID COMMUNICATION

Chronic hepatitis C is a common associated with hepatic granulomas

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Abstract

AIM: To determine the most frequent etiologies of hepatic epithelioid granulomas, and whether there was an association with chronic hepatitis C virus (HCV).

METHODS: Both a retrospective review of the pathology database of liver biopsies at our institution from 1996 through 2006 as well as data from a prospective study of hepatic fibrosis markers and liver biopsies from 2003 to 2006 were reviewed to identify cases of hepatic epithelioid granulomas. Appropriate charts, liver biopsy slides, and laboratory data were reviewed to determine all possible associations. The diagnosis of HCV was based on a positive HCV RNA.

RESULTS: There were 4578 liver biopsies and 36 (0.79%) had at least one epithelioid granuloma. HCV was the most common association. Fourteen patients had HCV, and in nine, there were no concurrent conditions known to be associated with hepatic granulomas. Prior interferon therapy and crystalloid substances from illicit intravenous injections did not account for the finding. There were hepatic epithelioid granulomas in 3 of 241 patients (1.24%) with known chronic HCV enrolled in the prospective study of hepatic fibrosis markers.

CONCLUSION: Although uncommon, hepatic granulomas may be part of the histological spectrum of chronic HCV. When epithelioid granulomas are found on the liver biopsy of someone with HCV, other clinically appropriate studies should be done, but if nothing else is found, the clinician can be comfortable with an HCV association.

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Key words: Hepatitis C; Liver; Granulomas; Liver biopsy

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INTRODUCTION

Epithelioid granulomas are found in 1%-10% of liver biopsy specimens[1-3]. They tend to fall into three broad categories which are systemic granulomatous disease, primary liver disease, or miscellaneous conditions that fit neither category. Hepatic granulomas are sometimes a surprise finding that may trigger an extensive search for an etiology. In some cases the granulomas are associated with a clinical picture which includes a high alkaline phosphatase and hepatomegaly; but these features are frequently absent. A significant percentage of liver biopsies performed today are for the staging of chronic hepatitis C. Granulomas have been reported with possible increased frequency in patients with hepatitis C with both mild disease as well as in explants[4-8]. It has been postulated that hepatitis C virus (HCV) may have a role in granuloma formation[9]. We decided to undertake a retrospective review of cases of hepatic granulomas at our institution during the last decade in order to determine the most frequent etiologies, and also to determine if there was an association with HCV.

MATERIALS AND METHODS

Patients studied

Following approval by our institutional review board, we used the data information system in our surgical pathology information system to identify all patients

Table 1 Patients with hepatic granuloma by diagnosis

Diagnosis	Number of patients
HCV	9
Histoplasmosis	4
Sarcoidosis	3
HIV alone	2
HCV/HBV	2
HCV/HIV	2
Mycobacterium avium	2
Primary biliary cirrhosis	2
Unknown	2
Tuberculosis	1
Coccidiomycosis	1
Cryptococcus	1
HBV	1
Hodgkin's lymphoma	1
Q Fever	1
Mucormycosis	1
Drug induced	1

from 1995 through 2005 that had a percutaneous or surgical liver biopsy, and also had the word granuloma or granulomatous in the final diagnosis or in the pathologists description. This data base includes all liver biopsies that are performed at our institution. The University of Texas Medical Branch is a tertiary care institution that consists of 5 hospitals and an outpatient center in Galveston, Texas, USA. Each year it serves patients from most of the counties in Texas, although two thirds of the patients are residents of the Texas Upper Gulf of Mexico Coast. One of the hospitals provides out patient and hospital care to a majority of the inmates in the Texas prison system. No explanted livers were included. We also excluded cases where the biopsy was performed at another institution, but slides were reviewed for a second opinion, and we also excluded several cases where biopsy of ossified nodules at surgery revealed old burned out or inactive granulomas. Autopsies were also excluded. Patients that had only lipogranulomas or small, poorly organized granulomas on their original pathology report were excluded from the study.

Since March 2003, the hepatology service at our institution has been in the midst of a prospective study of hepatic fibrosis markers in patients undergoing pre-treatment liver biopsies in chronic HCV. Some of the results of this study have been published[10,11]. This data base was searched as well for patients with evidence of hepatic granulomas on liver biopsy.

Parameters assessed

When appropriate, charts, reports, and the liver biopsy slides were reviewed. We tabulated information on special stains and cultures that were obtained on the specimens. All patients with hepatic granulomas had their biopsies examined with polarized light for crystalloid particles[12]. Clinical data was assessed to determine HCV, hepatitis B virus (HBV), and human immunodeficiency virus (HIV) status. The diagnosis of HCV was based on the presence of serum HCV RNA[13]. Information was also recorded regarding the patient's HCV genotype and routine liver tests. The stage of fibrosis based on the Batts Ludwig (F0-F4) staging system[14] was also noted.

Table 2 Patients with HCV only

Age (yr)	Gender	Fibrosis stage	Granuloma location	Comments
46	Female	F3	Portal tract	Multiple
54	Male	F2	Lobule	Multiple
46	Male	F2	Portal tract	Single
52	Female	F1	Diffuse	↑ alkaline phos
51	Female	F1	Portal tract	Single
45	Male	F1	Lobule	Multiple
67	Male	F1	Portal tract	Multiple, refractile Crystals
47	Male	F1	Portal tract	Multiple
37	Female	F2	Portal tract	Single

RESULTS

There were a total of 4578 liver biopsies performed at our institution during this time. There were 36 (0.79%) patients identified from the surgical pathology review that had at least one hepatic epithelioid granuloma (Table 1). Special stains were performed in 26 f and tissue cultures in five. The associated diagnoses are listed in Table 1. The most common association was HCV. Fourteen (36.1%) had HCV, and nine of these had no other clinical associations to explain the granulomas. Five of the HCV patients had other confounding associations including chronic hepatitis B, sarcoidosis, histoplasmosis, and HIV (2 patients). The etiologies with more than one case in the non HCV patients were sarcoidosis (3), histoplasmosis (4), primary biliary cirrhosis (3), HIV only (2), mycobacterium avis complex (2), and primary biliary cirrhosis (2), and unidentifiable (2).

There were 241 patients with chronic HCV that had liver biopsies while enrolled in the prospective hepatic fibrosis study during 2003-2006. Three (1.24%) had granulomas, and all of these had been identified in the above database search.

Patients with HCV and granulomas

Table 2 summarizes the results of the 9 patients with HCV, hepatic granulomas, and no other identifiable associations. All of the patients had special stains performed on their liver biopsies, and only one had a crystalloid substance noted on polarizing light. Eight of the patients had genotype 1, and the fibrosis stages F1, F2, and F3 were all present in at least one patient. None of the patients had cirrhosis. One patient with diffuse granulomas had an alkaline phosphatase consistently twice normal. Otherwise the liver function tests were typical of chronic HCV.

Characteristics of granulomas in HCV

The histologic findings in the patients with HCV only were variable. Figure 1 shows a large granuloma in an asymptomatic patient with mild elevation of ALT that had a staging liver biopsy. The granulomas were present in the

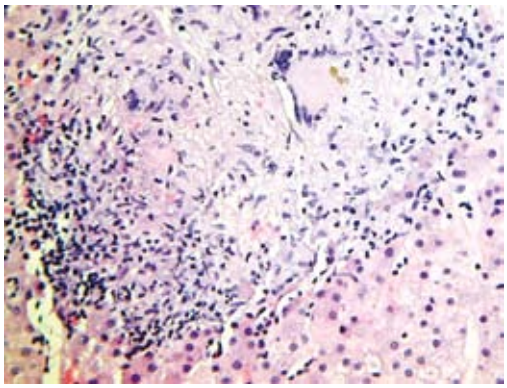

Figure 1 Large granuloma in the hepatic lobule noted in an asymptomatic patient with chronic HCV and mildly elevated ALT (HE, × 40).

portal area in 6 patients, the hepatic lobule in 2 patients, and diffusely in both the portal tract and lobule in one patient. In three patients, there was only a solitary granuloma in the portal tract, while in the others there were multiple granulomas. The case that had crystalloid substance on polarized light had multiple portal granulomas.

DISCUSSION

We found granulomas in less than 1% of our liver biopsies. Other reports have found granulomas in up to 10% of liver biopsies[1-3]. The frequency of detection as well as the frequency of various diagnoses can depend upon the geographic location of the center and the diseases endemic in that area as well as the diligence of the histopathology laboratory and the pathologists that prepare and examine the slides. Previous studies of hepatic granulomas have primarily been from eras before HIV and widespread organ transplantation, and before HCV became prominent. Although the percentages varied, sarcoidosis and tuberculosis tended to be most common associated diseases reported. A study from our institution from another era found that tuberculosis accounted for 53% and sarcoidosis 12% of the cases of hepatic epithelioid granulomas while in 20% no etiology could be determined[1]. In a large series of 565 cases of epithelioid hepatic granulomas in over 6000 biopsies accumulated over 4 decades, Klatskin[2] found that 36% had sarcoidosis, 12% had tuberculosis and 7% were undiagnosed. While our study indeed found 3 patients with sarcoidosis and another with *M. tuberculosis*, most cases were associated with HCV or HIV with associated infections.

There have been several reports of granulomas in the liver biopsies of patients with HCV and no other known systemic or hepatic diseases. This may not be unexpected since the most common reason for liver biopsies at most institutions today is staging of chronic HCV, and some unexplained granulomas have long been found on liver biopsies. On the other hand, the concurrence of HCV and granulomas may be frequent enough to not be simply explained by chance. A large recent retrospective review found epithelioid granulomas in 63 of 1662 liver biopsies[5]. While primary biliary cirrhosis (23.8%), sarcoidosis (11.1%), and unknown (11.1%) were the most common associations, HCV was associated with 9.5% of the cases. Emile *et al*[9] found that the explants of 5 of 52 patients undergoing liver transplantation for cirrhosis from HCV had epithelioid granulomas, and none of these patients had evidence of any other diseases such as tuberculosis or sarcoidosis. Goldin *et al*[4] found in a retrospective study that there were granulomas in the liver biopsy specimens of 14/155 patients with HCV compared with only 3/151 with HBV and 3/129 with alcoholic liver disease. Yamamoto *et al*[6] found unexplained granulomas in 2/273 (0.73%) liver biopsies of patients with chronic HCV. In a more recent study from Turkey, 8/605 (1.3%) of patients with chronic hepatitis C had unexplained granulomas on liver biopsy[7]. This is the same percentage that we found among chronic HCV patients in our prospective study of hepatic fibrosis markers that underwent liver biopsies over a three year period. Consequently, it has been postulated that hepatic granulomas may themselves be part of the histologic picture of chronic HCV, albeit uncommonly. Our finding that 9 of 36 patients with hepatic epithelioid granulomas had HCV as their only disease association would support this. We cannot rule out that some of these patients could have had other undiagnosed disorders such as sarcoidosis; but in follow up, none of them have manifested pulmonary or systemic findings to support another diagnosis.

Alpha interferon in regular or pegylated form has been used in the therapy of chronic HCV since the discovery of the virus[13]. It has been speculated that interferon itself can stimulate granuloma production, and there are several reported cases of the development of sarcoidosis in patients receiving interferon therapy[15-19]. This is thought to be related to the ability of interferon to stimulate a Th 1 immune response which is felt to be responsible for the granulomas in sarcoidosis[20]. Both the sarcoidosis and granulomas have usually regressed when interferon was stopped. It is unlikely that interferon usage played a major factor in our patients since only one subject with chronic HCV and granulomas had received interferon prior to liver biopsy.

Intravenous drug use is the most common route of transmission of chronic hepatitis C[21]. Since granulomas can be induced by foreign substances that might be used in a "street prepared" illicit drug mixture, we examined all the slides of patients with HCV and granulomas with polarized light, and a crystalloid substance was found in only one patient. Therefore, foreign body induced granulomas do not appear to be an explanation for most of the granulomas found in our patients with HCV.

We realize that there are problems interpreting the results of a retrospective study such as ours. The majority of the liver biopsies that we perform are on patients that are being staged prior to proposed treatment for chronic HCV. Therefore, the finding of otherwise unexplained granulomas in multiple patients with chronic HCV may not be that unusual or surprising. Nevertheless, in this study the most common association of hepatic granulomas was chronic HCV.

We would recommend if a patient with chronic HCV should have granulomas on liver biopsy that a search

for another disease should be made by utilizing special stains and other clinically appropriate tests such as a chest X-ray. However, if no other abnormality is found, the clinician should be comfortable associating the granuloma(s) with the chronic HCV.

COMMENTS

Background

Granulomas have long been curious findings on liver biopsies, and sometimes can trigger exhaustive searches for the etiology. Although there are many causes, sarcoidosis and tuberculosis have been the most frequent associations in previous series.

Research frontiers

Recent papers have reported finding granulomas in chronic hepatitis C virus (HCV) patients, and it has been speculated that granulomas may be an uncommon part of the immune response in chronic HCV. This study looked retrospectively at a decade of liver biopsies at the large institution to see what the most common disease associations with hepatic granulomas were. Authors also looked prospectively at the prevalence of granulomas in a series of chronic HCV patients undergoing staging liver biopsies.

Innovations and breakthroughs

The most common association of hepatic granulomas at the institution is chronic hepatitis C. The granulomas were both in the portal area and the lobule, and they were both single and multiple. Although present in only about 1% of liver biopsies of patients with hepatitis C, they should be considered as part of the histologic spectrum of the disease.

Applications

The finding of hepatic granulomas on the liver biopsy of someone with chronic HCV does not necessitate an extensive workup. Special stains and pertinent tests such as a chest x-ray should be done, but the clinician should be comfortable with the association if nothing is found.

Terminology

An epithelioid granuloma is a complex of transformed macrophages together with inflammatory cells and often multinucleated giant cells. It is a manifestation of delayed hypersensitivity.

Peer review

This is a very good retrospective examination of characteristics associated with hepatogranulomas, with the added strength of the prospective surveillance.

REFERENCES

1 **Guckian JC**, Perry JE. Granulomatous hepatitis. An analysis of 63 cases and review of the literature. *Ann Intern Med* 1966; **65**: 1081-1100

2 **Klatskin G**. Hepatic granulomata: problems in interpretation. *Mt Sinai J Med* 1977; **44**: 798-812

3 **Cunnigham D**, Mills PR, Quigley EM, Patrick RS, Watkinson G, MacKenzie JF, Russell RI. Hepatic granulomas: experience over a 10-year period in the West of Scotland. *Q J Med* 1982; **51**: 162-170

4 **Goldin RD**, Levine TS, Foster GR, Thomas HC. Granulomas and hepatitis C. *Histopathology* 1996; **28**: 265-267

5 **Gaya DR**, Thorburn D, Oien KA, Morris AJ, Stanley AJ. Hepatic granulomas: a 10 year single centre experience. *J Clin Pathol* 2003; **56**: 850-853

6 **Yamamoto S**, Iguchi Y, Ohomoto K, Mitsui Y, Shimabara M, Mikami Y. Epitheloid granuloma formation in type C chronic hepatitis: report of two cases. *Hepatogastroenterology* 1995; **42**: 291-293

7 **Ozaras R**, Tahan V, Mert A, Uraz S, Kanat M, Tabak F, Avsar E, Ozbay G, Celikel CA, Tozun N, Senturk H. The prevalence of hepatic granulomas in chronic hepatitis C. *J Clin Gastroenterol* 2004; **38**: 449-452

8 **Mert A**, Tabak F, Ozaras R, Tahan V, Senturk H, Ozbay G. Hepatic granulomas in chronic hepatitis C. *J Clin Gastroenterol* 2001; **33**: 342-343

9 **Emile JF**, Sebagh M, Feray C, David F, Reynes M. The presence of epithelioid granulomas in hepatitis C virus-related cirrhosis. *Hum Pathol* 1993; **24**: 1095-1097

10 **Snyder N**, Gajula L, Xiao SY, Grady J, Luxon B, Lau DT, Soloway R, Petersen J. APRI: an easy and validated predictor of hepatic fibrosis in chronic hepatitis C. *J Clin Gastroenterol* 2006; **40**: 535-542

11 **Snyder N**, Nguyen A, Gajula L, Soloway R, Xiao SY, Lau DT, Petersen J. The APRI may be enhanced by the use of the FIBROSpect II in the estimation of fibrosis in chronic hepatitis C. *Clin Chim Acta* 2007; **381**: 119-123

12 **Ishak KG**. Light microscopic morphology of viral hepatitis. *Am J Clin Pathol* 1976; **65**: 787-827

13 **National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002--June 10-12, 2002**. *Hepatology* 2002; **36**: S3-S20

14 **Batts KP**, Ludwig J. Chronic hepatitis. An update on terminology and reporting. *Am J Surg Pathol* 1995; **19**: 1409-1417

15 **Ryan BM**, McDonald GS, Pilkington R, Kelleher D. The development of hepatic granulomas following interferon-alpha2b therapy for chronic hepatitis C infection. *Eur J Gastroenterol Hepatol* 1998; **10**: 349-351

16 **Gitlin N**. Manifestation of sarcoidosis during interferon and ribavirin therapy for chronic hepatitis C: a report of two cases. *Eur J Gastroenterol Hepatol* 2002; **14**: 883-885

17 **Butnor KJ**. Pulmonary sarcoidosis induced by interferon-alpha therapy. *Am J Surg Pathol* 2005; **29**: 976-979

18 **Ubina-Aznar E**, Fernandez-Moreno N, Rivera-Irigoin R, Navarro-Jarabo JM, Garcia-Fernandez G, Perez-Aisa A, Vera-Rivero F, Fernandez-Perez F, Moreno-Mejias P, Mendez-Sanchez I, de Sola-Earle C, Sanchez-Cantos A. [Pulmonary sarcoidosis associated with pegylated interferon in the treatment of chronic hepatitis C] *Gastroenterol Hepatol* 2005; **28**: 450-452

19 **Menon Y**, Cucurull E, Reisin E, Espinoza LR. Interferon-alpha-associated sarcoidosis responsive to infliximab therapy. *Am J Med Sci* 2004; **328**: 173-175

20 **Alfageme Michavila I**, Merino Sanchez M, Perez Ronchel J, Lara Lara I, Suarez Garcia E, Lopez Garrido J. [Sarcoidosis following combined ribavirin and interferon therapy: a case report and review of the literature] *Arch Bronconeumol* 2004; **40**: 45-49

21 **Armstrong GL**, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. *Ann Intern Med* 2006; **144**: 705-714

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RAPID COMMUNICATION

Histological abnormalities of the small bowel mucosa in cirrhosis and portal hypertension

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Abstract

AIM: To study the small bowel (SB) mucosa on biopsy in cirrhotic patients with portal hypertension and in non-cirrhotic controls and grade findings according to the Marsh criteria.

METHODS: We prospectively enrolled 51 consecutive patients undergoing an upper endoscopy for their routine medical care. Twenty five patients with cirrhosis and portal hypertension were compared to 26 controls. We obtained coeliac serology and multiple upper small bowel biopsies on all 51 patients. A GI pathologist interpreted biopsies and graded findings according to the Marsh criteria. We assessed equivalence in Marsh grade between cirrhotic and non-cirrhotic controls using the Mann-Whitney test for equivalence.

RESULTS: Gender, ethnicity and age were similar between both groups. Marsh grades were equivalent between the groups. Grade of 0 was present in 96% and grade of 1 was present in 4% of both groups and there was no villus atrophy or decrease in villus/crypt ratio in patients with portal hypertension.

CONCLUSION: This study provides evidence for the lack of villus atrophy in patients with cirrhosis and portal hypertension, and supports the continuous reliance on the Marsh criteria when the diagnosis of coeliac disease is to be made in the presence of cirrhosis.

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Key words: Cirrhosis; Portal hypertension; Coeliac disease; Marsh criteria; Small bowel mucosa

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INTRODUCTION

Studies of small bowel (SB) mucosa in cirrhosis and portal hypertension report a diverse spectrum of histological abnormalities[1-5]. These include increased capillary angiogenesis, mucosal edema, decreased villus to crypt ratio, villus atrophy and decreased total absorptive surface. Villus abnormalities resemble the abnormalities of coeliac disease (CD) and this may affect the interpretation of small bowel biopsy, and lead to confusion when CD is to be excluded in patients with cirrhosis and portal hypertension.

The diagnosis of CD is increasingly considered and work up recommended during the evaluation of abnormal liver enzymes[6-7]. This is in part due to the heightened awareness of the association of CD with a variety of liver disorders. Most commonly described associated liver disorders include autoimmune hepatitis, primary biliary cirrhosis, non-alcoholic fatty liver disease, unexplained abnormal liver tests and cirrhosis[8-15]. The mechanisms of this association are not clear and the prevalence of CD in patients with liver disease is variable depending on the associated liver disease. For example, while CD affects 1% of the general population, one study reports that about 4% of 185 cirrhotic patients who had undergone liver transplantation were found to have CD, and 3 of 4 pa-

tients presenting with severe liver disease were diagnosed with CD and were remitted as possible candidates for liver transplantation when placed on a gluten free diet[16]. Approximately 40% of patients with CD have abnormal liver enzymes, and these return to normal in 75% to 95% when a gluten free diet is instituted[16,17]. Based on these and other studies, it is recommended that clinicians should have a low threshold for testing for CD in patients with abnormal liver blood tests[6].

The diagnosis of CD is based on initial screening with coeliac serological tests (endomysial EMA and human tissue transglutaminase hTTG antibodies); but histological examination of SB biopsy is required for establishing a definite diagnosis[6,10]. However, in patients with cirrhosis, the diagnosis of coexisting CD is a challenge because of the reported similar changes on SB mucosa in both cirrhosis and CD. CD may, therefore, be underdiagnosed in cirrhosis. Studies of SB biopsy in cirrhosis[1-5], have thus shed doubt on the validity of biopsy in the diagnosis of CD in cirrhotic patients; but reported findings are poorly characterized, lack standardized grading, and of unclear significance. The current study was undertaken to determine if SB biopsies in cirrhosis show features that might mimic CD. Findings would determine if SB biopsy should be used in the diagnosis of CD in patients with cirrhosis and portal hypertension.

The aim of the study was to prospectively assess the histological abnormalities of the SB mucosa in patients with and without cirrhosis and portal hypertension by grading findings according to the grading system defined by Marsh[6].

MATERIALS AND METHODS

Selection of patients and data collection

This is a prospective case control study approved by the Institutional Board Review at the Cleveland Clinic. Eighty consecutive patients scheduled for an upper endoscopy EGD at the Cleveland Clinic between 9/1/2005 to 11/30/2005 were identified. Medical records were reviewed. Of 80 patients, 25 with cirrhosis and portal hypertension and 26 without cirrhosis, portal hypertension or liver disease fulfilled inclusion and exclusion criteria and were enrolled in the study.

Records reviewed included age, ethnicity, gender, indications for upper endoscopy EGD, imaging studies and laboratory tests. Laboratory tests obtained within the preceding 6 mo of the date of enrollment were reviewed for liver transaminases, alkaline phosphatase, protime/INR, celiac serology panel, complete blood count and differential, iron saturation and ferritin, and viral hepatitis panel.

Patients were included if they were older than 18 years old and able to give informed consent. Patients with cirrhosis and undergoing EGD were included regardless of the etiology of cirrhosis. Patients without cirrhosis and undergoing EGD for acid reflux, abdominal pain, dysphagia or vomiting were included.

Cirrhosis was defined histologically according to Batts and Ludwig staging system[18]. In patients without a liver biopsy, diagnosis of cirrhosis and portal hypertension was based on a combination of clinical data (jaundice, cutaneous spider angiomas, muscle wasting, ascites, and palmar erythema), biochemical data (decreased serum albumin and prolonged protime), imaging study (nodular surface on ultrasound or CT scan), and manifestations of portal hypertension (low platelets, splenomegaly, esophageal varices, hepatic encephalopathy or ascites) in the setting of chronic liver disease.

Patients were excluded if they were pregnant, on dialysis, had a bleeding disorder or were actively bleeding at the time of endoscopy, taking anticoagulants or had INR greater than > 1.5 or platelet count less than $30 \times 10^3/mm^3$. Patients with a diagnosis of malabsorption, coeliac disease, patients taking corticosteroids or immunosuppressant drugs, patients with a history of Crohn's disease, organ transplant, graft versus host disease, food allergies, iron deficiency anemia, osteoporosis, ataxia, and autoimmune disorders that could potentially be associated with CD such as thyroid disorders, dermatitis herpetiformis and type 1diabetes were excluded. In the control group, additional exclusions encompass individuals with a history of chronic liver disease or a history of abnormal liver tests.

Informed consent for SB biopsy and for blood draw for coeliac serology panel was obtained on all 51 patients. Severity of cirrhosis was assessed by calculating Child Turcotte Pugh (CTP) and Model for End-Stage Liver Disease (MELD) scores for all cirrhotic patients.

Laboratory assessment

Coeliac serology panel included antibodies to human tissue transglutaminase (IgG and IgA for hTTG, QUANTA lite™ ELISA, Inova diagnostics, San Diego CA), endomysial antibodies (IgA EMA, Immunofluorescence Inova diagnostics San Diego CA), and total IgA levels by nephelometry (Beckman Coulter Immage/image 800 Immunochemistry system and Calibrator 1, Fullerton CA). Tests were consecutively analyzed in the immunology laboratory at the Cleveland Clinic. These tests are reported to be highly sensitive and specific in the diagnosis of CD in the general population with sensitivities and specificities above 85%, and they supplant the use of gliadin antibody testing as the preferred mean of serological detection[6]. Abnormal serology panel is any value for IgA EMA above 1:10 dilution, or any value for either IgG hTTG or IgA hTTG above 20 U.

Histological assessment

Upper endoscopies were performed by gastroenterologists at the Cleveland Clinic. The gastroenterologists were not blinded to the study, and they were asked to obtain at least 3 biopsies from the second part of the duodenum or beyond on all subjects. Biopsy specimens were placed in vials containing 10% of buffered formalin solution for fixation. Paraffin sections were prepared and stained by hematoxylin and eosin (HE) stain. Pathology slides were interpreted by a Cleveland Clinic pathologist (A.B.) experienced with the spectrum of mucosal changes in CD. The pathologist was blinded to names

and diagnosis. All slides were batched and read after samples from all 51 subjects collected and processed. The pathologist graded findings according to the Marsh grading system[6]. Marsh 0 is defined by normal mucosal and villus architecture, Marsh I is defined by normal villus architecture, but increased numbers of intraepithelial lymphocytes, Marsh Ⅱ shows increased intraepithelial lymphocytes, enlarged crypts and increased crypt cell division, Marsh Ⅲ is defined by villus atrophy, shortened blunt villi and enlarged hyperplastic crypts. Marsh Ⅳ demonstrates hypoplastic mucosa.

Statistical analysis

The sample size estimation was based on the inference that the standard deviation of the Marsh grade would be equal to 1 for both groups. The study was designed to establish equivalence in small bowel mucosa (defined as a difference in mean Marsh grades no greater than1) between cirrhotics and non-cirrhotics with a significance level of 0.05 and a power of at least 90%. Therefore, it was estimated that a total of 25 subjects would be required in each group.

Descriptive statistics, such as frequencies for categorical factors and mean (SD) for continuous factors, were computed for all variables. A Student's *t*-test was used to assess differences in age between cirrhotics with portal hypertension and non-cirrhotic controls. In addition, Pearson's χ^2 and Fisher's exact test were used to compare gender and race between the groups.

In order to assess whether the SB mucosal architecture in cirrhotic patients with portal hypertension was indistinguishable from the mucosal architecture in non-cirrhotic controls, the Mann-Whitney test for equivalence was used[19]. It tests the null hypothesis (H_0) of difference in SB mucosal architecture between groups versus the research hypothesis (H_a) of equivalent SB mucosal architecture between groups. A *P*-value < 0.05 was considered statistically significant. SAS version 9.1 software (SAS institute, Carey, NC) was used to carry out all analyses.

RESULTS

Study populations

A total of 51 patients were enrolled. Twenty five had cirrhosis and portal hypertension and 26 controls had no evidence of liver disease or portal hypertension. Fifty patients had normal coeliac serology and one patient in each group had abnormal biopsy.

Baseline demographic characteristics of patients who fulfilled inclusion criteria are shown in Table 1. The mean age was 57 (± 10) years in the portal hypertension with cirrhosis group and 52 (± 15) years in the control group. Fifty two percent were males and 92% were Caucasians in the former group *versus* 42% and 84% respectively in the control group. There was no evidence to suggest statistically significant differences in the demographic characteristics between the groups ($P > 0.05$).

The most common indications for upper endoscopy in cirrhotic patients with portal hypertension were screening for or banding of esophageal varices (100%). In the control group, the most common indications were acid reflux (30.8%), dysphagia (23.1%), epigastric pain (23.1%), and nausea and vomiting in (15.4%).

Table 1 Demographic characteristics of study subjects (mean ± SD) *n* (%)

	Cirrhotic	Non-cirrhotic	*P* value
n	25	26	-
Age	57.5 ± 9.7	51.9 ± 15.3	0.12
Gender			0.49
Male	13 (52)	11 (42.3)	
Female	12 (48)	15 (57.7)	
Ethnicity			0.67
Caucasian	23 (92)	22 (84.6)	
Other	2 (8)	4 (15.4)	

All 25 patients with cirrhosis had evidence of portal hypertension, esophageal varices, thrombocytopenia and an imaging study showing cirrhotic liver. Fifteen patients had a liver biopsy. Twelve patients had CTP score A, 11 had CTP score B and 2 had CTP score C. The mean platelet count was $89.76 \times 10^3/mm^3$, with a range between 45 and $148 \times 10^3/mm^3$. The mean MELD score was 10 with a range between 5 and 17. The most common etiologies for cirrhosis were nonalcoholic steatohepatitis (24%), hepatitis C (24%) and cryptogenic cirrhosis (24%).

Histological findings in patients with and without cirrhosis

There was strong evidence to suggest that based on Marsh grade, cirrhotics and non-cirrhotics have indistinguishable SB mucosa (Mann-Whitney test of equivalence: $P < 0.01$). One patient in each group (4%) had an abnormal small bowel biopsy and 96% of patients from each group had a normal small bowel biopsy (Figures 1 and 2). Both patients with abnormal biopsy were females with normal coeliac serology. The two women had Marsh grade I based on 3 SB biopsies obtained for each. One of these women was 57 years old, and had a liver biopsy that was consistent with nonalcoholic steatohepatitis and cirrhosis. Her platelet level was $130 \times 10^3/mm^3$, her MELD score was 10 and her CTP score was B. The other woman with Marsh grade I on SB biopsy did not have cirrhosis. She was 51 years old and was scheduled for EGD for epigastric pain and bloating.

DISCUSSION

Portal hypertension and cirrhosis due to a variety of parenchymal liver diseases are associated with malnutrition[20,21]. The pathophysiologic mechanisms are not totally understood and several factors may be involved. Malabsorption has been implicated and this was presumed to be related to changes in the SB villi[2,4,5]. For example, Such *et al*[4] investigated 6 patients with cirrhosis using jejunal biopsies, and studied the mucosa under electron microscopy. The authors observed that "the microvilli were reduced in number and appeared shorter

Figure 1 Percentage of patients with Marsh grade 0 and Marsh grade 1 in cirrhosis and no cirrhosis.

and thicker when compared to controls", and their conclusion was that "the total absorptive surface may be reduced in cirrhotic patients". Misra[2] found a significant decrease in villus/crypt ratio in cirrhotic patients when compared to healthy volunteers. On the other hand, Nagral[22] reports a significant number of patients with large vessels in duodenal mucosa of patients with portal hypertension in comparison with controls, but did not find a statistical difference in severity and type of infiltrate, edema of lamina propria or villus/crypt ratio between the groups. In the study of Barakat[5], abnormal villus changes were present in 11.4% of portal hypertensive patients. These were described as "shortened villi, decreased or even reversed villus to crypt ratio down to total villus atrophy". The authors implied that these changes might have an effect on the intestinal absorptive functions, and in turn, might have a share in the pathogenesis of nutritional derangements in portal hypertensive patients.

Results of these studies implicate SB villus shortening and atrophy as underlying factors in the malabsorption and malnutrition observed in cirrhosis. Such conclusions need further validation and other diseases that may affect the SB mucosa should be considered in the differential diagnosis. Furthermore, histological abnormalities were reported descriptively and without systematic classification. Lack of a proper classification of abnormalities was once described by Marsh[23] as follows: "The system of qualitative terminology is not only inappropriate but also seems to have paralyzed any new intellectual activity that might elucidate afresh the immunopathogenic basis of mucosal response". And in 1992, Marsh described the mucosal abnormalities of the SB by establishing a classification system that utilized inflammatory and atrophic grades. This grading system has since been adopted by clinicians and pathology researchers in the diagnosis and study of CD, and is the only available system to systematically grade mucosal abnormalities of the SB. In this unprecedented study, we prospectively analyzed and graded changes of SB mucosa on biopsy according to the unified and accepted grading system characterized by Marsh[6,10].

We found no difference in Marsh grade between the study groups. Our patients had either a grade of 0 or a grade of I . One patient in each group had a Marsh grade of I , but neither had abnormal coeliac serology. The presence of Marsh grade I or intraepithelial lymphocytes is a non-specific finding especially in the absence of coexistent abnormal serology. We did not find

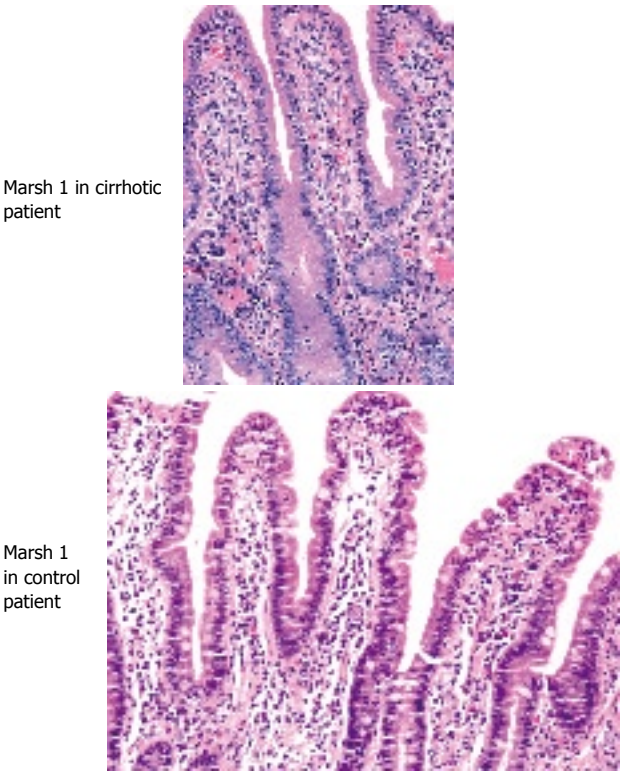

Figure 2 Small bowel mucosa in the cirrhotic and in the control patient, respectively.

any Marsh grades Ⅱ, Ⅲ or villus/crypt changes of CD among patients in either group. This provides evidence that histological abnormalities of the SB mucosa are equivalent between cirrhotic patients with portal hypertension and non-cirrhotic controls.

Since the etiology of cirrhosis has not been shown to influence the changes in mucosal architecture of the SB, we included all eligible cirrhotic patients regardless of the etiology of their cirrhosis. We excluded patients with clinical and laboratory evidence of malabsorption, patients with known SB mucosal disease and/or on therapy that included corticosteroids and immunosuppressants, and patients with autoimmune diseases that have the potential to be associated with CD or induce changes in the SB mucosa, because such cases may not necessarily yield the direct effects of cirrhosis on the SB mucosa.

We calculated the MELD and CTP scores on all cirrhotic patients. Although our study did not aim to examine whether the severity of liver disease would further exacerbate villus damage, we did not observe any correlation between Marsh grade and the degree of MELD and CTP scores. This has been reported previously[22].

An exact quantitative count for the intraepithelial lymphocytes IEL and immunohistochemical typing were not obtained because such stains are not utilized in routine clinical practice, and because one observer (pathologist A.B.) interpreted and analyzed all biopsy specimens.

Portal hypertensive enteropathy is receiving increased recognition in research studies of recent years. Studies have aimed at characterizing abnormalities on endoscopy, wireless capsule imaging, and SB biopsy. Small

bowel biopsy findings describe villus changes and villus atrophy. Contrary to previously reported studies, our study did not show shortened villi, reversal of villus to crypt ratio, villus atrophy, crypt hyperplasia or changes suggestive of CD in patients with portal hypertension. This concurs with the study of Nagral[22].

Our study is unique and different from other studies of the effects of cirrhosis and portal hypertension on the SB mucosa because we used the validated Marsh grading system to grade for abnormalities and we excluded subjects with CD and other diseases that would potentially affect the SB mucosa such as Crohn's and lymphoma *etc*, because we intended to study the sole effect of cirrhosis on the SB. SB villus changes and atrophy are characteristic, but are not specific for coeliac disease[24,25].

Coeliac serological tests have low sensitivities and specificities in chronic liver disease and cirrhosis[15,26-29] which emphasizes the importance of validating SB biopsy as the most important tool for the diagnosis of CD in this group of patients. Results of this study support the use of SB biopsy in patients with cirrhosis and portal hypertension when the diagnosis of CD is suspected. Furthermore, and since cirrhosis is not associated with significant inflammatory or atrophic changes of SB villi, we can extrapolate that there is a low probability that different results would be seen in the earlier stages of liver disease when cirrhosis has not yet developed.

Due to the estimated high prevalence of CD in patients with cirrhosis that is higher than in the general population[8,16,29,30], and the potential reversibility of liver dysfunction on a gluten free diet, we recommend prompt consideration and exclusion of SB mucosal diseases and CD (by using a combination of laboratory, clinical, pathologic and genetic examinations, and response to a gluten free diet), when shortened villi, reversal of villus to crypt ratio or villus atrophy are seen in patients with cirrhosis.

As to the mechanisms of malabsorption reported in patients with cirrhosis and portal hypertension, factors other than cirrhosis- related- villus changes should be considered. These may include bile salt deficiencies, motility disorders, protein losing enteropathy and other concomitant diseases of the SB. Further studies are needed.

In conclusion, our study provides evidence for the lack of villus atrophy in patients with cirrhosis and portal hypertension. Hence, small bowel biopsies can be interpreted reliably to exclude coeliac disease in these patients. Furthermore, findings of shortened villi and villus atrophy should trigger the exclusion of coeliac disease and other diseases of the small bowel in this group of patients.

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COMMENTS

Background

Cirrhosis affects the small bowel mucosa in ways not totally elucidated. Villus shortening and atrophy are described. But these findings are reported descriptively and without proper classification, which may affect the interpretation of small bowel biopsy when the diagnosis of coeliac disease is to be excluded in patients with cirrhosis. We aimed to study the small bowel mucosa on biopsy in cirrhotic patients with portal hypertension and in non-cirrhotic controls and grade findings according to the standardized grading system described by Marsh.

Research frontiers

Studies of recent years have shown that coeliac disease is increasingly recognized in association with chronic liver disease and cirrhosis, and coeliac disease is more common than previously thought but is under diagnosed. It is, therefore, recommended to exclude coeliac disease in patients who have cirrhosis and cryptogenic abnormality of liver tests, and the diagnostic tool of choice is a small bowel biopsy.

Innovations and breakthroughs

This study is different from other studies of the small bowel mucosa in cirrhosis and portal hypertension because we graded findings according to the standardized Marsh grading system and excluded subjects with diseases that could potentially affect the small bowel in order to assess the sole effect of portal hypertension on the small bowel mucosa. Additionally, we used the Mann-Whitney test of equivalence to support the equality of mucosal abnormalities in both study groups, and we estimated a sample size of 25 individuals in each group in order to confer a statistical power of at least 90%.

Applications

Today's medical practice emphasizes evidence based medicine. Our study provides evidence for the lack of villus atrophy in cirrhosis and portal hypertension; hence SB biopsies can be interpreted reliably to exclude coeliac disease in these patients and in future studies of the mechanisms of liver disease in patients with coeliac disease. Additionally, small bowel mucosal diseases should be excluded when villus shortening and atrophy are seen in patients with cirrhosis and portal hypertension

Peer review

This is a well designed study, and the data looks sound. Patients with liver disease of unknown etiology can have small bowel biopsies that reliably exclude coeliac disease as an association or cause of their hepatic disease.

REFERENCES

1 **Baraona E**, Orrego H, Fernandez O, Amenabar E, Maldonado E, Tag F, Salinas A. Absorptive function of the small intestine in liver cirrhosis. *Am J Dig Dis* 1962; **7**: 318-330

2 **Misra V**, Misra SP, Dwivedi M, Gupta SC. Histomorphometric study of portal hypertensive enteropathy. *Am J Clin Pathol* 1997; **108**: 652-657

3 **Astaldi G**, Strosselli E. Peroral biopsy of the intestinal mucosa in hepatic cirrhosis. *Am J Dig Dis* 1960; **5**: 603-612

4 **Such J**, Guardiola JV, de Juan J, Casellas JA, Pascual S, Aparicio JR, Sola-Vera J, Perez-Mateo M. Ultrastructural characteristics of distal duodenum mucosa in patients with cirrhosis. *Eur J Gastroenterol Hepatol* 2002; **14**: 371-376

5 **Barakat M**, Mostafa M, Mahran Z, Soliman AG. Portal hypertensive duodenopathy: clinical, endoscopic, and histopathologic profiles. *Am J Gastroenterol* 2007; **102**: 2793-2802

6 **AGA Institute Medical Position Statement on the Diagnosis and Management of Celiac Disease**. *Gastroenterology* 2006; **131**: 1977-1980

7 **Morisco F**, Pagliaro L, Caporaso N, Bianco E, Sagliocca L, Fargion S, Smedile A, Salvagnini M, Mele A. Consensus recommendations for managing asymptomatic persistent non-virus non-alcohol related elevation of aminotransferase levels: suggestions for diagnostic procedures and monitoring. *Dig Liver Dis* 2008; **40**: 585-598

8 **Bardella MT**, Valenti L, Pagliari C, Peracchi M, Fare M, Fracanzani AL, Fargion S. Searching for coeliac disease in

patients with non-alcoholic fatty liver disease. *Dig Liver Dis* 2004; **36**: 333-336
9 **Rubio-Tapia A**, Murray JA. The liver in celiac disease. *Hepatology* 2007; **46**: 1650-1658
10 **National Institutes of Health Consensus Development Conference Statement on Celiac Disease, June 28-30, 2004**. *Gastroenterology* 2005; **128**: S1-S9
11 **Kingham JG**, Parker DR. The association between primary biliary cirrhosis and coeliac disease: a study of relative prevalences. *Gut* 1998; **42**: 120-122
12 **Ludvigsson JF**, Elfstrom P, Broome U, Ekbom A, Montgomery SM. Celiac disease and risk of liver disease: a general population-based study. *Clin Gastroenterol Hepatol* 2007; **5**: 63-69.e1
13 **Volta U**, De Franceschi L, Lari F, Molinaro N, Zoli M, Bianchi FB. Coeliac disease hidden by cryptogenic hypertransaminasaemia. *Lancet* 1998; **352**: 26-29
14 **Hagander B**, Berg NO, Brandt L, Norden A, Sjolund K, Stenstam M. Hepatic injury in adult coeliac disease. *Lancet* 1977; **2**: 270-272
15 **Germenis AE**, Yiannaki EE, Zachou K, Roka V, Barbanis S, Liaskos C, Adam K, Kapsoritakis AN, Potamianos S, Dalekos GN. Prevalence and clinical significance of immunoglobulin A antibodies against tissue transglutaminase in patients with diverse chronic liver diseases. *Clin Diagn Lab Immunol* 2005; **12**: 941-948
16 **Kaukinen K**, Halme L, Collin P, Farkkila M, Maki M, Vehmanen P, Partanen J, Hockerstedt K. Celiac disease in patients with severe liver disease: gluten-free diet may reverse hepatic failure. *Gastroenterology* 2002; **122**: 881-888
17 **Bardella MT**, Fraquelli M, Quatrini M, Molteni N, Bianchi P, Conte D. Prevalence of hypertransaminasemia in adult celiac patients and effect of gluten-free diet. *Hepatology* 1995; **22**: 833-836
18 **Ludwig J**, Batts KP, Moyer TP, Poterucha JJ. Advances in liver biopsy diagnosis. *Mayo Clin Proc* 1994; **69**: 677-678
19 **Wellek S**. Testing Statistical Hypotheses of Equivalence. 1st ed. Boca Ration, London, New York, Washington, D.C.: Chapman & Hall/CRC Press, 2003: 106-111
20 **Henkel AS**, Buchman AL. Nutritional support in patients with chronic liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 202-209
21 **Hogenauer C**, Hammer HF. Maldigestion and Malabsorption. In: Feldman: Sleisenger & Fordtran's Gastrointestinal and Liver Disease. 8th ed. Saunders: An Imprint of Elsevier, 2006: 2200-2232
22 **Nagral AS**, Joshi AS, Bhatia SJ, Abraham P, Mistry FP, Vora IM. Congestive jejunopathy in portal hypertension. *Gut* 1993; **34**: 694-697
23 **Marsh MN**. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992; **102**: 330-354
24 **Green PH**, Cellier C. Celiac disease. *N Engl J Med* 2007; **357**: 1731-1743
25 **Farrell RJ**, Kelly CP. Celiac Sprue and Refractory Sprue. In: Feldman: Sleisenger & Fordtran's Gastrointestinal and Liver Disease. 8th ed. Saunders: An Imprint of Elsevier, 2006: 2277-2307
26 **Bizzaro N**, Tampoia M, Villalta D, Platzgummer S, Liguori M, Tozzoli R, Tonutti E. Low specificity of anti-tissue transglutaminase antibodies in patients with primary biliary cirrhosis. *J Clin Lab Anal* 2006; **20**: 184-189
27 **Vecchi M**, Folli C, Donato MF, Formenti S, Arosio E, de Franchis R. High rate of positive anti-tissue transglutaminase antibodies in chronic liver disease. Role of liver decompensation and of the antigen source. *Scand J Gastroenterol* 2003; **38**: 50-54
28 **Valera JM**, Hurtado C, Poniachik J, Abumohor P, Brahm J. [Study of celiac disease in patients with non-alcoholic fatty liver and autoimmune hepatic diseases] *Gastroenterol Hepatol* 2008; **31**: 8-11
29 **Lo Iacono O**, Petta S, Venezia G, Di Marco V, Tarantino G, Barbaria F, Mineo C, De Lisi S, Almasio PL, Craxi A. Anti-tissue transglutaminase antibodies in patients with abnormal liver tests: is it always coeliac disease? *Am J Gastroenterol* 2005; **100**: 2472-2477
30 **Volta U**. Pathogenesis and Clinical Significance of Liver Injury in Celiac Disease. *Clin Rev Allergy Immunol* 2009; **36:** 62-70

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RAPID COMMUNICATION

Expression of cell adhesion molecule CD44 in gastric adenocarcinoma and its prognostic importance

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Abstract

AIM: To evaluate the relation of cluster of differentiation 44 (CD44) expression with clinicopathological features of gastric adenocarcinoma, and also its effect on prognosis with an emphasis on the differences between intestinal and diffuse types.

METHODS: From 2000 to 2006, 100 patients with gastric adenocarcinoma, who had undergone total or subtotal gastrectomy without any prior treatment, were studied. Haematoxylin & eosin (HE) staining was used for histological evaluation, including the type (Lauren's classification) and grading of the tumor. The expression of CD44 in the gastric adenocarcinoma mucosa and the adjacent mucosa were determined by immunohistochemistry. The survival analysis was obtained using the Kaplan-Meier test.

RESULTS: Of 100 patients, 74 (74%) patients were male. The tumors were categorized as intestinal type (78%) or diffuse type (22%). Sixty-five percent of patients were CD44-positive. CD44 expression was not detected in normal gastric mucosa. Rather, CD44 was more commonly expressed in the intestinal subtype (P = 0.002). A significant relation was seen between the grade of tumor and the expression of CD44 (P = 0.014). The survival analysis showed a poor prognosis of patients with CD44-positive tumors (P = 0.008); and this was more prominent in the intestinal (P = 0.001) rather than diffuse type.

CONCLUSION: Cell adhesion molecule CD44 is highly expressed in gastric adenocarcinoma. CD44 expression is correlated with a poor prognosis in patients with the intestinal type of gastric adenocarcinoma. CD44 can, therefore, be utilized as a prognostic marker for this group of patients.

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Key words: Gastric cancer; Immunohistochemistry; Cluster of differentiation 44; Cell adhesion molecules; Survival rate

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INTRODUCTION

Gastric malignancy is one of the leading types of cancer

and is the second most common cause of cancer-related death in the world[1,2]. According to reports from Iran's Ministry of Health, gastric cancer is the most common fatal gastrointestinal malignancy, while cancer is the third most common cause of mortality in this country[3,4]. Adenocarcinoma is the most prevalent type of gastric cancer. According to Lauren's histological classification, it is subdivided into diffuse and intestinal pathologic subtypes, each having different epidemiological and prognostic features[5,6]. Multiple genetic and epigenetic alterations in oncogenes, tumor-suppressor genes, cell-cycle regulators, cell adhesion molecules and DNA repair genes are implicated in the multistep process of human stomach carcinogenesis[6,7], and different genetic pathways have been proposed for these two subtypes of gastric carcinoma[2]. Depth of invasion and lymph node metastasis result from the polygene, and their protein expression in gastric carcinoma. The key step of the basic and clinical research of gastric carcinoma is to discover the related etiological biomarkers.

The loss of normal cellular adhesion is a significant event in human cancer development. Metastasis is characterized by a loss of adhesion that allows cancer cells to invade, and leave the site of origin, subsequently adhering to other sites, such as lymph nodes, liver, or peritoneum[8]. Cluster of differentiation 44 (CD44) is a transmembrane glycoprotein involved in cellular adhesion. This polymorphic integral membrane glycoprotein, which is expressed by many cell types, serves as the principal transmembrane hyaluronate receptor. It is considered a determinant of metastatic and invasive behavior in different malignancies, such as lung carcinoma, malignant melanoma, leukemia, breast cancer, as well as gastrointestinal carcinomas[2,9,10]. On the other hand, contradictory reports concerning the biological role of CD44 in tumorigenesis and its clinical value in prognosis have also been presented[9]. The expression of CD44 potentiates tumor cells to adhere to the extracellular matrix through ligands such as hyaluronan and facilitates the efficient formation of cell colonies[11,12].

The reported frequency of CD44 expression in human gastric carcinoma specimens varies widely from 31% to 72%, most likely reflecting the differences in the study population[13,14]. The intensity of CD44 expression has been reported to correlate with an increased depth of invasion[13], although different correlations have been reported in the two histological types of this cancer.

In this study, the expression of CD44 in patients with gastric carcinoma was measured by immunohistochemical method, with an aim to evaluate the relation of CD44 expression with clinicopathological features and also its effect on prognosis with an emphasis on the differences between intestinal and diffuse types.

MATERIALS AND METHODS

From 2000 through 2006, 100 gastric adenocarcinoma patients, who underwent total or sub-total gastrectomy without any prior treatment such as chemotherapy or radiation therapy at Omid Oncology Hospital (Mashhad, Iran), were enrolled in this study. All patients had been residing in the north-eastern provinces of Iran at the time of surgery. Demographic data of all patients were recorded and TNM staging was performed according to AJCC (American Joint Committee on Cancer) staging by supra- and infra-diaphragmatic imaging studies. Follow-up data were also gathered from patients, including local and distant recurrence and metastasis. The study protocol was approved by the Clinical Research Ethics Committee of the Mashhad University of Medical Sciences.

Formalin-fixed and paraffin wax-embedded gastric adenocarcinoma specimens from these patients were selected from the pathology archive. Specimen blocks were stained with HE and histological typing was determined according to the Lauren's classification and tumor grade (well-differentiated, moderately-differentiated and poorly-differentiated). The pathologist reviewed all the blocks and chose one block with more tumoral tissue and less necrotic tissue for immunostaining. Specimens were cut into 4-μm thick sections, and the sections were dewaxed and processed for immunohistochemical (IHC) staining. The sections were stained using the streptavidin-biotin-peroxidase complex method (Dako LSAB2 system, Denmark). Mouse anti-CD44 monoclonal antibody (1:50 dilution; clone DF1485, Dakocytomation, Denmark) which is able to detect all isoforms of CD44, was employed as the primary antibody for 30 min. Internal lymphocytes were used as positive control, and normal gastric tissue as negative control. Also, for negative control, the primary antibody was omitted.

All sections were evaluated by the pathologist who was unaware of the clinical outcome of the patients. Tumors with more than 5% of CD44-positive cancer cells were regarded as positive. The results were reported as positive (cytoplasmic and/or membranous staining) or negative with the percentage of positive cells for each section.

Statistical analysis

The correlation between the CD44 expression status and clinicopathological variables was analyzed using parametric and non-parametric tests run by the statistical software SPSS version 13. Also a log-linear model by the statistical software SAS was used for more detailed analysis of the data. The survival analysis was performed by the Kaplan-Meier test. A P value less than 0.05 was considered statistically significant.

RESULTS

This study included 100 gastric adenocarcinoma cases (74 males and 26 females; male/female ratio: 2.8), with a mean age of 63.3 years (range 26-82 years). Data about stage, grade, histological types and location of tumors are summarized in Table 1. Forty-seven percent of patients were less than 65 years old. The mean age of patients with poorly differentiated tumors was

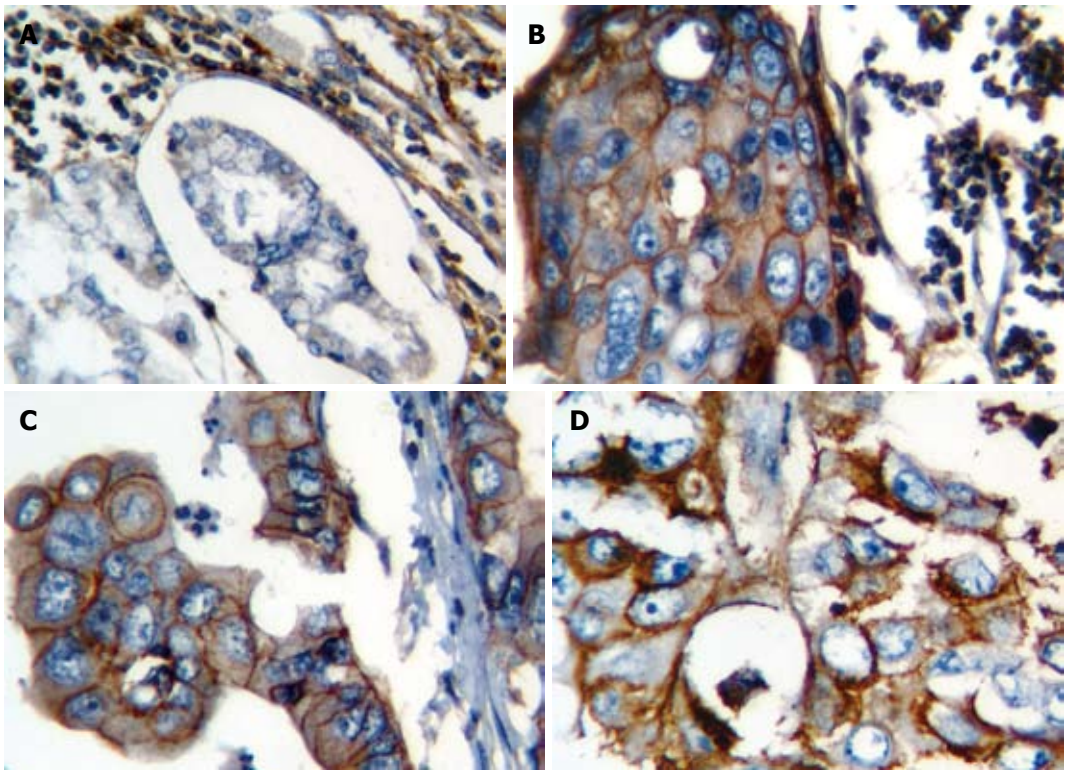

Figure 1 Representative example demonstrating the immunohistochemical staining of CD44 molecule in gastric adenocarcinoma tissue. A: Negative CD44 staining in adenocarcinoma glands and positive staining in the background lymphocytes (× 40); B: Membranous expression of CD44 in carcinoma cells with the stained lymphocytes as internal positive control in the background (× 40); C: Positive membranous staining of CD44 in carcinoma cells (x 40); D: Membranous and cytoplasmic expression of CD44 in carcinoma cells (× 40).

Table 1 Clinicopathological data of all patients

Data	Percentage (%)
Sex	
M	74
F	26
Age (yr)	
< 65	47
> 65	53
Location	
Cardia	37
Body & Fundus	33
Antrum	30
Histology type	
Intestinal	78
Diffuse	22
Grade	
Well-Diff	54
Mod-Diff	17
Poor-Diff	29
T	
T1	0
T2	5
T3	84
T4	11
N	
N0	16
N1	51
N2	30
N3	2
M	
M0	98
M1	2
Stage	
1	3
2	13
3	71
4	13

Diff: Differentiated; Mod: Moderately; Poor: Poorly; M: Male; F: Female.

significantly lower than other patients (55 years *vs* 65 years, $P = 0.019$). Stage Ⅳ disease was significantly more common among patients younger than 65 years ($P = 0.02$). All stage I cases were observed among patients older than 65 years. The diffuse-type cancer was two times higher among the patients younger than 65 years ($P = 0.021$).

Sixty-five percent of patients were CD44-positive and 35% were CD44-negative. CD44 staining was not detected in any adjacent normal gastric mucosa (Figure 1). Of 65 CD44-positive cases, 52 showed CD44 staining only on tumor cell surface membrane, while 13 showed both in cytoplasm and membrane.

In addition, a significant difference in CD44 expression was seen between the two histological subtypes: intestinal-type showed a significantly higher CD44 positivity (71%) compared to diffuse-type (42%) ($P = 0.002$). Moreover, poorly differentiated carcinomas showed a significantly less CD44 positivity, indicating a significant relation between CD44 expression, and the grade of tumor ($P = 0.014$; log linear, $P = 0.004$). Patients with stage 4 cancer expressed CD44 more than other stages, although this was not statistically significant. Other studied variables (sex, age and location of tumor) did not show any significant correlation with the expression of CD44.

Follow-up data were gathered for 71 patients. The median follow-up time was 6 mo. The overall survival was 16 mo. Regarding the results of survival analysis, there was no correlation between sex, age, location, type and the grade of tumor with prognosis. Poor outcome was seen among the patients with higher stage tumors (stages Ⅲ and Ⅳ, $P = 0.034$) compared to those with lower stages. In addition, the patients with CD44-positive tumors showed a poor prognosis (Log Rank, $P = 0.008$,

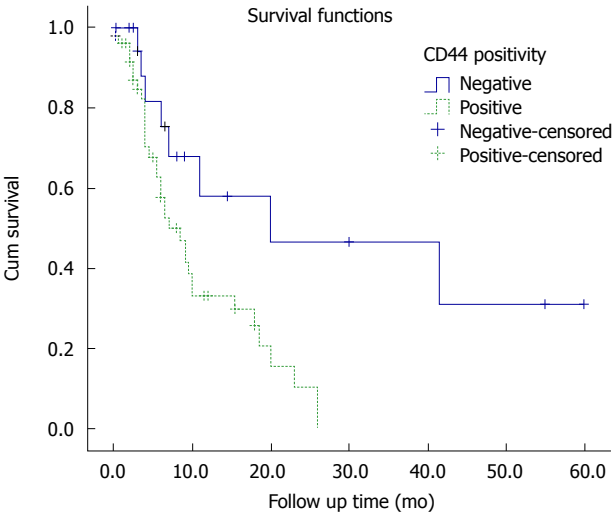

Figure 2 Kaplan-Meier curves of overall survival for CD44-positive and -negative gastric cancer cases.

Figure 2). Among patients with lower stage tumors, CD44 expression affected the prognosis regardless of tumor stage ($P = 0.040$). When analyzing the correlation of CD44 positivity, and intestinal/diffuse-type tumors separately, the results showed that CD44 only affected survival time in intestinal-type ($P = 0.001$), but not the diffuse-type tumors ($P = 0.7$).

DISCUSSION

The present study examined the expression of CD44 as an adhesion molecule in primary tumors by utilizing the IHC technique in 100 patients with gastric adenocarcinoma who had undergone surgery as the first step of management. The results showed that the expression rate of CD44 in tumoral tissue reached 65% in contrast to no expression of this molecule in the adjacent normal mucosa. CD44 was more commonly expressed in the intestinal-type tumor than in diffuse-type tumor, indicating that CD44 expression was related to the histological subtype of tumor, which is in agreement with previous studies[15,16]. In addition, a correlation was found between CD44 expression and tumor grade. But, no correlations between CD44 and other clinicopathological parameters were observed. Survival analysis showed that the grade and the histological type of gastric adenocarcinoma did not significantly affect survival. On the other hand, patients with higher stage adenocarcinoma showed a poor prognosis. CD44 was shown to affect survival, even more significantly than, and independently from the stage of the tumor. Moreover, CD44 positivity appeared to provide a poor prognosis only in patients with the intestinal-type gastric adenocarcinoma.

Since 1993 when Hieder *et al*[17] reported the expression of CD44 variants in gastric cancer, this adhesive molecule was recognized as a molecular marker related to the clinicopathological aspects of gastric cancer. CD44 is a member of a widely expressed family of adhesion receptors. This molecule was first described as a lymphocyte-homing receptor; however, it is expressed on a wide variety of cell types including mature T and B-cells[9,18-20]. Metastasizing tumor cells and recirculating (activated) lymphocytes share several properties, including motility and invasive behavior, an analogy which prompted the hypothesis that malignant cells might use molecules like CD44 for metastasizing[9]. *CD44* gene is located on chromosome 11p12-13, having at least 20 exons, of which ten are expressed in hematopoietic cells or the standard form of the gene. Other exons can be alternatively spliced to make up a wide variety of CD44 splice variants which have been found in various types of human malignancies, and have been considered markers in tumor progression and metastasis[15].

It has been supposed that the evaluation of the CD44 isoforms expression by the IHC method in cases of non-Hodgkin lymphoma, colon and renal cell carcinomas, as well as neuroblastomas may be a useful diagnostic parameter indicating invasive processes[21]. In 1994, Yokozaki *et al*[22] indicated that the detection of CD44 transcription variants can serve as a powerful tool for the diagnosis of gastric cancer. Many studies have indicated that the generation of CD44 splice variants, like V5 and V6, might be linked closely to gastric carcinoma tumorigenesis and differentiation, suggesting that these isoforms can be used as an indicator of tumor progression in the biopsies of patients with gastric carcinoma[23-28]. Moreover, it has been shown that patients with an over-expression of CD44 have a higher lymph node metastatic rate and invasion[27].

It is possible that different results among studies reflect the use of diverse antibodies having subtle variations in specificity. The problem is complicated more by the existence of numerous CD44 isoforms, which may have remarkable homology in their antigenic repertoire, thus increasing the possibility of cross-reactivity between the antibodies. Another reason for such discrepancies is probably the comparison of results having different techniques[9].

The correlation between clinicopathological parameters and CD44 expression in the tumor is known to be different between intestinal and diffuse types of gastric carcinoma[29,30]. A study showed that the intestinal-type was more frequently CD44s- and CD44v6-positive than the diffuse-type tumor[15], although the reactivity to these two antibodies did not correlate with histopathological and clinical prognostic factors in intestinal-type carcinoma[31]. More recently, Yamaguchi *et al*[16] found that the expression of CD44v6 protein was significantly higher in differentiated adenocarcinoma than in diffuse-type carcinoma. On the other hand, Saito *et al*[29] observed that CD44v6 appeared to play an important role in the invasion and metastasis of diffuse-type gastric carcinoma, but not in that of intestinal-type gastric carcinoma, and also demonstrated a significant correlation between CD44v6 expression and poor prognosis of diffuse-type gastric carcinoma.

The results of this study are consistent with previous findings, demonstrating that CD44 is mostly expressed

in the intestinal-type gastric cancer, and its expression is associated with poor prognosis. Thus, it can be concluded that the expression of CD44 is related to the phenotype of gastric malignancy, and may serve as a useful indicator of tumor metastasis, and may have a potential significance in diagnosing gastric cancer.

The mortality associated with gastric carcinoma is almost entirely caused by a subsequent metastatic disease. In fact, the prognostic assessment of gastric carcinoma still relies mainly on TNM staging, but the wide individual variability in prognosis is observed even in the same stages. The accurate prediction of the metastatic potential of the primary tumor, and hence the probable existence of undetected metastases, would be a critical factor in the management of patients with gastric carcinoma[14]. Our results emphasize that expression of CD44 is related to the prognosis of intestinal-type gastric cancer.

In conclusion, this study demonstrated that detection of CD44 protein in routinely fixed gastric carcinoma tissue by the IHC method can be used, along with other established parameters, to assess prognostic outcome, and particularly, to identify patients with a poor short-term prognosis. Furthermore, this suggests that, in the future, assessment of CD44 expression may guide the clinician in delineating a subset of patients with biologically unfavorable tumors who may profit from more intense post-operative adjuvant therapy.

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COMMENTS

Background

Gastric cancer is the 2nd most common cause of cancer-related death in the world. The mortality associated with gastric carcinoma is almost entirely caused by a subsequent metastatic disease. The accurate prediction of the metastatic potential of the primary tumor would be a critical factor in the management of patients with gastric carcinoma. Metastasis is characterized by a loss of adhesion that allows cancer cells to invade and leave the site of origin, subsequently adhering to other sites such as lymph nodes, liver, or peritoneum. Cluster of differentiation 44 (CD44), as an important glycoprotein involved in cellular adhesion, is considered a determinant of metastatic and invasive potential in different malignancies. There are different and even contradictory reports considering the role of this adhesive molecule in gastric carcinogenesis and metastasis.

Research frontiers

While there was no expression in surrounding normal tissue, CD44 expression rate in gastric adenocarcinoma was up to 65%, considering that the intestinal-type tumor expressed this marker, more common than diffuse-type tumor. Beside the prognostic effect of stage, CD44 was shown to affect survival, even more significantly than and independently from the stage of the tumor and this was more pronounced in intestinal-type.

Innovations and breakthroughs

For the first time in Iranian patients, this study demonstrated that detection of the CD44 protein in routinely fixed gastric carcinoma tissue by the IHC method can be used, along with other established parameters, to assess prognostic outcome, and particularly, to identify patients with a poor short-term prognosis.

Applications

In the future, assessment of CD44 expression may guide the clinician in delineating a subset of patients with biologically unfavorable tumors who may benefit from more intense postoperative adjuvant therapy.

Peer review

The results showed that the over-expression of cell adhesion molecule CD44 is correlated with a poor prognosis in patients with the intestinal-type gastric adenocarcinoma. CD44 can, therefore, be utilized as a prognostic marker for this group of patients.

REFERENCES

1 **Henson DE**, Dittus C, Younes M, Nguyen H, Albores-Saavedra J. Differential trends in the intestinal and diffuse types of gastric carcinoma in the United States, 1973-2000: increase in the signet ring cell type. *Arch Pathol Lab Med* 2004; **128**: 765-770

2 **Abbaszadegan MR**, Moaven O, Sima HR, Ghafarzadegan K, A'rabi A, Forghani MN, Raziee HR, Mashhadinejad A, Jafarzadeh M, Esmaili-Shandiz E, Dadkhah E. p16 promoter hypermethylation: a useful serum marker for early detection of gastric cancer. *World J Gastroenterol* 2008; **14**: 2055-2060

3 **Taghavi N**, Nasrollahzadeh D, Merat S, Yazdanbod A, Hormazdi M, Sotoudeh M, Semnani S, Eslami F, Marjani HA, Fahimi S, Khademi H, Malekzadeh R. Epidemiology of upper gastrointestinal cancers in Iran: a sub site analysis of 761 cases. *World J Gastroenterol* 2007; **13**: 5367-5370

4 **Yaghoobi M**, Rakhshani N, Sadr F, Bijarchi R, Joshaghani Y, Mohammadkhani A, Attari A, Akbari MR, Hormazdi M, Malekzadeh R. Hereditary risk factors for the development of gastric cancer in younger patients. *BMC Gastroenterol* 2004; **4**: 28

5 **Panani AD**. Cytogenetic and molecular aspects of gastric cancer: clinical implications. *Cancer Lett* 2008; **266**: 99-115

6 **Kountouras J**, Zavos C, Chatzopoulos D, Katsinelos P. New aspects of Helicobacter pylori infection involvement in gastric oncogenesis. *J Surg Res* 2008; **146**: 149-158

7 **Tamura G**. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. *World J Gastroenterol* 2006; **12**: 192-198

8 **Jothy S**. CD44 and its partners in metastasis. *Clin Exp Metastasis* 2003; **20**: 195-201

9 **Zavrides HN**, Zizi-Sermpetzoglou A, Panousopoulos D, Athanasas G, Elemenoglou I, Peros G. Prognostic evaluation of CD44 expression in correlation with bcl-2 and p53 in colorectal cancer. *Folia Histochem Cytobiol* 2005; **43**: 31-36

10 **Wang DR**, Chen GY, Liu XL, Miao Y, Xia JG, Zhu LH, Tang D. CD44v6 in peripheral blood and bone marrow of patients with gastric cancer as micro-metastasis. *World J Gastroenterol* 2006; **12**: 36-42

11 **Sneath RJ**, Mangham DC. The normal structure and function of CD44 and its role in neoplasia. *Mol Pathol* 1998; **51**: 191-200

12 **Kajita M**, Itoh Y, Chiba T, Mori H, Okada A, Kinoh H, Seiki M. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J Cell Biol* 2001; **153**: 893-904

13 **Setala L**, Lipponen P, Tammi R, Tammi M, Eskelinen M, Alhava E, Kosma VM. Expression of CD44 and its variant isoform v3 has no prognostic value in gastric cancer. *Histopathology* 2001; **38**: 13-20

14 **Yoo CH**, Noh SH, Kim H, Lee HY, Min JS. Prognostic significance of CD44 and nm23 expression in patients with stage II and stage IIIA gastric carcinoma. *J Surg Oncol* 1999; **71**: 22-28

15 **Dammrich J**, Vollmers HP, Heider KH, Muller-Hermelink HK. Importance of different CD44v6 expression in human gastric intestinal and diffuse type cancers for metastatic lymphogenic spreading. *J Mol Med* 1995; **73**: 395-401

16 **Yamaguchi A**, Goi T, Yu J, Hirono Y, Ishida M, Iida A, Kimura T, Takeuchi K, Katayama K, Hirose K. Expression

of CD44v6 in advanced gastric cancer and its relationship to hematogenous metastasis and long-term prognosis. *J Surg Oncol* 2002; **79**: 230-235
17 **Heider KH**, Dammrich J, Skroch-Angel P, Muller-Hermelink HK, Vollmers HP, Herrlich P, Ponta H. Differential expression of CD44 splice variants in intestinal- and diffuse-type human gastric carcinomas and normal gastric mucosa. *Cancer Res* 1993; **53**: 4197-4203
18 **Pure E**, Cuff CA. A crucial role for CD44 in inflammation. *Trends Mol Med* 2001; **7**: 213-221
19 **Yoo CH**, Noh SH. The Serum Assay of Soluble CD44 Standard, CD44 Variant 5, and CD44 Variant 6 in Patients with Gastric Cancer. *Cancer Res Treat* 2003; **35**: 3-8
20 **Marhaba R**, Zoller M. CD44 in cancer progression: adhesion, migration and growth regulation. *J Mol Histol* 2004; **35**: 211-231
21 **Gunthert U**, Stauder R, Mayer B, Terpe HJ, Finke L, Friedrichs K. Are CD44 variant isoforms involved in human tumour progression? *Cancer Surv* 1995; **24**: 19-42
22 **Yokozaki H**, Ito R, Nakayama H, Kuniyasu H, Taniyama K, Tahara E. Expression of CD44 abnormal transcripts in human gastric carcinomas. *Cancer Lett* 1994; **83**: 229-234
23 **Stock M**, Otto F. Gene deregulation in gastric cancer. *Gene* 2005; **360**: 1-19
24 **Chen JQ**, Zhan WH, He YL, Peng JS, Wang JP, Cai SR, Ma JP. Expression of heparanase gene, CD44v6, MMP-7 and nm23 protein and their relationship with the invasion and metastasis of gastric carcinomas. *World J Gastroenterol* 2004; **10**: 776-782
25 **Hsieh HF**, Yu JC, Ho LI, Chiu SC, Harn HJ. Molecular studies into the role of CD44 variants in metastasis in gastric cancer. *Mol Pathol* 1999; **52**: 25-28
26 **Joo M**, Lee HK, Kang YK. Expression of E-cadherin, beta-catenin, CD44s and CD44v6 in gastric adenocarcinoma: relationship with lymph node metastasis. *Anticancer Res* 2003; **23**: 1581-1588
27 **Liu YJ**, Yan PS, Li J, Jia JF. Expression and significance of CD44s, CD44v6, and nm23 mRNA in human cancer. *World J Gastroenterol* 2005; **11**: 6601-6606
28 **Chen GY**, Wang DR. The expression and clinical significance of CD44v in human gastric cancers. *World J Gastroenterol* 2000; **6**: 125-127
29 **Saito H**, Tsujitani S, Katano K, Ikeguchi M, Maeta M, Kaibara N. Serum concentration of CD44 variant 6 and its relation to prognosis in patients with gastric carcinoma. *Cancer* 1998; **83**: 1094-1101
30 **Castella EM**, Ariza A, Pellicer I, Fernandez-Vasalo A, Ojanguren I. Differential expression of CD44v6 in metastases of intestinal and diffuse types of gastric carcinoma. *J Clin Pathol* 1998; **51**: 134-137
31 **Hong RL**, Lee WJ, Shun CT, Chu JS, Chen YC. Expression of CD44 and its clinical implication in diffuse-type and intestinal-type gastric adenocarcinomas. *Oncology* 1995; **52**: 334-339

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RAPID COMMUNICATION

Continuous regional arterial infusion therapy with gabexate mesilate for severe acute pancreatitis

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Abstract

AIM: To evaluate the efficacy of continuous regional arterial infusion therapy (CRAI) with gabexate mesilate and antibiotics for severe acute pancreatitis (SAP).

METHODS: We conducted a prospective study on patients who developed SAP with or without CRAI. Out of 18 patients fulfilled clinical diagnostic criteria for SAP in Japan, 9 patients underwent CRAI, while 9 patients underwent conventional systemic protease inhibitor and antibiotics therapy (non-CRAI). CRAI was initiated within 72 h of the onset of pancreatitis. Gabexate mesilate (2400 mg/d) was continuously administered for 3 to 5 d. The clinical outcome including serum inflammation-related parameters were examined.

RESULTS: The duration of abdominal pain in the CRAI group was 1.9 ± 0.26 d, whereas that in the non-CRAI group was 4.3 ± 0.50. The duration of SIRS in the CRAI group was 2.2 ± 0.22 d, whereas that in the non-CRAI group was 3.2 ± 0.28. Abdominal pain and SIRS disappeared significantly in a short period of time after the initiation of CRAI using gabexate mesilate. The average length of hospitalization significantly differed between the CRAI and non-CRAI groups, 53.3 ± 7.9 d and 87.4 ± 13.9 d, respectively. During the first two weeks, levels of serum CRP and the IL6/IL10 ratio in the CRAI group tended to have a rapid decrease compared to those in the non-CRAI group.

CONCLUSION: The present results suggest that CRAI using gabexate mesilate was effective against SAP.

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Key words: Severe acute pancreatitis; Arterial infusion; Gabexate mesilate; Antibiotics

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INTRODUCTION

Severe acute pancreatitis (SAP) remains a lethal disease. It is defined as an inflammatory process of the pancreas with possible peripancreatic tissue, and multi-organ involvement inducing multi-organ dysfunction syndrome (MODS) with an increased mortality rate[1,2]. Continuous regional arterial infusion (CRAI) with protease inhibitor nafamostat mesilate and antibiotics has been proven to be effective as an initial therapy in Japan[2]. However, evidence supporting the benefit of CRAI in treating acute pancreatitis is insufficient, and its advisability according to the JPN guidelines for the management

of acute pancreatitis is classed as "Recommendation C"[3]. In the statement, it was described that CRAI with protease inhibitors and antibiotics may possibly reduce the mortality rate and incidence of infectious complications in necrotizing pancreatitis. Actually, until now, most cases have been treated with the protease inhibitor nafamostat mesilate. Here, we performed CRAI using gabexate mesilate to treat SAP, and investigated the clinical benefits including serum inflammation-related parameters such as cytokines and chemokines.

MATERIALS AND METHODS

Patients

The severity of acute pancreatitis was assessed within 48 h of admission according to the diagnostic criteria for the diagnosis of acute pancreatitis by the Research Committee for Intractable Diseases of the Pancreas in Japan by Ministry of Health, Labour and Welfare Japan (Tables 1 and 2)[4-6]. A total of 18 patients fulfilling clinical diagnostic criteria for SAP at six participating institutions were selected for the present study. Nine patients underwent CRAI (CRAI group), while 9 patients underwent conventional systemic protease inhibitor and antibiotics therapy (non-CRAI group). Each institution made the decision to perform CRAI or non-CRAI therapy, so the present study was not a randomized controlled trial. Clinical features of both groups were shown in Table 3. All 9 patients in the CRAI group were men, average age 48.0 ± 13.4 years (mean ± SD). The cause of SAP was alcohol (n = 5), gallstone (n = 1), hyperlipidemia (n = 1), post-endoscopic retrograde cholangiopancreatography (ERCP, n = 1), or unknown (n = 1). On the other hand, 4 of the 9 patients in the non-CRAI group were male and 5 were female (average age of group, 59.9 ± 15.1 years; mean ± SD). Regarding age at onset, no significant difference was observed between CRAI group and non-CRAI group (P = 0.0979). The causes of SAP patients in the non-CRAI group were gallstones (n = 4), alcohol (n = 3), post-ERCP (n = 1), or unknown (n = 1). All patients in both groups were diagnosed as stage 2 SAP. CRAI was initiated within 72 h of the onset of pancreatitis. A 5-Fr shepherd's catheter was placed in either the celiac artery (including the splenic and gastro-duodenal arteries) or in the supra-mesenteric artery, and gabexate mesilate (2400 mg/d) was continuously administered for 3-5 d. Antibiotics were administered every 12 h (panipenem in 5 patients, meropenem in 2 patients, imipenem in 1 patient, and piperacillin in 1 patient). Catheters were placed in the superior mesenteric, celiac, splenic, and gastroduodenal arteries of 3, 3, 2, and 1 patient, respectively. Complications in one patient comprised thrombosis of the superior mesenteric artery, and warfarin was administered. Carbapenem antibiotics were administered to all patients in the non-CRAI group.

Measured parameters

The duration of abdominal pain and of systemic inflammatory response syndrome (SIRS) as well as the length of hospitalization were recorded. As biochemical markers of pancreatitis, the levels of serum pancreatic amylase (P-amylase), the white blood counts (WBC), and C-reactive protein (CRP) were examined on day 0 (onset of pancreatitis), day 1, day 3, day 7, and day 14. ELISAs were performed to determine serum IL-6, IL-8, IL-10, TNF-α, and MCP-1 concentrations on day 0 (onset of pancreatitis), day 1, day 3, day 7, and 14. Samples were

Table 1 Criteria for grading the severity of acute pancreatitis in Japan[4]

Prognostic factors	Clinical signs	Laboratory data
Prognostic factor Ⅰ (2 points for each positive factor)	Shock Respiratory failure Mental disturbance Severe infection Hemorrhagic diathesis	BE ⩽ -3 mmol/L Ht ⩽ 30% (after hydration) BUN ⩾ 40 mg/dL or creatinine ⩾ 2.0 mg/dL
Prognostic factor Ⅱ (1 points for each positive factor)		PaO_2 ⩽ 60 mmHg (room air) FBS ⩾ 200 mg/dL Total protein ⩽ 60 g/L LDH ⩾ 700 IU/L Ca ⩽ 7.5 mg/dL Prothrombin time ⩾ 15 s Platelet count ⩽ $1 \times 10^5/mm^3$ CT grade Ⅳ or Ⅴ
Prognostic factor Ⅲ	SIRS score ⩾ 3 (2 points) Age ⩾ 70 yr (1 point)	

BE: Base excess; Ht: Hematocrit; BUN: Blood urea nitrogen; FBS: Fasting blood sugar; LDH: Lactate dehydrogenase; SIRS: Systemic inflammatory response syndrome. CT grade Ⅳ or Ⅴ: Presence of diffuse and uneven density in the pancreatic parenchyma or the presence of inflammatory changes extending beyond the border of the pancreas. Severity score: Sum of the points for the positive prognostic factors is defined as the severity score. Standardized criteria: Severe, presence of more than one prognostic factor Ⅰ, and/or the presence of more than two prognostic factor Ⅱ (severity score ⩾ 2 points); Moderate, presence of one prognostic factor Ⅱ (severity score = 1 point); Mild, acute pancreatitis without prognostic factor Ⅰ or Ⅱ (severity = 0 point).

Table 2 Stage classification of acute pancreatitis and mortality rate in 2003 in Japan[4]

Stage	Severity score	Severity	No. of patients (%)	Died	Mortality rate (%)
Stage 0	0 point	Mild	943 (53.3)	1	0.1
Stage 1	1 point	Moderate	280 (15.8)	2	0.7
Stage 2	2-8 points	Severe Ⅰ	455 (25.7)	17	3.7
Stage 3	9-14 points	Severe Ⅱ	63 (3.6)	16	25.4
Stage 4	⩾ 15 points	Most severe	27 (1.5)	16	59.3
Total			1786 (100)	52	2.9

In 2004, nationwide survey of patients with acute pancreatitis in Japan who visited the hospitals in the year 2003 (from January 1 to December 31) was performed by stratified random sampling method. From the first survey, the total number of patients treated for acute pancreatitis in Japan in the year 2003 was estimated as 35300 (95% confidence interval, 30500-40000). Clinical records of 1768 patients with acute pancreatitis were obtained in the second survey for analysis of etiology and outcome. Number of patients who died of acute pancreatitis or related complications.

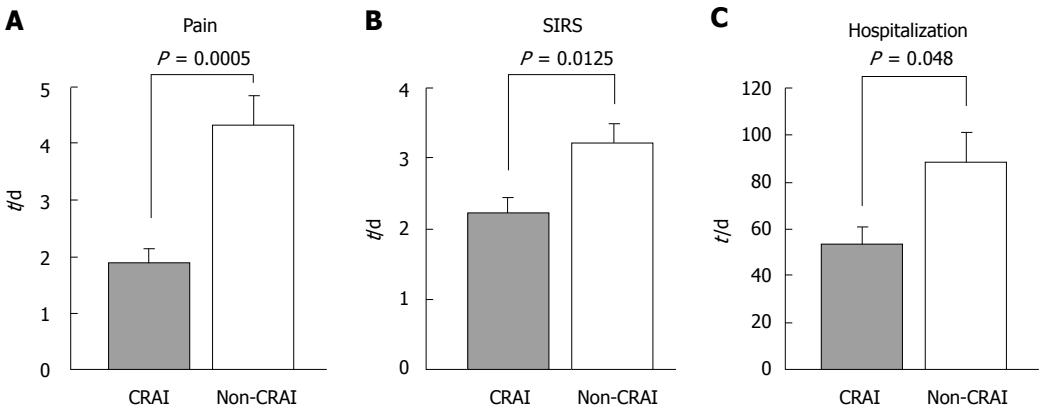

Figure 1 Changes in clinical parameters. The duration of abdominal pain (A) and of systemic inflammatory response syndrome (SIRS) (B) as well as the length of hospitalization (C) were investigated. Grey columns represent data for continuous regional arterial infusion using gabexate mesilate with antibiotics (CRAI-group) and white columns for non-CRAI group. Values are expressed as mean ± SE.

Table 3 The clinical features of patients

	CRAI group (n = 9)	Non-CRAI group (n = 9)
Mean age	48.0 ± 13.4	59.8 ± 15.1
Gender (male/female)	9/0	4/5
Cause of pancreatitis		
Alcoholic	5	3
Biliary	1	4
Hyperlipidemia	1	0
post-ERCP	1	1
Idiopathic	1	1
Severity score		
Mean score (range)	4.0 (2-7)	3.6 (2-7)

determined with commercially available kits according to the manufacturer's instructions for human IL-6, human IL-8, human IL-10, human TNF-α, and human MCP-1 (Biosource, Camarillo, CA, USA).

Statistical analysis

Results are expressed as mean ± SE. We analyzed the duration of abdominal pain, SIRS as well as the length of hospitalization using the proportional hazard model. Serum pancreatic enzymes, inflammation-related parameters, cytokines and chemokines were analyzed using the non-parametric Mann-Whitney U test. P values < 0.05 were considered significant. Pearson's correlation analysis was used to calculate correlations between the data.

RESULTS

Duration of abdominal pain, SIRS, and hospitalization

The duration of abdominal pain in the CRAI group was 1.9 ± 0.26 d (range, 1-3), whereas the duration in the non-CRAI group was 4.3 ± 0.50 (range, 3-8). Abdominal pain disappeared significantly in a short period of time after the initiation of CRAI with the protease inhibitor (P = 0.0005, Figure 1A). Similarly, SIRS disappeared significantly and shortly after the initiation of CRAI (P = 0.0125, Figure 1B). The duration of SIRS in the CRAI group was 2.2 ± 0.22 d (range, 1-3), whereas the duration in the non-CRAI group was 3.2 ± 0.28 (range, 2-4). The average length of hospitalization significantly differed between both groups, 53.3 ± 7.9 and 87.4 ± 13.9 d for the CRAI and non-CRAI, respectively. Patients in the CRAI group discharged significantly in a short period of time after the initiation of CRAI with gabexate mesilate (P = 0.048, Figure 1C).

Changes in serum inflammation-related parameters

P-amylase and WBC quickly decreased, with no significant differences between the groups (Figure 2A and B). During the first two weeks of therapy, levels of serum CRP in the CRAI group rapidly decreased (Figure 2C). IL-6 and IL-10 in the CRAI group rapidly decreased in the same manner as the IL-6/IL-10 ratio (Figure 2D). On the other hand, both CRP and IL-6/IL-10 in the non-CRAI group tended to decrease slowly with a 2-d delay in peak values compared to those in the CRAI group, with no significant differences between the groups. Levels of serum IL-8, TNF-α, and MCP-1 over time did not significantly differ between the two groups (data not shown).

DISCUSSION

Protease inhibitors are widely applied to treat acute pancreatitis in Japan; but since randomized controlled trials (RCTs) are difficult to conduct on patients with acute pancreatitis, only five RCTs have been examined gabexate mesilate[7-11]. The results of a meta-analysis of 4 among 5 trials were negative, and indicated that gabexate mesilate does not lower rates of surgical intervention or mortality. One of the reason was considered as follows; the protease inhibitors used to treat acute necrotizing pancreatitis cannot easily reach the pancreas when administered intravenously, and, because of ischemia or impaired microcirculation, they hardly penetrate into pancreatic tissue[12,13]. However, Chen *et al*[11] conducted an RCT and reported that continuous intravenous administration of high doses of gabexate mesilate (2400 mg/d) decreased the incidence of complications and mortality. On the other hand, since Takeda *et al*

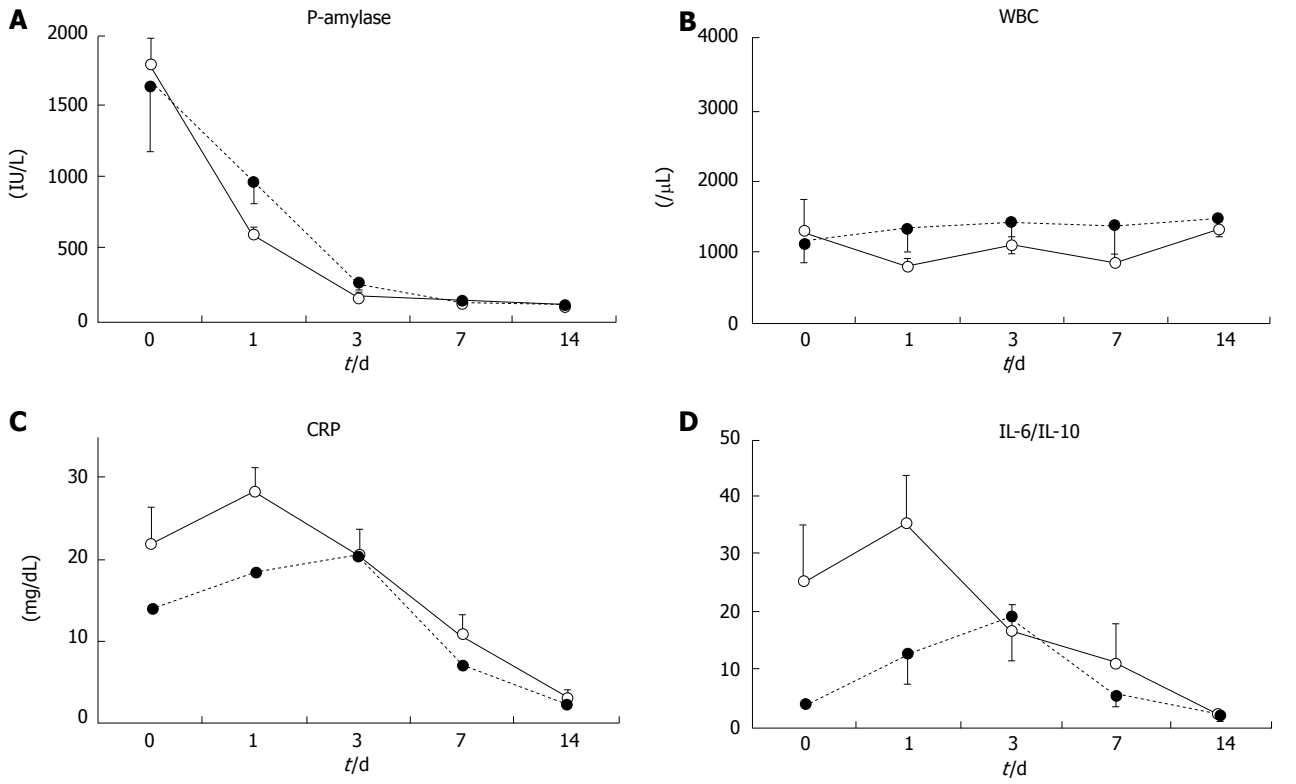

Figure 2 Changes in serum inflammation-related parameters. The levels of serum pancreatic amylase (P-amylase) (**A**), the white blood counts (WBC) (**B**), C-reactive protein (CRP) (**C**, and IL-6/IL-10 ratio **D**) were examined on days 0 (onset of pancreatitis), 1, 3, 7, and 14. Straight lines give data for continuous regional arterial infusion using gabexate mesilate with antibiotics (CRAI-group) and dotted lines for non-CRAI group. Values are expressed as mean ± SE. No significant differences between the groups were observed.

described arterial infusion with a protease inhibitor together with an antibiotic in Japan, severe acute pancreatitis has been treated by CRAI with nafamostat mesilate, and whereas an RCT has not been conducted, the usefulness of CRAI has been documented[14-17]. This strategy suppresses early inflammation and infection in pancreatic tissue, which controls subsequent systemic inflammation. The level of protease inhibitor in pancreatic tissues after CRAI using nafamostat was 5-fold higher than that delivered by intravenous injection, and trypsin activities in pancreatic tissues are significantly suppressed by CRAI[18]. On the other hand, the level of protease inhibitor in pancreatic tissues after CRAI using gabexate mesilate was 32-fold higher than that delivered by intravenous injection[19]. However, until now, CRAI using gabexate mesilate has not been examined sufficiently. In the present study, therefore, we investigated the usefulness of CRAI using gabexate mesilate for patients with SAP. The reasons for using gabexate mesilate were as follows: (1) Gabexate mesilate is the only intravenous protease inhibitor that has been proven effective in an RCT[11,20]. (2) Gabexate mesilate has a higher anticoagulant capacity than nafamostat mesilate[21]. (3) Gabexate mesilate induces less hyperkalemia even at high doses compared to nafamostat mesilate[22]. (4) In Japan, most studies on CRAI have used nafamostat mesilate, and more needs to be understood about gabexate mesilate.

All patients in this study had stage 2 pancreatitis, and since the severity was relatively mild, the patients were discharged in good health without requiring surgical intervention. The duration of pain, SIRS, and hospitalization was shorter for the CRAI group than the non-CRAI group. Previous studies of CRAI evaluated the mortality rates and surgical intervention in lethal SAP; but the present study suggested that CRAI is also effective against relatively milder forms of non-lethal SAP.

Blood cytokines and chemokines play important roles in the progression of severe acute pancreatitis. Local release of the proinflammatory cytokines, IL-18, TNF-α, and IL-1 upregulates IL-6. Levels of anti-inflammatory cytokines such as IL-10 also increase to maintain homeostasis. Excessive proinflammatory responses advance SIRS, and activated neutrophils and endothelial cells damage multiple organs. Ohmoto *et al*[23] reported that, during the healing process of acute pancreatitis, the IL-10/IL-6 ratio initially decreased, but increased as the pancreatitis improved. Put another way, IL-6/IL-10 ratio reveals an increase in a more severe stage of acute pancreatitis. We found here that IL-6 and IL-10 levels quickly increased and then decreased with therapy. The changes in the IL-6/IL-10 ratio were the same as those in CRP, but the ratio tended to decrease 2 d earlier in the CRAI group than in the non-CRAI group. These findings suggested that CRAI using gabexate mesilate effectively treats acute pancreatitis regarding biochemical features. On the other hand, changes in other

proinflammatory cytokines such as IL-8 and TNF-α were not significant. However, among patients with relatively mild stage 2 SAP in the present study, the release of these cytokines in tissues was insufficient to increase and reflect in their blood concentrations.

Essentially, a large-scale RCT should be necessary to verify the effects of CRAI; but to conduct such a study on patients with highly lethal SAP seems to be unethical in Japan. A future RCT might consider enrolling patients with stage 2 pancreatitis that is relatively mild and less fatal than in the present study. In conclusion, the present results suggest that CRAI using gabexate mesilate was effective against SAP in terms of yielding clinical benefits for patients with SAP.

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COMMENTS

Background

Severe acute pancreatitis (SAP) remains a lethal disease. Protease inhibitors are widely applied to treat acute pancreatitis in Japan; but the protease inhibitors used to treat acute necrotizing pancreatitis cannot easily reach the pancreas when administered intravenously, and, because of ischemia or impaired microcirculation, they hardly penetrate into pancreatic tissue. Recently, continuous regional arterial infusion (CRAI) with the protease inhibitor nafamostat mesilate and antibiotics has proven effective as an initial therapy. CRAI has been applied to treat SAP, but the evidence of its value is still scarce.

Research frontiers

The article focuses on the efficacy of CRAI using gabexate mesilate and antibodies for SAP.

Innovations and breakthroughs

The present study shows the efficacy of CRAI using gabexate mesilate for SAP, and the clinical benefits and sequential changes in serum inflammation-related parameters such as cytokines and chemokines. Abdominal pain and SIRS disappeared significantly in a short period of time after the initiation of CRAI with a protease inhibitor compared to non-CRAI. The average length of hospitalization significantly decreased with CRAI and patients discharged significantly in a shorter period of time after the initiation of CRAI with gabexate mesilate compared to non-CRAI.

Applications

CRAI using gabexate mesilate was shown to be effective against SAP in terms of clinical benefits for patients with SAP, and thus may provide a new strategy of treatment for SAP.

Peer review

Effect of continuous regional arterial infusion therapy with gabexate and antibiotics for SAP is very interesting clinical research. Known that SAP may have high mortality, some new modalities of therapy which improve prognosis of patients are welcome.

REFERENCES

1 **Bradley EL 3rd**. A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, Ga, September 11 through 13, 1992. *Arch Surg* 1993; **128**: 586-590
2 **Al Mofleh IA**. Severe acute pancreatitis: pathogenetic aspects and prognostic factors. *World J Gastroenterol* 2008; **14**: 675-684
3 **Takeda K**, Takada T, Kawarada Y, Hirata K, Mayumi T, Yoshida M, Sekimoto M, Hirota M, Kimura Y, Isaji S, Koizumi M, Otsuki M, Matsuno S. JPN Guidelines for the management of acute pancreatitis: medical management of acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2006; **13**: 42-47
4 **Otsuki M**, Hirota M, Arata S, Koizumi M, Kawa S, Kamisawa T, Takeda K, Mayumi T, Kitagawa M, Ito T, Inui K, Shimosegawa T, Tanaka S, Kataoka K, Saisho H, Okazaki K, Kuroda Y, Sawabu N, Takeyama Y. Consensus of primary care in acute pancreatitis in Japan. *World J Gastroenterol* 2006; **12**: 3314-3323
5 **Koizumi M**, Takada T, Kawarada Y, Hirata K, Mayumi T, Yoshida M, Sekimoto M, Hirota M, Kimura Y, Takeda K, Isaji S, Otsuki M, Matsuno S. JPN Guidelines for the management of acute pancreatitis: diagnostic criteria for acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2006; **13**: 25-32
6 **Ogawa M**, Hirota M, Hayakawa T, Matsuno S, Watanabe S, Atomi Y, Otsuki M, Kashima K, Koizumi M, Harada H, Yamamoto M, Nishimori I. Development and use of a new staging system for severe acute pancreatitis based on a nationwide survey in Japan. *Pancreas* 2002; **25**: 325-330
7 **Valderrama R**, Perez-Mateo M, Navarro S, Vazquez N, Sanjose L, Adrian MJ, Estruch J. Multicenter double-blind trial of gabexate mesylate (FOY) in unselected patients with acute pancreatitis. *Digestion* 1992; **51**: 65-70
8 **Yang CY**, Chang-Chien CS, Liaw YF. Controlled trial of protease inhibitor gabexelate mesilate (FOY) in the treatment of acute pancreatitis. *Pancreas* 1987; **2**: 698-700
9 **Buchler M**, Malfertheiner P, Uhl W, Scholmerich J, Stockmann F, Adler G, Gaus W, Rolle K, Beger HG. Gabexate mesilate in human acute pancreatitis. German Pancreatitis Study Group. *Gastroenterology* 1993; **104**: 1165-1170
10 **Messori A**, Rampazzo R, Scroccaro G, Olivato R, Bassi C, Falconi M, Pederzoli P, Martini N. Effectiveness of gabexate mesilate in acute pancreatitis. A metaanalysis. *Dig Dis Sci* 1995; **40**: 734-738
11 **Chen HM**, Chen JC, Hwang TL, Jan YY, Chen MF. Prospective and randomized study of gabexate mesilate for the treatment of severe acute pancreatitis with organ dysfunction. *Hepatogastroenterology* 2000; **47**: 1147-1150
12 **Inoue K**, Hirota M, Kimura Y, Kuwata K, Ohmuraya M, Ogawa M. Further evidence for endothelin as an important mediator of pancreatic and intestinal ischemia in severe acute pancreatitis. *Pancreas* 2003; **26**: 218-223
13 **Takeda K**, Mikami Y, Fukuyama S, Egawa S, Sunamura M, Ishibashi T, Sato A, Masamune A, Matsuno S. Pancreatic ischemia associated with vasospasm in the early phase of human acute necrotizing pancreatitis. *Pancreas* 2005; **30**: 40-49
14 **Takeda K**, Matsuno S, Sunamura M, Kakugawa Y. Continuous regional arterial infusion of protease inhibitor and antibiotics in acute necrotizing pancreatitis. *Am J Surg* 1996; **171**: 394-398
15 **Anai H**, Sakaguchi H, Uchida H, Matsuo N, Tanaka T, Yoshioka T, Ohishi H, Murao Y, Miyamoto S. Continuous arterial infusion therapy for severe acute pancreatitis: correlation between CT arteriography and therapeutic effect. *J Vasc Interv Radiol* 1999; **10**: 1335-1342
16 **Imaizumi H**, Kida M, Nishimaki H, Okuno J, Kataoka Y, Kida Y, Soma K, Saigenji K. Efficacy of continuous regional arterial infusion of a protease inhibitor and antibiotic for severe acute pancreatitis in patients admitted to an intensive care unit. *Pancreas* 2004; **28**: 369-373
17 **Takeda K**, Matsuno S, Ogawa M, Watanabe S, Atomi Y. Continuous regional arterial infusion (CRAI) therapy reduces the mortality rate of acute necrotizing pancreatitis: results of a cooperative survey in Japan. *J Hepatobiliary Pancreat Surg* 2001; **8**: 216-220
18 **Kakugawa Y**, Takeda K, Sunamura M, Kawaguchi S, Kobari M, Matsuno S. [Effect of continuous arterial infusion of protease inhibitor on experimental acute pancreatitis

induced by closed duodenal loop obstruction] *Nippon Shokakibyo Gakkai Zasshi* 1990; **87**: 1444-1450
19 **Satoh H**, Harada M, Tashiro S, Shiroya T, Imawaka H, Machii K. The effect of continuous arterial infusion of gabexate mesilate (FOY-007) on experimental acute pancreatitis. *J Med Invest* 2004; **51**: 186-193
20 **Pederzoli P**, Cavallini G, Falconi M, Bassi C. Gabexate mesilate vs aprotinin in human acute pancreatitis (GA. ME.P.A.). A prospective, randomized, double-blind multicenter study. *Int J Pancreatol* 1993; **14**: 117-124
21 **Takahashi Y**, Shibata A. The comparative study of nafamostat mesilate (FUT-175), gabexate mesilate (FOY) and heparin on anticoagulant and antifibrinolytic action. *Jpn J Clin Exp Med* 1988; **65**: 127-134
22 **Muto S**, Imai M, Asano Y. Effect of nafamostat mesilate on Na+ and K+ transport properties in the rabbit cortical collecting duct. *Br J Pharmacol* 1993; **109**: 673-678
23 **Ohmoto K**, Yamamoto S. Serum interleukin-6 and interleukin-10 in patients with acute pancreatitis: clinical implications. *Hepatogastroenterology* 2005; **52**: 990-994

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RAPID COMMUNICATION

Chronic gastrointestinal symptoms and quality of life in the Korean population

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Abstract

AIM: To evaluate the prevalence of chronic gastrointestinal symptoms and their impact on health-related quality of life (HRQOL) in the Korean population.

METHODS: A cross-sectional survey, using a reliable and valid Rome Ⅱ based questionnaire, was performed on randomly selected residents, between 18 and 69 years in age. All respondents were interviewed at their homes or offices by a team of interviewers. The impact of chronic gastrointestinal symptoms on HRQOL was assessed using the Korean version of the 36-item Short-Form general health survey (SF-36).

RESULTS: Of the 1807 eligible subjects, 1417 (78.4%: male 762; female 655) were surveyed. Out of the respondents, 18.6% exhibited at least one chronic gastrointestinal symptom. The prevalence of gastroesophageal reflux disease (GERD), defined as heartburn and/or acid regurgitation experienced at least weekly, was 3.5% (95% CI, 2.6-4.5). The prevalence of uninvestigated dyspepsia, irritable bowel syndrome (IBS) and chronic constipation based on Rome Ⅱ criteria were 11.7% (95% CI, 10.1-13.5), 2.2% (95% CI, 1.5-3.1), and 2.6% (95% CI, 1.8-3.5) respectively. Compared with subjects without chronic gastrointestinal symptoms (n = 1153), those with GERD (n = 50), uninvestigated dyspepsia (n = 166) and IBS (n = 31) had significantly worse scores on most domains of the SF-36 scales.

CONCLUSION: The prevalence of GERD, uninvestigated dyspepsia and IBS were 3.5%, 11.7% and 2.2% respectively, in the Korean population. The health-related quality of life was significantly impaired in subjects with GERD, uninvestigated dyspepsia and IBS in this community.

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Key words: Chronic gastrointestinal symptom; Gastroesophageal reflux disease; Dyspepsia; Irritable bowel syndrome; Qaulity of life

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INTRODUCTION

Functional gastrointestinal disorders (FGIDs) are highly prevalent in different geographic populations and cause a variety of gastrointestinal symptoms that greatly inconvenience the affected individuals[1,2]. According to a household survey in the United States, 69% of those questioned reported as having at least one of twenty functional gastrointestinal symptoms in the preceding three months[1]. In other population-based surveys, the prevalence of non-ulcer dyspepsia was approximately 30% and irritable bowel syndrome was 15%[2,3]. The prevalence of functional gastrointestinal disorders has been reported to differ based on the geographical region and race. Although the prevalence of such disorders in

Koreans is thought to be different from that in the Westerners, there has been no randomly sampled population-based study on the prevalence of chronic gastrointestinal symptoms in the Korean population.

The health-related quality of life (HRQOL) is a patient-focused concept, referring to an impairment of functional status (physical or mental) and the sense of well-being. As such, it is an important measure of the impact of chronic diseases. Several studies have shown that the HRQOL is significantly reduced in patients with chronic gastrointestinal symptoms in referral settings, compared with the general population, as well as in patients with other chronic conditions, such as GERD and asthma. However, there is a lack of evidence to support a decrease in the HRQOL in the general population having chronic GI symptoms[4,5].

The aims of the present study were to evaluate the prevalence of chronic gastrointestinal symptoms according to the Rome Ⅱ criteria, and to determine whether the HRQOL is impaired in subjects having chronic gastrointestinal symptoms in the Korean community.

MATERIALS AND METHODS

Subjects

The study was carried out in Asan-si, Chungchungnam-do Province from January 3, 2000 to February 28, 2001. Asan-Si is a small city located in the middle of South Korea. The population comprises approximately 180 000 people, and is a combination of urban and farming community. Sociodemographic factors including age, sex, educational background and economic level of Asan-si are similar to the overall Korean population, based on the information obtained from the Korean National Statistical Office (http://www.nso.go.kr). We assumed a symptom prevalence of 40%; thus at least 1024 interviews were required to achieve the required precision of ± 3% with 95% confidence. Therefore, the target sample size was 1400. A cross-sectional survey was performed on randomly selected Asan-si residents, between 18 and 69 years of age. A lengthy consultation was made with the regional health office (RHO) to prepare the survey. For instance, the general regional information provided by the RHO helped to organize visits and complete other administrative activities. Ten out of Asan-si's 17 districts, with population size over 500 were randomly selected. Within each of these districts, a total of 200 households from every third street were chosen. The size, gender and age of all household members were obtained from the RHO database before the visit. Gender and age were used to stratify the households and one person in each household was selected by random sampling, before a list of selected individuals was compiled in each stratum. The regional health office checked the eligibility of the selected individuals by examining their medical records, followed by a telephone interview. Exclusion criteria included pregnant or lactating women, history of surgery of the GI tract including partial resection of the stomach and/or resection of the small/large intestine (subjects with an appendectomy were included), major psychosis, mental retardation or dementia, significant illness that may render them unable to complete the questionnaire, or had other nationalities. The revised list was used for actual household visit after excluding the ineligible individuals. Since only housewives and elderly individuals would be available during the day, we pre-scheduled the time and order of the house visits with the help of the regional health office.

Questionnaire

We developed a bowel symptom questionnaire to identify chronic gastrointestinal symptoms in adults, based on the Rome Ⅱ criteria. The complete questionnaire was 22 pages long and included 130 questions, of which 103 were used to diagnose gastroesophageal reflux and chronic gastrointestinal symptoms. The bowel symptoms questionnaire was modified from the Mayo version of Bowel Symptoms Questionnaire[6] and several items were adopted from the Rome Ⅱ criteria. The validation process consisted of forward and backward translation as well as confirming the patient's ability to understand. However, we did not perform test-retest reliability, because this was not a self-reported questionnaire, and trained interviewers visited homes and helped the subjects to complete the questionnaire. During the survey, a face-to-face interview was conducted to help describe certain parts of the Bowel Symptoms Questionnaire. Specifically, heartburn is not a term in the Korean language; therefore the symptoms had to be verbally described and a diagram indicating the location of the burning sensation was added to the questionnaire in order to help ensure that the subjects fully comprehended the medical conditions under investigation. The remaining 27 questions enquired about the sociodemographic characteristics, self-reported height and weight, physician visits, past illnesses, social habits (smoking, alcohol, and medication use), impact on everyday life, possible anxiety induced by the problem, as well as lifestyle changes that may have been relevant.

HRQOL was assessed by the Korean version of SF-36 (SF-36-K). We previously assessed the reliability, discriminant validity and concurrent validity of the SF-36-K before conducting this study. The SF-36-K was fully applicable and well understood by Korean patients, as well as healthy subjects. The reliability was assessed by using the test-retest method, and the internal consistency method. The test-retest method showed high correlation between the two tests. Cronbach's correlation alpha of all 8 subscales of the SF-36-K was > 0.73 (range, 0.73-0.96). Discriminant validity was supported by comparing the SF-36 score in 179 healthy subjects and 44 patients with gastrointestinal disorders. All 8 domains of the SF-36 were well correlated with subscores of the WHO Quality of Life Scale-K and the Psychological Well-Being Index. The SF-36 is a widely used general health profile questionnaire with 36 questions comprising eight scales: physical functioning (e.g. walking, lifting), role functioning-physical (limitations in ability to perform usual activities), role functioning-emotional (impact of emotional problems on work or

daily activities), social functioning (impact of health or emotional problems on social activities), bodily pain (level of bodily pain or discomfort), mental health (anxiety, depression, sense of psychological well-being), vitality (energy level or fatigue), and general health perceptions (global evaluations of health). The SF-36 is scored from 0 to 100 with higher scores indicating a better HRQOL.

Survey design

We conducted a cross-sectional survey of gastrointestinal symptoms and their impact on the quality of life (QOL) in Korean subjects in cooperation with the public health center. Before the survey, the questionnaire was explained to the health-care personnel of the Public Health Center to outline the purpose of the study and to request their participation. All subjects were interviewed in person at their homes or offices by a team of interviewers, all trained by the same physician (C.M.G.). The consistency and completeness of the completed questionnaire was checked after each interview.

Statistics

The 1807 eligible individuals interviewed can be considered as a representative sample of the Asan-si population. We calculated the prevalence of GERD and chronic gastrointestinal symptoms according to the Rome Ⅱ criteria. The prevalence is presented in percentages with 95% exact confidence intervals (CI). Comparisons among groups were performed by the χ^2 test or Fisher's exact test for categorical data and *t*-test for continuous data. The association between prevalence rate and age in each group was tested by logistic regression model. Differences of SF-36 sub-scale scores between groups were estimated by ANCOVA model with adjustment of covariates. Pair-wise differences between groups without chronic GI symptoms and groups with chronic GI symptoms (GERD, UD and IBS) were tested by Bonferroni adjusted *t*-test. Gender, age, economic level and education variables were used for adjustment. A multiple regression model was performed for SF-36 sub-scale scores for individuals who had chronic GI symptoms. Gender, age, smoking, religion, education, economic level, physician visit and overlapping symptom variables were used as predictors. Statistical analyses were performed using SAS (SAS, Cary, NC, USA).

RESULTS

Response rate and subject characteristics

Among the randomly selected 2024 subjects, a total of 217 were not eligible to participate in the study. One hundred and twenty individuals were no longer living in Asan-si and 97 could not be interviewed due to physical or mental disorders. Out of the 1807 eligible subjects, 314 could not be contacted after three attempts, and 76 refused to participate. A total of 1417 (78.4%) of the 1807 eligible subjects returned the completed survey. Of these respondents, 762 were male (53.8%) and 655 were female (46.2%), with a mean age of 44 years. The demographic and socioeconomic features of the respondents are shown in Table 1.

Table 1 Characteristics of the study population *n* (%)

Variable	Male (*n* = 762)	Female (*n* = 655)	Total (*n* = 1417)
Age			
18-29	163 (11.5)	151 (10.7)	314 (22.2)
30-39	132 (9.3)	110 (7.8)	242 (17.1)
40-49	169 (11.9)	146 (10.3)	315 (22.2)
50-59	150 (10.6)	116 (8.2)	266 (18.8)
60-69	148 (10.4)	132 (9.3)	280 (19.7)
Median age (yr)	45	44	44
Mean age (yr)	44.1 ± 14.4	43.7 ± 15.1	43.9 ±14.8
BMI (kg/m^2)	22.8	22.0	22.4
Ever tobacco smoker (%)	67.8[a]	4.0	38.3
Alcohol use (> 75 g/wk) (%)	36.4[a]	2.6	20.7
Marital status (single)[1] (%)	26.5	28.7	27.5
Employed (%)	85.4[a]	45.6	67.0
Presence of religion (%)	50.5[b]	65.0	57.2
High school graduate (%)	57.0[a]	49.2	53.4
Economic status (%)			
High	6.4	6.3	6.4
Middle	76.1	79.8	77.8
Low	17.5	13.9	15.8

[1]Marital status was divided into single or married/living as a couple. Single status was extended to include unmarried persons, divorced individuals and those with a deceased spouse [a]$P < 0.01$, [b]$P < 0.001$ compared to female.

Prevalence of chronic gastrointestinal symptoms

The prevalence of weekly episodes of heartburn and acid regurgitation was 2.0% (95% CI, 1.2-2.7) and 2.0% (95% CI, 1.3-2.8) respectively. The prevalence of GERD, defined as heartburn and/or acid regurgitation experienced at least weekly, was 3.5% (95% CI, 2.6-4.5). The prevalence of specific chronic gastrointestinal symptoms, according to the Rome Ⅱ criteria is summarized in Table 2. At least one chronic gastrointestinal symptom was present in 18.6% of the 1417 respondents. The most prevalent chronic gastrointestinal symptom was uninvestigated dyspepsia (11.7%; 95% CI, 10.1-13.5). According to the subtypes of dyspepsia, dysmotility-like dyspepsia was the most prevalent (69.9%), followed by ulcer-like dyspepsia (28.3%) and non-specific dyspepsia (1.8%). Thirty one subjects (2.2%) fulfilled the Rome Ⅱ criteria for the diagnosis of IBS (Table 2). Of these, 13 subjects (42%) were classified as diarrhea-predominant, and 12 (39%) constipation-predominant IBS. The remaining 6 subjects (19%) fell into the alternating IBS subgroup. There were no differences in the overall prevalence of IBS based on gender; however, compared to male subjects, females reported more frequent constipation-predominant IBS (IBS-C). Females also reported more frequent chronic constipation. There were no gender-based differences in the prevalence of the other chronic gastrointestinal symptoms (Table 2).

Age-specific prevalence of GERD, uninvestigated dyspepsia, and IBS are shown in Figure 1, and the odds ratio with 95% confidence interval are shown in Table 3. The prevalence of GERD and dyspepsia showed significant differences between different age groups (logistic regression, $P < 0.01$).

Physician visit and medication use

In the present population-based study, 50.5% of the

Table 2 Prevalence of chronic gastrointestinal symptoms, according to the Rome Ⅱ criteria

Chronic gastrointestinal symptoms	Respondent (*n* = 1417) *n*	% (95% exact CI)	Male (*n* = 762) %	Female (*n* = 655) %
Globus	7	0.5 (0.2-1.0)	0.4	0.7
Chronic dysphagia	7	0.5 (0.2-1.0)	0.3	0.7
Rumination	0	0.0 (0.0-0.3)	0	0
Chronic chest pain	21	1.5 (0.9-2.3)	1.6	1.4
Chronic heartburn	24	1.7 (1.1-2.5)	1.8	1.6
Uninvestigated dyspepsia	166	11.7 (10.1-13.5)	10.8	12.8
IBS	31	2.2 (1.5-3.1)	1.8	2.6
IBS-D	13	0.9 (0.5-1.6)	1	0.8
IBS-C	12	0.8 (0.4-1.5)	0.1	1.7[a]
IBS-A	6	0.4 (0.2-0.9)	0.7	0.2
Chronic bloating	57	4.0 (3.1-5.2)	2.9	5.3
Chronic constipation	37	2.6 (1.8-3.6)	0.5	5.0[a]
Chronic diarrhea	11	0.8 (0.4-1.4)	0.8	0.8
Chronic incontinence	18	1.3 (0.8-2.0)	1.6	1.1

IBS: Irritable bowel syndrome; IBD-D: Diarrhea-predominant IBS; IBS-C: Constipation- predominant IBS; IBS-A: Alternating constipation and diarrhea IBS. [a]$P < 0.05$ (Fisher's exact test) compared to male.

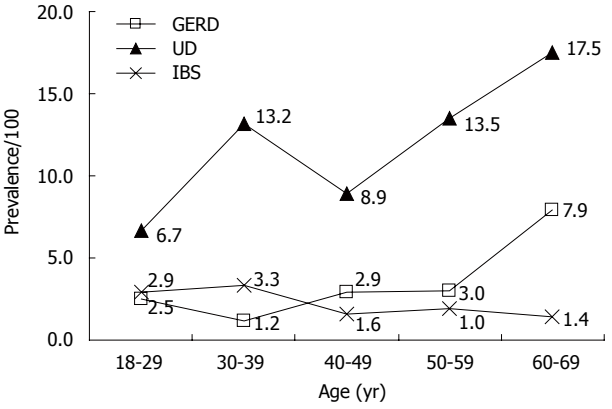

Figure 1 Age specific prevalence rate (per 100) of GERD, UD and IBS in Asan-si, Korea. GERD: Gastroesophageal reflux disease; UD: Uninvestigated dyspepsia; IBS: Irritable bowel syndrome.

subjects surveyed had experienced at least one gastrointestinal symptom in the previous year, and 10.9% had reported visiting a physician due to their gastrointestinal symptoms. With regard to the use of medications, 5.7% of the respondents took non-steroidal anti-inflammatory drugs (NSAIDs), and 5.5% had taken anti-acid agents or antacids in the past year. More women took NSAIDs and constipation medications (Table 4).

Impact of chronic gastrointestinal symptoms on health-related quality of life

Of the 1417 respondents, 1153 individuals did not experience any chronic GI symptoms, while 198 subjects had features suggestive of GERD, UD or IBS, or a combination of these symptoms. There was no significant difference between the two groups with respect to gender, smoking, martial status and BMI ($P > 0.05$). However, age, education level and the number of physician visits were statistically different ($P < 0.001$). The mean age of subjects with chronic GI symptoms and subjects without chronic GI symptoms was 47.8 ± 14.9 and 43.0 ± 14.6, respectively. Subjects with chronic GI symptoms had a lower education level, and also visited a physician more frequently for gastrointestinal symptoms than subjects without chronic GI symptoms. SF-36 subscale scores were calculated for four groups: (1) subjects without chronic gastrointestinal symptoms, (2) subjects with GERD, (3) subjects with uninvestigated dyspepsia, and (4) subjects with IBS. The adjusted mean scores for gender, age, education and economic level, as well as the accompanying *P*-values for the eight domains of the SF-36 are summarized in Table 5. Compared with those not experiencing chronic gastrointestinal symptom, subjects with GERD exhibited significantly worse scores on all except two domains (social-functioning and role-emotional). Subjects with uninvestigated dyspepsia and IBS had significantly worse scores on all domains compared with those not having chronic gastrointestinal symptoms (Table 5). Multiple regression analysis of the association between the HRQOL and covariates was performed on subjects with chronic GI symptoms (n = 198) (Table 6). All covariates shows VIF smaller than 10, thus no covariates were excluded as the cause of multi-

Table 3 Association between age and chronic gastrointestinal symptoms

Age	GERD	UD	IBS
18-29	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)
30-39	0.48 (0.13-1.82)	2.12 (1.19-3.77)	1.15 (0.44-3.04)
40-49	1.14 (0.44-3.00)	1.39 (0.77-2.50)	0.56 (0.18-1.68)
50-59	1.23 (0.46-3.33)	2.28 (1.30-4.02)	0.68 (0.22-2.04)
60-69	3.30 (1.44-7.54)	3.00 (1.75 -5.14)	0.50 (0.15-1.63)

Values are shown as odds ratio and 95% confidence interval. GERD, gastroesophageal reflux disease; UD, uninvestigated dyspepsia; IBS, irritable bowel syndrome.

Table 4 Details of physician visits and use of medications *n* (%)

Variables	Men (*n* = 762)	Women (*n* = 655)	Total (*n* = 1417)
Experience (%) of any GI symptoms	366 (48.0)	351 (53.6)[a]	50.50
Experience (%) of visiting a physician due to GI symptoms	80 (10.5)	75 (11.5)	155 (10.9)
1-2/yr	42 (5.5)	47 (7.2)	89 (6.3)
3-5/yr	12 (1.6)	12 (1.8)	24 (1.7)
6-10/yr	8 (1.0)	4 (0.6)	12 (0.8)
> 10/yr	18 (2.4)	12 (1.8)	30 (2.1)
Use of medications			
NSAIDs	27 (3.5)	54 (8.2)[b]	81 (5.7)
Antacids	35 (4.6)	28 (4.3)	63 (4.5)
H_2RA	11 (1.4)	4 (0.6)	15 (1.1)
Medications for constipation	17 (2.2)	49 (7.5)[b]	66 (4.7)
Antihypertensive medications	17 (2.2)	18 (2.7)	35 (2.5)

[a]$P < 0.05$, [b]$P < 0.01$ compared to male.

Table 5 Comparison of SF-36 subscales between subjects without chronic GI symptoms, and those with GERD, UD and IBS

SF-36 Subscale	Subjects without CGIS (*n* = 1153)	GERD (*n* = 50)	UD (*n* = 166)	IBS (*n* = 31)
Physical functioning	87.6 ± 5.1	76.0 ± 6.2[b]	81.7 ± 5.4[b]	82.0 ± 66.8[a]
Role physical	80.1 ± 10.3	64.9 ± 12.4[b]	68.1 ± 10.9[b]	67.3 ± 13.7[a]
Bodily pain	89.3 ± 6.7	74.6 ± 8.1[b]	76.5 ± 7.1[b]	77.7 ± 8.9[b]
General health	68.8 ± 7.0	49.3 ± 8.5[b]	52.7 ± 7.5[b]	50.0 ± 9.4[b]
Vitality	57.6 ± 7.5	50.0 ± 9.0[a]	51.0 ± 7.9[b]	45.9 ± 10.0[b]
Social functioning	92.0 ± 4.9	88.3 ± 5.9	86.2 ± 5.2[b]	86.7 ± 6.6[a]
Role emotional	86.2 ± 10.4	80.7 ± 12.5	77.7 ± 11.0[b]	71.7 ± 13.8[b]
Mental health	77.7 ± 6.2	67.9 ± 7.5[b]	71.6 ± 6.6[b]	66.1 ± 8.3[b]

Note: Age, gender, education level and economic level adjusted mean and 95% confidence interval of SF-36 subscale. CGIS: Chronic GI symptom; GERD: Gastroesophageal reflux disease; UD: Uninvestigated dyspepsia; IBS: Irritable bowel syndrome. Comparisons were performed between GERD *vs* no CGIS, dyspepsia *vs* no CGIS, and IBS *vs* no CGIS. *P* value was adjusted by Bonferroni method. [a]$P < 0.05$ compared to subjects without chronic gastrointestinal symptom; [b]$P < 0.01$ compared to subjects without chronic gastrointestinal symptom.

Table 6 Multiple regression results: estimated coefficient of predictors of each SF-36 subscale domain

Variable	PF	RP	BP	GH	VT	SF	RE	MH
Female sex	-4.6	-9.6	-4.7	-5.3[b]	-4.3[a]	-3.6	-8.2	-1.8
Age[1]	-5.7[c]	-3.2	-1.3	-4.6[b]	-3.2[a]	-1.4	-1.2	-3.5[b]
Ever smoking	2.5	3	2	-2.6	2.3	2.4	3	1.3
Religion	4.1	9.7	-0.1	1.3	6.5[a]	-3.5	-3.5	2.3
< high school education	-6.7[a]	-5.8	-3	2.6	3.1	0.4	3.8	4.2
Low economic class	-2.8	-14.0[a]	-5.8	-3.3	-0.2	-3.9	-17.3	-0.2
Number of physician visit[2]	0.6	-4.8[a]	-4.8[c]	-4.1[b]	-1.9	-0.9	-4.8[a]	-3.5[c]
Presence of overlapping symptoms	-4.7	-3.5	-8.3[a]	-6.9	1.6	-3.9	-5.5	-8.3[b]
R^2	0.375	0.134	0.165	0.19	0.09	0.09	0.124	0.177

PF: Physical function; RP: Role-physical; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social function; RE: Role-emotion; MH: Mental health. [1]Age variables are categorized by 10 yr intervals; [2]Number of physician visits for gastrointestinal symptoms: 0/yr(0), 1-2/yr(1), 3-5/yr(2), 6-10/yr(3), > 10/yr(4). [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

collinearity. The results of overall *F*-test were significant for all models ($P < 0.05$). Female gender, old age, a low level of education (< high school education), a low economic class, number of physician visits within the past year, and overlapping chronic gastrointestinal symptoms were associated with reduction in the SF-36 scales.

DISCUSSION

The present population-based study describes the prevalence of chronic gastrointestinal symptoms and their impact on the HRQOL in the Korean population.

Heartburn and acid regurgitation are specific symptoms of GERD, and a diagnosis of GERD can be made on the basis of these symptoms alone without further diagnostic tests[7]. When defined as "at least weekly heartburn and/or acid regurgitation", the prevalence of GERD in the West ranges between 10% and 20%, whereas in Asia the prevalence is reported to be < 5%[8]. In the present study, the prevalence of GERD was found to be 3.5% which is much lower than that in Western countries. According to two previous population studies in Korea, the prevalence of GERD was 5% and 7.1%, respectively[9,10]. It is unclear why the prevalence of GERD is lower in Korea compared to the West. Differences in the intake of dietary fat, body build, genetic factors, and the prevalence of *H pylori* infection are possible contributing factors[11]. The prevalence of dyspepsia has been shown to vary considerably between different populations. Although our data may represent the presence of valid epidemiological differences, it is also possible that the varying definitions used in different population-based studies may have contributed to this discrepancy. In the present study, the prevalence of uninvestigated dyspepsia by Rome Ⅱ criteria was 11.7%. These results are similar to a study from Taiwan, which reported prevalence of functional dyspepsia (FD) by the Rome Ⅱ criteria as 11.8%[12]. Population-based studies in Australia and Mexico have reported prevalence rates of dyspepsia by Rome Ⅱ criteria of 24.4%[13] and 8.0%[14], respectively. Based on Rome Ⅱ criteria, a patient presenting with upper abdominal pain or discomfort that is exclusively relieved by defecation and/or is associated with a change in the bowel pattern is defined as IBS rather than dyspepsia plus IBS[15]. Accordingly, many of the previously Rome I-defined dyspepsia subjects should be reclassified as IBS, rather than IBS with dyspepsia. Furthermore, patients with predominant heartburn should be excluded. Although, the present study could not demonstrate the prevalence of dyspepsia defined by Rome I criteria, the prevalence of dyspepsia by Rome Ⅱ criteria was lower compared to our previous study using the Rome I criteria (11.7% *vs* 15.5%)[9]. Reduction in prevalence has also been reported in other studies that directly compared the prevalence of dyspepsia using Rome I and Rome Ⅱ criteria[12,13,16]. Several individuals who did not meet Rome Ⅱ criteria for dyspepsia were taking medications such as antacids, prokinetic agents and digestives for a long time. If medication alone was indicative of dyspepsia, our study indicates that its prevalence would be 16%. It is also possible that some subjects had GERD and not dyspepsia, or there was an overlap. One of these factors may also account for the low prevalence of GERD in the Korean population. Over-the-counter medications may explain the underestimation of the prevalence of other functional GI disorders. Conversely, a number of drugs can theoretically induce bowel symptoms. However, data supporting the role of drugs, aside from NSAIDs, in the development of bowel symptoms in the general population are lacking[17,18].

The prevalence of IBS in Asian population-based studies has generally been lower compared to in the

West, regardless of the criteria applied. The prevalence of IBS by Rome Ⅱ criteria has been reported to vary from 4.7% to 25% in the West and from 3.7% to 19.1% in Asia[19]. In the present study, the prevalence of IBS by Rome Ⅱ criteria was 2.2%. In addition, the overall prevalence of IBS was similar among men and women; however, the prevalence of constipation-predominant IBS was higher in women. There is typically a significant female predominance with respect to hospital visits by IBS patients in Western countries, but this trend is not consistent with community studies and has been attributed to gender differences in health care utilization[19]. In a recent systematic review of 13 studies on IBS, based on Rome Ⅱ criteria, 7 studies found a higher prevalence of IBS in females and 4 studies found no gender difference, as in the present study[19]. A possible explanation for the lower female to male ratio in IBS in Koreans may be that men encounter more socioeconomic problems, causing increased stress and, as a consequence, an increased level of IBS[20].

In the present study, we also examined the impact of GERD, and two common chronic gastrointestinal symptoms (uninvestigated dyspepsia and IBS) on the HRQOL. Although a number of studies suggest that the HRQOL is significantly reduced in patients with chronic gastrointestinal symptoms in a referral setting, the data are conflicting[21], and very few studies have evaluated the impact of chronic gastrointestinal symptoms on HRQOL in the general population. In the present study, we observed that the quality of life was significantly impaired in subjects with GERD, uninvestigated dyspepsia and IBS. These findings are consistent with previous studies[5,22-24].

The present study showed that old age, female gender, the number of physician visits per year and presence of overlapping symptoms were associated with a negative impact on several domains of the SF-36. In general, old age was associated with a less favorable assessment of their personal health, pessimistic health appraisal, social isolation and unemployment[25]. Few studies have investigated whether women and men with chronic gastrointestinal symptoms differ with respect to the HRQOL measures. In a study based on referral center and primary care patients, Simren *et al*[26] observed that women with IBS had lower HRQOL compared to men with IBS. In another study, Lee *et al*[27] also found that women with IBS reported lower HRQOL scores. In the present study, a greater number of hospital visits was associated with a poorer HRQOL, which is in agreement with a US population-based study[28]. Fifty five percents of subjects with IBS had sought health care in the past year, and subjects with IBS had significantly lower IBS-QOL scores in the mental health and social functioning domains[28].

The strengths of the present study were inclusion of a random population sample, and the use of personal interviews with the subjects. As a result, we obtained a high response rate (78.4%), avoided a significant response bias, and had a negligible number of missing values. Since the interviewers associated with this study were trained before the commencement of contact with the subjects, a uniform survey was possible due to the fact that the interviewers could explain each aspect of the questionnaire fully, especially to subjects who were old, had low education level, or those who could not understand certain items. Sociodemographic factors including age, gender, educational background and economic level of the individuals in Asan-si were similar to the Korean population, based on the information obtained from the Korean National Statistical Office. Asan-si is a combined urban and farming community. We selected 10 out of the 17 districts of Asan-si. Five districts were randomly chosen in urban areas and the remaining five were selected in rural areas, which may have limited the generalization of the study. However, no differences were found between the demographic factors and the prevalence of GI symptoms in the 10 districts, and between rural and urban areas.

In summary, we evaluated the prevalence of chronic gastrointestinal symptoms in the Korean general population and demonstrated a significant impact of chronic gastrointestinal symptoms on the HRQOL. Dyspepsia was found to be the most common chronic gastrointestinal symptom, and the prevalence of GERD and IBS was lower compared to in the West. The presence of chronic gastrointestinal symptoms was found to have a negative impact on the HRQOL. This negative impact was greater in females, the elderly, individuals of lower economic class, and in subjects with higher number of physician visits, and overlapping symptoms.

REFERENCES

1 **Drossman DA**, Li Z, Andruzzi E, Temple RD, Talley NJ, Thompson WG, Whitehead WE, Janssens J, Funch-Jensen P, Corazziari E. U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. *Dig Dis Sci* 1993; **38**: 1569-1580

2 **Agreus L**, Svardsudd K, Nyren O, Tibblin G. Irritable bowel syndrome and dyspepsia in the general population: overlap and lack of stability over time. *Gastroenterology* 1995; **109**: 671-680

3 **Jones RH**, Lydeard SE, Hobbs FD, Kenkre JE, Williams EI, Jones SJ, Repper JA, Caldow JL, Dunwoodie WM, Bottomley JM. Dyspepsia in England and Scotland. *Gut* 1990; **31**: 401-405

4 **Chang L**. Review article: epidemiology and quality of life in functional gastrointestinal disorders. *Aliment Pharmacol Ther* 2004; **20** Suppl 7: 31-39

5 **Halder SL**, Locke GR 3rd, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Impact of functional gastrointestinal disorders on health-related quality of life: a population-based case-control study. *Aliment Pharmacol Ther* 2004; **19**: 233-242

6 **Talley NJ**, Phillips SF, Wiltgen CM, Zinsmeister AR, Melton LJ 3rd. Assessment of functional gastrointestinal disease: the bowel disease questionnaire. *Mayo Clin Proc* 1990; **65**: 1456-1479

7 **Locke GR 3rd**, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997; **112**: 1448-1456

8 **Dent J**, El-Serag HB, Wallander MA, Johansson S. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut* 2005; **54**: 710-717

9 **Choo KY**, Choi MG, Choi H, Lee DS, Kim JI, Kim SS, Bhang CS, Park SH, Kim JK, Han SW, Choi KY, Chung IS, Chung KW, Sun HS. The prevalence of gastrointestinal symptoms in a rural community in Korea. *Kor J Neurogastroenterol Motil* 2000; **6**: 31-43

10 **Yang SY**, Lee OY, Bak YT, Jun DW, Lee SP, Lee SH, Park GT, Yoon BC, Choi HS, Hahm JS, Lee MH, Lee DH. Prevalence of gastroesophageal reflux disease symptoms and uninvestigated dyspepsia in Korea: a population-based study. *Dig Dis Sci* 2008; **53**: 188-193

11 **Cho YS**, Choi MG, Jeong JJ, Chung WC, Lee IS, Kim SW, Han SW, Choi KY, Chung IS. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Asan-si, Korea. *Am J Gastroenterol* 2005; **100**: 747-753

12 **Lu CL**, Lang HC, Chang FY, Chen CY, Luo JC, Wang SS, Lee SD. Prevalence and health/social impacts of functional dyspepsia in Taiwan: a study based on the Rome criteria questionnaire survey assisted by endoscopic exclusion among a physical check-up population. *Scand J Gastroenterol* 2005; **40**: 402-411

13 **Westbrook JI**, Talley NJ. Empiric clustering of dyspepsia into symptom subgroups: a population-based study. *Scand J Gastroenterol* 2002; **37**: 917-923

14 **Schmulson M**, Ortiz O, Santiago-Lomeli M, Gutierrez-Reyes G, Gutierrez-Ruiz MC, Robles-Diaz G, Morgan D. Frequency of functional bowel disorders among healthy volunteers in Mexico City. *Dig Dis* 2006; **24**: 342-347

15 **Talley NJ**, Stanghellini V, Heading RC, Koch KL, Malagelada JR, Tytgat GN. Functional gastroduodenal disorders. *Gut* 1999; **45** Suppl 2: II37- II42

16 **Thompson WG**, Irvine EJ, Pare P, Ferrazzi S, Rance L. Functional gastrointestinal disorders in Canada: first population-based survey using Rome II criteria with suggestions for improving the questionnaire. *Dig Dis Sci* 2002; **47**: 225-235

17 **Bytzer P**, Hallas J. Drug-induced symptoms of functional dyspepsia and nausea. A symmetry analysis of one million prescriptions. *Aliment Pharmacol Ther* 2000; **14**: 1479-1484

18 **Ofman JJ**, Maclean CH, Straus WL, Morton SC, Berger ML, Roth EA, Shekelle PG. Meta-analysis of dyspepsia and nonsteroidal antiinflammatory drugs. *Arthritis Rheum* 2003; **49**: 508-518

19 **Kang JY**. Systematic review: the influence of geography and ethnicity in irritable bowel syndrome. *Aliment Pharmacol Ther* 2005; **21**: 663-676

20 **Han SH**, Lee OY, Bae SC, Lee SH, Chang YK, Yang SY, Yoon BC, Choi HS, Hahm JS, Lee MH, Lee DH, Kim TH. Prevalence of irritable bowel syndrome in Korea: population-based survey using the Rome II criteria. *J Gastroenterol Hepatol* 2006; **21**: 1687-1692

21 **El-Serag HB**, Olden K, Bjorkman D. Health-related quality of life among persons with irritable bowel syndrome: a systematic review. *Aliment Pharmacol Ther* 2002; **16**: 1171-1185

22 **Koloski NA**, Talley NJ, Boyce PM. The impact of functional gastrointestinal disorders on quality of life. *Am J Gastroenterol* 2000; **95**: 67-71

23 **Xiong LS**, Chen MH, Chen HX, Xu AG, Wang WA, Hu PJ. A population-based epidemiologic study of irritable bowel syndrome in South China: stratified randomized study by cluster sampling. *Aliment Pharmacol Ther* 2004; **19**: 1217-1224

24 **Chen M**, Xiong L, Chen H, Xu A, He L, Hu P. Prevalence, risk factors and impact of gastroesophageal reflux disease symptoms: a population-based study in South China. *Scand J Gastroenterol* 2005; **40**: 759-767

25 **Sobhonslidsuk A**, Silpakit C, Kongsakon R, Satitpornkul P, Sripetch C, Khanthavit A. Factors influencing health-related quality of life in chronic liver disease. *World J Gastroenterol* 2006; **12**: 7786-7791

26 **Simren M**, Abrahamsson H, Svedlund J, Bjornsson ES. Quality of life in patients with irritable bowel syndrome seen in referral centers versus primary care: the impact of gender and predominant bowel pattern. *Scand J Gastroenterol* 2001; **36**: 545-552

27 **Lee OY**, Mayer EA, Schmulson M, Chang L, Naliboff B. Gender-related differences in IBS symptoms. *Am J Gastroenterol* 2001; **96**: 2184-2193

28 **Williams RE**, Black CL, Kim HY, Andrews EB, Mangel AW, Buda JJ, Cook SF. Determinants of healthcare-seeking behaviour among subjects with irritable bowel syndrome. *Aliment Pharmacol Ther* 2006; **23**: 1667-1675

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RAPID COMMUNICATION

Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia

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Abstract

AIM: To investigate the efficacy and safety of n-3 polyunsaturated fatty acids (PUFA) from seal oils for patients with nonalcoholic fatty liver disease (NAFLD) associated with hyperlipidemia.

METHODS: One hundred and forty-four patients with NAFLD associated with hyperlipidemia were included in the 24-wk, randomized, controlled trial. The patients were randomized into two groups. Group A (n = 72) received recommended diet and 2 g n-3 PUFA from seal oils, three times a day. Group B (n = 72) received recommended diet and 2 g placebo, three times a day. Primary endpoints were fatty liver assessed by symptom scores, liver alanine aminotransferase (ALT) and serum lipid levels after 8, 12, 16, and 24 wk. Hepatic fat infiltration was detected by ultrasonography at weeks 12 and 24 after treatment.

RESULTS: A total of 134 patients (66 in group A, 68 in group B) were included in the study except for 10 patients who were excluded from the study. After 24 wk of treatment, no change was observed in body weight, fasting blood glucose (FBG), renal function and blood cells of these patients. Total symptom scores, ALT and triglyceride (TG) levels decreased more significantly in group A than in group B ($P < 0.05$). As expected, there was a tendency toward improvement in aspartate aminotransferase (AST), γ-glutamyltranspeptidase (GGT), and total cholesterol (TCHO) and high-density lipoprotein (HDL) cholesterol levels ($P < 0.05$) after administration in the two groups. However, no significant differences were found between the two groups. The values of low-density lipoprotein (LDL) were significantly improved in group A ($P < 0.05$), but no significant change was found in group B at different time points and after a 24-wk treatment. After treatment, complete fatty liver regression was observed in 19.70% (13/66) of the patients, and an overall reduction was found in 53.03% (35/66) of the patients in group A. In contrast, in group B, only five patients (7.35%, 5/68) achieved complete fatty liver regression (P = 0.04), whereas 24 patients (35.29%, 24/68) had a certain improvement in fatty liver (P = 0.04). No serious adverse events occurred in all the patients who completed the treatment.

CONCLUSION: Our results indicate that n-3 PUFA from seal oils is safe and efficacious for patients with NAFLD associated with hyperlipidemia and can improve their total symptom scores, ALT, serum lipid levels and normalization of ultrasonographic evidence. Further study is needed to confirm these results.

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Key words: Nonalcoholic fatty liver disease; Polyunsaturated fatty acids; Seal oil; Hyperlipidemia; Therapy

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents a

spectrum of conditions characterized by an excessive accumulation of hepatic fat in the absence of alcohol consumption[1]. In Western countries and some regions of China, the prevalence of nonalcoholic steatohepatitis (NASH) and NAFLD is 1%-5% and 15%-39%, respectively[2]. In most of patients, NAFLD follows a relatively benign course and remains stable for years[1]. However, it was recently reported that many cases of cryptogenic liver cirrhosis may be related to unrecognized NASH[3]. Thus, treatment should be reserved for patients at risk of developing severe liver diseases. Medical therapy for NAFLD and NASH has been disappointing to date[4]. Although a number of treatment modalities are available, they cannot prevent the progression of early liver disease to its advanced stage, and the only recommended therapies are dietary modification and weight loss[1,5]. It has been shown that n-3 polyunsaturated fatty acids (PUFA) is effective on NAFLD[6-8]. In this study, we evaluated the efficacy and safety of n-3 PUFA from seal oils in ameliorating serum lipids and liver enzymes in patients with NAFLD associated with hyperlipidemia.

MATERIALS AND METHODS

Patients

One hundred and forty-four patients with NAFLD associated with mixed dyslipidemia were studied as outpatients in the Tonggji Hospital, Tongji University, from September 2006 to June 2008. Written informed consent was obtained from all patients and the study was approved by the Ethics Committee in Tonggji Hospital of Tongji University.

The inclusion criteria were as follows: age between 18 and 65 years, lack of excessive alcohol ingestion confirmed by careful questioning by the primary physician and dietitians (consumption of less than 70 g alcohol in female and 140 g in male per week), elevated serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) above the normal limit, but under 5 times the upper limit of the normal range for $\geqslant$ 6 mo before the study. Diagnosis of dyslipidemia was based on the presence of one or more of the following findings: fasting serum total cholesterol (TCHO) > 5.7 mmol/L, serum triglyceride (TG) > 1.8 mmol/L and high-density lipoprotein cholesterol (HDL-C) < 0.8 mmol/L, fatty liver diagnosed by abdominal ultrasonography, and ability to give informed consent. Exclusion criteria included overuse of alcohol, viral hepatitis, hemochromatosis, Wilson's disease, autoimmune hepatitis, α-1 antitrypsin deficiency, primary sclerosing cholangitis or primary biliary cirrhosis; history of any other hepatic, gastrointestinal, renal, cardiovascular, neurological or hematological disorders, psychiatric disorder which might impair the ability of patients to provide written informed consent; as well as pregnancy, breastfeeding, or lack of effective birth control in women at child-bearing age. In addition, patients were excluded if they were on any medications that could influence liver function during the observation period or involved in any clinical trial before the study.

Study design

All the patients meeting the criteria for enrollment agreed to participate in the study. The patients were randomized into two groups to receive a 24-wk treatment. Group A (n = 72) received recommended diet and 2 g n-3 PUFA from seal oils (Shanghai Hengsheng Biology & Medicine CO. Ltd, Shanghai, China), three times a day. Group B (n = 72) received recommended diet and 2 g placebo (Shanghai Hengsheng Biology & Medicine CO. Ltd, Shanghai, China), three times a day. Recommended diet was composed of 50% carbohydrates, 20% protein and 30% fat in accordance with the American Heart Association diet[9]. All obese and overweight patients were advised to lose their weight with a restriction of daily caloric intake to 25-30 kcal/kg per day[9]. All medications the patients received during the 24-wk treatment period were recorded. At the time of enrolment and when the study was completed, body temperature, body mass index (BMI), blood pressure and heart rate were detected and liver ultrasonography was performed. Laboratory tests included serum ALT, AST, γ-glutamyltranspeptidase (GGT), TG, TCHO, HDL-C, LDL-C, FBS, and complete blood cell counts.

During the 24-wk treatment period, total symptom scores, liver enzymes and fasting lipids were monitored at weeks 8, 12, 16, and 24. Hepatic fat infiltration was detected by upper abdominal ultrasonography at weeks 12 and 24. Symptoms included liver discomfort or pain, weakness, abdominal distention, and nausea. The severity of each clinical symptom was scored using a 4-point scale as follows: 0 score = asymptomatic, 1 score = mild, 2 scores = moderate, 3 scores = severe. All patients were investigated after 12 h fasting and underwent ultrasonography for liver steatosis. Ultrasound scans were performed by a trained operator who was blind to the treatment of participants. The severity of steatosis was also scored using a 4-point scale as follows[10]: grade 0 = normal echogenicity, grade 1 = slight, grade 2 = moderate, grade 3 = severe.

Statistical analysis

The data were presented as mean ± SD and analyzed using SPSS11.5 for Windows (SPSS, Chicago, IL, USA). Statistical analysis for baseline characteristics of the study groups was performed using χ^2 test and t-test. Student's t-test and Wilcoxon signed rank test were used to evaluate the changes in biochemical parameters before and after treatment. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the patients

Of the 144 patients enrolled in this study, 134 completed the protocol and were included in the analysis. The baseline clinical and demographic data about the two

Table 1 Baseline characteristics of groups A and B (mean ± SD)

	Group A (n = 66)	Group B (n = 68)	P vaule
Age (yr)	45.00 ± 10.91	44.03 ± 11.30	0.74
Sex ratio (male/female)	47/19	50/18	0.76
Body height (cm)	169.07 ± 7.96	169.71 ± 7.89	0.88
Weight (kg)	75.71 ± 10.99	75.16 ± 11.33	0.93
BMI (kg/m^2)	26.37 ± 3.12	25.96 ± 2.70	0.57
HR (/min)	75.57 ± 6.24	75.89 ± 6.58	0.32
Duration of NAFLD (mo)	22.32 ± 38.82	13.65 ± 20.00	0.55
SBP (mmHg)	126.59 ± 10.97	125.89 ± 9.87	0.58
DBP (mmHg)	82.26 ± 7.50	81.59 ± 8.02	0.81
HB (g/L)	143.31 ± 16.49	145.53 ± 15.52	0.58
RBC (× 10^{12}/L)	4.71 ± 0.57	4.76 ± 0.61	0.84
WBC (× 10^9/L)	6.16 ± 1.58	6.52 ± 1.45	0.18
Platelet (× 10^9/L)	205.79 ± 49.43	195.78 ± 53.33	0.53
BUN (mmol/L)	5.30 ± 1.67	5.19 ± 1.57	0.63
Creatinine (μmol/L)	72.56 ± 15.27	76.69 ± 15.35	0.2
FBG (mmol/L)	5.89 ± 1.20	5.46 ± 1.82	0.43
Total symptom scores	1.87 ± 1.18	1.79 ± 0.45	0.23
Steatosis degree 0/1/2/3 (%)	0/30/56/14	0/37/48/15	0.63

BMI: Body mass index; HR: Heart rate; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HB: Haematoglobin; RBC: Red blood cells; WBC: White blood cells; BUN: Blood urea nitrogen; FBG: Fasting blood glucose.

groups are shown in Table 1. Patients in the two groups were matched for age, sex, body height and weight, BMI, HR, course of disease, blood pressure, and blood tests including transaminase and lipid concentrations. The ultrasound stages of steatosis were also paired (P = 0.63). No patient was classified as grade 0. In group A, 37% of the patients were classified as grade1, 48% as grade 2, and 15% as grade 3, respectively. In group B, 30% of the patients were classified as grade 1, 56% as grade 2, and 14% as grade 3, respectively.

Findings in the two groups before and after treatment

No significant difference was observed in dietary compliance, BMI, blood pressure, HR, HB, RBC, WBC, platelet, serum BUN and creatinine(Cr) between the two groups. At the end of a 24-wk treatment period, a significant improvement in several liver and lipid parameters was observed between the two groups. In particular, total symptom scores, ALT and TG levels decreased more significantly (P < 0.01) in Group A (total symptom score = 1.87 ± 1.18 to 0.42 ± 0.72, ALT = 62.79 ± 35.92 U/L to 39.27 ± 18.94 U/L, TG= 3.94 ± 2.69 mmol/L to 2.08 ± 1.03 mmol/L) than in Group B (total symptom score = 1.87 ± 1.18 to 0.42 ± 0.72, ALT = 79.76 ± 50.59U/L to 42.32 ± 22.23U/L, TG = 3.80 ± 2.85 mmol/L to 2.33 ± 1.42 mmol/L) (Table 2). As compared to the pretreatment values, total symptom score, ALT and TG levels at weeks 8, 12, and 16 decreased significantly in the two groups after treatment (P < 0.01).

As expected, there was a tendency toward improvement in AST, GGT, TCHO, and HDL levels (P < 0.05) in the two groups after treatment. However, no significant difference was observed in the two groups.

Table 2 Liver enzymes, lipid parameters and ultrasound findings before and after treatment in groups A and B (mean ± SD)

Variables	Group A (n = 66)	Group B (n = 68)
At baseline		
Total symptom scores	1.87 ± 1.18	1.79 ± 1.45
Serum ALT (U/L)	62.79 ± 35.92	79.76 ± 50.59
Serum AST (U/L)	38.13 ± 20.99	50.09 ± 39.05
Serum GGT (U/L)	72.53 ± 52.48	79.96 ± 5.27
Serum TCHO (mmol/L)	6.30 ± 0.83	5.91 ± 1.16
Serum TG (mmol/L)	3.94 ± 2.69	3.80 ± 2.85
Serum HDL-C (mmol/L)	1.01 ± 0.24	1.05 ± 0.33
Serum LDL-C (mmol/L)	3.26 ± 0.98	3.19 ± 0.92
Steatosis degree 0/1/2/3 (%)	0/30/56/14	0/37/48/15
At week 8		
Total symptom scores	1.00 ± 0.96^{b,c}	1.23 ± 1.21^b
Serum ALT (U/L)	50.81 ± 35.24^{b,c}	61.09 ± 40.30^b
Serum AST (U/L)	35.25 ± 21.20^a	39.84 ± 23.55^a
Serum GGT (U/L)	57.82 ± 50.22^a	79.97 ± 87.16
Serum TCHO (mmol/L)	5.81 ± 0.81^{a,c}	5.90 ± 0.97^a
Serum TG (mmol/L)	2.86 ± 1.49^b	3.17 ± 2.80^b
Serum HDL-C (mmol/L)	1.04 ± 0.19	1.13 ± 0.24
Serum LDL-C (mmol/L)	3.16 ± 0.80^a	3.16 ± 0.85
At week 12		
Total symptom scores	0.66 ± 0.87^{b,d}	0.97 ± 1.10^b
Serum ALT (U/L)	47.48 ± 33.30^{b,c}	55.17 ± 43.15^b
Serum AST (U/L)	30.69 ± 16.80^a	37.26 ± 19.57^a
Serum GGT (U/L)	46.94 ± 35.38^b	72.87 ± 73.30^a
Serum TCHO (mmol/L)	5.66 ± 1.18^a	5.74 ± 1.14^a
Serum TG (mmol/L)	2.47 ± 1.75^{a,c}	2.80 ± 2.57^a
Serum HDL-C (mmol/L)	1.12 ± 0.24^a	1.15 ± 0.28^a
Serum LDL-C (mmol/L)	3.10 ± 0.98^a	3.14 ± 0.85
Steatosis degree 0/1/2/3 (%)	9/64/21/6^a	9/59/26/6^a
At week 16		
Total symptom scores	0.40 ± 0.60^{b,d}	0.77 ± 1.07^b
Serum ALT (U/L)	45.06 ± 34.23^{b,c}	44.13 ± 33.15^b
Serum AST (U/L)	30.18 ± 15.40^a	32.03 ± 16.51^a
Serum GGT (U/L)	44.34 ± 39.50^b	62.86 ± 78.00^a
Serum TCHO (mmol/L)	5.69 ± 0.99^a	5.68 ± 0.99^a
Serum TG (mmol/L)	2.26 ± 1.26^{b,d}	2.64 ± 2.90^b
Serum HDL-C (mmol/L)	1.13 ± 0.22^a	1.22 ± 0.28^a
Serum LDL-C (mmol/L)	3.12 ± 0.83^a	3.12 ± 0.87
At week 24		
Total symptom scores	0.42 ± 0.72^{b,d}	0.53 ± 0.97^b
Serum ALT (U/L)	39.27 ± 18.94^{b,d}	42.32 ± 22.23^b
Serum AST (U/L)	30.45 ± 12.67^a	30.25 ± 14.21^a
Serum GGT (U/L)	42.47 ± 26.84^b	58.43 ± 36.21^b
Serum TCHO (mmol/L)	5.08 ± 0.76^a	5.21 ± 1.22^a
Serum TG (mmol/L)	2.08 ± 1.03^{b,d}	2.33 ± 1.42^b
Serum HDL-C (mmol/L)	1.25 ± 0.25^a	1.20 ± 0.21^a
Serum LDL-C (mmol/L)	3.12 ± 0.84^a	3.11 ± 0.78
Steatosis degree 0/1/2/3 (%)	20/64/12/4^{b,d}	7/51/36/6^a

TCHO: Total cholesterol; TG: Triglycerides; HDL-C: High density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol. ^aP < 0.05, ^bP < 0.01 *vs* baseline; ^cP < 0.05, ^dP < 0.01 *vs* group B.

Significant improvements were found in serum LDL-C levels of group A (P < 0.05), but not found in group B after treatment.

Ultrasonography showed a normal liver echopattern at the end of treatment in 19.70% (13/66) of the patients, and an overall reduction in 53.03% (35/66) of the patients in group A. In contrast, only five patients (7.35%, 5/68) achieved complete regression (P = 0.04), whereas 24 patients (35.29%, 24/68) had a certain reduction (P = 0.04) in group B. No change was

observed in the remaining 64.71% of patients.

Gastrointestinal complaints of increased fecal frequency, epigastria, and defecation were occasionally noted in 8 of the 134 patients; but these adverse effects were not significantly different in the two groups. The patients recovered when they completed the treatment. Most of the patients who completed the treatment had no adverse events, indicating that they can tolerate the treatment. No severe adverse event was observed.

DISCUSSION

NAFLD is a chronic disease with multiple consequences. The spectrum of this disease ranges from simple steatosis to NASH, which may lead to liver fibrosis and cirrhosis[11-13]. It has also been well established that NAFLD is intimately related to various clinical and biological markers of the insulin resistance syndrome[14-16]. The pathogenesis of NASH is multifactorial, including insulin resistance, excessive intracellular fatty acids, oxidant stress, mitochondrial dysfunction and innate immunity[17]. However, the pathogenesis of NAFLD/NASH is yet to be clearly elucidated. Since the most prevailing general theory is the "two-hit" hypothesis proposed by Day and James in 1998[16], most treatment modalities should be focused on improving the "two-hit" hypothesis or insulin resistance[18].

Currently, therapeutic options are limited. The present "gold standard" for NAFLD is weight reduction, or more precisely, a reduction in central obesity so as to reverse insulin resistance[19-21]. Standard practice advocates weight loss and exercise. Such "lifestyle adjustment" or anti-obesity measures (including bariatric surgery when required) can improve insulin sensitivity with only a modest weight loss (2-8 kg)[22-24], which is difficult for most patients to achieve.

It was reported that dietary supplementation with fatty acids, such as fish and fish oils, can improve NAFLD associated with hyperlipidemia by modifying the function of platelets and leukocytes[25-26]. Suggested modes of action are through their modulation of eicosanoid synthesis and reduction in plasma TG concentration. The fat composition of seal oils differs significantly from that of fish. In marine mammals, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are found mainly at the sn-1 and sn-3 positions of TG, whereas in fish, these fatty acids are positioned in sn-2, which may display the better effects on NAFLD associated with hyperlipidemia than fish oils.

Increased fat intake with an excessive amount of n-6 fatty acids can promote NAFLD. However, n-3 PUFA can ameliorate experimental NAFLD[27]. It was reported that n-3 PUFA from different animals is effective against NAFLD[28-30]. In this study, we evaluated the efficacy and safety of n-3 fatty acids from seal oils in 144 patients with NAFLD associated with hyperlipidemia. The results showed that treatment of NAFLD patients with hyperlipidemia with n-3 PUFA from seal oils significantly reduced their total symptom score, ALT and lipid levels, and normalized ultrasonographic evidence compared to treatment with the recommended diet alone.

As expected, there was a tendency toward improvement in AST, GGT, TC, and HDL levels ($P < 0.05$) in the two groups after treatment. However, no significant difference was seen in the two groups. Spadaro *et al*[29] also reported that serum GGT, HDL, ALT, and lipid levels are decreased after treatment. In the present study, the values of LDL were significantly improved in group A ($P < 0.05$), but not in group B at different time points and after a 24-wk treatment period. Ultrasonography showed complete fatty liver regression in 19.70% (13/66) of the patients, and an overall reduction in 53.03% (35/66) of the patients in group A. In contrast, only five patients (7.35%, 5/68) achieved complete regression ($P = 0.04$), whereas 24 patients (35.29%, 24/68) had a certain reduction ($P = 0.04$) in group B. No change was observed in the remaining 64.71% of patients.

Tanaka *et al*[31] reported that treatment with EPA, one of the major components of n-3 PUFA, seems to be safe and efficacious for patients with NASH, largely due to its anti-inflammatory and anti-oxidative properties. On the other hand, n-3 PUFA could reduce VLDL production, resulting in decreased serum triglyceride levels[32-34]. These findings are consistent with our findings, such as improvement in total symptom score, ALT and lipid levels and normalization of ultrasonographic evidence in patients with NAFLD associated with hyperlipidemia. Improvement in serum biochemistry parameters was also observed in group B, indicating that restricted diet and exercise can reverse insulin resistance at a certain extent[35-37]. In the present study, the two drugs (placebo and seal oils) appeared to be safe and effective in patients with NAFLD associated with hyperlipidemia and no severe side effects were observed during treatment.

The gold standard for diagnosis of NAFLD is liver biopsy, but it is not frequently performed in NAFLD patients due to its low acceptance rate[38]. In our study, ultrasonography was performed to detect and monitor changes in the liver since it is sensitive, cheap, invasive and easy to perform. However, lack of histological findings is a major drawback of this investigation.

In conclusion, treatment of NAFLD associated with hyperlipidemia with PUFA from seal oils seems to be safe and efficacious, and can improve the total symptom score, ALT and lipid levels and normalization of ultrasonographic evidence. Further study is needed to confirm these results.

COMMENTS

Background

Recent reports suggest that many cases of cryptogenic liver cirrhosis may be related to unrecognized nonalcoholic steatohepatitis (NASH); however, medical therapy for nonalcoholic fatty liver disease (NAFLD) and NASH has been disappointing to date. The only recommended therapies are dietary modification and weight loss. N-3 polyunsaturated fatty acids (PUFA) seems to be efficacious on treating NAFLD from animal and some small samples human studies.

Research frontiers

The present "gold standard" for treatment of NAFLD is a reduction in central obesity so as to reverse insulin resistance. Several small samples randomized trails have suggested n-3 PUFA from different animals were effective in the treatment of NAFLD; we explored whether seal oil is efficacious and safe in large samples NAFLD patients.

Innovations and breakthroughs

Total symptom scores in NAFLD patients, and large samples were observed besides biochemical indicators and ultrasonography in this study. Seal oils n-3 PUFA can improve liver enzyme, serum lipid levels and normalization of ultrasonographic evidence.

Applications

Seal oils PUFA administration seems to be safe and efficacious for patients with NAFLD associated with hyperlipidemia as well as the "lifestyle adjustment" or anti-obesity measures.

Terminology

NAFLD refers to the presence of hepatic steatosis not associated with a significant intake of ethanol. Insulin resistance is central to the pathogenesis of NAFLD; thus obesity, diabetes, and the metabolic syndrome are frequently associated with the disease.

Peer review

This is an interesting study. Further details need to be given as to the precise ultrasound scoring system that was used to assess the resolution of hepatic steatosis. Was there an objective scoring system used or was this all subjective? The paper is otherwise well written and merits publication.

REFERENCES

1 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231

2 **Zhou YJ**, Li YY, Nie YQ, Ma JX, Lu LG, Shi SL, Chen MH, Hu PJ. Prevalence of fatty liver disease and its risk factors in the population of South China. *World J Gastroenterol* 2007; **13**: 6419-6424

3 **Nagata K**, Suzuki H, Sakaguchi S. Common pathogenic mechanism in development progression of liver injury caused by non-alcoholic or alcoholic steatohepatitis. *J Toxicol Sci* 2007; **32**: 453-468

4 **Moscatiello S**, Marzocchi R, Villanova N, Bugianesi E, Marchesini G. Which treatment for nonalcoholic fatty liver disease? *Mini Rev Med Chem* 2008; **8**: 767-775

5 **Okita M**, Hayashi M, Sasagawa T, Takagi K, Suzuki K, Kinoyama S, Ito T, Yamada G. Effect of a moderately energy-restricted diet on obese patients with fatty liver. *Nutrition* 2001; **17**: 542-547

6 **Hatzitolios A**, Savopoulos C, Lazaraki G, Sidiropoulos I, Haritanti P, Lefkopoulos A, Karagiannopoulou G, Tzioufa V, Dimitrios K. Efficacy of omega-3 fatty acids, atorvastatin and orlistat in non-alcoholic fatty liver disease with dyslipidemia. *Indian J Gastroenterol* 2004; **23**: 131-134

7 **Song BJ**, Moon KH, Olsson NU, Salem N Jr. Prevention of alcoholic fatty liver and mitochondrial dysfunction in the rat by long-chain polyunsaturated fatty acids. *J Hepatol* 2008; **49**: 262-273

8 **Svegliati-Baroni G**, Candelaresi C, Saccomanno S, Ferretti G, Bachetti T, Marzioni M, De Minicis S, Nobili L, Salzano R, Omenetti A, Pacetti D, Sigmund S, Benedetti A, Casini A. A model of insulin resistance and nonalcoholic steatohepatitis in rats: role of peroxisome proliferator-activated receptor-alpha and n-3 polyunsaturated fatty acid treatment on liver injury. *Am J Pathol* 2006; **169**: 846-860

9 **Zelber-Sagi S**, Nitzan-Kaluski D, Goldsmith R, Webb M, Blendis L, Halpern Z, Oren R. Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): a population based study. *J Hepatol* 2007; **47**: 711-717

10 **Graif M**, Yanuka M, Baraz M, Blank A, Moshkovitz M, Kessler A, Gilat T, Weiss J, Walach E, Amazeen P, Irving CS. Quantitative estimation of attenuation in ultrasound video images: correlation with histology in diffuse liver disease. *Invest Radiol* 2000; **35**: 319-324

11 **Cortez-Pinto H**, Jesus L, Barros H, Lopes C, Moura MC, Camilo ME. How different is the dietary pattern in non-alcoholic steatohepatitis patients? *Clin Nutr* 2006; **25**: 816-823

12 **Oliveira CP**, Coelho AM, Barbeiro HV, Lima VM, Soriano F, Ribeiro C, Molan NA, Alves VA, Souza HP, Machado MC, Carrilho FJ. Liver mitochondrial dysfunction and oxidative stress in the pathogenesis of experimental nonalcoholic fatty liver disease. *Braz J Med Biol Res* 2006; **39**: 189-194

13 **Alwayn IP**, Gura K, Nose V, Zausche B, Javid P, Garza J, Verbesey J, Voss S, Ollero M, Andersson C, Bistrian B, Folkman J, Puder M. Omega-3 fatty acid supplementation prevents hepatic steatosis in a murine model of nonalcoholic fatty liver disease. *Pediatr Res* 2005; **57**: 445-452

14 **Mendez-Sanchez N**, Arrese M, Zamora-Valdes D, Uribe M. Treating nonalcoholic fatty liver disease. *Liver Int* 2007; **27**: 1157-1165

15 **Harrison SA**, Kadakia S, Lang KA, Schenker S. Nonalcoholic steatohepatitis: what we know in the new millennium. *Am J Gastroenterol* 2002; **97**: 2714-2724

16 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845

17 **Ma X**, Li Z. Pathogenesis of nonalcoholic steatohepatitis (NASH). *Chin J Dig Dis* 2006; **7**: 7-11

18 **Preiss D**, Sattar N. Non-alcoholic fatty liver disease: an overview of prevalence, diagnosis, pathogenesis and treatment considerations. *Clin Sci* (Lond) 2008; **115**: 141-150

19 **Larter CZ**, Farrell GC. Insulin resistance, adiponectin, cytokines in NASH: Which is the best target to treat? *J Hepatol* 2006; **44**: 253-261

20 **Palasciano G**, Moschetta A, Palmieri VO, Grattagliano I, Iacobellis G, Portincasa P. Non-alcoholic fatty liver disease in the metabolic syndrome. *Curr Pharm Des* 2007; **13**: 2193-2198

21 **Boppidi H**, Daram SR. Nonalcoholic fatty liver disease: hepatic manifestation of obesity and the metabolic syndrome. *Postgrad Med* 2008; **120**: E01-E07

22 **Oh MK**, Winn J, Poordad F. Review article: diagnosis and treatment of non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2008; **28**: 503-522

23 **Schreuder TC**, Verwer BJ, van Nieuwkerk CM, Mulder CJ. Nonalcoholic fatty liver disease: An overview of current insights in pathogenesis, diagnosis and treatment. *World J Gastroenterol* 2008; **14**: 2474-2486

24 **Krasnoff JB**, Painter PL, Wallace JP, Bass NM, Merriman RB. Health-related fitness and physical activity in patients with nonalcoholic fatty liver disease. *Hepatology* 2008; **47**: 1158-1166

25 **Vognild E**, Elvevoll EO, Brox J, Olsen RL, Barstad H, Aursand M, Osterud B. Effects of dietary marine oils and olive oil on fatty acid composition, platelet membrane fluidity, platelet responses, and serum lipids in healthy humans. *Lipids* 1998; **33**: 427-436

26 **Puri P**, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, Sargeant C, Contos MJ, Sanyal AJ. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 2007; **46**: 1081-1090

27 **El-Badry AM**, Graf R, Clavien PA. Omega 3 - Omega 6: What is right for the liver? *J Hepatol* 2007; **47**: 718-725

28 **Allard JP**, Aghdassi E, Mohammed S, Raman M, Avand G, Arendt BM, Jalali P, Kandasamy T, Prayitno N, Sherman M, Guindi M, Ma DW, Heathcote JE. Nutritional assessment and hepatic fatty acid composition in non-alcoholic fatty liver disease (NAFLD): a cross-sectional study. *J Hepatol* 2008; **48**: 300-307

29 **Spadaro L**, Magliocco O, Spampinato D, Piro S, Oliveri C, Alagona C, Papa G, Rabuazzo AM, Purrello F. Effects of n-3 polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease. *Dig Liver Dis* 2008; **40**: 194-199

30 **Capanni M**, Calella F, Biagini MR, Genise S, Raimondi L, Bedogni G, Svegliati-Baroni G, Sofi F, Milani S, Abbate R, Surrenti C, Casini A. Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in

patients with non-alcoholic fatty liver disease: a pilot study. *Aliment Pharmacol Ther* 2006; **23**: 1143-1151

31 **Tanaka N**, Sano K, Horiuchi A, Tanaka E, Kiyosawa K, Aoyama T. Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis. *J Clin Gastroenterol* 2008; **42**: 413-418

32 **Murano Y**, Funabashi T, Sekine S, Aoyama T, Takeuchi H. Effect of dietary lard containing higher alpha-linolenic acid on plasma triacylglycerol in rats. *J Oleo Sci* 2007; **56**: 361-367

33 **Vernaglione L**, Cristofano C, Chimienti S. Omega-3 polyunsaturated fatty acids and proxies of cardiovascular disease in hemodialysis: a prospective cohort study. *J Nephrol* 2008; **21**: 99-105

34 **Elizondo A**, Araya J, Rodrigo R, Poniachik J, Csendes A, Maluenda F, Diaz JC, Signorini C, Sgherri C, Comporti M, Videla LA. Polyunsaturated fatty acid pattern in liver and erythrocyte phospholipids from obese patients. *Obesity* (Silver Spring) 2007; **15**: 24-31

35 **Ueno T**, Sugawara H, Sujaku K, Hashimoto O, Tsuji R, Tamaki S, Torimura T, Inuzuka S, Sata M, Tanikawa K. Therapeutic effects of restricted diet and exercise in obese patients with fatty liver. *J Hepatol* 1997; **27**: 103-107

36 **Huang MA**, Greenson JK, Chao C, Anderson L, Peterman D, Jacobson J, Emick D, Lok AS, Conjeevaram HS. One-year intense nutritional counseling results in histological improvement in patients with non-alcoholic steatohepatitis: a pilot study. *Am J Gastroenterol* 2005; **100**: 1072-1081

37 **Harrison SA**, Fincke C, Helinski D, Torgerson S, Hayashi P. A pilot study of orlistat treatment in obese, non-alcoholic steatohepatitis patients. *Aliment Pharmacol Ther* 2004; **20**: 623-628

38 **Wieckowska A**, McCullough AJ, Feldstein AE. Noninvasive diagnosis and monitoring of nonalcoholic steatohepatitis: present and future. *Hepatology* 2007; **46**: 582-589

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RAPID COMMUNICATION

ERCC1 polymorphism, expression and clinical outcome of oxaliplatin-based adjuvant chemotherapy in gastric cancer

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Abstract

AIM: To determine the influence of excision repair cross complementing group 1 (*ERCC1*) codon 118 polymorphism and mRNA level on the clinical outcome of gastric cancer patients treated with oxaliplatin-based adjuvant chemotherapy.

METHODS: Eighty-nine gastric cancer patients treated with oxalipatin-based adjuvant chemotherapy were included in this study. *ERCC1* codon 118 C/T polymorphism was tested by polymerase chain reaction-ligation detection reaction (PCR-LDR) method in peripheral blood lymphocytes of those patients; and the intratumoral *ERCC1* mRNA expression was measured using reverse transcription PCR in 62 patients whose tumor tissue specimens were available.

RESULTS: No significant relationship was found between *ERCC1* codon 118 polymorphism and *ERCC1* mRNA level. The median relapse-free and overall survival period was 20.1 mo and 28.4 mo, respectively. The relapse-free and overall survivals in patients with low levels of *ERCC1* mRNA were significantly longer than those in patients with high levels ($P < 0.05$), while there was no significant association found between *ERCC1* 118 genotypes and the disease prognosis. Multivariate analysis also showed that *ERCC1* mRNA level was a potential predictor for relapse and survival in gastric cancer patients treated with oxaliplatin-based adjuvant chemotherapy ($P < 0.05$).

CONCLUSION: *ERCC1* codon 118 polymorphism has no significant impact on *ERCC1* mRNA expression, and the intratumoral *ERCC1* mRNA level but not codon 118 polymorphism may be a useful predictive parameter for the relapse and survival of gastric cancer patients receiving oxaliplatin-based adjuvant chemotherapy.

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Key words: Gastric cancer; Adjuvant chemotherapy; Excision repair cross complementing group 1; Gene polymorphism

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INTRODUCTION

In China, gastric cancer is the leading cause of cancer deaths, accounting for nearly one-fourth of all cancer deaths. Surgery is the primary modality for managing early-stage and locally-advanced disease. However, even after gastrectomy, the majority of patients develop local or distant recurrence[1]. Adjuvant chemotherapy for gastric cancer has been under clinical investigation for more than four decades. Fluoropyrimidines, platinum-drugs and taxanes were shown to be effective in the treatment of gastric cancer. However, the response rates of these drugs or their combinations were less than 50%[2,3]. There is no standard regimen for postoperative treatment at the moment. Having an effective assay to predict the response to a given chemotherapeutic protocol beforehand would greatly enhance the success rate as well as the life quality of the patients.

The nucleotide excision repair (NER) system plays a significant role in repairing a variety of distorting

lesions, including platinum-drug induced DNA adducts. Oxaliplatin is a platinum-based therapeutic agent that has shown anti-tumor activities in gastric cancer. Resistance to oxaliplatin has been attributed to enhanced tolerance and repair of DNA damage through the NER pathway. As an excision nuclease within the NER pathway, excision repair cross complementing group 1 (*ERCC1*) has been reported to play a major role in the response to platinum-based chemotherapy. Studies have shown that the higher the *ERCC1* expression levels, the less sensitive the tumors to platinum therapies[4-7]. Recently, a single nucleotide polymorphism at codon 118 (C→T) was reported to be associated with altered *ERCC1* mRNA levels[8] and clinical outcome in cancer patients treated with platinum-based chemotherapy[9-12]. However, the results about the relationship among *ERCC1* codon 118 polymorphism, *ERCC1* mRNA level and platinum sensitivity are controversial. In this study, we investigated whether the *ERCC1* codon 118 polymorphism could influence *ERCC1* mRNA expression, and whether the polymorphism, and the intratumoral *ERCC1* mRNA expression have the prognostic value for the gastric cancer patients receiving oxaliplatin-based adjuvant treatment.

MATERIALS AND METHODS

Patients

From June 2001 to March 2006, 89 patients with histologically confirmed gastric cancer were enrolled in this study at the 4th Affiliated Hospital of Suzhou University. Inclusion criteria included: (1) patients without early recurrence or incurable resection, (2) patients receiving no other adjuvant treatment, such as radiotherapy or immunotherapy, and (3) patients with their performance status score of 0-1 and a life expectancy over 6 mo. All those patients received radical surgery, and then were treated with at least four cycles of oxaliplatin-based adjuvant treatment, including 70 with 5-FU/leucovorin/oxaliplatin (FOLFOX4: oxaliplatin 85 mg/m^2 and leucovorin 400 mg/m^2 followed on days 1 and 2 by 5-FU 400 mg/m^2 intravenous (IV) bolus, then 600 mg/m^2 IV over 22-h continuous infusion, and repeated every 2 wk), 9 with 5-FU/leucovorin/oxaliplatin/other regimens (taxanes or hydroxycamptothecin) (paclitaxel 135 mg/m^2 or docetaxol 75 mg/m^2 on day 1, hydroxycamptothecin 8 mg/m^2 on days 1-5; and the usage of 5-FU and leucovorin was the same as that in FOLFOX4). If patients had hematologic toxic effects of -grade 3 or grade 4 or nonhematologic toxic effects of grades 2-4, their daily dose was reduced properly.

Blood samples were collected in EDTA-containing tubes from gastric cancer patients before surgery or chemotherapy, and tumor tissue samples were obtained during surgery, and stored in liquid nitrogen until preparation of RNA extracts. Follow-up of those patients was made at 3-mo intervals after chemotherapy at outpatient clinics or by routine phone calls. This study was approved by the ethics and research committee of our hospital.

Table 1 The sequences of primers and probes

Primers or probes	Sequences (5'-3')	Length of product (bp)
actin-U	AGAAGATGACCCAGATCATGTT	290
actin-L	CTTAATGTCACGCACGATTTCC	
ERCC1-U	TACCACAACCTGCACCCAGACTAC	321
ERCC1-L	CTGACTGTCCGTTTTGTTGACTGA	
ERCC1-118-U	GGTCATCCCTATTGATGGCTTCTG	154
ERCC1-118-L	AGCTCACCTGAGGAACAGGGCACAG	
ERCC1-118-P	p-TTGCGCACGAACTTCAGTACGGGAT GGGACACTAATCGGAGGATTA-FAM	
ERCC1-118-T	CTACGGAG GATTATGAGGAGCTGCGT CGCCAAATTCCCAGGGCACA	92
ERCC1-118-C	CTACGAAATCAGGAGGATTATGAGGA GACGTCGCCAAATTCCCAGGG CACG	97

Genotyping of ERCC1 codon 118 polymorphism

Genomic DNA was isolated from peripheral blood lymphocytes using Axygene genomic DNA purification kit (Axygen Biotechnology, China). The primers and probes are listed in Table 1. Genotyping of *ERCC1* codon 118 was performed using polymerase chain reaction-ligation detection reaction (PCR-LDR) method as described previously[13].

Relative quantitative analysis of ERCC1 mRNA using reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 62 tumor tissues using Trizol (Invitrogen Corporation, CA) according to the manufacturer's instructions. The amount of total RNA was estimated by ultraviolet absorbance at 260 nm, and the quality was determined by agarose gel electrophoresis in the presence of formaldehyde. cDNA strand synthesis was performed using a reverse transcription system (Promega Corporation, US).

ERCC1 and an internal reference gene (β-*actin*) cDNA fragments were amplified separately by PCR in triplicates. The PCRs were carried out in a total volume of 25 μL including 2 μL cDNA, 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.5 μmol/L each primer, 1 U hot-start Taq DNA polymerase (QIAgen). Cycling parameters were as follows: 95℃ for 15 min; 35 cycles of 94℃ for 40 s, 52℃ for 30 s, and 72℃ for 30 s; and a final extension step at 72℃ for 10 min. PCR products were analyzed by 2% agarose gel electrophoresis, and ethidium bromide staining following by visualization with ultraviolet illumination using a gel imaging analyzing system. *ERCC1* amplification products were calculated as a ratio of the gray scale of *ERCC1* to that of β-*actin*.

Statistical analysis

Data analysis was performed using SPSS 13.0 for Windows. *ERCC1* levels were categorized into a low and high value using the median concentration as a cut-off point. The relationship between the genotype frequencies, mRNA, levels and clinical characteristics were assessed by χ^2 or Fisher's exact probability tests. The Mann-Whitney *U* test was used to assess the correlation between *ERCC1* genotypes and mRNA levels. Relapse-free survival (RFS) was defined as the

Table 2 Relationship among *ERCC1* genotypes, mRNA expression and clinical characteristics of gastric cancer

Clinical characteristics	*n*	*ERCC1* polymorphism		χ^2	*P*	*ERCC1* mRNA		χ^2	*P*
		C/C (*n* = 45)	C/T + T/T (*n* = 44)			High value (*n* = 31)	Low value (*n* = 31)		
Age (yr)									
⩾ 58	48	25	23	0.096	0.833	18	18	0	1.000
< 58	41	20	21			13	13		
Gender									
Male	66	31	35	1.318	0.334	20	24	0.253	0.402
Female	23	14	9			11	7		
TNM stage									
I - II	19	13	6	3.082	0.079	6	6	0	1.000
III-IV	70	31	39			25	25		
Grading									
G2	45	20	24	0.552	0.527	15	19	1.042	0.444
G3	44	21	20			16	12		

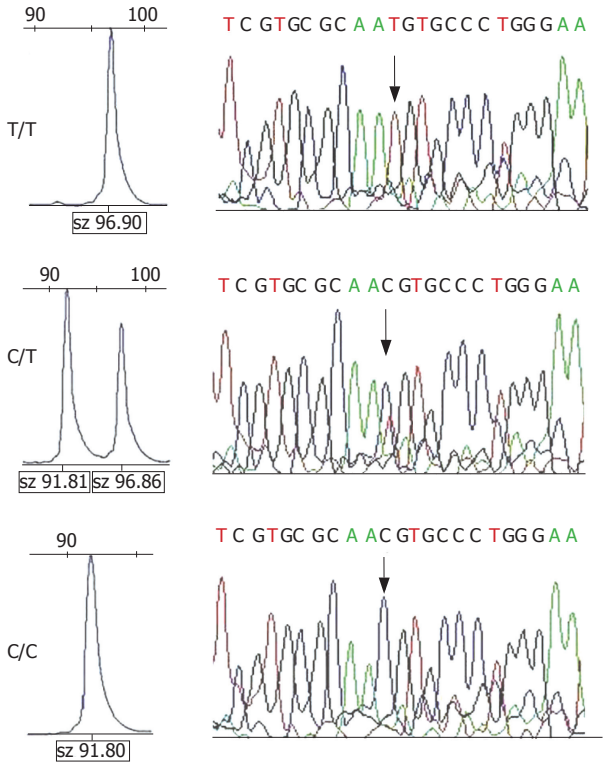

Figure 1 Genotyping results of *ERCC1* codon 118 polymorphism electrophoresis results of PCR-LDR products with different genotypes and its sequencing results. LDR products of *ERCC1* 118 C/C and T/T were 92 and 97 base pairs. The SNP sites are indicated by the arrowhead. The results were completely matched to the corresponding results derived from PCR-LDR.

time interval between the date of surgery and the date of confirmed relapse or the date of last follow-up. Overall survival (OS) was defined as the time between surgery and death. Survival curves were generated by the Kaplan-Meier method, and verified by the log-rank test. Cox proportional hazards regression analysis was used to estimate odds ratios (ORs) and their 95% confidence intervals (CIs), representing the overall relative risk of relapse and death associated with *ERCC1* polymorphism or expression, and to adjust for potential confounding variables. All of the values were two-sided and statistical significance was defined as $P < 0.05$.

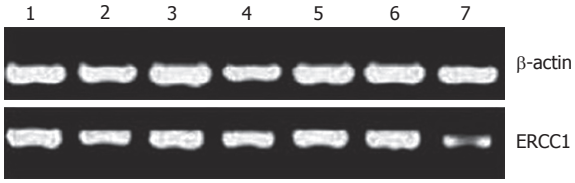

Figure 2 RT-PCR results of *ERCC1* mRNA in gastric cancer tissues.

RESULTS

ERCC1 genotypes and mRNA expression

A total of 89 patients were analyzed. Their demographic and disease characteristics are shown in Table 2. The allelic discrimination data from PCR-LDR assay were confirmed by direct sequencing of representative PCR products (Figure 1). Of the 89 patients, the frequencies of *ERCC1* codon 118 C/C, C/T and T/T were 50.6% (45/89), 42.7% (38/89) and 6.7% (6/89); and the allele frequencies of A and T were 71.9% and 28.1%, respectively. Genotype distribution of *ERCC1* codon 118 was consistent with the Hardy-Weinberg equilibrium among patients ($\chi^2 = 0.288$, $P > 0.05$).

Gastric cancer tissue samples were available in 62 patients. The intratumoral expression of *ERCC1* mRNA in those tissues was tested by semi-quantitative RT-PCR (Figure 2). A marked inter-individual variation in *ERCC1* mRNA expression in the 62 samples was observed: *ERCC1*/β-*actin* ratios ranged from 0.087 to 1.006 with a median value of 0.672. The median value was assigned as the cut-off value to divide those 62 patients into two groups with high or low *ERCC1* mRNA values.

No significant relationship was found between *ERCC1* expression and *ERCC1* codon 118 genotypes (the median *ERCC1* expression was 0.680 for C/C and 0.665 for C/T + T/T; $Z = -0.592$, $P = 0.554$) (Figure 3).

No significant association was found between age, gender, stage or grading and *ERCC1* codon 118 polymorphism or mRNA levels, except that a trend was found between the polymorphism and stage ($P = 0.079$) (Table 2).

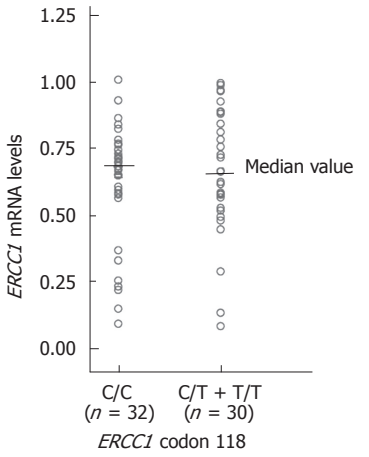

Figure 3 Relationship between *ERCC1* mRNA levels and codon 118 polymorphism.

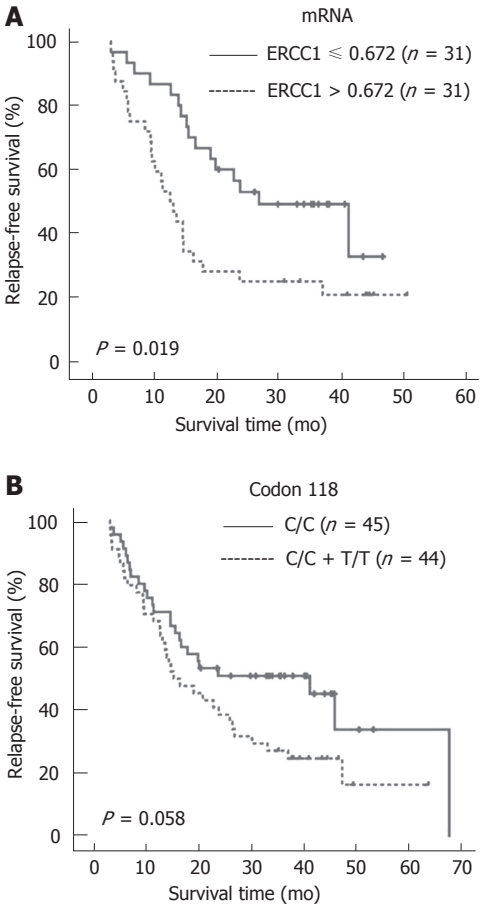

Figure 4 Relapse-free survival curves of gastric cancer patients according to *ERCC1* mRNA expression and *ERCC1* 118 C/T polymorphism relapse-free survival curves according to *ERCC1* mRNA expression (A) or *ERCC1* 118 polymorphism (B). The relapse-free survival in patients with high levels of *ERCC1* mRNA (> 0.672) was significantly poorer than that in patients with low levels (⩽ 0.672) ($P < 0.05$), while there was no significant difference between patients with *ERCC1* 118 C/C and variant genotypes (T/T or C/T).

Associations between ERCC1 polymorphism, mRNA levels and clinical outcome

Patients with the C/C genotype showed a trend towards correlation with prolonged RFS when compared to those with the C/T + T/T genotypes (22.0 mo *vs* 16.5 mo, χ^2 = 3.602, $P = 0.058$, Figure 4A). The median OS was 29.8 (95% CI = 20.9-83.1) mo for the patients with the C/C genotype, and 26.4 (95% CI = 22.1-34.7) mo in those with the C/T or T/T genotype ($\chi^2 = 1.548$, $P = 0.214$) (Figure 5A). The median RFS was 23.8 mo in patients with low *ERCC1* values, but only 13.2 mo in patients with high *ERCC1* levels ($\chi^2 = 5.464$, $P = 0.019$) (Figure 4B). A significant difference in OS also was found between the groups with low *ERCC1* levels and high *ERCC1* levels (29.6 mo *vs* 18.7 mo, $\chi^2 = 4.546$, $P = 0.033$) (Figure 5B).

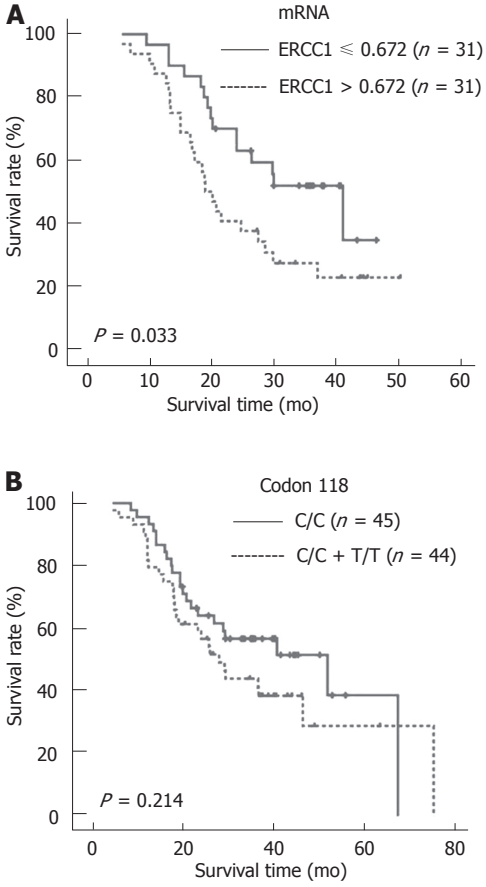

Figure 5 Overall survival curves of gastric cancer patients according to *ERCC1* mRNA expression and *ERCC1* 118 C/T polymorphism survival curves according to *ERCC1* mRNA levels (A) or *ERCC1* 118 polymorphism (B). The overall survival in patients with low level of *ERCC1* mRNA was significantly longer than that in patients with high levels ($P < 0.05$), while there was no significant difference found between patients with *ERCC1* 118 C/C and variant genotypes (T/T or C/T).

Cox multivariate analysis showed that, after adjustment for age, gender, stage and grading, a high *ERCC1* mRNA level appeared to be an independent risk factor for RFS (adjusted OR = 2.493, 95% CI: 1.291-4.814, $P = 0.006$) and OS (adjusted OR = 2.449, 95% CI: 1.264-4.743, $P = 0.008$). No significant association was found between *ERCC1* codon 118 genotypes and RFS (adjusted OR = 1.644, 95% CI = 0.954-2.833, $P = 0.074$) or OS (adjusted OR = 1.310, 95% CI = 0.727-2.358, $P = 0.369$).

DISCUSSION

Optimal chemotherapeutic treatment would allow clinicians to maximize the benefits of cancer

chemotherapy. Successful adjuvant chemotherapy following gastrectomy is crucial for a favorable outcome in gastric cancer. However, few prognostic and predictive markers have been identified to individualize treatment, maximize therapeutic effect. The *ERCC1* expression and codon 118 polymorphism have been reported to influence platinum-based drug sensitivity in advanced or metastatic cancers. The aim of this study was to determine whether *ERCC1* codon 118 polymorphism could influence the intratumoral *ERCC1* mRNA level and predict the clinical outcome of gastric cancer patients treated with oxaliplatin-based adjuvant chemotherapy.

Some studies suggested that impaired DNA repair within the tumor could lead to the decreased removal of platinum-DNA adducts and, therefore, increased clinical response to platinum chemotherapy. *ERCC1* mRNA level has been shown to correlate with nucleotide excision repair capacity. Chinese hamster ovary cells, which do not express a functional ERCC1 protein, are more susceptible to platinum-drugs than the parental cell line with normal ERCC1[4]. So it is naturally expected that the higher the levels of *ERCC1* expression, the less susceptible the tumors to platinum agents. Recently, a synonymous polymorphism at codon 118 converting a common codon usage (AAC) to an infrequent one (AAT), both coding for asparagine, has been associated with reduced mRNA and protein levels[8]. However, the assumed relationship between *ERCC1* codon 118 polymorphism and expression was not always observed[14]. In this study, the *ERCC1* mRNA levels in patients with C/C genotype was higher than that in patients with C/T or T/T genotype; but the difference failed to reach statistical significance ($P > 0.05$), which suggested that the polymorphism may have limited impact on *ERCC1* mRNA levels. In addition, the possibility that *ERCC1* codon 118 polymorphism is in linkage disequilibrium with other *ERCC1* mutations or polymorphisms that directly affect its expression also cannot be ruled out. Other possible reasons may be the relatively small sample size of the present study, and the quantitative method for *ERCC1* mRNA expression used in this study. In the future, using more accurate real-time quantitative RT-PCR assay on the large number of patients may help us to give more persuasive data on the putative association.

Although *ERCC1* codon 118 polymorphism has been extensively studied for its involvement in carcinogenesis[15,16], the predictive value of the polymorphism on platinum chemotherapy has not been studied thoroughly. The functional importance of this polymorphism is still under debate. A limited number of studies suggest that the favorable prognosis seems associated with the T allele[12,17-19]; but controversial results also exist[9-11,20,21]. Viguier *et al*[12] and Martinez-Balibrea *et al*[19] found that colorectal cancer patients with the *ERCC1* 118 T/T genotype were more likely to respond to oxaliplatin-based chemotherapy than carriers of the other genotypes. The favorable effect of T/T genotype also was found in lung cancer[22], pancreatic cancer[23], and ovarian cancer[18] patients treated with platinum-based chemotherapy. However, other studies on lung cancer[10] and colorectal cancer[9,11,20,21] showed opposite results. In addition, several studies demonstrated that no clear association was found between *ERCC1* codon 118 polymorphism and platinum sensitivity[24-26]. In a recent study on advanced gastric cancer treated with fluorouracil/cisplatin palliative chemotherapy, a tendency to higher response rate was found in patients with C allele ($P = 0.09$). In this study, patients with the C/C genotype also showed a trend to prolonged RFS when compared to those with the other genotypes, while no significant relationship was found between *ERCC1* codon 118 genotypes and OS. The small sample size ($n = 89$) of the present study might remain a limitation to clarify the exact role of *ERCC1* codon 118 polymorphism. Other possible reasons for controversial results may include genotyping in normal or tumor tissues, variable doses and schedules of platinum-based therapy, different ethnic populations, variable tumor stage and different kind of cancers.

A limitation of the presented study is that we only analyzed germline genotype. The germline genotypes offer better clinical accessibility and applicability, compared to tumor tissue, which presents difficulties in obtaining and handling samples. To analyze somatic genotype from tumor tissues was not easy. It is difficult to purify malignant cells from miscellaneous normal cells in clinical tumor tissue even using laser microdissection. The classification of a certain gene polymorphism may be hampered in a mixture of normal and malignant cells, which has been clearly illustrated by loss of heterozygosity. The correlation between germline genotype from peripheral blood and tumor tissue should be considered. To the best of our knowledge, there are no related reports on the impact of LOH on *ERCC1* polymorphism in gastric cancer. Hence, the possible influence of LOH on *ERCC1* genotyping should be discussed in the future.

Relative consensus conclusions were obtained regarding the effect of *ERCC1* expression on platinum-drug sensitivity. A multi-centers study on non–small-cell lung cancer found that cisplatin-based adjuvant chemotherapy significantly prolonged survival among patients with ERCC1-negative tumors, but not among patients with ERCC1-positive tumors[5]. In advanced gastric cancer patients treated with 5-FU and oxaliplatin, favorable response rate and survival were also found in patients without ERCC1 protein expression[7]. Other studies showed that the low intratumoral *ERCC1* mRNA expression was associated with favorable clinical outcomes after treatment with platinum-based chemotherapy in lung cancer[27,28], colorectal cancer[29], gastric cancer[30-32], ovarian cancer[33], bladder cancer[34], head and neck cancer[35]. A recent phase Ⅲ trial in non-small-cell lung cancer also demonstrated that assessment of intratumoral *ERCC1* mRNA expression is feasible in the clinical setting and predicts response to cisplatin[36]. However, most of those studies focused on the influence of *ERCC1* expression on the effect of platinum-drug in advanced or metastatic diseases, little was known about its effect on platinum-drug adjuvant chemotherapy. Our

results suggested that low *ERCC1* mRNA level appeared to be an independent prognostic factor for better prognosis, which is consistent with the results observed in advanced gastric cancer[30-32].

In conclusion, *ERCC1* codon 118 polymorphism has no significant effect on *ERCC1* mRNA expression; and the intratumoral *ERCC1* mRNA level, but not *ERCC1* codon 118 polymorphism may be an important prognostic marker for the clinical outcome of gastric cancer patients treated with oxaliplatin-based adjuvant chemotherapy. Detection of the intratumoral *ERCC1* mRNA expression may give meaningful clinical information with respect to the rational choice platinum compound in the treatment of gastric cancer.

COMMENTS

Background

Oxaliplatin is one of the most effective agents against gastric cancer; its efficacy rate differs greatly among patients. Resistance to oxaliplatin has been attributed to enhanced tolerance and repair of DNA damage. Excision repair cross complementing group 1 (ERCC1) has been reported to play a major role in the response to platinum chemotherapy, but little is know about the effect of *ERCC1* codon 118 polymorphism and expression on clinical outcome of oxaliplatin-based adjuvant chemotherapy in gastric cancer.

Research frontiers

ERCC1 has been reported to play a major role in the response to platinum chemotherapy. Studies have shown that the higher the *ERCC1* expression levels, the less sensitive the tumors to platinum therapies. Recently, a single nucleotide polymorphism at codon 118 (C→T) was reported to be associated with altered *ERCC1* mRNA levels and clinical outcome in cancer patients treated with platinum-based chemotherapy. However, the results about the relationship among *ERCC1* codon 118 polymorphism, *ERCC1* mRNA level and platinum sensitivity are controversial.

Innovations and breakthroughs

No significant relationship was found between *ERCC1* codon 118 polymorphism and *ERCC1* mRNA levels. It is found that *ERCC1* mRNA level but not codon 118 polymorphism was a potential indicator in predicting the relapse and survival of gastric cancer patients treated with oxaliplatin-based adjuvant chemotherapy. To our knowledge, it is the first report to study the effect of intratumoral *ERCC1* expression and *ERCC1* codon 118 polymorphism on clinical outcome of oxaliplatin-based adjuvant chemotherapy in Chinese patients with gastric cancer.

Applications

Controlled, prospective clinical trials are required to confirm our results and to establish the advantage of pre-treatment tumor biopsy for *ERCC1* screening, which permits a more rational decision on whether to precede an oxaliplatin-based adjuvant chemotherapy. So patients who are unlikely to respond may spare unnecessary toxicity and can be treated with alternative drugs.

Terminology

Oxaliplatin is a widely applied medicine for chemotherapy of gastrointestinal cancer. ERCC1 is an important enzyme for DNA repair.

Peer review

This is a good study. Over all the study has clinical relevance for LDLT programmes.

REFERENCES

1 **Macdonald JS**. Treatment of localized gastric cancer. *Semin Oncol* 2004; **31**: 566-573
2 **Carrato A**, Gallego-Plazas J, Guillen-Ponce C. Adjuvant therapy of resected gastric cancer is necessary. *Semin Oncol* 2005; **32**: S105-S108
3 **Hejna M**, Wöhrer S, Schmidinger M, Raderer M. Postoperative chemotherapy for gastric cancer. *Oncologist* 2006; **11**: 136-145
4 **Bramson J**, Panasci LC. Effect of ERCC-1 overexpression on sensitivity of Chinese hamster ovary cells to DNA damaging agents. *Cancer Res* 1993; **53**: 3237-3240
5 **Olaussen KA**, Dunant A, Fouret P, Brambilla E, André F, Haddad V, Taranchon E, Filipits M, Pirker R, Popper HH, Stahel R, Sabatier L, Pignon JP, Tursz T, Le Chevalier T, Soria JC. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med* 2006; **355**: 983-991
6 **Fujii T**, Toyooka S, Ichimura K, Fujiwara Y, Hotta K, Soh J, Suehisa H, Kobayashi N, Aoe M, Yoshino T, Kiura K, Date H. ERCC1 protein expression predicts the response of cisplatin-based neoadjuvant chemotherapy in non-small-cell lung cancer. *Lung Cancer* 2008; **59**: 377-384
7 **Kwon HC**, Roh MS, Oh SY, Kim SH, Kim MC, Kim JS, Kim HJ. Prognostic value of expression of ERCC1, thymidylate synthase, and glutathione S-transferase P1 for 5-fluorouracil/oxaliplatin chemotherapy in advanced gastric cancer. *Ann Oncol* 2007; **18**: 504-509
8 **Yu JJ**, Mu C, Lee KB, Okamoto A, Reed EL, Bostick-Bruton F, Mitchell KC, Reed E. A nucleotide polymorphism in ERCC1 in human ovarian cancer cell lines and tumor tissues. *Mutat Res* 1997; **382**: 13-20
9 **Stoehlmacher J**, Park DJ, Zhang W, Yang D, Groshen S, Zahedy S, Lenz HJ. A multivariate analysis of genomic polymorphisms: prediction of clinical outcome to 5-FU/oxaliplatin combination chemotherapy in refractory colorectal cancer. *Br J Cancer* 2004; **91**: 344-354
10 **Isla D**, Sarries C, Rosell R, Alonso G, Domine M, Taron M, Lopez-Vivanco G, Camps C, Botia M, Nuñez L, Sanchez-Ronco M, Sanchez JJ, Lopez-Brea M, Barneto I, Paredes A, Medina B, Artal A, Lianes P. Single nucleotide polymorphisms and outcome in docetaxel-cisplatin-treated advanced non-small-cell lung cancer. *Ann Oncol* 2004; **15**: 1194-1203
11 **Park DJ**, Zhang W, Stoehlmacher J, Tsao-Wei D, Groshen S, Gil J, Yun J, Sones E, Mallik N, Lenz HJ. ERCC1 gene polymorphism as a predictor for clinical outcome in advanced colorectal cancer patients treated with platinum-based chemotherapy. *Clin Adv Hematol Oncol* 2003; **1**: 162-166
12 **Viguier J**, Boige V, Miquel C, Pocard M, Giraudeau B, Sabourin JC, Ducreux M, Sarasin A, Praz F. ERCC1 codon 118 polymorphism is a predictive factor for the tumor response to oxaliplatin/5-fluorouracil combination chemotherapy in patients with advanced colorectal cancer. *Clin Cancer Res* 2005; **11**: 6212-6217
13 **Huang ZH**, Hua D, Li LH, Zhu JD. Prognostic role of p53 codon 72 polymorphism in gastric cancer patients treated with fluorouracil-based adjuvant chemotherapy. *J Cancer Res Clin Oncol* 2008; **134**: 1129-1134
14 **Park DJ**, Stoehlmacher J, Zhang W, Tsao-Wei D, Groshen S, Zahedy S, Gil J, Mallik N, Lenz HJ. ERCC1 polymorphism is associated with differential ERCC1 mRNA levels. American Association for Cancer Research's 93rd Annual Meeting. 2002 April 6-10
15 **Zhou W**, Liu G, Park S, Wang Z, Wain JC, Lynch TJ, Su L, Christiani DC. Gene-smoking interaction associations for the ERCC1 polymorphisms in the risk of lung cancer. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 491-496
16 **Yin J**, Vogel U, Guo L, Ma Y, Wang H. Lack of association between DNA repair gene ERCC1 polymorphism and risk of lung cancer in a Chinese population. *Cancer Genet Cytogenet* 2006; **164**: 66-70
17 **Suk R**, Gurubhagavatula S, Park S, Zhou W, Su L, Lynch TJ, Wain JC, Neuberg D, Liu G, Christiani DC. Polymorphisms in ERCC1 and grade 3 or 4 toxicity in non-small cell lung cancer patients. *Clin Cancer Res* 2005; **11**: 1534-1538
18 **Steffensen KD**, Waldstrøm M, Jeppesen U, Brandslund I, Jakobsen A. Prediction of response to chemotherapy by ERCC1 immunohistochemistry and ERCC1 polymorphism in ovarian cancer. *Int J Gynecol Cancer* 2008; **18**: 702-710
19 **Martinez-Balibrea E**, Abad A, Aranda E, Sastre J, Manzano JL, Díaz-Rubio E, Gómez-España A, Aparicio J, García T, Maestu I, Martínez-Cardús A, Ginés A, Guino E.

Pharmacogenetic approach for capecitabine or 5-fluorouracil selection to be combined with oxaliplatin as first-line chemotherapy in advanced colorectal cancer. *Eur J Cancer* 2008; **44**: 1229-1237

20 **Ruzzo A**, Graziano F, Loupakis F, Rulli E, Canestrari E, Santini D, Catalano V, Ficarelli R, Maltese P, Bisonni R, Masi G, Schiavon G, Giordani P, Giustini L, Falcone A, Tonini G, Silva R, Mattioli R, Floriani I, Magnani M. Pharmacogenetic profiling in patients with advanced colorectal cancer treated with first-line FOLFOX-4 chemotherapy. *J Clin Oncol* 2007; **25**: 1247-1254

21 **Ryu JS**, Hong YC, Han HS, Lee JE, Kim S, Park YM, Kim YC, Hwang TS. Association between polymorphisms of ERCC1 and XPD and survival in non-small-cell lung cancer patients treated with cisplatin combination chemotherapy. *Lung Cancer* 2004; **44**: 311-316

22 **Zhou W**, Gurubhagavatula S, Liu G, Park S, Neuberg DS, Wain JC, Lynch TJ, Su L, Christiani DC. Excision repair cross-complementation group 1 polymorphism predicts overall survival in advanced non-small cell lung cancer patients treated with platinum-based chemotherapy. *Clin Cancer Res* 2004; **10**: 4939-4943

23 **Kamikozuru H**, Kuramochi H, Hayashi K, Nakajima G, Yamamoto M. ERCC1 codon 118 polymorphism is a useful prognostic marker in patients with pancreatic cancer treated with platinum-based chemotherapy. *Int J Oncol* 2008; **32**: 1091-1096

24 **Tibaldi C**, Giovannetti E, Vasile E, Mey V, Laan AC, Nannizzi S, Di Marsico R, Antonuzzo A, Orlandini C, Ricciardi S, Del Tacca M, Peters GJ, Falcone A, Danesi R. Correlation of CDA, ERCC1, and XPD polymorphisms with response and survival in gemcitabine/cisplatin-treated advanced non-small cell lung cancer patients. *Clin Cancer Res* 2008; **14**: 1797-1803

25 **Ruzzo A**, Graziano F, Kawakami K, Watanabe G, Santini D, Catalano V, Bisonni R, Canestrari E, Ficarelli R, Menichetti ET, Mari D, Testa E, Silva R, Vincenzi B, Giordani P, Cascinu S, Giustini L, Tonini G, Magnani M. Pharmacogenetic profiling and clinical outcome of patients with advanced gastric cancer treated with palliative chemotherapy. *J Clin Oncol* 2006; **24**: 1883-1891

26 **Keam B**, Im SA, Han SW, Ham HS, Kim MA, Oh DY, Lee SH, Kim JH, Kim DW, Kim TY, Heo DS, Kim WH, Bang YJ. Modified FOLFOX-6 chemotherapy in advanced gastric cancer: Results of phase II study and comprehensive analysis of polymorphisms as a predictive and prognostic marker. *BMC Cancer* 2008; **8**: 148

27 **Lord RV**, Brabender J, Gandara D, Alberola V, Camps C, Domine M, Cardenal F, Sánchez JM, Gumerlock PH, Tarón M, Sánchez JJ, Danenberg KD, Danenberg PV, Rosell R. Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. *Clin Cancer Res* 2002; **8**: 2286-2291

28 **Ceppi P**, Volante M, Novello S, Rapa I, Danenberg KD, Danenberg PV, Cambieri A, Selvaggi G, Saviozzi S, Calogero R, Papotti M, Scagliotti GV. ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. *Ann Oncol* 2006; **17**: 1818-1825

29 **Shirota Y**, Stoehlmacher J, Brabender J, Xiong YP, Uetake H, Danenberg KD, Groshen S, Tsao-Wei DD, Danenberg PV, Lenz HJ. ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. *J Clin Oncol* 2001; **19**: 4298-4304

30 **Metzger R**, Leichman CG, Danenberg KD, Danenberg PV, Lenz HJ, Hayashi K, Groshen S, Salonga D, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Konda B, Leichman L. ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. *J Clin Oncol* 1998; **16**: 309-316

31 **Matsubara J**, Nishina T, Yamada Y, Moriwaki T, Shimoda T, Kajiwara T, Nakajima TE, Kato K, Hamaguchi T, Shimada Y, Okayama Y, Oka T, Shirao K. Impacts of excision repair cross-complementing gene 1 (ERCC1), dihydropyrimidine dehydrogenase, and epidermal growth factor receptor on the outcomes of patients with advanced gastric cancer. *Br J Cancer* 2008; **98**: 832-839

32 **Wei J**, Zou Z, Qian X, Ding Y, Xie L, Sanchez JJ, Zhao Y, Feng J, Ling Y, Liu Y, Yu L, Rosell R, Liu B. ERCC1 mRNA levels and survival of advanced gastric cancer patients treated with a modified FOLFOX regimen. *Br J Cancer* 2008; **98**: 1398-1402

33 **Dabholkar M**, Vionnet J, Bostick-Bruton F, Yu JJ, Reed E. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *J Clin Invest* 1994; **94**: 703-708

34 **Bellmunt J**, Paz-Ares L, Cuello M, Cecere FL, Albiol S, Guillem V, Gallardo E, Carles J, Mendez P, de la Cruz JJ, Taron M, Rosell R, Baselga J. Gene expression of ERCC1 as a novel prognostic marker in advanced bladder cancer patients receiving cisplatin-based chemotherapy. *Ann Oncol* 2007; **18**: 522-528

35 **Handra-Luca A**, Hernandez J, Mountzios G, Taranchon E, Lacau-St-Guily J, Soria JC, Fouret P. Excision repair cross complementation group 1 immunohistochemical expression predicts objective response and cancer-specific survival in patients treated by Cisplatin-based induction chemotherapy for locally advanced head and neck squamous cell carcinoma. *Clin Cancer Res* 2007; **13**: 3855-3859

36 **Cobo M**, Isla D, Massuti B, Montes A, Sanchez JM, Provencio M, Viñolas N, Paz-Ares L, Lopez-Vivanco G, Muñoz MA, Felip E, Alberola V, Camps C, Domine M, Sanchez JJ, Sanchez-Ronco M, Danenberg K, Taron M, Gandara D, Rosell R. Customizing cisplatin based on quantitative excision repair cross-complementing 1 mRNA expression: a phase III trial in non-small-cell lung cancer. *J Clin Oncol* 2007; **25**: 2747-2754

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CASE REPORT

Acalculous cholecystitis due to *Salmonella enteritidis*

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Abstract

Acute acalculous cholecystitis (AAC) is defined as an acute inflammation of the gallbladder in the absence of stones. We herein report a case of a young man who developed AAC after a *Salmonella enteritidis* gastrointestinal infection.

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Key words: Cholecystitis; Young adult; Infectious; *Salmonella enteritidis*

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INTRODUCTION

Acute acalculous cholecystitis (AAC) is defined as an acute inflammation of the gallbladder in the absence of stones. Traditionally, it was considered a fatal disease almost exclusively of critical ill patients; however, there are recent reports of cases of AAC affecting less severe patients with good prognosis treated with antibiotics, in the absence of cholecystectomy.

We herein report a case of a young man who developed AAC after a *Salmonella enteritidis* gastrointestinal infection.

CASE REPORT

A 27-year-old man was admitted to hospital with abdominal pain, diarrhoea, persistent vomiting and 38℃ temperature. On physical examination, he was febrile, but in a good state of health. His abdomen was mildly tender to palpation with guarding in his lower right area. Laboratory tests disclosed a white cell count of 5300 × 1000/μL with 50% neutrophils and 32% lymphocytes, and haemoglobin and platelets were normal. The biochemical studies including liver and renal tests, electrolyte panel and coagulation profile, were normal. An abdominal X-ray film showed gas in several loops of a moderately dilated small bowel, and an abdominal sonography disclosed marked mucosal thickening in the right quadrant affecting ileon loops, cecum and ascending colon with small lymph node enlargement; the remainder of the abdominal contents, including the gallbladder were normal. Serology for *Salmonella typhi* H and O, Yersinia and Shigella were negative, as were blood cultures. The coproculture obtained on admission was positive for *Salmonella enteritidis*.

The patient was treated with intravenous fluids, analgesics and antipyretics and became afebrile on the second day; in a week time, the abdominal pain subsided and he was able to restart oral diet so he was discharged from hospital.

The following day he returned to the Emergency Department due to epigastric and right hypochondria pain, nausea and fever. He had no diarrhoea. On physical examination, he presented a temperature of 38℃, a tender upper abdomen, and Murphy's sign. The laboratory tests showed mild normocytic-normochromic anaemia with 7900 × 1000/μL white cells. The biochemical tests were normal. A new abdominal sonography disclosed normal intestinal loops, but his gallbladder was distended and presented a markedly thick wall (7 mm) with no stones, and was surrounded by a little fluid collection.

He was then administered intravenous antibiotics

(ciprofloxacine and metronidazole) and showed no signs of fever on the second day. The abdominal pain slowly subsided. Blood, urine and faeces cultures taken on admission showed negative results. He was discharged 10 d later on oral antibiotics and controlled as an outpatient. A new abdominal ultrasound disclosed a normal gallbladder without lithiasis or sludge.

DISCUSSION

AAC accounts for 5%-14% of all cases of acute cholecystitis[1,2]. Patients tend to be predominantly male and older than 50 years of age.

The pathogenesis of AAC is not well defined as the precise mechanism is unknown to date. It seems that several factors such as ischemia, infection and bile changes are involved. Ryu *et al*[1] found that patients with visceral atherosclerosis may be at increased risk for acute acalculous cholecystitis due to an impaired mucosal resistance. Systemic sepsis with release of mediators and bile stasis with alterations in the chemical composition of bile are another implicated potential pathogenic mechanisms involved. Multiple risk factors such as previous surgery and trauma or burn injury have been associated, but none of them were present in our patient.

However, as in our patient, AAC may also occur from secondary infection of the gallbladder following a systemic infection by bacteria[3], virus[4], parasites[5] or fungi.

AAC due to primary bacterial infection is rare. Several cases have been reported complicating *Salmonella typhi* infection[3,6] and after non-typhoidal salmonellosis[7,8] as well.

During the past two decades, an increase in the number of *Salmonella enteritidis* isolates has been observed even in developed countries[9], and there are also rare complications of this common disease described in medical literature[10] Some of these complications are extra-intestinal such as septic arthritis[11] or meningitis[12], but most of them are intra-abdominal[13] due to blood or lymphatic spread of the gbacteria.

Among the latter, AAC is infrequent and can occur even weeks after the diarrhoea has stopped[13] (our patient was discharged from hospital asymptomatic and developed symptoms 24 h later). The diagnosis is based on clinical symptoms, and ultrasound provides the definite diagnosis.

Salmonella enteritidis can be absent in blood cultures and be cultivated in faeces and bile[8,14]. The bacterium, like any other intestinal pathogen, can not only reach the gallbladder through blood drainage but also directly from the bowel along the bile ducts, as could have been the case in our patient.

Most cases described in literature experienced a bad outcome due to gallbladder gangrene, and perforation[2]. Even with early cholecystectomy in good surgical candidates[2], or cholecystostomy or endoscopy nasobiliary drainage in bad ones[15], the outcomes were bad. However, this has changed as the disease is now described in less severely ill patients with no adverse prognosis factors. In this setting, a 4-6 wk course of broad spectrum antibiotics, as indicated in our patient, is recommended. If symptoms cease and a control ultrasound shows a non-dilated gallbladder with a thin wall, cholecystectomy is not needed.

In conclusion, this case shows that AAC, a rare complication of *Salmonella enteritidis*, can also be present in non-critically ill patients. In this setting, the prognosis is better, cholecystectomy is not always needed and patients treated with a long course of wide spectrum antibiotics can obtain a good prognosis.

REFERENCES

1 **Ryu JK**, Ryu KH, Kim KH. Clinical features of acute acalculous cholecystitis. *J Clin Gastroenterol* 2003; **36**: 166-169
2 **Kalliafas S**, Ziegler DW, Flancbaum L, Choban PS. Acute acalculous cholecystitis: incidence, risk factors, diagnosis, and outcome. *Am Surg* 1998; **64**: 471-475
3 **Avalos ME**, Cerulli MA, Lee RS. Acalculous acute cholecystitis due to Salmonella typhi. *Dig Dis Sci* 1992; **37**: 1772-1775
4 **Basar O**, Kisacik B, Bozdogan E, Yolcu OF, Ertugrul I, Koklu S. An unusual cause of acalculous cholecystitis during pregnancy: hepatitis A virus. *Dig Dis Sci* 2005; **50**: 1532
5 **Anthoine-Milhomme MC**, Chappuy H, Cheron G. Acute acalculous cholecystitis in a child returning from the Ivory Coast. *Pediatr Emerg Care* 2007; **23**: 242-243
6 **Axelrod D**, Karakas SP. Acalculous cholecystitis and abscess as a manifestation of typhoid fever. *Pediatr Radiol* 2007; **37**: 237
7 **Garrido-Benedicto P**, Gonzalez-Reimers E, Santolaria-Fernandez F, Rodriguez-Moreno F. Acute acalculous cholecystitis due to Salmonella. *Dig Dis Sci* 1994; **39**: 442-443
8 **Sese Torres J**, Morlans Molina G, Capdevila Cirera A, Valls Camp X, Herrero Reche A. [Acute alithiasic cholecystitis caused by infectious gastroenteritis] *Med Clin* (Barc) 1985; **84**: 672
9 **Mishu B**, Koehler J, Lee LA, Rodrigue D, Brenner FH, Blake P, Tauxe RV. Outbreaks of Salmonella enteritidis infections in the United States, 1985-1991. *J Infect Dis* 1994; **169**: 547-552
10 **Ochoa J**, Ricarte E, Carrasco M, Simon MA, Cabello J, Yanguela JM. [Complications of acute gastroenteritis caused by Salmonella no typhi] *Rev Esp Enferm Apar Dig* 1989; **75**: 262-266
11 **Meldrum R**, Feinberg JR. Septic arthritis of the ankle due to Salmonella enteritidis: a case report. *South Med J* 2004; **97**: 77-79
12 **Aissaoui Y**, Azendour H, Balkhi H, Haimeur C, Atmani M. [Postoperative meningitis caused by an unusual etiological agent: Salmonella enteritidis] *Neurochirurgie* 2006; **52**: 547-550
13 **Fernandez Rodriguez R**, Moreno Sanchez D, Martinez Fernandez R, Medina Asensio J, Ferrero Collado A. [Enterocolitis caused by Salmonella enteritidis complicated by acute cholecystitis without lithiasis] *Rev Esp Enferm Apar Dig* 1988; **74**: 477-479
14 **Sese J**, Mas J, Pujol R, Capdevila A. [Acute non-calculous Salmonella enteriditis cholecystitis, diagnosis by percutaneous puncture] *Med Clin* (Barc) 1986; **87**: 564-565
15 **Owen CC**, Jain R. Acute Acalculous Cholecystitis. *Curr Treat Options Gastroenterol* 2005; **8**: 99-104

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CASE REPORT

Splenic rupture following colonoscopy

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Abstract

Colonoscopy is a safe and routinely performed diagnostic and therapeutic procedure for different colorectal diseases. Although the most common complications are bleeding and perforation, extracolonic or visceral injuries have also been described. Splenic rupture is a rare complication following colonoscopy, with few cases reported. We report a 60-year-old female who presented to surgical consultation 8 h after a diagnostic colonoscopy. Clinical, laboratory and imaging findings were suggestive for a massive hemoperitoneum. At surgery, an almost complete splenic disruption was evident, and an urgent splenectomy was performed. After an uneventful postoperative period, she was discharged home. Splenic injury following colonoscopy is considered infrequent. Direct trauma and excessive traction of the splenocolic ligament can explain the occurrence of this complication. Many times the diagnosis is delayed because the symptoms are due to colonic insufflation, so the most frequent treatment is an urgent splenectomy. A high index of suspicion needs an early diagnosis and adequate therapy.

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Key words: Colonoscopy; Splenic injury; Splenic rupture; Splenectomy

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INTRODUCTION

Colonoscopy is a safe and routinely performed diagnostic and therapeutic procedure for different large bowel diseases. The most common complications include bleeding (1%) and perforation (0.1%-0.2%), and the chance of complication increases if any therapeutic actions are added, such as polypectomy or dilation[1,2]. Extracolonic or visceral injuries, including pneumothorax, pneumomediastinum, acute appendicitis, retroperitoneal abscess and others, are far less common[3]. Splenic injury is a rare complication of colonoscopy with few cases described; the first one was reported in 1974[4]. Even when some patients with late presentation have been mentioned[5,6], most of them developed symptoms a few hours after colonoscopy, and the majority of them underwent emergency surgery. We report a case of splenic rupture following colonoscopy, treated with urgent splenectomy.

CASE REPORT

A 60-year old female, with no significant medical history, underwent a diagnostic colonoscopy in another center. During the procedure, two rectal polyps (5 mm each) were resected. It was not difficult to reach the ileocecal valve. Endoscopy was performed under intravenous sedation (midazolam, 5 mg iv). She was observed in the recovery room for 2 h and then discharged home. Eight hours later, the patient came to our institution, complaining of diffuse abdominal pain and distension. On examination, she was pale, with a pulse rate of 100 beats per minute and her blood pressure was 103/52 mmHg. She had nonspecific abdominal tenderness, but no peritoneal signs. A colonic perforation after colonoscopy was suspected. The patient was resuscitated with vigorous intravenous fluid administration at the intermediate care unit. Her chest and abdominal X-ray showed no free air (Figure 1). Blood tests showed a hematocrit of 18% (hemoglobin, 6 g/dL). After an adequate haemodynamic stabilization and transfusion of 3 units of packed red cells, an abdominal

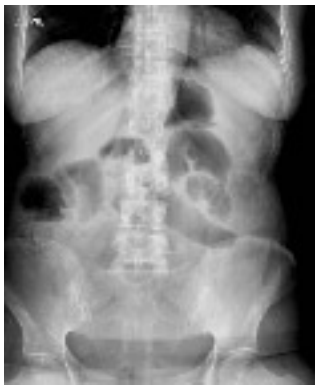

Figure 1 Abdominal X-ray showing no free air.

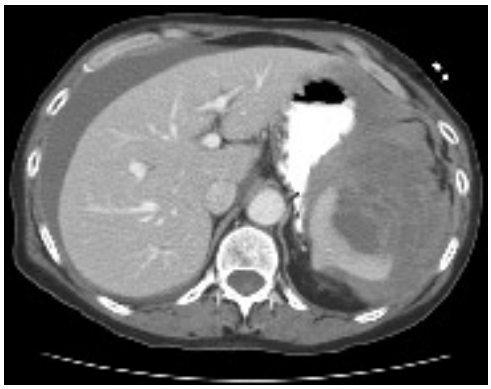

Figure 2 Hemoperitoneum and perisplenic hematoma.

CT scan demonstrated free fluid with a density suggesting blood, and a 12.5 cm × 9.6 cm left subphrenic perisplenic hematoma (Figure 2). No pneumoperitoneum was evident. At laparotomy, about three liters of intraperitoneal blood, and an almost complete splenic disruption were evident, so a splenectomy was performed. There were no perisplenic adhesions. No colon wounds or tears were seen. The postoperative course was uneventful, and she was discharged home on postoperative day 4. Specimen histologic examination revealed nothing but haemorragic parenchyma and inflammatory response, without any underlying splenic disease.

DISCUSSION

Splenic injury following colonoscopy is considered very infrequent. Only 40 cases have been reported[3-15]. To our knowledge, our case is the first case coming from South America. We believe that this is a rare but, most of the times, an under published complication of colonoscopy, so the real incidence might be higher. Although no specific causes have been established, the mechanism of injury may be related to direct trauma or preferently, excessive traction on the splenocolic ligament, which results in evulsion of the splenic capsule and different grades of parenchymal disruption[3,5,7]. A polypectomy or a biopsy was performed in most of reported cases[8]. Splenomegaly, inflammatory bowel disease, pancreatitis and intrabdominal post-surgical adhesions have been mentioned as predisposing factors, due to a suspected decreased mobility between the spleen and colon[8-10]. However, these are not constant findings at surgery[9]. The addition of external abdominal pressure during colonoscopy has also been proposed as a risk factor for the development of this complication[7,8]. Usually, the clinical presentation occurs within the first 24 h after colonoscopy[7,8]; but many times the diagnosis is delayed because the symptoms are attributed to colonic insufflation[3]. Finally, the diagnosis is made in a critically ill patient, with the onset of hypotension and acute anemia. There are also some reported cases with a late presentation (from more than 24 h to 10 d) and mild symptoms[5,6]. CT scan is the imaging modality of choice[11,12], which determines the extent of splenic damage, and demonstration of hemoperitoneum. This information, added to the clinical setting, may help decide on the therapeutic option. In most series, splenectomy is the most frequent treatment of choice[3-9]. Very few cases have been treated with transfusion of hemocomponents, broad spectrum antibiotics and close hemodynamic monitoring[10,13,14]. Another therapeutic action successfully described is splenic artery embolization[15]. The use of this "conservative" treatment must be in direct relation with the hemodynamic status of each case, and in the expertise of a multidisciplinary medical team. In our case, the patient was treated with an urgent splenectomy, and had an uneventful postoperative period.

We believe that this is an unusual and probably under reported complication of colonoscopy. As colonoscopy is performed widely in different centers, the medical team should be aware of the possibility of a splenic injury after colonoscopy and a high level of suspicion needs an early diagnosis and adequate treatment.

REFERENCES

1 **Macrae FA**, Tan KG, Williams CB. Towards safer colonoscopy: a report on the complications of 5000 diagnostic or therapeutic colonoscopies. *Gut* 1983; **24**: 376-383
2 **Schwesinger WH**, Levine BA, Ramos R. Complications in colonoscopy. *Surg Gynecol Obstet* 1979; **148**: 270-281
3 **Espinal EA**, Hoak T, Porter JA, Slezak FA. Splenic rupture from colonoscopy. A report of two cases and review of the literature. *Surg Endosc* 1997; **11**: 71-73
4 **Wherry DC**, Zehner H Jr. Colonoscopy-fiberoptic endoscopic approach to the colon and polypectomy. *Med Ann Dist Columbia* 1974; **43**: 189-192
5 **Taylor FC**, Frankl HD, Riemer KD. Late presentation of splenic trauma after routine colonoscopy. *Am J Gastroenterol* 1989; **84**: 442-443
6 **Merchant AA**, Cheng EH. Delayed splenic rupture after colonoscopy. *Am J Gastroenterol* 1990; **85**: 906-907
7 **Janes SE**, Cowan IA, Dijkstra B. A life threatening complication after colonoscopy. *BMJ* 2005; **330**: 889-890
8 **Ahmed A**, Eller PM, Schiffman FJ. Splenic rupture: an unusual complication of colonoscopy. *Am J Gastroenterol* 1997; **92**: 1201-1204
9 **Al Alawi I**, Gourlay R. Rare complication of colonoscopy. *ANZ J Surg* 2004; **74**: 605-606
10 **Tsoraides SS**, Gupta SK, Estes NC. Splenic rupture after colonoscopy: case report and literature review. *J Trauma* 2007; **62**: 255-257
11 **Zenooz NA**, Win T. Splenic rupture after diagnostic

colonoscopy: a case report. *Emerg Radiol* 2006; **12**: 272-273
12 **Johnson C**, Mader M, Edwards DM, Vesy T. Splenic rupture following colonoscopy: two cases with CT findings. *Emerg Radiol* 2006; **13**: 47-49
13 **Heath B**, Rogers A, Taylor A, Lavergne J. Splenic rupture: an unusual complication of colonoscopy. *Am J Gastroenterol* 1994; **89**: 449-450
14 **Rockey DC**, Weber JR, Wright TL, Wall SD. Splenic injury following colonoscopy. *Gastrointest Endosc* 1990; **36**: 306-309
15 **Stein DF**, Myaing M, Guillaume C. Splenic rupture after colonoscopy treated by splenic artery embolization. *Gastrointest Endosc* 2002; **55**: 946-948

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CASE REPORT

Therapeutic barium enema for bleeding colonic diverticula: Four case series and review of the literature

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Abstract

The prevalence of diverticular diseases of the colon, including severe and persistent bleeding in Eastern countries, has increased in the last decades. The bleeding from colonic diverticula is the most common cause of acute lower gastrointestinal bleeding. Herein, we report four cases of severe and persistent bleeding of colonic diverticular disease that could be treated with a high concentration barium enema. These four cases showed a similar pattern of bleeding whose source could not be identified. Colonoscopy revealed fresh blood in the entire colon and many diverticula were noted throughout the colon. No active bleeding source was identified, but large adherent clots in some diverticula were noted. After endoscopic and angiographic therapies failed, therapeutic barium enema stopped the severe bleeding. These patients remained free of re-bleeding in the follow-up period (range 17-35 mo) after the therapy. We report the four case series of therapeutic barium enema and reviewed the literature pertinent to this procedure.

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Key words: Therapeutic barium enema; Colonic diverticula; Diverticular bleeding

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INTRODUCTION

Diverticular hemorrhage is a common cause of lower gastrointestinal bleeding[1-3]. Most colonic diverticula are asymptomatic and remain uncomplicated. However, it was reported that severe diverticular hemorrhage occurs in 3%-5% of patients with diverticula[4,5]. Most cases of diverticular bleeding resolve themselves. However, massive bleeding of diverticula often requires endoscopic or angiographic therapy. As in some cases, the source of bleeding cannot be identified or multiple sites of bleeding are found, endoscopic or angiographic treatment is not so effective for bleeding. Although surgical treatment has been performed for persistent bleeding, the patients are often elderly and, therefore, at a high risk for surgery.

Most of the reported series of therapeutic barium enema are from Western countries. However, the prevalence of diverticular diseases of the colon, including severe and persistent bleeding in the Eastern countries, has increased in the last decades. We present herein four patients with severe and persist bleeding due to colonic diverticular disease that were treated with high concentration barium enemas, and have reviewed the literature pertinent to this procedure.

CASE SERIES

Case 1

A 63-year-old man was hospitalized for a several-day history of painless passage of bright red blood per rectum. He had hypertension and diabetes mellitus, and had been taking medication for hypertension for ten years. He had no history of receiving non-steroidal anti-inflammatory drugs, low-dose aspirin, and anticoagulants. His past history revealed two bleeding episodes from colonic diverticula that resolved on their own. Physical examination revealed no abdominal tenderness. Laboratory tests showed severe anemia.

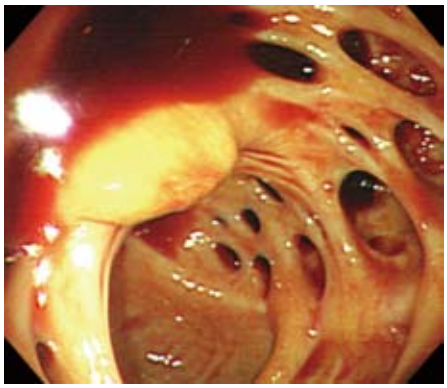

Figure 1 Endoscopic appearance of bleeding diverticula with adherent clots in the ascending colon.

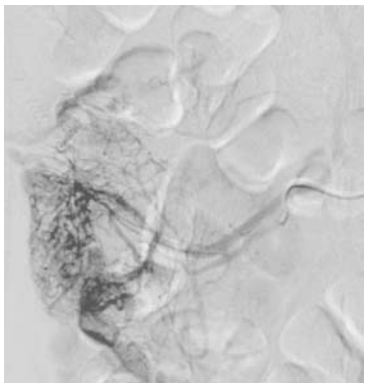

Figure 2 Superior mesenteric arteriogram showing hypervascularity in the ascending colon.

Colonoscopy revealed fresh blood in the entire colon and many diverticula with adherent clots were noted throughout the colon (Figure 1). No active bleeding source was identified; but large adherent clots in some diverticula were noted. Endoscopic placement of metallic clips was done for some diverticula with large adherent clots suspicious of being a bleeding source. On the next day, the patient had severe hematochezia again and required additional transfusion of 4 units of packed red cells. Scintigraphic examination failed to identify the bleeding site. Emergency angiography was performed. Although superior mesenteric arteriography could not reveal any extravasation, hypervascularity was noted on the right side of the colon (Figure 2). Vasopressin infusion was administered; but the patient continued to bleed, and required 4 units of blood. We discussed the situation with the patient and his family, and explained the subsequent treatment modalities, including surgical treatment or optional therapeutic barium enema. After obtaining informed consent, we performed therapeutic barium enema. Barium (concentration: 200%, volume: about 400 mL) including 50 000 units of thrombin was administered per rectum, and the leading edge of the contrast medium was followed up to the ascending colon by fluoroscopy. The enema tip was withdrawn one hour after confirming that the diverticula in the ascending colon were filled with barium (Figure 3). On the next day, we confirmed that multiple diverticula were filled with barium by abdominal X-ray examination. Ten days after the therapy, we also confirmed that the multiple diverticula at the right side of the colon were filled with barium. The patient was discharged without any further bleeding or complications, and surgical treatment was avoided. The patient remained free of re-bleeding more than 3 years after the therapy.

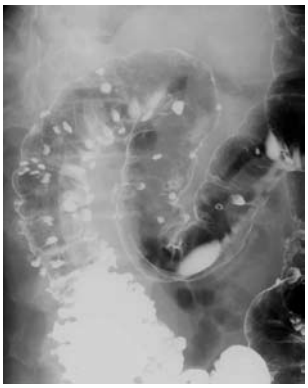

Figure 3 X-ray revealing diverticula in the ascending colon filled with barium.

Case 2

A-67-year old man with diverticula, noted on prior screening colonoscopy, was transferred to our hospital with sudden painless massive rectal bleeding. While in hospital, he developed painless hematochezia and severe anemia requiring transfusion of 2 units of packed red cells. He had hypertension, and had been taking medication for hypertension for five years. He had a history of receiving non-steroidal anti-inflammatory drugs for lumbago. Colonoscopy revealed fresh blood in the entire colon and many diverticula throughout the colon. Endoscopic placement of metallic clips was done for some diverticula suspicious of being a bleeding source. Two days later, the patient had severe hematochezia again and required additional transfusion of 2 units of packed red cells. Emergency angiography could not reveal any extravasation. Therapeutic barium enema was performed and the patient remained free of re-bleeding two years after the therapy.

Case 3

A-76-year old man visited the Emergency Department for a three-day history of diarrhea and painless hematochezia. He had hypertension and had been taking medication for hypertension. Colonoscopy revealed fresh blood throughout the colon and many diverticula in the ascending and sigmoid colon. Endoscopic placement of metallic clips was done for some diverticula suspicious of being a bleeding source. Three days later, the patient began bleeding again, and required transfusion of 2 units of packed red cells. The patient and his family wished to avoid intervention, and agreed to the therapeutic barium enema. Active bleeding was stopped after the therapeutic barium enema. He remained free of re-bleeding 20 mo after the therapy.

Case 4

A-63-year old man was hospitalized for a two-day history

Table 1 Cases undergoing therapeutic barium enema for diverticular hemorrhage

Case	1	2	3	4
Gender	M	M	M	M
Age (yr)	63	67	76	63
Associated diseases	HT,DM	HT	HT	None
NSAID, L-Asp, Anticoagulants	None	NSAID	None	None
Previous bleeding episodes (*n*)	2	2	1	1
Location	Throughout the colon	Throughout the colon	Ascending sigmoid	Ascending
Appearance	AC, FB	FB	FB	AC
Hemorrhagic site	NI	NI	NI	NI
Follow-up period (mo)	35	23	20	17
Recurrent bleeding	None	None	None	None

NI: Not identified; AC: Adherent clot; FB: Fresh blood; HT: Hypertension; DM: Diabetes mellitus; NSAID: Nonsteroidal anti-inflammatory drug; L-Asp: Low-dose aspirin.

of painless passage of bright red blood per rectum. Colonoscopy revealed fresh blood in the entire colon and many diverticula with adherent clots in the ascending colon. Endoscopic placement of metallic clips was done for some diverticula suspicious of being a bleeding source. During the following hospitalization days, the patient experienced 3 further episodes of bleeding and received 3 units of packed red cells. The patient wished to avoid intervention and agreed to receive therapeutic barium enema. There was no evidence of re-bleeding 17 mo after the therapy.

DISCUSSION

The prevalence of diverticular diseases of the colon in the Eastern countries has increased in the last decades, and the increasing prevalence reflects changes in the life-style and eating habits. A previous study on 6849 patients undergoing barium enema examination during an 8-year period (from 1985 to 1992) revealed an increase in the frequency from 10.7% in 1985 to 17.8% in 1992[6] Another study concluded that diverticular diseases of the right colon have increased steadily in Japan, suggesting that diverticulitis and bleeding may continue to increase[7]. As many reports stated, the prevalence of colonic diverticula and their related severe bleeding have increased recently in the Eastern countries including Japan[6-8].

Colonic diverticula are usually asymptomatic. However, in some cases, acute and chronic inflammation, hemorrhage, and perforation develop as complications of this disease. Bleeding from colonic diverticula is the most common cause of acute lower gastrointestinal bleeding[1-3]. It was reported that acute lower intestinal bleeding occurs in up to 3%-5% of colonic diverticula[4,5]. Most cases of diverticular bleeding resolve themselves and diverticular bleeding stops spontaneously in 70%-80% of cases[9]. However, some patients require evaluation by colonoscopy and angiography, or surgical treatment to stop their bleeding. There are some case reports on various techniques of treatment with colonoscopy for diverticular bleeding including heater probes, epinephrine injection therapy, argon plasma coagulation, and endo-clip application[10-16]. Endoscopic treatment can be useful when a source of lower gastrointestinal bleeding is identified. However, when the source of bleeding cannot be identified or multiple sites of bleeding are found, endoscopic treatment is not effective for stopping bleeding. The specific location of bleeding points is very important for therapeutic colonoscopy of bleeding diverticula. Intermittent diverticular hemorrhage can also lead to incomplete endoscopic therapy.

Angiography is recognized as an accurate diagnostic modality for detecting the site of active gastrointestinal bleeding. Mesenteric angiography is indicated when the flow is estimated to be greater than 0.5-1 mL/min, and offers a potential for selective vasopressin infusion or arterial embolization if the bleeding site is identified[16]. However, angiography frequently fails to reveal the source of gastrointestinal hemorrhage. In addition, in p patients with multiple sites of bleeding or intermittent and quiescent bleeding, it is difficult to treat diverticular hemorrhage by angiographic intervention. Furthermore, angiography is an invasive modality with complications such as arterial dissection and occlusion, bowel infarction, myocardial infarction with vasopressin infusion, and renal failure due to contrast medium[1,17].

In our four cases, colonoscopy demonstrated large amounts of fresh blood throughout the colon and many diverticula with adherent blood clots; but it was difficult to identify the active bleeding site, and the specific bleeding points. Endoscopic and angiographic therapies failed to identify the bleeding points, and could not stop bleeding. As an optional therapy, therapeutic barium enema was performed for severe bleeding to avoid surgical treatment. Table 1 summarizes the four cases of persistent and severe diverticular bleeding in whom the active bleeding sites could not be identified and therapeutic barium enema was effective and no re-bleeding was detected. Each case had one or two persistent bleeding episodes previously and underwent repeated endoscopic treatment. However, these patients remained well and had no re-bleeding after barium enema treatment for 17-35 mo. In all cases, the concentration of barium was 200%, the volume was 400 mL, and the enema tip was withdrawn one hour after the therapy (Table 1). In our four cases, although no severe complications of therapeutic barium enema (such as perforation) occurred, we have to take into account the

Table 2 Previous reports on therapeutic barium enema for diverticular hemorrhage

Author, year	*n*	Location	Concentration of barium	Successful cases	Recurrent bleeding
Adams *et al* 1970	28	NR	NR (20%)[1]	26	9
Chorost *et al* 2000	1	TO	20%	1	0
Koperna *et al* 2001	63	NR	NR	53	10
Matsuhashi *et al* 2003	1	AC, TC, SC	200% (with 1 mg of epinephrine)	1	0
Our cases 2008	4	TO AC, SC (2 cases) AC	200%	4	0

[1]Mentioned in the discussion section. TO: Throughout the colon; AC: Ascending colon; NR: Not reported; SC: Sigmoid colon; TC: Transverse colon.

possibility of perforation in patients with diverticulitis.

Some case reports and clinical studies of therapeutic barium enema for diverticular hemorrhage have been reported (Table 2)[8,18-20]. A previous case report[8] presented successful treatment with a high concentration of barium with 1 mg of epinephrine. In that case, epinephrine was added to the solution for vasoconstriction; but the possible adverse effects of epinephrine such as sudden hypertension were not ruled out. Another case report[18] presented successful treatment with a 20% barium sulphate solution at a height of 0.9 m for 5 min. In 1970, Adams *et al*[19] demonstrated that 26 of 28 acute bleeding episodes were arrested by therapeutic barium enema with a 20% concentration of barium. That study also stated that the only single complication was laceration of the rectal mucosa by the enema tube[19]. A previous clinical study[20] evaluated the efficacy of barium enema therapy for severe diverticular bleeding and concluded that therapeutic barium enema is the treatment of choice for the first bleeding episode, while surgical resection should be performed if re-bleeding occurs. In that report, the failure rate of conservative treatment and therapeutic barium enema with consequent re-bleeding was 43.4% and 15.9%, respectively[20]. Furthermore, an investigation suggested that complications develop more often in patients after colonic resection than in those after barium enema therapy, and that the mortality after surgery is significantly higher than that following therapeutic barium enema[20].

Most of the reported cases of therapeutic barium enema were from the Western countries. However, the prevalence of diverticular diseases of the colon in the Eastern countries including Japan has increased recently. From our present experiences, therapeutic barium enema is also effective for right side diverticula which are typically located in the Eastern countries.

It is difficult to clarify the mechanism underlying the effect of therapeutic barium enema. Adams *et al*[19] mentioned two potential factors, namely the pressure by the barium solution producing tamponade of the bleeding vessel, and the direct hemostatic action by the barium sulfate. The effect of barium on bleeding in the gastrointestinal tract is also described in a previous report[21]. That report mentioned that tap-water enema is better, as it contains no anticoagulants, and is more effective in producing clot formation than most barium suspensions.

A clinical study on surgery for complicated colonic diverticula concluded that in patients with multiple bleeding sites or severe ongoing hemorrhage from a source that cannot be localized despite endoscopic and angiographic assessment, subtotal or total colectomy may be imperative[22]. However, in view of the mortality of surgical therapy, optional and non-invasive therapies such as therapeutic barium enema are needed in the cases that require colonic resection.

In conclusion, the prevalence of diverticular diseases of the colon has increased in the last decades not only in the Western countries, but also in the Eastern countries. Barium enema therapy is effective for diverticular hemorrhage when the active bleeding site could not be identified by colonoscopy. When no other therapeutic techniques are available, barium enema therapy may be useful as an optional therapy which may avoid surgical therapy. As far as we know, there are few reports on randomized trials of treatment of colonic diverticular bleeding. Because of the limited number of clinical case series, further randomized controlled trials of treatment are required to clarify the role of therapeutic barium enema in bleeding diverticula.

REFERENCES

1 **Vernava AM 3rd**, Moore BA, Longo WE, Johnson FE. Lower gastrointestinal bleeding. *Dis Colon Rectum* 1997; **40**: 846-858
2 **Longstreth GF**. Epidemiology and outcome of patients hospitalized with acute lower gastrointestinal hemorrhage: a population-based study. *Am J Gastroenterol* 1997; **92**: 419-424
3 **Zuckerman GR**, Prakash C. Acute lower intestinal bleeding. Part II: etiology, therapy, and outcomes. *Gastrointest Endosc* 1999; **49**: 228-238
4 **Stollman NH**, Raskin JB. Diagnosis and management of diverticular disease of the colon in adults. Ad Hoc Practice Parameters Committee of the American College of Gastroenterology. *Am J Gastroenterol* 1999; **94**: 3110-3121
5 **McGuire HH Jr**, Haynes BW Jr. Massive hemorrhage for diverticulosis of the colon: guidelines for therapy based on bleeding patterns observed in fifty cases. *Ann Surg* 1972; **175**: 847-855
6 **Miura S**, Kodaira S, Shatari T, Nishioka M, Hosoda Y, Hisa

TK. Recent trends in diverticulosis of the right colon in Japan: retrospective review in a regional hospital. *Dis Colon Rectum* 2000; **43**: 1383-1389
7 **Nakada I**, Ubukata H, Goto Y, Watanabe Y, Sato S, Tabuchi T, Soma T, Umeda K. Diverticular disease of the colon at a regional general hospital in Japan. *Dis Colon Rectum* 1995; **38**: 755-759
8 **Matsuhashi N**, Akahane M, Nakajima A. Barium impaction therapy for refractory colonic diverticular bleeding. *AJR Am J Roentgenol* 2003; **180**: 490-492
9 **McGuire HH Jr**. Bleeding colonic diverticula. A reappraisal of natural history and management. *Ann Surg* 1994; **220**: 653-656
10 **Prakash C**, Chokshi H, Walden DT, Aliperti G. Endoscopic hemostasis in acute diverticular bleeding. *Endoscopy* 1999; **31**: 460-463
11 **Simpson PW**, Nguyen MH, Lim JK, Soetikno RM. Use of endoclips in the treatment of massive colonic diverticular bleeding. *Gastrointest Endosc* 2004; **59**: 433-437
12 **Jensen DM**, Machicado GA, Jutabha R, Kovacs TO. Urgent colonoscopy for the diagnosis and treatment of severe diverticular hemorrhage. *N Engl J Med* 2000; **342**: 78-82
13 **Mauldin JL**. Therapeutic use of colonoscopy in active diverticular bleeding. *Gastrointest Endosc* 1985; **31**: 290-291
14 **Hokama A**, Uehara T, Nakayoshi T, Uezu Y, Tokuyama K, Kinjo F, Saito A. Utility of endoscopic hemoclipping for colonic diverticular bleeding. *Am J Gastroenterol* 1997; **92**: 543-546
15 **Bloomfeld RS**, Rockey DC, Shetzline MA. Endoscopic therapy of acute diverticular hemorrhage. *Am J Gastroenterol* 2001; **96**: 2367-2372
16 **Ramirez FC**, Johnson DA, Zierer ST, Walker GJ, Sanowski RA. Successful endoscopic hemostasis of bleeding colonic diverticula with epinephrine injection. *Gastrointest Endosc* 1996; **43**: 167-170
17 **Peter DJ**, Dougherty JM. Evaluation of the patient with gastrointestinal bleeding: an evidence based approach. *Emerg Med Clin North Am* 1999; **17**: 239-261, x
18 **Chorost MI**, Fruchter G, Kantor AM, Wu J, Ghosh BC. The therapeutic barium enema revisited. *Clin Radiol* 2001; **56**: 856-858
19 **Adams JT**. Therapeutic barium enema for massive diverticular bleeding. *Arch Surg* 1970; **101**: 457-460
20 **Koperna T**, Kisser M, Reiner G, Schulz F. Diagnosis and treatment of bleeding colonic diverticula. *Hepatogastroenterology* 2001; **48**: 702-705
21 **Miller RE**, Skucas J, Violante MR, Shapiro ME. The effect of barium on blood in the gastrointestinal tract. *Radiology* 1975; **117**: 527-530
22 **Funariu G**, Bintintan V, Seicean R. Urgent surgery for complicated colonic diverticula. *J Gastrointestin Liver Dis* 2006; **15**: 37-40

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CASE REPORT

Polysplenia syndrome with preduodenal portal vein detected in adults

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Abstract

Polysplenia syndrome, defined as the presence of multiple spleens of almost equal volume, is a rare condition involving congenital anomalies in multiple organ systems. We report this anomaly in a 41-year-old female who underwent a left lateral sectionectomy due to recurrent cholangitis and impacted left lateral duct stones. Polysplenia syndrome with preduodenal vein was diagnosed preoperatively by computed tomography (CT) and surgery was done safely. Although the polysplenia syndrome with preduodenal portal vein (PDPV) in adult is rarely encountered, surgeons need to understand the course of the portal vein and exercise caution in approaching the biliary tract.

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Key words: Polysplenia; Polysplenia syndrome; Preduodenal portal vein; Intrahepatic duct stones; Congenital anomaly

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INTRODUCTION

Polysplenia syndrome is a rare disease that occurs in patients with two or more spleens of identical sizes and various organ anomalies[1]. Reports indicate that most patients with polysplenia syndrome die before 5 years of age because the disease is often associated with congenital anomalies, such as cardiovascular anomalies[2]. Severe cardiovascular anomalies include interruption of the suprarenal inferior vena cava, atrioventricular septal defects, ipsilateral pulmonary venous drainage, ventricular outflow tract obstruction, dextrocardia and abnormal great vessel relationships[3]. Some patients with polysplenia syndrome have a normal heart or only minor cardiac defects, are often diagnosed incidentally in patients being treated for other disease[4]. However, they may harbor anomalies in abdominal organs or the gastrointestinal tract, one example of which is a preduodenal portal vein (PDPV)[5]. A PDPV can be diagnosed early as duodenal obstruction in infants, but is often found incidentally or during surgery when there are no symptoms[6].

One of the ways to prevent injuries to the hepatic portal system during surgery is to diagnosis polyspenia syndrome accompanied by a PDPV prior to surgery. We treated a patient with polysplenia syndrome, which was diagnosed during a left lateral sectionectomy to treat intrahepatic duct stones, and we report our findings along with related studies.

CASE REPORT

A 41-year-old female presented for evaluation of right upper quadrant pain of 1 mo duration. At a local hospital, she was noted to have polysplenia and left lateral intrahepatic duct stones without intrahepatic duct dilatation of segment 4. She was referred to our institution for further evaluation and treatment. The physical examination revealed no abnormalities. An abdominal computed tomography (CT) showed intrahepatic duct stones with polysplenia and the portal vein was located anteriorly to the duodenum (Figure 1). Laboratory findings revealed

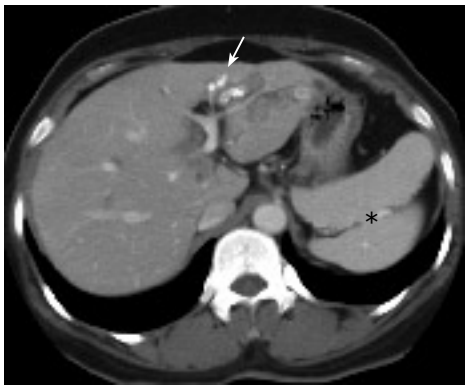

Figure 1 Abdominal computed tomography scan. Polysplenia (asterisks) are present in the left upper quadrant and it shows the left lateral intrahepatic duct stones with atrophy (arrow).

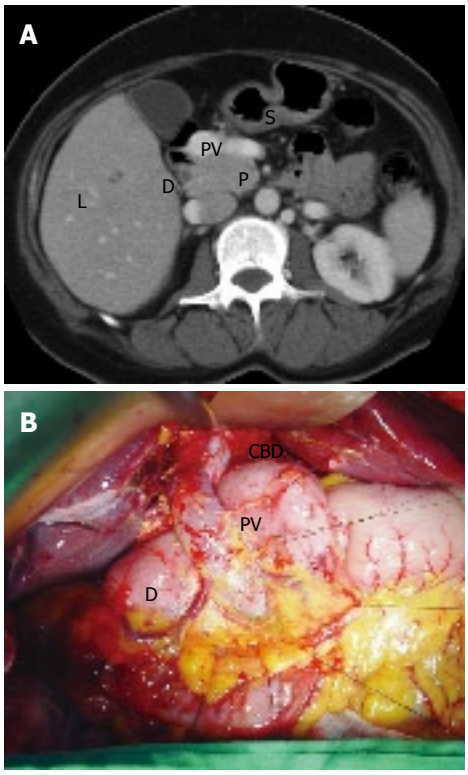

Figure 2 The location and presentation in surgery of the portal vein. A: The portal vein (PV) was located ventrally to the duodenum (D) and pancreas (P), liver (L) and stomach (S) are in the normal position; B: At surgery, the portal vein (PV) was seen running anteriorly across the duodenal first portion (D), the common bile duct (C) was identified left to the portal vein.

elevated levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and lactate dehydrogenase. Echocardiography and chest radiography were normal. The diagnosis of intraheptic duct stones with polyspenia syndrome was established.

The surgical procedure for intrahepatic duct stones began with a thorough exploration of the abdomen. The portal vein was detected in front of the first part of the duodenum. The portal vein passed upward, right to the common bile duct, and bifurcated into the left and right portal branches in the porta hepatis (Figure 2). Both the common bile duct and the right hepatic artery were identified to the left of the portal vein. The left hepatic artery originated from the left gastric artery. The presence of two spleens was confirmed on the left side of the upper abdomen, along with the greater curvature of the stomach. A nasogastric tube passed through duodenum without any difficulty. We could not locate the stenotic duodenal region posterior to the portal vein. The shape of the liver and pancreas were normal; the small bowel and colon were normally positioned.

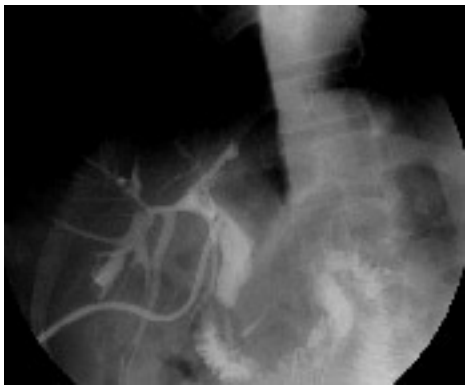

Figure 3 T-tube cholangiography shows variations of right intrahepatic bile duct.

A left lateral sectionectomy with a cholecystectomy was performed safely. An intraoperative choledochoscopy through the segment 3 bile duct was done for removal of the right intrahepatic duct stone, and we confirmed no residual stones existed in the bile duct based on T-tube cholangiography (Figure 3). The postoperative course was uneventful, and the patient was discharged 12 d postoperatively.

DISCUSSION

As the name of the condition implies, polysplenia syndrome refers to patients with two or more spleens. Studies also include descriptions of cases that involve a number of very small spleens, a multilobular spleen with tiny accessory spleen, and an undivided spleen[2,7].

The spleen develops during the 5th embryonic week from the splenic primordia, originating from the dorsal mesogastrium. The initial splenic primordia are then created as incisures on the left side of the dorsal mesogastrium. When the incisures fail to fuse, they create two or more spleens[8]. The blood flow of an embryo makes a transition from symmetric to asymmetric around the 25th d to determine the visceral sidedness, and it has been suggested that this is when cono-truncal anomalies and anomalies of atrioventricular canal occur[9]. At the same time, PDPV also develop. Venous blood is drained from the primitive gut, consisting of two vitelline veins of the yolk sac. The veins implement communication in the liver (cranial communication), behind (middle communication) and in front of (caudal communication) the duodenum. When the caudal and cranial communications are lost during the 9-mm embryo stage, the S-shape portal vein is created. It is believed that the PDPV anomaly is developed during this stage if cranial

and middle communications are lost[10].

Although the exact cause of polysplenia is unknown, studies have suggested that it is caused by various factors including embryogenic, genetic, and teratogenic components[11]. Since splenic anomalies (splenic agenesis, hypogenesis and polysplenia) are often accompanied by anomalies in the cardiovascular tract and other abdominal organs, it can be inferred that the spleen plays a significant role during the early embryonic stage[12].

Reports indicate that polysplenia syndrome occurs in both genders with an identical frequency[5]. Known cardiovascular anomalies include absence or hypoplasia of the suprarenal inferior vena cava (with or without azygos or hemiazygos continuation), levoisomerism of the right bronchial tree, dextrocardia, ventricular septal defects, and the absence of the coronary sinuses, most patients die before 5 years of age due to the accompanying cardiovascular anomalies. Patients without cardiac anomalies may reach adulthood, accounting for 10%-15% of cases of polysplenia. Since most adult patients do not exhibit any symptoms, polysplenia syndrome is often diagnosed incidentally during other procedures[5]. Even adult patients can have anomalies in abdominal organs including visceral heterotaxia with a right-sided stomach, a left-sided or large midline liver, right-sided spleen, malrotation of the intestine, a short pancreas, and anomalies of the inferior vena cava. Cases with only PDPV, as with our patient, are recognized as very minor anomalous cases. Even though intestinal obstruction is often displayed in such cases, there were no anomalies of the digestion system for our patient. First described by Knight HO in 1921, PDPV is a congenital anomaly that involves the portal vein passing in front of the duodenum[13]. A PDPV can be associated with duodenal atresia, stenosis, web, annular pancreas and malrotation, and surgery may be required for treatment[14]. However, since the portal vein is a thin-walled, low-pressure vessel, it is highly unlikely that PDPV alone can cause duodenal obstruction. Patients can survive to reach adulthood without any symptoms, and the anomaly is often found during examinations or surgeries to treat other diseases.

Polysplenia syndrome can be detected relatively easily with diagnostic imaging including abdominal CT and magnetic resonance imaging (MRI)[15]. In our case, polysplenia syndrome was diagnosed incidentally while examining the patient who was complaining of pain in the right upper abdomen and fever. Known causes of atrophy of the lateral segment of the liver in PDPV include selective portal vein obstruction, biliary duct obstruction, partial obstruction of the portal vein associated with distention of the hepatic bile duct, long-standing malnutrition and cachexia, or toxic and vascular influences[14]. However, in the case of our patient, it is likely that the atrophy was caused by intrahepatic duct stones. Reports indicate that most cases of PDPV in adults involve surgery for cholelithiasis, making us believe that PDPV could be a cause for cholelithiasis[14]. When surgery is required, care must be exercised, especially for procedures involving the upper abdomen. If PDPV is not detected prior to surgery, it can cause severe complications, such as hemorrhage and vascular ligation. Such accidents can be prevented by performing careful diagnostic imaging in advance, such as CT, and especially noting the possibility of PDPV in cases of polysplenia syndrome.

REFERENCES

1 **Griffiths JD**, Marshall VC. Torsion of the spleen in the polysplenia syndrome. *Aust N Z J Surg* 1984; **54**: 571-573
2 **Gayer G**, Hertz M, Strauss S, Zissin R. Congenital anomalies of the spleen. *Semin Ultrasound CT MR* 2006; **27**: 358-369
3 **Roguin N**, Hammerman H, Korman S, Riss E. Angiography of azygos continuation of inferior vena cava in situs ambiguus with left isomerism (polysplenia syndrome). *Pediatr Radiol* 1984; **14**: 109-112
4 **Gayer G**, Apter S, Jonas T, Amitai M, Zissin R, Sella T, Weiss P, Hertz M. Polysplenia syndrome detected in adulthood: report of eight cases and review of the literature. *Abdom Imaging* 1999; **24**: 178-184
5 **Peoples WM**, Moller JH, Edwards JE. Polysplenia: a review of 146 cases. *Pediatr Cardiol* 1983; **4**: 129-137
6 **Ooshima I**, Maruyama T, Ootsuki K, Ozaki M. Preduodenal portal vein in the adult. *J Hepatobiliary Pancreat Surg* 1998; **5**: 455-458
7 **Abut E**, Akkaya L, Uysal U, Arman A, Guveli H, Bolukbas C, Kurdas OO. Selective spleen scintigraphy in the diagnosis of polysplenia syndrome. *Br J Radiol* 2004; **77**: 698-700
8 **Gayer G**, Zissin R, Apter S, Atar E, Portnoy O, Itzchak Y. CT findings in congenital anomalies of the spleen. *Br J Radiol* 2001; **74**: 767-772
9 **Miyabara S**, Sugihara H, Kamio A, Oota K, Abe H, Kato S. Atypical polysplenia only with the hepatic segment of inferior vena in a middle-aged. *Acta pathol Jpn* 1984; **34**: 111-116
10 **Muneta S**, Sakai S, Fukuda H, Imamura Y, Matsumoto I. Polysplenia syndrome with various visceral anomalies in an adult: embryological and clinical considerations. *Intern Med* 1992; **31**: 1026-1031
11 **de la Monte SM**, Hutchins GM. Sisters with polysplenia. *Am J Med Genet* 1985; **21**: 171-176
12 **Nakada K**, Kawaguchi F, Wakisaka M, Nakada M, Enami T, Yamate N. Digestive tract disorders associated with asplenia/polysplenia syndrome. *J Pediatr Surg* 1997; **32**: 91-94
13 **Knight HO**. An anomalous portal vein with its surgical dangers. *Ann Surg* 1921; **74**: 697-699
14 **Ishizaki Y**, Tanaka M, Okuyama T. Surgical implications of preduodenal portal vein in the adult. Case report and review of the literature. *Arch Surg* 1994; **129**: 773-775
15 **Kobayashi H**, Kawamoto S, Tamaki T, Konishi J, Togashi K. Polysplenia associated with semiannular pancreas. *Eur Radiol* 2001; **11**: 1639-1641

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CASE REPORT

Splenic inflammatory pseudotumor mimicking angiosarcoma

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Abstract

Splenic tumors are rare. Differentiation of the tumors before operation is of great value regarding the outcome. A case of a 32-year-old man with a splenic inflammatory pseudotumor (IPT) mimicking splenic angiosarcoma is described. The tumor was highly suspected of being splenic angiosarcoma based on radiological findings preoperatively. However, after splenectomy, histopathological examinations revealed splenic IPT. Splenic IPT and angiosarcoma are rare and often pose diagnostic difficulties because the clinical and radiological findings are obscure. Due to large differences in prognosis, we briefly reviewed the clinical, radiological, and pathological features of both of the tumors.

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Key words: Splenic inflammatory pseudotumor; Splenic angiosarcoma; Spleen tumor

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INTRODUCTION

Although splenic tumors are rare, differentiation of the tumors before operation is of great value. Among them, splenic inflammatory pseudotumor (IPT) is a benign tumor characterized microscopically by a proliferation of inflammatory cells[1]; but splenic angiosarcoma is a dismal malignancy of vascular origin[2]. We usually differentiate them before operation by imaging studies. In this report, we present a case of splenic IPT mimicking angiosarcoma on radiological findings.

CASE REPORT

A 32-year-old man was a carrier of hepatitis B virus for years with regular follow-up at outpatient clinics. A splenic mass was incidentally detected by sonography. Physical examinations were unremarkable. The patient had no systemic complaints except mild vague discomfort over the epigastric region. Biochemical and hematological investigations were all within normal ranges except for slightly elevated serum glutamate-oxaloacetate transaminase (SGOT). Tumor markers including CEA, AFP, CA-199, and CA-125 were all negative. Sonography of the spleen showed a well-defined encapsulated tumor, 6.3 × 6.1 cm in diameter, with hyperechoic density in the central portion (Figure 1). Non-contrast and contrast abdominal computed tomography (CT) showed a mass over the spleen with high density in the central portion and multiple diffuse low-attenuation nodules in liver parenchyma (Figure 2A and B). Magnetic resonance imaging (MRI) was done for differentiation and revealed a mass lesion around 6 cm in the spleen, which showed partially dense intensity in T2-weighted image (Figure 3A) and peripheral nodule enhancement with gadolinium-contrast filling and pooling in T1 contrast-enhancement dynamic study (Figure 3B-D).

Overall, there were two parts of different signal

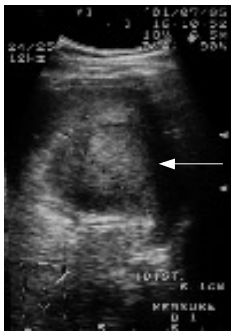

Figure 1 Sonography showed a well-defined encapsulated tumor, 6.3 cm x 6.1 cm in size, over the spleen with hyperechoic density in the central portion (arrow) of the tumor.

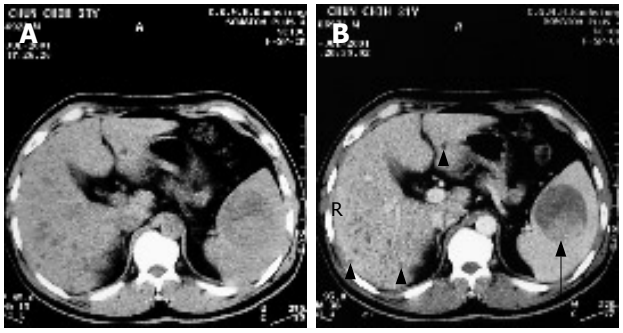

Figure 2 Abdominal CT. A: Non-contrast CT showed a well-defined tumor over the spleen; B:Contrast CT showed partial enhancement of the tumor with high density in the dorsal portion of the tumor (arrow) and multiple diffuse low-attenuation nodules in liver parenchyma (arrowhead).

intensity comprising the tumor at the spleen. The major part of the circumscribed lesion showed persistent low-signal intensity with some stippling enhancement from MRI dynamic contrast-enhanced series, while the minor portion located at the dorsal aspect showed similar signal-intensity change as the normal spleen parenchyma either at pre- or post-contrast phases, which is suggestive of hypervascular lesion, such as angiosarcoma.

Diffuse multiple tiny cystic lesions were noted in bilateral lobes of the liver. Biliary hamartoma was first considered. However, differential diagnosis should have included multiple hepatic cysts, micro-abscesses or even metastases. Therefore, primary splenic angiosarcoma with the possibility of multiple liver metastasis was the first consideration.

Surgical intervention was indicated, and exploratory laparotomy was performed thereafter. During the operation, the spleen was removed smoothly and liver hypertrophy with multiple tiny cystic lesions over the liver surface was noted. There was no evidence of malignancy in the frozen sections examined.

The specimen of spleen measured 11 × 8.5 × 6 cm in size and weighed 240 g (Figure 4A). Grossly, there was a well-circumscribed tumor inside the spleen, measuring 5.5 × 5.0 × 4.0 cm in size (Figure 4B). There were two parts of different appearances comprising the tumor. The major part of the circumscribed lesion showed yellowish appearance with some stippling red spots scattered over the cut surface, while the minor portion located at the dorsal aspect showed similar appearance as the normal spleen parenchyma.

Microscopically, the sections of the specimen were

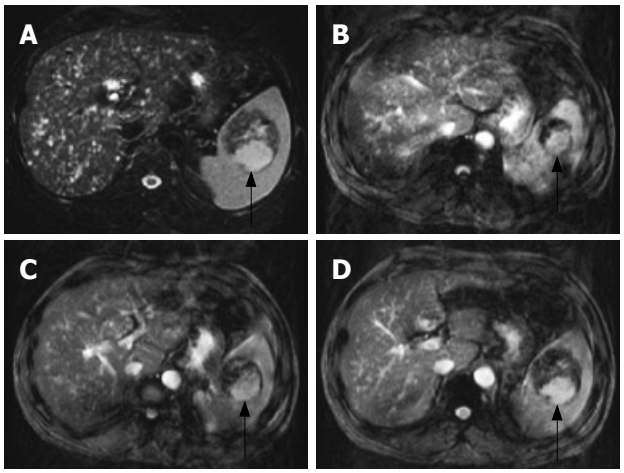

Figure 3 MRI. A: In T2-weighted image, a well-circumscribed tumor (arrow) in the spleen, which showed partially dense intensity; B-D: In T1 contrast-enhancement dynamic study, peripheral nodule enhancement with gadolinium-contrast filling and pooling were noted in the tumor.

composed of fibrosis, focal sclerosis, plump spindle cells and vascular proliferation (Figure 5). An admixture of inflammatory cells included lymphocytes, plasma cells and neutrophils, in which lymphocytes were predominant. The major part of the tumor consisted of more fibrosis and focally sclerotic change, while the minor part consisted of more vascular proliferation and more inflammatory cell infiltration. The sections of the specimen were compatible with the picture of IPT.

The patient's postoperative course was uneventful, and he has been well for 3 years following surgery.

DISCUSSION

The term "inflammatory pseudotumor" was first proposed in 1954, since these lesions are composed of inflammatory cells. Inflammatory pseudotumors have been reported in several anatomic locations, such as orbit, respiratory tract, gastrointestinal tract, and liver[3]. However, splenic involvement is extremely rare. The first 2 cases of splenic inflammatory pseudotumor were reported in 1984. About one-half of the lesions are discovered incidentally during work-up for other malignancies after splenectomy for other conditions such as idiopathic thrombocytopenic purpura, or at autopsy[4]. Clinical symptoms are not specific to the disease, with left upper quadrant or epigastric pain. Splenomegaly is usually present. Laboratory investigations may reveal fever, anemia, hypergammaglobulinemia, thrombocytosis, or hypersplenism; however, more than one-third of the cases reported showed no evidence of any abnormality in laboratory investigations[1]. Other patients have signs of immune thrombocytopenic purpura[4]. However, our patient only had history of hepatitis B with mild vague abdominal discomfort.

There are several radiological modalities suggested for diagnosing splenic IPT, including ultrasonography, CT, and MRI. Ultrasonography usually shows a low echoic mass in most cases[5]. CT is the radiological

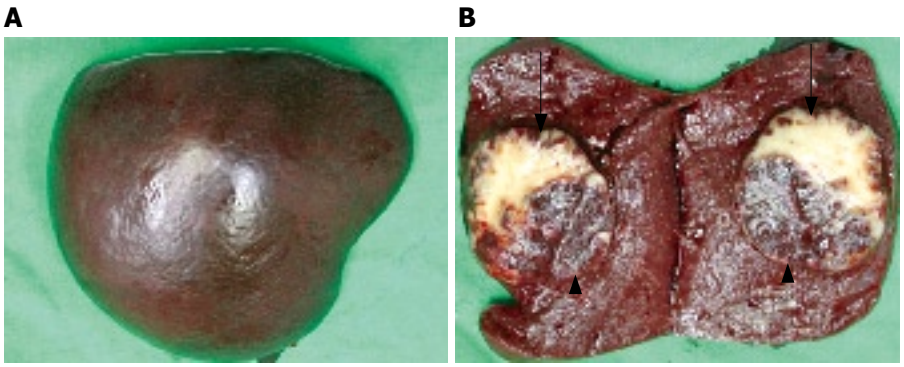

Figure 4 The specimen of spleen. The removed spleen (A) was 11 cm x 8.5 cm x 6 cm in size. A well-circumscribed tumor (B), measuring 5.5 cm x 5 cm x 4 cm, over the central portion of the spleen was noted. There were two parts of different appearances comprising the tumor. The major part of the circumscribed lesion (arrow) showed yellowish appearance with some stippling red spots scattered over the cut surface. While the minor portion (arrowhead) located at dorsal aspect showed similar appearance as the normal spleen parenchyma.

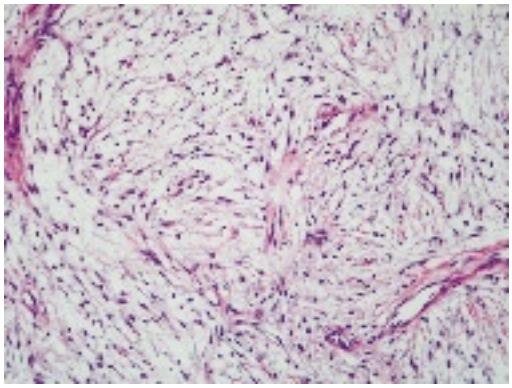

Figure 5 Histologic findings (hematoxylin and eosin stain, original magnification x 200). The sections of the specimen are composed of fibrosis, focal sclerosis, plump spindle cells and vascular proliferation.

test that most often demonstrates the presence of a splenic lesion and usually demonstrates a low-density mass in both the non-enhanced and enhanced modes. Although CT is quite sensitive, this modality is not specific in differentiating between splenic IPT and other malignancies[3]. MRI findings usually show an iso- or low-intensity mass on the T1-weighted images and a low-intensity mass on the T2-weighted images, while also demonstrating a low-intensity in the early phase and a high-intensity mass in the delayed phase of a dynamic study. However, the same criticism can be said about MRI with T1- and T2-weighted imaging for the poor specificity in differentiating pseudotumors from other malignancies[3].

Gross features in all reported cases of splenic IPT are described as a solitary well-circumscribed lesion with a white or tan cut surface that compresses the adjacent splenic parenchyma. Histological examinations of specimens are composed of acute and chronic inflammatory cells infiltrating a stroma of mesenchymal myofibroblastic spindle cells. The inflammatory cells are predominantly plasma cells, mature lymphocytes, and rarely eosinophils.

Etiology and pathogenesis of IPT remain unknown. Some characteristics described support an immunologic disorder[4]. Pathologic features of pseudotumor may be related to the production of mediators in inflammation, and note that interleukin-1 can produce local lesions and systemic manifestations with IPT. In addition, granulomatous inflammation process, focal parenchymal necrosis with hemorrhage, disturbance of blood supply or bacterial or viral infection have all been speculated in the pathogenesis of splenic IPT[6].

If a primary splenic tumor is suspected, splenectomy is required for diagnostic purposes and for therapy. Laparoscopic surgery for benign splenic tumors is considered to be a valid procedure. However, if the splenic lesions have a malignant potential, laparoscopic surgery elevates the risk of either intra-abdominal dissemination or skin metastasis. Therefore, if malignant splenic tumor can not be ruled out, laparoscopic surgery is not suggested[7].

Angiosarcoma is a malignancy of vascular origin, and is characterized by masses of endothelial cells with cellular atypia and anaplasia[2]. Angiosarcoma may occur anywhere in the body, but most often in the skin, soft tissues, breast and liver. Splenic involvement is exceedingly rare. Clinical manifestations include abdominal discomforts, splenomegaly and signs of immune thrombocytopenic purpura[2], which were not specific to the disease. Pathogenesis remains obscure. Prognosis is extremely poor, with a 6-month survival rate of 20%[8]. The tumor commonly metastasizes to the liver, lung, bone, lymph nodes, omentum, or peritoneum.

There are also several radiological modalities suggested for diagnosing splenic angiosarcoma including sonography, CT, MRI and angiography. Unlike splenic pseudotumor, sonographic findings in splenic angiosarcoma show a heterogeneous mass or multiple reflective areas[8,9]. CT may demonstrate hypoattenuating lesions on nonenhanced scans. Areas of high attenuation on noncontrast CT may represent acute hemorrhage or hemosiderin deposits. Contrast enhancement of angiosarcoma may show some enhancements similar to that of hepatic hemangioma[2]. The MRI appearance of splenic hemangioma is similar to that of hemangioma of the liver. The lesion will be hypo- or isointense on T1-weighted MR images and hypertense on T2-weighted MR images. T1-weighted images obtained after contrast administration may demonstrate a difference between the cystic and solid components[2]. The most specific modality to differentiate splenic IPT from angiosarcoma is angiography. Angiographic findings of angiosarcoma show a hypervascular tumor with contrast pooling, and that of splenic IPT usually show avascular or hypovascular tumor[10].

In the present case, sonography showed hyperechoic density in the central portion of the tumor. CT also

showed a high density area and partial enhancement in the central portion of splenic tumor with multiple diffuse low-attenuation nodules in liver. MRI revealed a mass, which showed partial dense intensity in T2-weighted image. In T1 mode with gadolinium-enhancement, peripheral nodule with contrast filling and pooling, which was compatible with a picture of angiosarcoma, was noted. Therefore, the first impression of angiosarcoma with suspected liver metastasis was reasonable preoperatively. The reason why splenic IPT could mimic the picture of angiosarcoma could be explained by the hypervascular component within the minor part of the tumor, which absorbed the contrast medium in radiological examination. Accordingly, the ITP should be kept in mind in the differential diagnosis of splenic space-occupying lesions even if the imaging modality does not favor it.

REFERENCES

1 **Ozkara SK**, Gurbuz Y, Ercin C, Muezzinoglu B, Turkmen M. Inflammatory pseudotumor of the spleen. *Virchows Arch* 2001; **438**: 629-631

2 **Thompson WM**, Levy AD, Aguilera NS, Gorospe L, Abbott RM. Angiosarcoma of the spleen: imaging characteristics in 12 patients. *Radiology* 2005; **235**: 106-115

3 **Chen WH**, Liu TP, Liu CL, Tzen CY. Inflammatory pseudotumor of the spleen. *J Chin Med Assoc* 2004; **67**: 533-536

4 **Hatsuse M**, Murakami S, Haruyama H, Inaba T, Shimazaki C. Inflammatory pseudotumor of the spleen complicated by idiopathic thrombocytopenic purpura. *Ann Hematol* 2005; **84**: 619-620

5 **Hayasaka K**, Soeda S, Hirayama M, Tanaka Y. Inflammatory pseudotumor of the spleen: US and MRI findings. *Radiat Med* 1998; **16**: 47-50

6 **Neuhauser TS**, Derringer GA, Thompson LD, Fanburg-Smith JC, Aguilera NS, Andriko J, Chu WS, Abbondanzo SL. Splenic inflammatory myofibroblastic tumor (inflammatory pseudotumor): a clinicopathologic and immunophenotypic study of 12 cases. *Arch Pathol Lab Med* 2001; **125**: 379-385

7 **Tsugawa K**, Hashizume M, Migou S, Kawanaka H, Sugimachi K, Irie H, Maeda T, Akaboshi K. Laparoscopic splenectomy for an inflammatory pseudotumor of the spleen: operative technique and case report. *Hepatogastroenterology* 1998; **45**: 1887-1891

8 **Hai SA**, Genato R, Gressel I, Khan P. Primary splenic angiosarcoma: case report and literature review. *J Natl Med Assoc* 2000; **92**: 143-146

9 **Ha HK**, Kim HH, Kim BK, Han JK, Choi BI. Primary angiosarcoma of the spleen. CT and MR imaging. *Acta Radiol* 1994; **35**: 455-458

10 **Moriyama S**, Inayoshi A, Kurano R. Inflammatory pseudotumor of the spleen: report of a case. *Surg Today* 2000; **30**: 942-946

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CASE REPORT

Rare pulmonary and cerebral complications after transarterial chemoembolization for hepatocellular carcinoma: A case report

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Author contributions: Zhao H and Lou JY contributed equally to this work; Zhao H, Lou JY, Wang HQ, Fan QQ, Chen XX, observed and treated the patient with rare complications; Zhao H and Lou JY wrote the paper.

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Abstract

We report a rare case of acute pulmonary and cerebral complication after transarterial chemoembolization (TACE) for inoperable hepatocellular carcinoma. The case involved a large tumor and hepatic vein invasion. Nonspecific pulmonary and cerebral symptoms such as acute dyspnoea and transient consciousness loss developed in the patient, a 49-year-old woman, following the TACE due to pulmonary and cerebral oil embolism. The chest and brain conditions of this patient improved after some supportive therapies and nursing interventions. She also subsequently completed the other three procedures of TACE.

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Key words: Hepatocellular carcinoma; Chemoembolization; Therapeutic; Pulmonary embolism; Cerebral embolism

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death in China where the majority of HCC patients have underlying hepatic B virus (HBV) infection and cirrhosis, and most cases are unresectable due to late stage and multifocali. Transarterial chemoembolization (TACE) is one of the most common treatment modalities as a palliative and preoperative method for patients with advanced HCC, which can improve the resection rate of HCC and prolong the survival time of patients. HCC has a tendency to invade the portal and hepatic veins, which may result in formation of hepatic arterio-venous shunts. Some rare complications such as remote ectopic embolism can be caused by this kind of abnormal shunts. In this paper, we report a rare pulmonary and cerebral complication of HCC, which is probably associated with hepatic vein invasion.

CASE REPORT

A 49-year-old woman was admitted to the Department of Radiology of the Second Affiliated Hospital of Zhejiang University in October 2004 with right upper quadrant pain and weight loss. She was a hepatitis B virus carrier. Her α-fetoprotein level was 1185.3 ng/mL. Ultrasonography and computed tomography (CT) revealed a 10-cm mass in the posterior segments of the right liver lobe. A 1.5-cm mass was also found in the left lateral segment. These clinical signs indicated that the patient had inoperable HCC and Child-Pugh class A cirrhosis. TACE was offered to the patient. Angiogram demonstrated no obvious hepatic arterio-venous shunt, but multiple smaller masses in both lobes of the liver. An emulsion of oxaliplatin, pirarubicin, hydroxycamptothecin and lipiodol were prepared, 35 mL and 3 mL of the mixture were administered intra-arterially to the right and left hepatic artery, respectively. The patient experienced right upper quadrant pain after TACE and had an uneventful recovery. One month later, a second TACE procedure was performed *via* the right hepatic artery and 40 mL of the mixture was administered. On the next day, she experienced sudden acute dyspnoea and the peripheral oxygen saturation decreased to 90%. The chest X-ray showed some

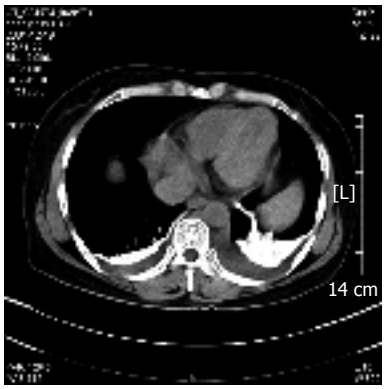

Figure 1 Chest CT scan revealing multiple iodized oil-like high-density materials in parenchyma of the lung.

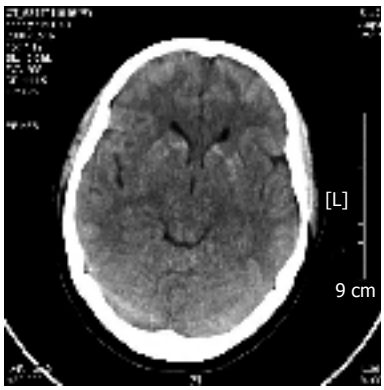

Figure 2 Non-contrast enhanced CT scanning showing multiple disseminated hyper-intense lesions in the brain, consistent with deposition of iodized oil.

increased reticular shadows in the left lung, especially in the lower zones, and a chest CT scan revealed multiple iodized oil-like high-density materials in parenchyma of the lung (Figure 1). After 10 mg dexamethasone i.v. and other supportive therapies were administered, the respiratory symptom was attenuated. Two days later, the patient suffered from a serious headache and transient consciousness loss, accompanying nausea and vomiting followed by confusion, lower extremity weakness. Non-contrast enhanced CT scanning showed multiple disseminated hyper-intense lesions in the brain, consistent with deposition of iodized oil (Figure 2). One week later, her respiratory and neurologic symptoms disappeared completely, and she was discharged. The patient also consequently completed the other three TACE procedures, during which no similar symptoms occurred.

DISCUSSION

TACE has various severe complications, including acute hepatic failure, intrahepatic biloma, pseudoaneurysm formation, and ectopic infarction, which occur in less than 1% of patients. Pulmonary embolism is a rare complication of TACE. Xia *et al*[1] reported a total of 2012 TACE procedures in 1348 patients, but pulmonary embolism occurred only in case. Sporadic cases of cerebral embolism after TACE have also been reported[2-5]. No reports are available on pulmonary embolism accompanying cerebral embolism after TACE. We encountered a rare pulmonary and cerebral complication of HCC, which was probably associated with hepatic vein invasion. The patient had nonspecific respiratory and neurological symptoms, including cough, dyspnoea, headache, transient loss of consciousness, confusion, and weak extremities. Chest X-ray and CT scanning showed some positive findings, indicating deposition of iodized oil, and the diagnosis of pulmonary and cerebral embolism was confirmed clinically.

The underlying mechanisms of pulmonary and cerebral embolism after TACE are still obscure. Hepatic arterio-venous shunt, which is associated with hepatic vein invasion of HCC, may be the reasonable explanation for pulmonary embolism. Vascular abnormalities, referred to as pulmonary arterio-venous shunt, can be found in patients with advanced liver disease[6]. If patients have pulmonary embolism after TACE, the oil emboli may also pass through the pulmonary arterio-venous shunt and enter the systemic circulation. In this case, the patient suffered from pulmonary and cerebral embolism subsequently. Thus, we hypothesize that iodized oil passed through the hepatic arterio-venous shunt, and then traveled to the cerebral artery through intrapulmonary arterio-venous shunt There are also some other hypotheses including intracardiac right-to-left shunt (e.g. patent foramen ovale) and right-to-left shunt *via* the arteriovenous anastomosis between the right inferior phrenic artery (IPA) and the intrapulmonary vasculature. However, there is no evidence to support the theory of intracardiac or IPA shunts in this patient.

Although pulmonary and cerebral embolism or infarctions are rare complications of TACE in patients with HCC, we should be aware of this kind of situations when we observe complications of TACE. When angiogram shows any hepatic arterio-venous shunts, we should decrease the dose of lipiodol during the procedure and pay great attentions to the respiratory and neurological symptoms post-operatively, which may be caused by ectopic embolism. The manifestations caused by lipiodol are different from those caused by thrombus, because lipiodol diffuses to peripheral blood vessels while thrombus usually obstructs the main branch of arteries.

A thorough patient assessment should be performed by nurses before the procedure. Some risk factors including a large size of HCC, hepatic vein invasion of HCC, live cirrhosis, congenital cardiovascular disease, and chronic pulmonary disease should be noticed before the procedure. If the patients have risk factors for pulmonary and cerebral complications of TACE, nurses should give a reassessment, extra education, psychological support.

In addition to post-embolization syndrome (PES), with its symptoms manifested as fever, pain, nausea, and vomiting, there are also some severe or rare complications of TACE, including acute hepatic failure,

intrahepatic biloma, pseudoaneurysm formation, ectopic infarction, *etc*. Nurses should keep in mind that a small number of patients after TACE will suffer from some severe or rare complications. Our patient developed a dry cough and dyspnea in the first day after TACE, followed by severe headache, nausea, vomiting, weak extremities and lost consciousness transiently in the third day. Nurses immediately provided nursing interventions such as semi-reclining position, inhaling oxygen, administration of steroids and dehydrating agents, and intensive monitoring of the patient's vital signs. She had a good recovery and was discharged. If some symptoms such as cough, chest pain, chest distress, headache, nausea, and vomiting occur in patients after TACE, nurses should make physical examination and notify the physician to exclude pulmonary and cerebral complications.

When the diagnosis of pulmonary embolism is confirmed, some nursing actions must be taken immediately. Nurses should keep the patient's airway open, and have oxygen inhaled to maintain the patient's basic respiratory function. Vital signs, blood oxygen saturation, mental status, and some laboratory values must be monitored and documented. If the patient has signs of respiratory failure, intensive care and mechanical ventilation must be provided promptly. Tiny lipiodol particles usually diffuse and stay in the peripheral bronchus and alveoli, which can damage gas exchange and cause special inflammations. Steroids, bronchodiators and prophylactic antibiotics should also be given.

The aim of nursing care during an acute phase of cerebral embolism after TACE is to minimize cerebral damage. Nurses should frequently observe the level of consciousness, pupil size and reaction to light, patient's response to commands, movement and strength, patient's vital signs, *etc*. To reduce the intracranial pressure, some nursing routines must be given such as keeping the head of bed above 30º, restriction of fluids, oxygen inhaling and administration of steroids and dehydrating agents. Sedatives and tranquilizers, which can depress the respiratory center and obscure neurological observations, should not be given except for some specific situations such as epileptic seizure attack.

In summary, even though pulmonary embolism and cerebral embolism are rare complications of TACE, we should be aware of these rare complications in patients with high risk factors and reduce the dose of iodized oil or stop the procedure.

REFERENCES

1 **Xia J**, Ren Z, Ye S, Sharma D, Lin Z, Gan Y, Chen Y, Ge N, Ma Z, Wu Z, Fan J, Qin L, Zhou X, Tang Z, Yang B. Study of severe and rare complications of transarterial chemoembolization (TACE) for liver cancer. *Eur J Radiol* 2006; **59**: 407-412

2 **Yoo KM**, Yoo BG, Kim KS, Lee SU, Han BH. Cerebral lipiodol embolism during transcatheter arterial chemoembolization. *Neurology* 2004; **63**: 181-183

3 **Wu RH**, Tzeng WS, Chang CM. Iodized oil embolization to brain following transcatheter arterial embolization of liver. *J Gastroenterol Hepatol* 2005; **20**: 1465-1467

4 **Takao H**, Makita K, Doi I, Watanabe T. Cerebral lipiodol embolism after transcatheter arterial chemoembolization of hepatocellular carcinoma. *J Comput Assist Tomogr* 2005; **29**: 680-682

5 **Matsumoto K**, Nojiri J, Takase Y, Egashira Y, Azama S, Kato A, Kitahara K, Miyazaki K, Kudo S. Cerebral lipiodol embolism: a complication of transcatheter arterial chemoembolization for hepatocellular carcinoma. *Cardiovasc Intervent Radiol* 2007; **30**: 512-514

6 **Lange PA**, Stoller JK. The hepatopulmonary syndrome. *Ann Intern Med* 1995; **122**: 521-529

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CASE REPORT

Port site and distant metastases of gallbladder cancer after laparoscopic cholecystectomy diagnosed by positron emission tomography

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Abstract

We report port site and distant metastases of unsuspected gallbladder cancer after laparoscopic cholecystectomy diagnosed by positron emission tomography (PET) in two patients. Patient 1, a 72-year-old woman was diagnosed as cholelithiasis and cholecystitis and received laparoscopic cholecystectomy. Unsuspected gallbladder cancer was discovered with histological result of well-differentiated squamous cell carcinoma of the gallbladder infiltrating the entire wall. A PET scan using F-18-fluorodeoxyglucose (FDG-PET) before radical resection revealed residual tumor in the gallbladder fossa and recurrence at port site and metastases in bilateral hilar lymph nodes. Patient 2, a 69-year-old woman underwent laparoscopic cholecystectomy more than one year ago with pathologically confirmed unsuspected adenosquamous carcinoma of stage pT1b. At 7-mo follow-up after surgery, the patient presented with nodules in the periumbilical incision. Excisional biopsy of the nodule revealed adenosquamous carcinoma. The patient was examined by FDG-PET, demonstrating increased FDG uptake in the right lobe of the liver and mediastinal lymph nodes consistent with metastatic disease. This report is followed by a discussion about the utility of FDG-PET in the gallbladder cancer.

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Key words: Gallbladder cancer; Positron emission tomography; Fluorodeoxyglucose

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INTRODUCTION

The vast majority of cholecystectomies are currently performed laparoscopically, and unsuspected gallbladder cancer can be discovered incidentally following 1% of routine cholecystectomies[1]. There is a suspicion that recurrence of the tumor in the abdominal incision is more common after laparoscopic operations. Several possible factors probably involved in the development of such metastases have been proposed[2,3]. Resection of the recurrent malignancy developed in the port sites is warranted, and may lead to survival benefit only when the port site metastases is the only manifestation of recurrent disease. The preoperative accurate evaluation of recurrent gallbladder cancer is essential for reasonable treatment. We report two cases of port site and distant metastases of unsuspected gallbladder cancer after laparoscopic cholecystectomy diagnosed by FDG-PET.

CASE REPORT

Patient 1

A 72-year-old woman presented with right upper quadrant pain and fever. She had a history of cholelithiasis documented by ultrasound, and intermittent attacks of biliary colic over 2 years. She was diagnosed with cholelithiasis and cholecystitis. She received laparoscopic

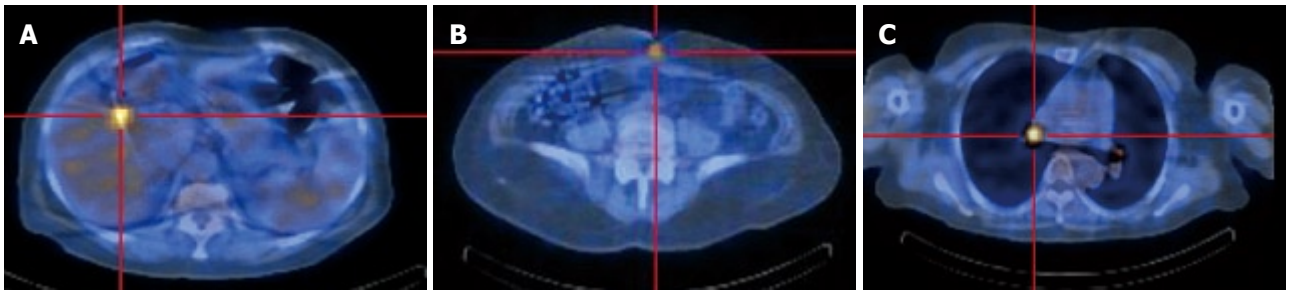

Figure 1 Increased uptake of the radiopharmaceutical in patient 1 (FDG-PET). A:Gallbladder fossa; B: Periumbilical area; C: Bilateral hilar lymph nodes.

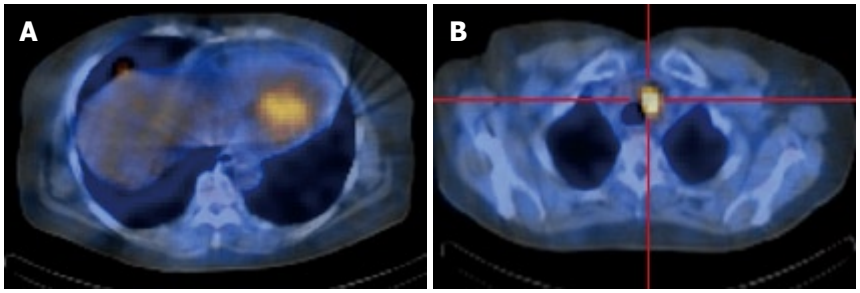

Figure 2 Increased uptake of the radiopharmaceutical in patient 2 (FDG-PET). A:Right lobe of the liver; B: Mediastinal lymph nodes.

cholecystectomy and the gallbladder was noted to be edematous and thick-walled, with multiple stones. Histological evaluation revealed an unsuspected well differentiated squamous cell carcinoma of the gallbladder infiltrating the entire wall. More than one month after surgery, she visited our hospital for further radical surgery. Palpable nodules in the periumbilical incision were found by physical examination during admission. A FDG-PET scan was performed, demonstrating increased uptake of the radiopharmaceutical in the gallbladder fossa and periumbilical area as well as bilateral hilar lymph nodes (Figure 1). The lesions were interpreted as residual tumor in the gallbladder fossa and recurrence at port site and metastases in bilateral hilar lymph nodes. Tru-cut biopsy confirmed metastatic squamous cell carcinoma similar to the previous histology. The patient refused further treatment and was discharged.

Patient 2

A 69-year-old woman with a history of intermittent right upper quadrant pain over 11 years underwent laparoscopic cholecystectomy more than 1 year ago. The histological examination revealed an unsuspected adenosquamous carcinoma of stage pT1b (tumor invades into muscularis). At 7-mo follow-up after surgery, the patient presented with nodules in the periumbilical incision interpreted as inflammation or postoperative change. The nodule enlarged progressively, and she visited our hospital 19 mo after surgery. Abdominal CT scan revealed a small nodule in the right lobe of the liver, which was difficult to interpret. Excisional biopsy of the nodule in the periumbilical incision was performed and histological examination revealed adenosquamous carcinoma. The patient was examined by FDG-PET, demonstrating increased FDG uptake in the right lobe of the liver and mediastinal lymph nodes consistent with metastatic disease (Figure 2). Chest computed tomography demonstrated enlarged mediastinal lymph nodes consistent with metastases. The patient has subsequently been treated with Gemzar and oxaliplatin with regression of tumor. She died of a non-cancer related cause 4 mo after the second operation.

DISSCUSSION

PET is a noninvasive scanning method to assess metabolism *in vivo* by means of positron-emitting radiolabeled tracers. This is in contrast with conventional imaging modalities, including ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI) which evaluate structural or anatomical changes[3]. The tracer to measure cellular metabolism commonly used in PET is FDG. FDG is a glucose analogue that is phosphorylated in the cells, but not further metabolized. Most malignant tumors show increased uptake of FDG because malignant transformation and growth of tumor cells is associated with overexpression of glucose transporters and increased hexokinase activity[4].

FDG-PET imaging has been increasingly used to identify and stage various tumors. The majority of these studies show FDG-PET to be superior to traditional imaging in the differential diagnosis of malignancy. This has been particularly notable in the evaluation of recurrent or metastatic disease[5-7]. A few studies have evaluated the use of FDG-PET in the assessment of biliary system tumors or gallbladder carcinoma[4,8-10]. In the study of Anderson *et al*, nine of 14 gallbladder cancer patients had residual carcinoma at the time of PET[9]. FDG-PET was useful in our cases to delineate recurrent gallbladder cancer, and its extent and had an important clinical impact on the selection of proper treatment. In both cases, FDG-PET detected residual tumor or port site and distant metastatic diseases and

changed the surgical plan of radical resection with intent for cure. This report emphasizes that FDG-PET may play an important role in the posttherapy follow-up of gallbladder cancer. Taking the accumulation of FDG in the malignant gallbladder cancer cells into consideration, FDG-PET can be considered as a complementary preoperative staging method. In cases in which it is easy to understaging gallbladder cancer before surgery such as peritoneal seeding, small hepatic metastases, and small regional lymph nodes involvement, FDG-PET may be able to provide important diagnostic information to obtain a correct presurgical staging, and sometimes lead to the change of treatment.

Gallbladder cancer is a relatively rare disease that has no specific symptoms or signs, and the clinical presentations of gallbladder cancer, and gallstone disease are commonly difficult to distinguish. The only effective treatment for carcinoma of the gallbladder is operative resection, and an open technique is preferred. Unfortunately, as is often the case, the lack of presurgical differential diagnosis hampers the planning of surgery. Recently, a few published articles have studied the utility of FDG-PET in gallbladder cancer focusing on not only posttherapy follow-up and preoperative staging, but also the establishment of the benign or malignant natures of gallbladder lesions. Koh *et al*[10] reported that FDG- PET provided reliable differential diagnoses, identifying gallbladder carcinoma with 75% sensitivity, 87.5% specificity, and 81.3% accuracy. Anderson *et al*[9] report that the sensitivity of this modality was 78% in their series of 14 gallbladder cancer cases. In the study of Antonio *et al*, which comprises a series of 16 patients, FDG-PET showed a sensitivity of 0.80, a specificity of 0.82 in diagnosing gallbladder cancer[4]. These studies revealed that FDG-PET can provide important information for establishing the nature of gallbladder lesions especially when in conjunction with conventional modalities.

Because FDG is taken up not only by malignant tumor cells, but also by activated inflammatory cells, benign inflammatory or infectious lesions typically without obvious increase of FDG uptake under some circumstances can produce false positive results[11,12]. The most common reason for false positive FDG-PETs is an inflammatory lesion. Xanthogranulomatous cholecystitis and polypoid lesion with adenomyomatosis are also the common reasons caused false positive result[4,10,11]. PET imaging must be interpreted with caution in patients with known severe inflammatory or granulomatous disease. Nishiyama *et al*[12] illustrated the relationship between the severity of inflammation and the specificity of PET, and proposed that patients with signs of acute inflammation should be excluded from examination. If the PET scans are performed under conditions with no or low-grade inflammation, an accurate diagnosis of acute or chronic cholecystitis as a benign lesion may be possible.

Although PET was sensitive for the detection of gallbladder cancer, some false negative findings also occurred. The limited sensitivity of FDG-PET for small lesions may have several causes[10,12]. Some factors illustrate the intrinsic limitations of PET resolution for small lesions: activity in small lesions may be underestimated because of the partial-volume effect, movement artifacts caused by nongated breath holding, or physiologic liver FDG uptake. PET scanning performed under suboptimal conditions can also decrease the sensitivity: patient fasting may be too short, and lead to an unnecessarily high liver FDG uptake; the duration of FDG administration and data acquisition may be too short. In diabetic patients, the rate of FDG accumulation in the tumor is decreased, impaired the sensitivity of FDG-PET[10]. In patients with mucinous adenocarcinoma of the gallbladder, a false-negative result has also been reported, probably secondary to poor cellular density[4]. To increase the sensitivity for small lesions, the underestimation due to the partial-volume effect may be reduced by improving the spatial resolution of PET; movement artifacts may be reduced by breath gating of the measurement, and by avoiding reintroduction of the patient to the scanner; PET scanning can be well performed under optimal conditions. Nishiyama *et al* adopted dual-time-point FDG-PET to evaluate the nature of gallbladder lesions, and demonstrated that delayed FDG-PET was more helpful than early FDG-PET in the evaluation of malignancy, because of the increased uptake by lesions, and the increased lesion-to-background contrast[12]. Recent hybrid PET-CT systems provide structural and functional information simultaneously, and may offer early and accurate staging with an improved specificity[13,14].

In conclusion, despite the relatively small number of gallbladder cancer patients, received FDG-PET scan, this imaging may play an important role in the differential diagnosis, staging, restaging, and posttherapy follow-up of gallbladder cancer.

REFERENCES

1 **Akyurek N**, Irkorucu O, Salman B, Erdem O, Sare M, Tatlicioglu E. Unexpected gallbladder cancer during laparoscopic cholecystectomy. *J Hepatobiliary Pancreat Surg* 2004; **11**: 357-361

2 **Lundberg O**. Port site metastases after laparoscopic cholecystectomy. *Eur J Surg Suppl* 2000; 27-30

3 **Lomis KD**, Vitola JV, Delbeke D, Snodgrass SL, Chapman WC, Wright JK, Pinson CW. Recurrent gallbladder carcinoma at laparoscopy port sites diagnosed by positron emission tomography: implications for primary and radical second operations. *Am Surg* 1997; **63**: 341-345

4 **Rodriguez-Fernandez A**, Gomez-Rio M, Llamas-Elvira JM, Ortega-Lozano S, Ferron-Orihuela JA, Ramia-Angel JM, Mansilla-Rosello A, Martinez-del-Valle MD, Ramos-Font C. Positron-emission tomography with fluorine-18-fluoro-2-deoxy-D-glucose for gallbladder cancer diagnosis. *Am J Surg* 2004; **188**: 171-175

5 **Herder GJ**, Kramer H, Hoekstra OS, Smit EF, Pruim J, van Tinteren H, Comans EF, Verboom P, Uyl-de Groot CA, Welling A, Paul MA, Boers M, Postmus PE, Teule GJ, Groen HJ. Traditional versus up-front [18F] fluorodeoxyglucose-positron emission tomography staging of non-small-cell lung cancer: a Dutch cooperative randomized study. *J Clin Oncol* 2006; **24**: 1800-1806

6 **Wiering B**, Krabbe PF, Jager GJ, Oyen WJ, Ruers TJ. The impact of fluor-18-deoxyglucose-positron emission

tomography in the management of colorectal liver metastases. *Cancer* 2005; **104**: 2658-2670
7 **Sperti C**, Pasquali C, Fiore V, Bissoli S, Chierichetti F, Liessi G, Pedrazzoli S. Clinical usefulness of 18-fluorodeoxyglucose positron emission tomography in the management of patients with nonpancreatic periampullary neoplasms. *Am J Surg* 2006; **191**: 743-748
8 **Wakabayashi H**, Akamoto S, Yachida S, Okano K, Izuishi K, Nishiyama Y, Maeta H. Significance of fluorodeoxyglucose PET imaging in the diagnosis of malignancies in patients with biliary stricture. *Eur J Surg Oncol* 2005; **31**: 1175-1179
9 **Anderson CD**, Rice MH, Pinson CW, Chapman WC, Chari RS, Delbeke D. Fluorodeoxyglucose PET imaging in the evaluation of gallbladder carcinoma and cholangiocarcinoma. *J Gastrointest Surg* 2004; **8**: 90-97
10 **Koh T**, Taniguchi H, Yamaguchi A, Kunishima S, Yamagishi H. Differential diagnosis of gallbladder cancer using positron emission tomography with fluorine-18-labeled fluoro-deoxyglucose (FDG-PET). *J Surg Oncol* 2003; **84**: 74-81
11 **Fletcher JW**, Djulbegovic B, Soares HP, Siegel BA, Lowe VJ, Lyman GH, Coleman RE, Wahl R, Paschold JC, Avril N, Einhorn LH, Suh WW, Samson D, Delbeke D, Gorman M, Shields AF. Recommendations on the use of 18F-FDG PET in oncology. *J Nucl Med* 2008; **49**: 480-508
12 **Nishiyama Y**, Yamamoto Y, Fukunaga K, Kimura N, Miki A, Sasakawa Y, Wakabayashi H, Satoh K, Ohkawa M. Dual-time-point 18F-FDG PET for the evaluation of gallbladder carcinoma. *J Nucl Med* 2006; **47**: 633-638
13 **Rodriguez-Fernandez A**, Gomez-Rio M, Medina-Benitez A, Moral JV, Ramos-Font C, Ramia-Angel JM, Llamas-Elvira JM, Ferron-Orihuela JA, Lardelli-Claret P. Application of modern imaging methods in diagnosis of gallbladder cancer. *J Surg Oncol* 2006; **93**: 650-664
14 **Casneuf V**, Delrue L, Kelles A, Van Damme N, Van Huysse J, Berrevoet F, De Vos M, Duyck P, Peeters M. Is combined 18F-fluorodeoxyglucose-positron emission tomography/computed tomography superior to positron emission tomography or computed tomography alone for diagnosis, staging and restaging of pancreatic lesions? *Acta Gastroenterol Belg* 2007; **70**: 331-338

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Meetings

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Transplantation

International Gastroenterological Congresses 2008
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EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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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

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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

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Contents

Contents

World Journal of Gastroenterology
Volume 14 **Number** 42 **November 14**, 2008

World Journal of Gastroenterology
Volume 14 **Number** 42 **November** 14, 2008

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EDITORIAL

Evolution of systemic therapy of advanced hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) commonly occurs in hepatitis B endemic areas, especially in Asian countries. HCC is highly refractory to cytotoxic chemotherapy. This resistance is partly related to its tumor biology, pharmacokinetic properties, and both intrinsic and acquired drug resistance. There is no convincing evidence thus far that systemic chemotherapy improves overall survival in advanced HCC patients. Other systemic approaches, such as hormonal therapy and immunotherapy, have also disappointing results. Recently, encouraging results have been shown in using sorafenib in the treatment of advanced HCC patients. In this review, we concisely summarize the evolution of developments in the systemic therapy of advanced HCC.

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Key words: Advanced hepatocellular carcinoma; Chemotherapy; Doxorubicin; Sorafenib

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth ranking cancer in the world, with more than 80% of cases occurring in Asia[1]. The most common causes of HCC are hepatitis B and C viral infections. Chronic hepatitis B viral infection is prevalent in Asian countries and accounts for most cases of HCC. In contrast, chronic hepatitis C viral infection is more common in Western countries. In recent years, there is an increasing incidence of HCC in Western countries, primarily due to increase in prevalence of hepatitis C viral infection.

Current effective treatments for HCC include liver resection, transplantation, various local ablative and trans-arterial therapies. Surgical resection and liver transplantation are the main curative treatments. Unfortunately, only around 20% patients, mostly diagnosed by regular screening, may benefit from these surgical therapies. Most other patients either present late with advanced tumor or have severe underlying cirrhosis, precluding any surgical or even loco-regional therapies. Previously, these patients can only be palliated by chemotherapy or best supportive treatment alone.

CHEMOTHERAPY TREATMENT OF HCC

HCC is a relatively chemo-resistant tumor and is highly refractory to cytotoxic chemotherapy. This resistance is partly related to its tumor biology, pharmacokinetic properties, and both intrinsic and acquired drug resistance.

In terms of HCC tumor biology, the liver is a defensive organ that responds to the damages in a unique manner. Non-hepatic epithelial cells progress through the cell cycle until they either die or arrest/repair; whereas growth-arrested (i.e. G0) hepatocytes proliferate in response to damage[2,3]. In fact, HCC cells show a higher apoptotic capacity in earlier stages of carcinogenesis. Nonetheless, in advanced stages, they gradually develop the resistance to apoptosis. This anti-apoptotic phenotype is associated with the development and progression of HCC[4]. More importantly, it also partly explains why HCC cells are resistant to cytotoxic chemotherapy. Moreover, p53 mutation is the most commonly encountered alternations in HCC. Both hepatitis viruses and chemicals are implicated in the etiology of p53 mutations during the molecular

Table 1 Pivotal chemotherapy trials in advanced HCC

Chemotherapy	Study	No. of patients	Response rate
Doxorubicin			
Pegylated liposomal doxorubicin	Halm	16	0
Doxorubicin	Johnson	32	44
Doxorubicin, 5-FU, mitomycin C	Al-Idrissi	40	13
Doxorubicin, bleomycin	Ravry	60	16
Doxorubicin, cisplatin	Lee	37	18.9
Doxorubicin, 5-FU, cisplatin, interferon (PIAF)	Yeo	91	20.9
Cisplatin			
Cisplatin	Falkson	35	17
Cisplatin, interferon	Ji	30	13.3
Cisplatin, 5-FU, mitoxantrone	Ikeda	51	27
Gemcitabine			
Gemcitabine	Fuchs	30	0
Gemcitabine	Yang	28	18
Gemcitabine, oxaliplatin	Taieb	21	19
Epirubicin, etoposide			
Epirubicin	Hochster	18	17
Etoposide	Melia	24	13
Epirubicin, etoposide	Bobbio-Pallavicini	36	39
Others			
Mitoxantrone	Lai	20	0
Paclitaxel	Chao	20	0
Irofulven	Falcon-Lizaraso	29	7
Irinotecan	O'Reilly	14	7
Nolatrexed	Stuart	26	8
T138067	Leung	21	10
Capecitabine	Patt	37	11
5-FU, interferon	Patt	28	14

pathogenesis of HCC[5]. As chemotherapeutic agents require p53 to induce apoptosis, tumors with a disruption in p53 pathway are thus resistant to chemotherapy. Furthermore, DNA topoisomerase Ⅱ alpha is over-expressed and up-regulated in HCC cell lines. Since doxorubicin targets DNA topoisomerase Ⅱ, over-expression of the protein in HCC may account for HCC resistance to doxorubicin-based therapy[6]. Regarding its pharmacokinetic properties, the liver plays a pivotal role in the metabolism of both endogenous and exogenous substances inside the body *via* the CYP450 enzyme system[7]. In cirrhotic patients, the total liver mass is reduced, and distortion of the liver architecture leads to significant intra-hepatic shunting and reduced extraction of protein-bound substances. Moreover, cirrhosis also affects the absorption, plasma protein binding, distribution and renal excretion of drugs. Therefore, cirrhosis has a significant impact on the pharmacokinetics of systemic therapy for HCC. Lastly, HCC cells have intrinsic drug resistance mediated by an enhanced cellular drug efflux mechanism, which is usually enacted through the drug transporter family of the ATP-binding cassette proteins that include MDR1, p-glycoprotein and the multidrug resistance protein[8,9]. Moreover, co-expression of p53 and p-glycoprotein also contribute to HCC drug resistance[9].

There is no convincing evidence, thus far, that systemic chemotherapy improves overall survival in advanced HCC patients[10]. Table 1 summarizes the results of pivotal chemotherapy studies of HCC. Single-agent doxorubicin has been shown to produce a response rate of about 10%-15%, but with no proven survival benefit[11]. It has been widely used and regarded as the standard systemic treatment for advanced HCC until recently. Significant grade 3 or 4 toxicities, especially neutropenia, are encountered in patients treated with doxorubicin[12]. Other chemotherapeutic agents, such as epirubicin, cisplatin, 5-fluorouracil, etoposide, and their combinations, have been studied with low response rates and no survival benefit[13]. Similarly, the newer generation of chemotherapeutic agents, such as gemcitabine, irinotecan and pegylated liposomal doxorubicin, also show disappointing results[14,15]. Combination chemotherapy has been employed in the treatment of advanced HCC. Although some of the combination regimes have shown promising activity in phase Ⅱ studies, most of them fail to demonstrate any survival advantage in randomized phase Ⅲ studies[16,17]. Especially, the combination of cisplatin, interferon-alpha-2b, doxorubicin and fluorouracil (PIAF) was under intense investigation at one time. In the phase Ⅱ study, Leung *et al*[18] showed on average 26% partial response, with 4 patients achieving a complete pathological response. Nevertheless, in the phase Ⅲ study, although this combination had achieved higher response rates than other combinations, there was no demonstrable survival benefit and there were considerable toxicities[19]. Recently, a phase Ⅱ study using a combination of gemcitabine and oxaliplatin demonstrated an 18% response rate and 76% of patients had the disease under control[20]. Similarly, another phase Ⅱ study showed a 6% response rate and a 72% disease control rate by employing a 3-wk cycle of capecitabine and oxaliplatin in the treatment of advanced HCC patients[21]. However, as with the results from the PIAF study, these 'promising' data need to be further validated in the ongoing randomized phase Ⅲ trials before they can be employed in routine clinical practice.

HORMONAL THERAPY

Estrogen receptor, progesterone receptor and androgen receptor are expressed in HCC[22,23]. Thus, hormonal agents were used to treat advanced HCC. Among various hormonal agents used for the treatment of advanced HCC patients, tamoxifen was frequently employed in the past, due to its good tolerability and oral administration. However, several prospective randomized controlled trials failed to demonstrate overall survival benefit in treating advanced HCC patients with tamoxifen[24-26]. Moreover, a recent meta-analysis conducted by Nowak *et al*[27] also showed no survival advantage.

IMMUNOTHERAPY OF HCC

Interferon is frequently employed in the treatment of viral hepatitis. However, its role in the treatment of HCC remains controversial. Studies conducted by Lai

et al[28,29] had shown encouraging efficacy with a 30% response rate and overall survival benefit from using a high dose of interferon (2.5×10^7-5.0×10^8 IU/m^2, three times weekly) to treat advanced HCC patients. However, there were significant treatment-related toxicities in patients who received high-dose interferon. On the other hand, when a lower dosage (3×10^6 IU/m^2, three times weekly) of interferon was used instead, there was no demonstrable clinical benefit[30].

SOMATOSTATIN ANALOG TREATMENT

The somatostatin analog octreotide and the long-acting form lanreotide are used in treating HCC, due to the presence of somatostatin receptors in HCC cells[31]. A randomized study, conducted by Kouroumalis *et al*[32], showed survival benefits in employing subcutaneous octreotide in the treatment of advanced HCC patients. Nevertheless, the study conducted by Becker *et al*[33] and Yuen *et al*[34] did not show any survival benefit in using lanreotide in the treatment of advanced HCC patients'.

THALIDOMIDE

Thalidomide was originally introduced in the 1960s as a sedative[35]. It was later re-evaluated for its anti-neoplastic effect in the 1990s. Its mechanism of action is poorly understood and complex, including anti-angiogenesis *via* the inhibition of VEGF, tumor necrosis factor-alpha and modulation of other inflammatory cytokines[36]. It was used in treating advanced HCC patients, mainly due to its anti-angiogenic property. However, single-agent thalidomide[37,38] and its combinations with epirubicin or interferon[39,40] only produced a response rate of 3.1%-6.3% with a median survival of 2.7-6.8 mo. In view of its limited activity and frequent association with treatment-related toxicities, it is now seldom included in the treatment algorithm for advanced HCC patients.

SORAFENIB FOR ADVANCED HCC

Growth factors and related receptors are often overexpressed and/or dysregulated in HCC. Clinical trials indicate that growth factor receptors and their related signalling pathways play important roles in HCC cancer etiology and progression, thus providing rational targets for innovative cancer therapies[41]. Among various growth factor pathways, the activation of the Raf/mitogen-activated protein kinase-extracellular signal-regulated kinase (MAPK) pathway[42,43] plays a pivotal role in promoting the tumor growth. Sorafenib is an oral multi-kinase inhibitor that blocks tumor proliferation by targeting the Raf/MAPK/ERK signaling pathway; it also has anti-angiogenic properties attained by targeting the tyrosine kinase VEGR-2, VEGFR-3 and PDGF receptor β[44]. Recently a large randomized phase Ⅲ study, the SHARP trial[45], was conducted and in this study, 602 patients with biopsy-proven advanced HCC were randomized to receive either sorafenib (400 mg twice daily, $n = 299$) or a placebo ($n = 303$). The results demonstrated a significant improvement in both OS (median 10.7 mo *versus* 7.9 mo) and TTP (median 5.5 mo *versus* 2.8 mo) in the sorafenib group *versus* the placebo group. These results represented a 44% increase in OS (hazards ratio, 0.69; $P = 0.00058$) and 73% prolongation in the TTP (hazards ratio, 0.58; $P = 0.000007$). Sorafenib was generally well-tolerated and serious adverse events only occurred in 13% of patients. This trial represents the first randomized systemic therapy trial that demonstrates the overall survival benefit of systemic treatment in patients with advanced HCC thus far. Similar to the study design of SHARP study, an Oriental sorafenib study was conducted to investigate the efficacy and tolerability of using single agent sorafenib in treating advanced HCC patients in hepatitis-B endemic Asian population[46]. In this study, the median OS of patient on sorafenib was 6.2 mo which was significantly better than 4.1 mo achieved in patients on placebo ($P = 0.0155$). Again, sorafenib was well-tolerated in Asian patient population. However, the commonest toxicities encountered in Asian patient population were hand-foot skin reactions (10.1%) instead of diarrhea (39%) in the SHARP trial.

CONCLUSION

The management of patients with advanced HCC has been a disappointing issue for decades. The recent development of single agent sorafenib, in the treatment of advanced HCC patients, indeed represents an important advance in this challenging disease. Although these two pivotal studies have demonstrated good activity and tolerability in treating advanced HCC patients with sorafenib, there are still many unresolved issues regarding the optimal use of sorafenib. In particular, most patients enrolled in these two pivotal trials had Child-Pugh A cirrhosis with favorable clinical parameters. Therefore, the benefits and safety profile of sorafenib in unselected advanced HCC patients, especially those with Child-Pugh B/C patients or other poor prognostic factors, are still unknown. Moreover, there are currently no reliable clinical parameters or biomarkers which may predict the response to sorafenib. In view of the high cost of sorafenib and potential toxicities associated with sorafenib, reliable biomarkers that can potentially guide the use of sorafenib in the treatment of advanced HCC patients is desperately needed.

Moreover, in the era of targeted therapy, proper patient selection, treatment assessment and endpoints are vital to the success of the clinical trials. This is especially true in HCC, where radiological assessment is difficult, because of poor delineation of the tumor in the liver. Recently, the HCC expert panel meeting recommended adopting a modification of RECIST criteria to assess tumor response[47]. Also, the time to progression was recommended by the panel as the primary endpoint in phase two trials testing targeted therapy in HCC.

The modest improvement, as demonstrated in these two pivotal trials; is still not optimal, as most patients

still have relatively short survival times when compared to patients with other solid tumors, such as colorectal cancer. Therefore, there is a need for researchers to unravel more of the underlying hepato-carcinogenesis mechanism and key molecular targets for therapeutic intervention. Moreover, another future focus will be on how to best combine the other targeting agents or chemotherapeutic agents in order to incrementally improve the survival of advanced HCC patients.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001; **94**: 153-156
2 **Taub R**. Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol* 2004; **5**: 836-847
3 **Epstein RJ**, Leung TW. Reversing hepatocellular carcinoma progression by using networked biological therapies. *Clin Cancer Res* 2007; **13**: 11-17
4 **Fabregat I**, Roncero C, Fernandez M. Survival and apoptosis: a dysregulated balance in liver cancer. *Liver Int* 2007; **27**: 155-162
5 **Hussain SP**, Schwank J, Staib F, Wang XW, Harris CC. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene* 2007; **26**: 2166-2176
6 **Watanuki A**, Ohwada S, Fukusato T, Makita F, Yamada T, Kikuchi A, Morishita Y. Prognostic significance of DNA topoisomerase IIalpha expression in human hepatocellular carcinoma. *Anticancer Res* 2002; **22**: 1113-1119
7 **Lee WM**. Drug-induced hepatotoxicity. *N Engl J Med* 2003; **349**: 474-485
8 **Ng IO**, Liu CL, Fan ST, Ng M. Expression of P-glycoprotein in hepatocellular carcinoma. A determinant of chemotherapy response. *Am J Clin Pathol* 2000; **113**: 355-363
9 **Park JG**, Lee SK, Hong IG, Kim HS, Lim KH, Choe KJ, Kim WH, Kim YI, Tsuruo T, Gottesman MM. MDR1 gene expression: its effect on drug resistance to doxorubicin in human hepatocellular carcinoma cell lines. *J Natl Cancer Inst* 1994; **86**: 700-705
10 **Palmer DH**, Hussain SA, Johnson PJ. Systemic therapies for hepatocellular carcinoma. *Expert Opin Investig Drugs* 2004; **13**: 1555-1568
11 **Lai EC**, Choi TK, Cheng CH, Mok FP, Fan ST, Tan ES, Wong J. Doxorubicin for unresectable hepatocellular carcinoma. A prospective study on the addition of verapamil. *Cancer* 1990; **66**: 1685-1687
12 **Gish RG**, Porta C, Lazar L, Ruff P, Feld R, Croitoru A, Feun L, Jeziorski K, Leighton J, Gallo J, Kennealey GT. Phase III randomized controlled trial comparing the survival of patients with unresectable hepatocellular carcinoma treated with nolatrexed or doxorubicin. *J Clin Oncol* 2007; **25**: 3069-3075
13 **Zhu AX**. Systemic therapy of advanced hepatocellular carcinoma: how hopeful should we be? *Oncologist* 2006; **11**: 790-800
14 **O'Reilly EM**, Stuart KE, Sanz-Altamira PM, Schwartz GK, Steger CM, Raeburn L, Kemeny NE, Kelsen DP, Saltz LB. A phase II study of irinotecan in patients with advanced hepatocellular carcinoma. *Cancer* 2001; **91**: 101-105
15 **Halm U**, Etzrodt G, Schiefke I, Schmidt F, Witzigmann H, Mossner J, Berr F. A phase II study of pegylated liposomal doxorubicin for treatment of advanced hepatocellular carcinoma. *Ann Oncol* 2000; **11**: 113-114
16 **Ikeda M**, Okusaka T, Ueno H, Takezako Y, Morizane C. A phase II trial of continuous infusion of 5-fluorouracil, mitoxantrone, and cisplatin for metastatic hepatocellular carcinoma. *Cancer* 2005; **103**: 756-762
17 **Lee J**, Park JO, Kim WS, Park SH, Park KW, Choi MS, Lee JH, Koh KC, Paik SW, Yoo BC, Joh J, Kim K, Jung CW, Park YS, Im YH, Kang WK, Lee MH, Park K. Phase II study of doxorubicin and cisplatin in patients with metastatic hepatocellular carcinoma. *Cancer Chemother Pharmacol* 2004; **54**: 385-390
18 **Leung TW**, Patt YZ, Lau WY, Ho SK, Yu SC, Chan AT, Mok TS, Yeo W, Liew CT, Leung NW, Tang AM, Johnson PJ. Complete pathological remission is possible with systemic combination chemotherapy for inoperable hepatocellular carcinoma. *Clin Cancer Res* 1999; **5**: 1676-1681
19 **Yeo W**, Mok TS, Zee B, Leung TW, Lai PB, Lau WY, Koh J, Mo FK, Yu SC, Chan AT, Hui P, Ma B, Lam KC, Ho WM, Wong HT, Tang A, Johnson PJ. A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. *J Natl Cancer Inst* 2005; **97**: 1532-1538
20 **Louafi S**, Boige V, Ducreux M, Bonyhay L, Mansourbakht T, de Baere T, Asnacios A, Hannoun L, Poynard T, Taieb J. Gemcitabine plus oxaliplatin (GEMOX) in patients with advanced hepatocellular carcinoma (HCC): results of a phase II study. *Cancer* 2007; **109**: 1384-1390
21 **Boige V**, Raoul JL, Pignon JP, Bouche O, Blanc JF, Dahan L, Jouve JL, Dupouy N, Ducreux M. Multicentre phase II trial of capecitabine plus oxaliplatin (XELOX) in patients with advanced hepatocellular carcinoma: FFCD 03-03 trial. *Br J Cancer* 2007; **97**: 862-867
22 **Nagasue N**, Ito A, Yukaya H, Ogawa Y. Estrogen receptors in hepatocellular carcinoma. *Cancer* 1986; **57**: 87-91
23 **Boix L**, Bruix J, Castells A, Fuster J, Bru C, Visa J, Rivera F, Rodes J. Sex hormone receptors in hepatocellular carcinoma. Is there a rationale for hormonal treatment? *J Hepatol* 1993; **17**: 187-191
24 **Castells A**, Bruix J, Bru C, Ayuso C, Roca M, Boix L, Vilana R, Rodes J. Treatment of hepatocellular carcinoma with tamoxifen: a double-blind placebo-controlled trial in 120 patients. *Gastroenterology* 1995; **109**: 917-922
25 **Tamoxifen in treatment of hepatocellular carcinoma: a randomised controlled trial**. CLIP Group (Cancer of the Liver Italian Programme) *Lancet* 1998; **352**: 17-20
26 **Chow PK**, Tai BC, Tan CK, Machin D, Win KM, Johnson PJ, Soo KC. High-dose tamoxifen in the treatment of inoperable hepatocellular carcinoma: A multicenter randomized controlled trial. *Hepatology* 2002; **36**: 1221-1226
27 **Nowak A**, Findlay M, Culjak G, Stockler M. Tamoxifen for hepatocellular carcinoma. *Cochrane Database Syst Rev* 2004; CD001024
28 **Lai CL**, Lau JY, Wu PC, Ngan H, Chung HT, Mitchell SJ, Corbett TJ, Chow AW, Lin HJ. Recombinant interferon-alpha in inoperable hepatocellular carcinoma: a randomized controlled trial. *Hepatology* 1993; **17**: 389-394
29 **Lai CL**, Wu PC, Lok AS, Lin HJ, Ngan H, Lau JY, Chung HT, Ng MM, Yeoh EK, Arnold M. Recombinant alpha 2 interferon is superior to doxorubicin for inoperable hepatocellular carcinoma: a prospective randomised trial. *Br J Cancer* 1989; **60**: 928-933
30 **Llovet JM**, Sala M, Castells L, Suarez Y, Vilana R, Bianchi L, Ayuso C, Vargas V, Rodes J, Bruix J. Randomized controlled trial of interferon treatment for advanced hepatocellular carcinoma. *Hepatology* 2000; **31**: 54-58
31 **Verhoef C**, van Dekken H, Hofland LJ, Zondervan PE, de Wilt JH, van Marion R, de Man RA, IJzermans JN, van Eijck CH. Somatostatin receptor in human hepatocellular carcinomas: biological, patient and tumor characteristics. *Dig Surg* 2008; **25**: 21-26
32 **Kouroumalis E**, Skordilis P, Thermos K, Vasilaki A, Moschandrea J, Manousos ON. Treatment of hepatocellular carcinoma with octreotide: a randomised controlled study. *Gut* 1998; **42**: 442-447
33 **Becker G**, Allgaier HP, Olschewski M, Zahringer A, Blum HE. Long-acting octreotide versus placebo for treatment of advanced HCC: a randomized controlled double-blind study. *Hepatology* 2007; **45**: 9-15

34 **Yuen MF**, Poon RT, Lai CL, Fan ST, Lo CM, Wong KW, Wong WM, Wong BC. A randomized placebo-controlled study of long-acting octreotide for the treatment of advanced hepatocellular carcinoma. *Hepatology* 2002; **36**: 687-691
35 **Somers GF**. Pharmacological properties of thalidomide (alpha-phthalimido glutarimide), a new sedative hypnotic drug. *Br J Pharmacol Chemother* 1960; **15**: 111-116
36 **Eleutherakis-Papaiakovou V**, Bamias A, Dimopoulos MA. Thalidomide in cancer medicine. *Ann Oncol* 2004; **15**: 1151-1160
37 **Hsu C**, Chen CN, Chen LT, Wu CY, Yang PM, Lai MY, Lee PH, Cheng AL. Low-dose thalidomide treatment for advanced hepatocellular carcinoma. *Oncology* 2003; **65**: 242-249
38 **Yau T**, Chan P, Wong H, Ng KK, Chok SH, Cheung TT, Lam V, Epstein RJ, Fan ST, Poon RT. Efficacy and tolerability of low-dose thalidomide as first-line systemic treatment of patients with advanced hepatocellular carcinoma. *Oncology* 2007; **72** Suppl 1: 67-71
39 **Zhu AX**, Fuchs CS, Clark JW, Muzikansky A, Taylor K, Sheehan S, Tam K, Yung E, Kulke MH, Ryan DP. A phase II study of epirubicin and thalidomide in unresectable or metastatic hepatocellular carcinoma. *Oncologist* 2005; **10**: 392-398
40 **Schwartz JD**, Sung M, Schwartz M, Lehrer D, Mandeli J, Liebes L, Goldenberg A, Volm M. Thalidomide in advanced hepatocellular carcinoma with optional low-dose interferon-alpha2a upon progression. *Oncologist* 2005; **10**: 718-727
41 **Hopfner M**, Schuppan D, Scherubl H. Growth factor receptors and related signalling pathways as targets for novel treatment strategies of hepatocellular cancer. *World J Gastroenterol* 2008; **14**: 1-14
42 **Huynh H**, Nguyen TT, Chow KH, Tan PH, Soo KC, Tran E. Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: its role in tumor progression and apoptosis. *BMC Gastroenterol* 2003; **3**: 19
43 **Guan J**, Chen XP, Zhu H, Luo SF, Cao B, Ding L. Involvement of extracellular signal-regulated kinase/mitogen-activated protein kinase pathway in multidrug resistance induced by HBx in hepatoma cell line. *World J Gastroenterol* 2004; **10**: 3522-3527
44 **Wilhelm SM**, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post LE, Bollag G, Trail PA. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004; **64**: 7099-109
45 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Haussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390
46 **Cheng A**, Kang Y, Chen Z, Tsao C, Qin S, Kim J, Burock K, Zou J, Voliotis D, Guan ZZ. Randomized phase III trial of sorafenib versus placebo in Asian patients with advanced hepatocellular carcinoma. *J Clin Oncol* (Meeting Abstracts) 2008; **26**: 4509
47 **Llovet JM**, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, Sherman M, Schwartz M, Lotze M, Talwalkar J, Gores GJ. Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008; **100**: 698-711

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OBSERVER

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Segmental colitis associated with diverticulosis syndrome

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Abstract

An inflammatory process that involves the sigmoid colonic segment associated with diverticular disease (SCAD) appears to be a distinct clinical and pathological disorder. It has been described in older adults, often presenting with rectal bleeding. Most of the patients seem to respond to treatment only with a 5-aminosalicylate, but some spontaneously resolve with no treatment. Endoscopic evaluation usually shows a non-specific inflammatory process localized in the sigmoid colon alone that may resolve completely with histologically normal colonic mucosa. Repeated symptomatic events with discrete episodes of segmental colitis may occur, but most patients have an entirely benign clinical course. Definition of the underlying molecular events that occur with SCAD may be critically important in understanding the critical elements present in a colonic inflammatory process that can completely resolve without pharmacological or biological treatment.

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Key words: Segmental colitis; Diverticulosis; Crohn's disease; Natural history; Ulcerative colitis; Colon cancer

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INTRODUCTION

Segmental colonic involvement with a well circumscribed inflammatory process may occur in different forms of infectious colitis or inflammatory bowel disease. In infectious disease, multiple different sites within the colon are likely to be involved, sometimes with small bowel changes. In ulcerative colitis, the rectum or rectosigmoid segment is generally involved with disease extending in a continuous pattern proximally within the colon for a variable distance. During colonoscopic examination in ulcerative colitis, this inflammatory process seems to be well demarcated, while in others, less so. In Crohn's disease, any segment of the colon may be involved, most often cecum and/or ascending colon. Concomitant small intestinal disease is also present. In recent years, other less common, but distinct types of inflammatory disease have become better recognized. One segmental form associated with diverticulosis, usually involving only the sigmoid colon, is the so-called "SCAD syndrome", or more precisely, segmental colitis associated with diverticulosis (SCAD)[1-5].

CLINICAL FEATURES OF SCAD

This form of segmental involvement, especially if only the sigmoid colon is affected, frequently presents with bloody stools. In some, but not all, abdominal pain, cramping discomfort, or diarrhea may occur. Males are generally affected more often than females[6], and usually, most suffer from the initial onset of symptoms after 40 or 50 years of age, somewhat different from the usual female predominant distribution of Crohn's disease, including Crohn's disease of the colon[6]. In Crohn's disease, as well, studies have shown that over 80% of adults are usually diagnosed well before age 40[6-8]. Like other forms of idiopathic inflammatory bowel diseases, the cause and pathogenesis needs to be better characterized and elucidated[1,5,9]. Endoscopic examination reveals a localized sigmoid inflammatory process with normal rectal and proximal colonic mucosa. Detailed biopsy examination of the entire colonic mucosa shows a localized non-specific mucosal inflammatory process only in the sigmoid colon[6,10].

SELECTION BIAS

In some, an earlier diagnosis of a resected colonic neoplasm (i.e. adenoma) is not infrequent[6]. As a

result, a form of selection bias may be present as this subgroup of older patients may be simply more aware of the significance of rectal bleeding and their particular increased risk for recurrent adenomas or colon cancer. Therefore, many often seek expedient medical investigation to define their symptoms and alleviate their anxiety. The good news of detection of inflammatory mucosal disease, rather than colonic cancer, is the virtually immediate relief of their concerns.

LONG-TERM NATURAL HISTORY

Even better news is that most diagnosed with this segmental form of colitis, if followed over several years, remain well, usually resolving completely within a few weeks or months without recurrence[6]. Repeat endoscopic studies, including biopsies, reveal no evidence of residual inflammatory change. Some may have been initially treated with oral 5-aminosalicylates or other symptomatic agents. However, it is not certain that these medications are really necessary since some have spontaneously resolved with no traditional pharmacologic measures. Occasionally, other drugs have been used, including steroids, suggesting that there may be a spectrum of disease severity observed over time. In some cases, discrete episodes of recurrent symptoms and endoscopic changes have been defined and resection of the sigmoid colon was very rarely done. To date, no particular predisposition to colonic neoplastic disease, including invasive carcinoma, has been documented.

CLINICAL SIGNIFICANCE

This entity has special significance. First and most important, this inflammatory process is most often self-limited. Typically, the clinical course of SCAD is benign and it resolves without further recurrence or need for treatment. Due to similarities with other forms of inflammatory bowel disease, particularly Crohn's disease, the implications of an inaccurate diagnosis are evident. A case of segmental colitis involving the sigmoid colon, labeled as Crohn's colitis, might lead the treating physician or clinical trial investigator (in the case of new agents) to conclude that a positive outcome was due to the treatment provided rather than the natural history of an otherwise clinically benign inflammatory process. Anecdotal treatment success, including open label treatment trials of new agents for Crohn's disease, may be quite difficult to evaluate, especially if there are claims of "complete mucosal healing". Second, utilization of modern classification methods used for entities, such as Crohn's disease, that define age of diagnosis, location within the intestinal tract and clinical behavior may lead to further recognition of such entities so as to permit more precise treatment[7,8]. Finally, a "new" or distinct colonic inflammatory process that usually spontaneously resolves, such as SCAD, raises a critical need to better understand the molecular events that might be present allowing a long-term benign disease course. More precise treatments for other inflammatory colonic diseases aimed at cure, rather than remission, might then emerge.

REFERENCES

1 **Sladen GE**, Filipe MI. Is segmental colitis a complication of diverticular disease? *Dis Colon Rectum* 1984; **27**: 513-514
2 **Peppercorn MA**. Drug-responsive chronic segmental colitis associated with diverticula: a clinical syndrome in the elderly. *Am J Gastroenterol* 1992; **87**: 609-612
3 **Gore S**, Shepherd NA, Wilkinson SP. Endoscopic crescentic fold disease of the sigmoid colon: the clinical and histopathological spectrum of a distinctive endoscopic appearance. *Int J Colorectal Dis* 1992; **7**: 76-81
4 **Imperiali G**, Meucci G, Alvisi C, Fasoli R, Ferrara A, Girelli CM, Rocca F, Saibeni S, Minoli G. Segmental colitis associated with diverticula: a prospective study. Gruppo di Studio per le Malattie Infiammatorie Intestinali (GSMII). *Am J Gastroenterol* 2000; **95**: 1014-1016
5 **Goldstein NS**, Leon-Armin C, Mani A. Crohn's colitis-like changes in sigmoid diverticulitis specimens is usually an idiosyncratic inflammatory response to the diverticulosis rather than Crohn's colitis. *Am J Surg Pathol* 2000; **24**: 668-675
6 **Freeman HJ**. Natural history and long-term clinical behavior of segmental colitis associated with diverticulosis (SCAD syndrome). *Dig Dis Sci* 2008; **53**: 2452-2457
7 **Freeman HJ**. Application of the Montreal classification for Crohn's disease to a single clinician database of 1015 patients. *Can J Gastroenterol* 2007; **21**: 363-366
8 **Freeman HJ**. Granuloma-positive Crohn's disease. *Can J Gastroenterol* 2007; **21**: 583-587
9 **Evans JP**, Cooper J, Roediger WE. Diverticular colitis - therapeutic and aetiological considerations. *Colorectal Dis* 2002; **4**: 208-212
10 **West AB**, Losada M. The pathology of diverticulosis coli. *J Clin Gastroenterol* 2004; **38**: S11-S16

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REVIEW

Optical coherence tomography in detection of dysplasia and cancer of the gastrointestinal tract and bilio-pancreatic ductal system

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Abstract

Optical coherence tomography (OCT) is an optical imaging modality that performs high-resolution, cross-sectional, subsurface tomographic imaging of the microstructure of tissues. The physical principle of OCT is similar to that of B-mode ultrasound imaging, except that it uses infrared light waves rather than acoustic waves. The *in vivo* resolution is 10-25 times better (about 10 μm) than with high-frequency ultrasound imaging, but the depth of penetration is limited to 1-3 mm, depending upon tissue structure, depth of focus of the probe used, and pressure applied to the tissue surface. In the last decade, OCT technology has evolved from an experimental laboratory tool to a new diagnostic imaging modality with a wide spectrum of clinical applications in medical practice, including the gastrointestinal (GI) tract and pancreatic-biliary ductal system. OCT imaging from the GI tract can be done in humans by using narrow-diameter, catheter-based probes that can be inserted through the accessory channel of either a conventional front-view endoscope, for investigating the epithelial structure of the GI tract, or a side-view endoscope, inside a standard transparent ERCP catheter, for investigating the pancreatico-biliary ductal system. Esophagus and the esophago-gastric junction has been the most widely investigated organ so far; more recently, also duodenum, colon and pancreatico-biliary ductal system have been extensively investigated. OCT imaging of the gastrointestinal wall structure is characterized by a multiple-layer architecture that permits an accurate evaluation of the mucosa, lamina propria, muscularis mucosae, and part of the submucosa. The technique may be, therefore, used to identify pre-neoplastic conditions of the GI tract, such as Barrett's epithelium and dysplasia, and evaluate the depth of penetration of early-stage neoplastic lesions. OCT imaging of the pancreatic and biliary ductal system could improve the diagnostic accuracy for ductal epithelial changes and the differential diagnosis between neoplastic and non-neoplastic lesions.

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Key words: Optical coherence tomography; Barrett's epithelium; Dysplasia; Adenocarcinoma; Gastrointestinal tract; Pancreatico-biliary ductal system

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INTRODUCTION

Optical coherence tomography (OCT) is an optical imaging modality, introduced in 1991[1], that performs high-resolution, cross-sectional, subsurface tomographic imaging of the microstructure in materials and biologic systems by measuring backscattered or backreflected infrared light.

The physical principle of OCT is similar to that of B-mode ultrasound imaging, except that the intensity of infrared light, rather than sound waves, is measured. OCT devices use a low-power infrared light with a wavelength ranging from 750 to 1300 nm in which the only limiting factor is the scattering of light. Scattering occurs when the light interacts with tissue surface and the image formation depends upon the difference in optical backscattering properties of the tissue. OCT images are generated from measuring the echo time delay and the intensity of back-scattered light[2,3]. Wavelengths of

the infrared light used in OCT are one to two orders of magnitude higher than ultrasound wavelength, so OCT technology can yield a lateral and axial spatial resolution of about 10 μm, which is 10 to 25 fold better than that of available high-frequency ultrasound imaging[4]. The spatial resolution of OCT images is nearly equivalent to that of histologic sections. The depth of penetration of OCT imaging is approximately 1-3 mm, depending upon tissue structure, depth of focus of the probe used, and pressure applied to the tissue surface. Although the progressive increase in ultrasound resolution is accompanied by a corresponding decrease in depth of penetration, a similar trade-off between resolution and depth of penetration does not occur in OCT imaging.

In OCT, two-dimensional cross-sectional images of tissue microstructure are constructed by scanning the optical beam and performing multiple axial measurements of backscattered light at different transverse positions. The resulting data set is a two-dimensional array that represents the displayed as a grey-scale or false-color image.

Three types of scanning patterns are available for OCT imaging: radial[5,6], longitudinal[7,8], and transverse[9]. The radial-scan probe directs the OCT beam radially, giving images that are displayed in a "radar-like", circular plot. Radial scanning can easily image large areas of tissue by moving the probe back over the tissue surface and has the highest definition when the probe is inserted within a small diameter lumen, because the OCT images become progressively coarser when a large-diameter lumen is scanned, due to the progressive increase of pixel spacing with increasing the distance between the probe and the tissue. The linear and transverse probes scan the longitudinal and transverse positions of the OCT beam at a fixed angle, generating rectangular images of longitudinal and transverse planes at a given angle with respect to the probe. Linear scanning has the advantage that pixel spacing in the transverse direction is uniform and can better image a definite area of the scanned tissue, especially in presence of large-diameter and non-circular lumens, where maintaining constant distance from the probe to the surface over the entire circumferential scan may be impossible. Transverse scanning modality provides a better depth of field. Depth of field is the range of distances from the probe over which optimal resolution of scanning can be obtained; current OCT scans permit imaging depths of up to 2-3 mm in tissues, by using probes with different focuses.

In the last decade, OCT technology has evolved from an experimental laboratory tool to a new diagnostic imaging modality with a wide spectrum of clinical applications in medical practice, including the gastrointestinal (GI) tract and pancreatico-biliary ductal system.

OCT TECHNIQUE FOR GI TRACT AND PANCREATICO-BILIARY DUCTAL SYSTEM IMAGING

OCT imaging from the GI tract can be done in humans by using narrow-diameter, catheter-based probes. The probe can be inserted through the accessory channel of either a conventional front-view endoscope, for investigating the epithelial structure of the GI tract, or a side-view endoscope, inside a standard transparent ERCP catheter, for investigating the pancreatico-biliary ductal system.

OCT scanning can be done by maintaining the probe placed lightly or firmly on the wall of the GI tract. When the probe is placed lightly on the mucosal surface, the depth of penetration is limited mainly to the superficial submucosa; by this way superficial epithelium, lamina propria, and the upper part of submucosa are clearly visualized. When the probe is placed firmly against the mucosal surface, submucosa and muscularis propria can be clearly visualized, but details of the superficial layers of the mucosa are lost. When the OCT probe is held in strict contact with the tissue surface, as occurs when it is inserted across strictures of the pancreatico-biliary ductal system, the superficial epithelium may appear compressed and difficult to evaluate.

Several *in vitro* studies demonstrated the feasibility of OCT in the GI tract: In these studies the GI tract wall was identified as a multiple layer structure characterized by a sequence of hyper- and hypo-reflective layers, with a variable homogenicity of the back-scattered signal[8-10]. Neoplastic and normal tissue also showed different light backscattering patterns[11-13].

Subsequent studies were, therefore, performed in *ex vivo* tissue specimens and aimed at comparing OCT imaging with histology, to assess the reliability of the OCT technique to identify and recognize the GI and pancreatico-biliary wall structure. OCT was shown to clearly differentiate the layer structure of the wall[14].

In vivo studies confirmed the possibility of OCT to recognize the multiple-layer structure of the GI wall[15,16]; the possibility to introduce the OCT probe into a standard transparent catheter for cannulation during an ERCCP procedure permits the epithelial layers of the pancreato-biliary ductal system and sphincter of Oddi to be investigated. From a clinical point of view, OCT imaging of the pancreatic and biliary ductal system could improve the diagnostic accuracy for ductal epithelial changes and the differential diagnosis between neoplastic and non-neoplastic lesions, since in several conditions, X-ray morphology obtained by ERCP and other imaging techniques may be non-diagnostic, and the sensitivity of intraductal brush cytology during ERCP procedures is highly variable.

In our studies, a near-focus OCT probe (Pentax, Lightlab Imaging, Westford, MA, USA) was used, with a penetration depth of about 1 mm and a resolution of approximately 10 μm. The probe operates at 1.2-1.4 μm center wavelength (nominal value: 1.3 μm), with a scan frequency ranging from 1000 to 4000 kHz (nominal value: 3125 kHz). Radial and longitudinal scanning resolutions have an operating range in tissue of 15-20 μm (nominal value: 18 μm), and 21-27 μm (nominal value: 24 μm), respectively. Infrared light is delivered to the imaging site through a single optical fiber 0.006 diameter. OCT probe is assembled in a catheter with an outer

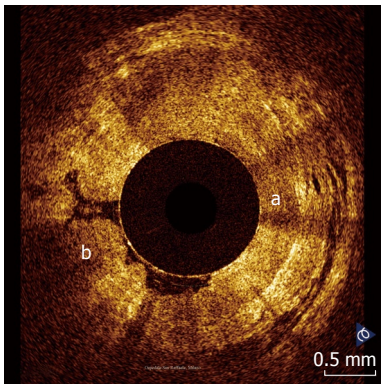

Figure 1 OCT pattern of squamo-columnar junction. The horizontal, layered architecture of the esophageal wall (a) appears clearly distinguishable from the vertical crypt-and-pit architecture of the gastric wall (b).

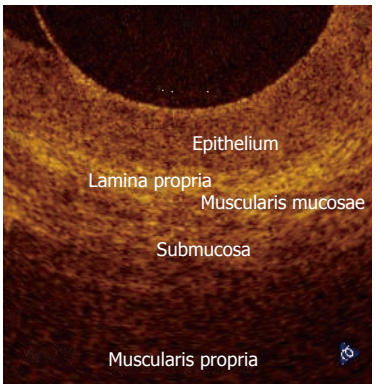

Figure 2 Magnification of a OCT image showing normal esophageal wall. The OCT image shows a multiple-layer structure characterized by a superficial weakly scattering (hypo-reflective) layer, corresponding to the squamous epithelium, a highly scattering (hyper-reflective) layer corresponding to the lamina propria, a weakly scattering layer corresponding to the muscularis mucosae, difficult to recognize, a moderately scattering layer corresponding to the submucosa, and a weakly scattering, deep layer corresponding to muscolaris propria.

diameter of 1.2 mm; the catheter-based probe consists of a rotating probe encased in a transparent outer sheath, which remains stationary while the rotating probe has a pullback movement of 1 mm/s, with an acquisition rate of 10 frames per second. Using this technique, a segment of tissue 5.5 cm long can be filmed over a 55-s period.

OCT RECOGNITION OF GI TRACT AND PANCREATICO-BILIARY WALL STRUCTURE IN NORMAL CONDITIONS

GI tract

The esophagus and the esophago-gastric junction has been the most widely investigated organ so far. Esophago-gastric junction appears at OCT investigation clearly recognizable because the stomach wall shows a different OCT pattern, characterized by the presence of a vertical crypt-and-pit architecture of the mucosa that changes abruptly to the horizontal, layered tissue architecture of the esophageal squamous epithelium (Figure 1).

In normal conditions, OCT imaging of esophageal wall recognizes a multiple-layer structure characterized by a superficial weakly scattering (hypo-reflective) layer, corresponding to the squamous epithelium, a highly scattering (hyper-reflective) layer corresponding to the lamina propria, a weakly scattering layer corresponding to the muscularis mucosae, a moderately scattering layer corresponding to the submucosa, and a weakly scattering, deep layer corresponding to muscularis propria (Figure 2). The latter layer is not even recognizable *in vivo*, depending on the depth of penetration of the OCT probe used. Submucosal glands and vessels have also been identified[5-8]. In a recent *ex vivo* study the muscularis mucosae was distinctly recognized during OCT investigation by using a Ti: Sapphire laser light source[16].

Overall, the normal esophageal wall architecture shows at OCT imaging a clearly recognizable horizontal, layered structure.

OCT images of the gastric mucosa are characterized by less contrast, depending upon the crypt-and-pit architecture of the glandular epithelium[2,8,12]. Four layers can be identified from the surface: The glandular epithelium, muscularis mucosae, submucosa with blood vessels, and muscularis propria[9]. Inflammation, as occurs in gastritis, has been reported to produce greater backscattering of the signal and a more pronounced crypt-and-pit pattern architecture, compared with normal tissue[17].

In the duodenum and small intestine OCT clearly recognizes the mucosa and submucosa with the vascular structure[5,15]. OCT identified intestinal villous morphology and the degree of atrophy with 100% agreement compared to histology in a study by Hsiung *et al*[18], who analyzed OCT images *ex vivo* on fresh surgical specimens from the small intestine compared with histology. The ability of OCT imaging to recognize the villous pattern and its alterations could be used to identify celiac disease in real time during standard upper GI endoscopy in patients undergoing endoscopy for conditions often related to a misdiagnosed celiac disease, such iron deficiency anemia, osteoporosis, diabetes mellitus, or autoimmune disorders, and select dyspeptic patients who need biopsies for detecting the disease[19,20].

In the colon, mucosa and submucosa can also be seen with strong correlation with histology. Mucosa appears as a hyper-reflective layer; submucosa as a hypo-reflective layer with horizontal striations, and the OCT appearance is related to its composition, which could be of assistance in the diagnosis of chronic inflammatory conditions involving the submucosa. Dynamic application of pressure of the OCT probe on the tissue reveals compressibility of both mucosa and submucosa, that may be another criteria for identifying chronic inflammation and fibrosis[19]. The detection of transmural inflammation serves to distinguish patients with Crohn's disease from those with ulcerative colitis; the detection of dysplasia may help in the follow-up of long-standing chronic inflammatory diseases[21]. In presence of dysplasia, the detection of lower boundary of the mucosa may help in identifying the extension of dysplastic changes.

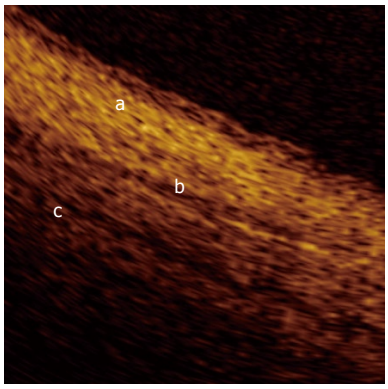

Figure 3 Magnification of an OCT image from the normal common bile duct wall. From the surface of the duct, up to a depth of 1 mm, the following layers are recognizable: the single layer of epithelial cells, approximately 0.04-0.06 mm thick, visible as a superficial, hypo-reflective band (a); the connective-muscular layer surrounding the epithelium, visible as a hyper-reflective layer approximately 0.34-0.48 mm thick (b); the connective layer visible as a hypo-reflective layer with longitudinal relatively hyper-reflective strips (smooth muscle fibers) (c).

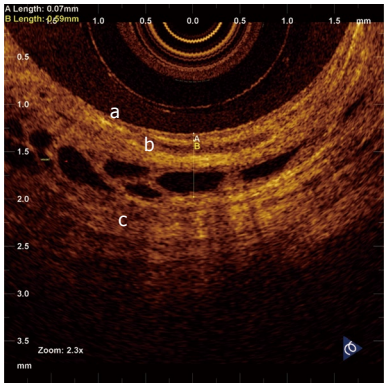

Figure 4 Magnification of an OCT image from the normal sphincter of Oddi wall. From the surface of the duct, up to a depth of 1 mm, the following layers are recognizable: The single layer of epithelial cells, approximately 0.04-0.08 mm thick, visible as a superficial, hypo-reflective band (a); the connective-muscular layer surrounding the epithelium, visible as a hyper-reflective layer approximately 0.23-0.37 mm thick (b); the connective layer visible as a hypo-reflective layer with longitudinal relatively hyper-reflective strips (smooth muscle fibers) (c). Within the intermediate and outer layer vessels are also recognizable, visualized as non-reflecting areas surrounded by a hypo-reflective endothelium. Margins between the intermediate and outer layer are poorly recognizable, due to the irregular distribution of connective and muscular structure.

Pancreatico-biliary ductal system

To date, visualization of the epithelium of the main pancreatic duct has been obtained mainly post-mortem[22] and *ex vivo* in humans[23,24], while *in vivo* it comes from one study in animals[25] and another in humans[26]. Normal biliary ductal system has been investigated in humans, *ex vivo* in a study[23] and *in vivo*, in two ERCP-based studies[27,28]. Sphincter of Oddi structure has also been investigated in normal and pathological conditions either in *ex vivo* or *in vivo* studies[23,27].

In a recent study by our group[26], OCT imaging of main pancreatic duct, common bile duct and sphincter of Oddi normal structure has been shown to be able to provide features that were similar to those observed

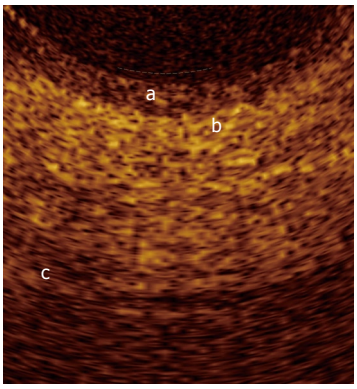

Figure 5 Magnification of an OCT image from the normal main pancreatic duct wall. From the surface of the duct, up to a depth of 1 mm, the following layers are recognizable: The single layer of epithelial cells, approximately 0.04-0.08 mm thick, visible as a superficial, hypo-reflective band (a); the connective-fibro-muscular layer surrounding the epithelium, visible as a hyper-reflective layer approximately 0.36-0.56 mm thick (b); the connective and acinar structure close to the ductal wall epithelium, visible as a hypo-reflective layer (c).

in the corresponding histological specimens in 80% of sections; the agreement between OCT and histology in the definition of normal wall was good (81.8%). OCT images identified three differentiated layers up to a depth of about 1 mm. From the surface of the duct, it was possible to recognize an inner hypo-reflective layer corresponding to the single layer of epithelial cells close to the lumen, an intermediate homogeneous hyper-reflective layer corresponding to the fibro-muscular layer surrounding the epithelium, and an outer, less definite, hypo-reflective layer corresponding to the smooth muscular structure within a connective tissue in the common bile duct and at the level of the sphincter of Oddi, and connective-acinar structure in the main pancreatic duct (Figures 3-5).

The three different layers showed a linear, regular surface and each layer had a homogeneous back-scattered signal in every frame; however, the differentiation between the intermediate and outer layer appeared more difficult than between the inner and intermediate layer. The thickness of the inner and intermediate layers measured by OCT was similar to those measured by histology; the muscular and connective-acinar structure was visible until the working depth of penetration into the tissue of the near-focus probe (about 1 mm).

Smooth muscle structure appeared at OCT scanning as hyper-reflective, longitudinal strips within a context of hypo-reflective tissue and was particularly recognizable at the level of sphincter of Oddi. Veins, arteries and secondary pancreatic ducts were also identifiable by OCT, characterized by hypo- or non-reflective, well delimited areas.

The images acquired in this study provided information on tissue architectural morphology that could have only previously be obtained with conventional biopsy. These results suggest that OCT could become a powerful imaging technology, enabling high-resolution diagnostic images to be obtained from the pancreato-biliary system during a diagnostic ERCP procedure.

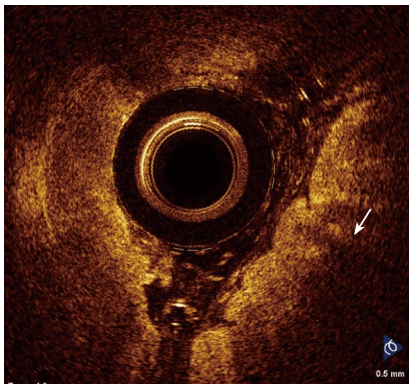

Figure 6 Barrett's esophagus (arrow). OCT features predictive for the presence of intestinal metaplasia are: The absence of the layered structure of the normal squamous epithelium and the presence of the vertical crypt-and-pit morphology of normal gastric mucosa; a disorganized architecture with inhomogeneous backscattering of the signal and an irregular mucosal surface; the presence of submucosal glands characterized by a markedly hypo-reflective tissue below the epithelial surface.

OCT RECOGNITION OF GI TRACT AND PANCREATICO-BILIARY WALL STRUCTURE IN DYSPLASTIC AND NEOPLASTIC CONDITIONS

At present, the exact cause of the disorganized architecture and altered light-scattering associated with dysplastic tissue by OCT imaging is unknown. A number of factors have been suggested, including subcellular morphological changes, altered fibrovascular stroma and abnormal mucin content associated with neoplastic tissue change, proliferation of cells leading to a loss of epithelial and stromal orientation, and altered cytological features such as an increased nuclear-to-cytoplasm ratio that may alter infrared light back-scattering[9,29].

In its current form and resolution, OCT will likely localize areas displaying architectural distortion to guide biopsy.

GI tract

Most of the so far published studies used OCT imaging to detect dysplasia and early cancer within Barrett's epithelium. Since the penetration depth of OCT does not exceed 1-2 mm, the technique could be useful, not only in detecting dysplasia, but also in staging superficial cancers that are difficult to stage accurately with ultrasound endoscopy. The technique appears, therefore, of crucial importance in the management of the disease.

Barrett's epithelium is characterized by the presence of specialized intestinal metaplasia within the esophageal mucosa. The hallmark histologic feature of specialized intestinal metaplasia is the presence of goblet cells. Identification of Barrett's epithelium is of clinical relevance since the lesion requires endoscopic follow-up, being a recognized precancerous condition. In clinical practice Barrett's epithelium is generally identified by performing multiple biopsies within the areas of gastric metaplasia, either in a random manner or after a previous vital staining with methylene blue (MB).

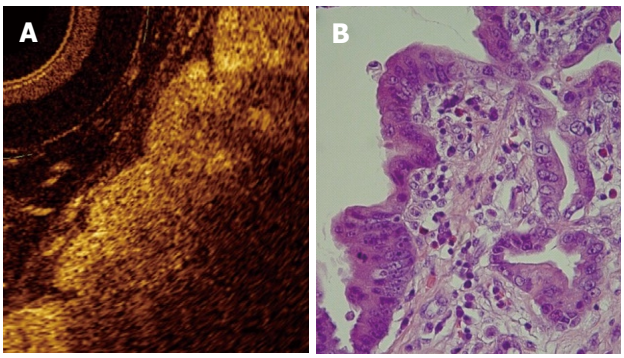

Figure 7 OCT image showing high-grade dysplasia (A) compared to histology (B). Because the degree of reflectivity depends upon nuclear size, a markedly inhomogenous and hypo-reflective back-scattering of the signal should indicate the presence of high-grade dysplasia.

Although the inter-subject variability of OCT imaging of normal squamous epithelium and gastric mucosa appears to be low, the OCT imaging of Barrett's epithelium demonstrated a greater variability in the previous published studies. OCT features predictive for the presence of intestinal metaplasia are: (1) the absence of the layered structure of the normal squamous epithelium and the presence of the vertical crypt-and-pit morphology of normal gastric mucosa; (2) a disorganized architecture with inhomogeneous back-scattering of the signal and an irregular mucosal surface; and (3) the presence of submucosal glands characterized at the OCT imaging as pockets of low reflectance below the epithelial surface[30-32] (Figure 6). When these OCT criteria were applied to images acquired prospectively, the criteria were found to be 97% sensitive and 92% specific for specialized intestinal metaplasia, with a PPV of 84%. The presence of the crypt-and-pit architecture may render difficult to discriminate between intestinal metaplasia and normal or inflamed gastric mucosa[9].

Unfortunately, up to now, attempts to identify OCT patterns characteristic for dysplasia, mainly the high-grade type, have been substantially disappointing. The increased nuclear-to-cytoplasmic ratio occurring in dysplasia may alter the light reflection characteristics, giving a more inhomogeneous back-scattering of the signal (Figure 7). Because the degree of reflectivity depends upon nuclear size, a markedly homogenous and hypo-reflective back-scattering of the signal should indicate the presence of high-grade dysplasia; moreover, it is possible that by quantitating the OCT signal as a function of depth, OCT would be able to characterize high-grade dysplasia within intestinal metaplasia tissue. Poneros *et al*[17], by using two parameters of tissue reflectivity as an indicator of dysplasia, retrospectively diagnosed high-grade dysplasia with 100% sensitivity and 85% specificity. Such an accurate analysis of the degree of signal reflectivity requires to avoid areas with incorrect artifact signal properties: This may be obtained by the identification of a precisely defined area with homogeneous signal reflectance, an adequate catheter-tissue contact, and a reduction of motion

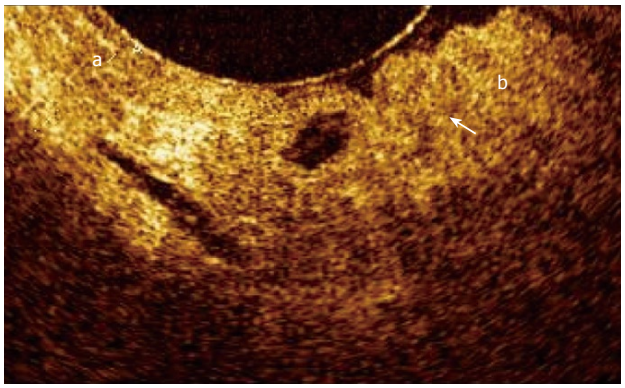

Figure 8 OCT image showing normal oesophageal mucosa (a) and Barrett's epithelium (b) with focal high-grade dysplasia (arrow).

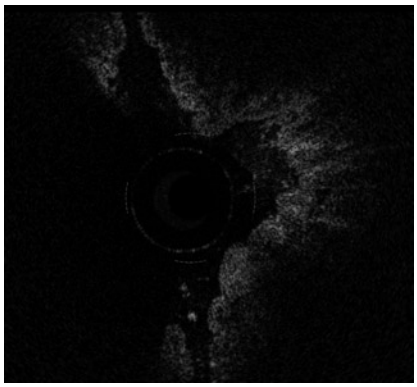

Figure 9 Magnified OCT imaging of early stage adenocarcinoma raised within Barrett's epithelium. The lack of the regular esophageal wall layered morphology and a markedly heterogeneous back-reflectance of the signal characterize the neoplastic lesion, which is confined within the epithelium.

artifacts. More recently, the morphological appearance of the OCT images, rather than the quantitative analysis of the OCT signal in the image, were used for the diagnosis and grading of dysplasia; for this purpose an endoscope fitted with an EMR standard cap was used, to stabilize the mucosal surface and avoid movement from esophageal peristalsis and transmitted cardiac and respiratory motion. In this study sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy for dysplasia were respectively, 68%, 82%, 53%, 89%, and 78%[33].

However, with the current available OCT devices, the recognition of dysplasia within intestinal metaplasia and mainly the differentiation between low- and high-grade dysplasia appears difficult[34] (Figure 8).

OCT features characteristic for adenocarcinoma arising from Barrett's epithelium are the lack of the regular esophageal wall layered morphology and a markedly heterogeneous back-reflectance of the signal[35,36] (Figure 9). These features permit to clearly identify the lesion and differentiate between the neoplastic and non-neoplastic tissue in advanced disease.

Figure 10 shows and compares OCT findings of normal esophageal mucosa, Barrett's epithelium, dysplasia, and adenocarcinoma.

Despite some studies were conducted about the use

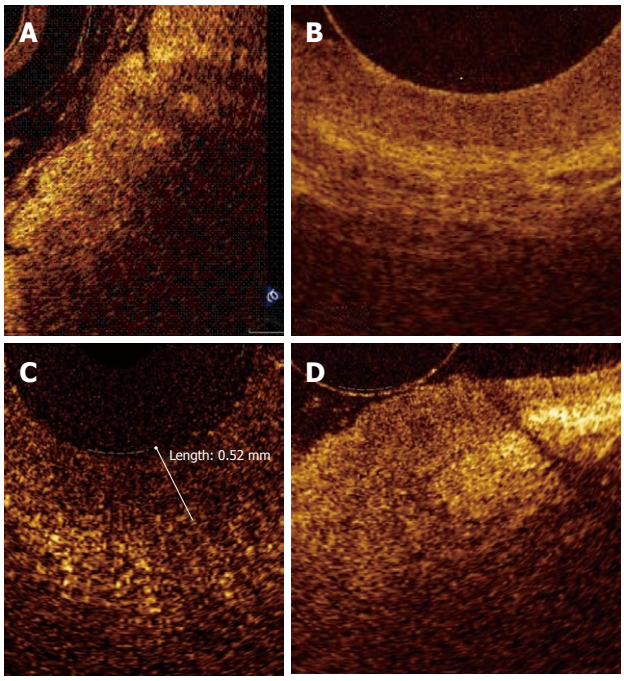

Figure 10 Comparison of OCT findings of normal esophageal mucosa (A), Barrett's epithelium (B), dysplasia (C), and adenocarcinoma (D).

of the OCT in the stomach and small intestine, no data are actually available about its use in detecting dysplasia.

Only few data are present in literature concerning the use of the OCT to detect dysplasia or cancer in the colon[37-39]. In a study by Pfau *et al*[39] on 24 patients, 30 dysplastic adenomas and 14 hyperplastic polyps were studied; the real-time OCT investigation showed that adenomas were more disorganized than the hyperplastic polyps, with a significantly more disorganized structure ($P = 0.0005$). Moreover, the infrared-light back-scattering of the adenomatous polyps appeared more hypo-reflective than hyperplastic ($P = 0.0007$). By using a computer-generated method to quantify the degree of scattering of individual pixels within a specified area in each image (60 × 60 pixels), it was found that the mean differences in light scattering were significantly greater between adenomatous and normal tissue (mean difference = 45.81), than between hyperplastic polyps and normal tissue (mean difference = 14.86). The real-time OCT infra-red light back-scattering score of polyps was also demonstrated to be a significant predictor of an adenomatous status. However, differently from the dysplasia occurring within Barrett's epithelium, in the study done by these authors defined OCT parameters histologically proven to detect colonic dysplasia were not found.

Pancreatico-biliary ductal system

Pathological pancreatic ductal system has been investigated by our group in humans in two *ex vivo* studies[14,24] performed on multiple surgical pancreatic specimens obtained from patients with pancreatic head adenocarcinoma.

In chronic inflammatory changes involving the main pancreatic duct, OCT still showed conserved three-layer

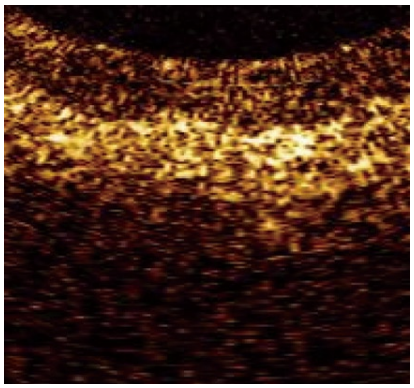

Figure 11 Magnified OCT images from section of main pancreatic duct showing low-grade dysplasia. The surface between the inner and intermediate layers appeared irregular. Dysplasia presents strong hyper-reflectance of the intermediate layer, particularly in the part closest to the inner layer. The outer layer did not differ from other non-malignant conditions and appeared homogeneously hypo-reflective.

architecture. However, the inner, hypo-reflective layer appeared slightly larger than normal and the intermediate layer appeared more hyper-reflective than in normal tissue; this is probably because of the dense mononuclear cell infiltrate. The back-scattered signal was heterogeneous with marked hypo- or hyper-reflectance in some sections. The agreement between OCT and histology in the definition of MPD chronic inflammatory changes was poor (27.7%).

The OCT pattern in presence of dysplasia of the main pancreatic duct epithelium was characterized by an inner layer markedly thickened, strongly hypo-reflective and heterogeneous; this OCT finding is probably due to the initial structural disorganization (increased mitosis and altered nucleus/cytoplasm ratio). The surface between the inner and intermediate layers appeared irregular. As in chronic inflammatory tissue, dysplasia too gave strong hyper-reflectance of the intermediate layer, particularly in the part closest to the inner layer. The outer layer did not differ from other non-malignant conditions and appeared homogeneously hypo-reflective (Figure 11). However, in chronic pancreatitis and dysplasia, only 62% of cases OCT and histology were concordant. The K statistic used to assess agreement between the two procedures was equal to 0.059 for non-neoplastic MPD wall appearance.

Overall, normal wall structure and chronic inflammatory or low-grade dysplastic changes cannot be distinguished in 38% of the sections because the architecture of the layers and surface light reflection did not show a characteristic OCT pattern.

In all sections with histologically proven adenocarcinoma, OCT showed a totally subverted MPD wall architecture. The three layers of the ductal wall and their linear, regular surface, normally giving a homogeneous back-scattered signal, were not recognizable. The margins between the connective-fibromuscular layer and acinar tissue were unidentifiable. The back-scattering of the signal appeared strongly heterogeneous, with minute, multiple, non-reflective

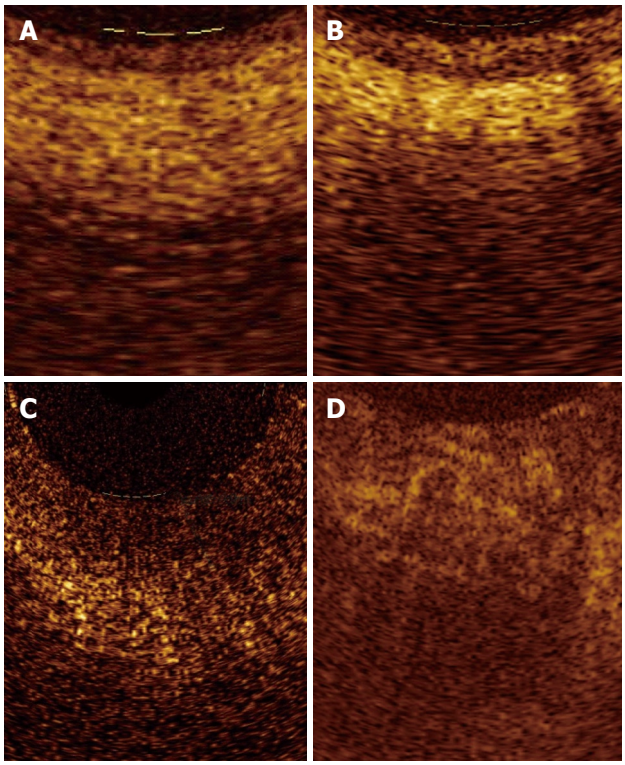

Figure 12 Magnified OCT images from sections with either normal (A), tumor-associated chronic inflammation (B), low-grade dysplasia (C), and adenocarcinoma (D) tissue.

areas in the disorganized pancreatic microstructure. Of sections with adenocarcinoma, OCT and histology were 100% concordant.

Figure 12 shows magnified OCT images from sections of main pancreatic duct with normal tissue, chronic pancreatitis, low-grade dysplasia, and adenocarcinoma.

Totally subverted wall architecture was also observed by OCT in presence of neoplastic tissue within the common bile duct[40] (Figure 13).

Studies *in vivo* were performed in animals[25] and humans[27]. We evaluated the diagnostic accuracy of OCT for the diagnosis of carcinoma, during ERCP, in a series of patients with MPD strictures of unknown etiology. In this study, the accuracy of OCT for detection of neoplastic tissue was 100%, compared with 66.7 % for intraductal brush cytology. The study showed that OCT is feasible during an ERCP procedure and was superior to brush cytology in distinguishing non-neoplastic from neoplastic lesions[26].

In conclusion, OCT appears a promising technique for real-time, high-resolution, cross-sectional imaging of the inner layer of the wall of the GI and pancreatobiliary tract, during the routine endoscopy. The technique recognizes with high definition the mucosa, muscularis mucosae and submucosa, and seems particularly useful in the study of the esophageal mucosa; given its superior resolution compared with other imaging modalities such as endoscopic ultrasonography (EUS) or catheter-probe EUS (CPEUS), OCT has great potential as a powerful adjunct to standard endoscopy in identification and

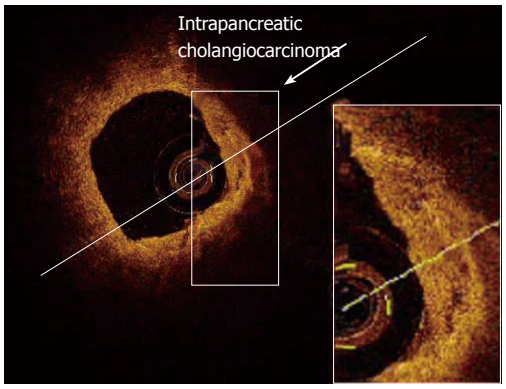

Figure 13 Magnified OCT images of neoplastic tissue within the intrapancreatic portion of the common bile duct.

surveillance of Barrett's epithelium, in order to detect high-grade dysplasia and adenocarcinoma at early stage and identify cases in whom mucosectomy becomes a curative procedure.

In the pancreatico-biliary ductal system, OCT can be used to discriminate between non-neoplastic and neoplastic tissue when strictures of unknown etiology are identified during an ERCP procedure, being its diagnostic accuracy higher than reported for intraductal brush cytology.

However, despite the promising studies reported in literature, with the current available OCT devices the recognition of dysplasia within intestinal metaplasia and mainly the differentiation between low- and high-grade dysplasia appears difficult.

On the other hand, since OCT has a penetration depth that does not exceed the 2 mm, it has a greater capability of diagnosing adenocarcinoma confined within mucosa and submucosa and could, therefore, be useful in staging superficial cancers that are difficult to stage accurately by EUS.

Features characteristic for adenocarcinoma within Barrett's epithelium are the lack of the regular layered morphology of the esophageal wall and a markedly heterogeneous back-reflectance of the signal. However, further studies are needed to evaluate whether OCT can identify and stage the lesion at an early stage.

OCT appears more promising in the differential diagnosis between non-neoplastic and neoplastic lesions arising within the pancreatico-biliary ductal system, since the ductal wall layered structure can be recognized easier and clearer.

At present, it seems to be fairly premature to affirm that OCT plays a role in the real-time diagnosis of dysplasia *in vivo*. However, improvements in both axial and lateral resolutions to the subcellular level (< 5 μm) together with the development of better light sources and optics, may allow dysplastic cells to be better identified in the future. Doppler OCT could also offer a unique ability to provide detailed subsurface imaging of mucosal microvascular networks.

REFERENCES

1 **Huang D**, Swanson EA, Lin CP, Schuman JS, Stinson WG, Chang W, Hee MR, Flotte T, Gregory K, Puliafito CA. Optical coherence tomography. *Science* 1991; **254**: 1178-1181
2 **Fujimoto JG**. Optical coherence tomography for ultrahigh resolution in vivo imaging. *Nat Biotechnol* 2003; **21**: 1361-1367
3 **Swanson EA**, Huang D, Hee MR, Fujimoto JG, Lin CP, Puliafito CA. High-speed optical coherence domain reflectometry. *Optics Letters* 1992; **17**; 151-153
4 **Das A**, Sivak MV Jr, Chak A, Wong RC, Westphal V, Rollins AM, Willis J, Isenberg G, Izatt JA. High-resolution endoscopic imaging of the GI tract: a comparative study of optical coherence tomography versus high-frequency catheter probe EUS. *Gastrointest Endosc* 2001; **54**: 219-224
5 **Sivak MV Jr**, Kobayashi K, Izatt JA, Rollins AM, Ung-Runyawee R, Chak A, Wong RC, Isenberg GA, Willis J. High-resolution endoscopic imaging of the GI tract using optical coherence tomography. *Gastrointest Endosc* 2000; **51**: 474-479
6 **Shen B**, Zuccaro G Jr. Optical coherence tomography in the gastrointestinal tract. *Gastrointest Endosc Clin N Am* 2004; **14**: 555-571
7 **Zuccaro G**, Gladkova N, Vargo J, Feldchtein F, Zagaynova E, Conwell D, Falk G, Goldblum J, Dumot J, Ponsky J, Gelikonov G, Davros B, Donchenko E, Richter J. Optical coherence tomography of the esophagus and proximal stomach in health and disease. *Am J Gastroenterol* 2001; **96**: 2633-2639
8 **Bouma BE**, Tearney GJ, Compton CC, Nishioka NS. High-resolution imaging of the human esophagus and stomach in vivo using optical coherence tomography. *Gastrointest Endosc* 2000; **51**: 467-474
9 **Poneros JM**, Brand S, Bouma BE, Tearney GJ, Compton CC, Nishioka NS. Diagnosis of specialized intestinal metaplasia by optical coherence tomography. *Gastroenterology* 2001; **120**: 7-12
10 **Tearney GJ**, Brezinski ME, Southern JF, Bouma BE, Boppart SA, Fujimoto JG. Optical biopsy in human gastrointestinal tissue using optical coherence tomography. *Am J Gastroenterol* 1997; **92**: 1800-1804
11 **Pitris C**, Jesser C, Boppart SA, Stamper D, Brezinski ME, Fujimoto JG. Feasibility of optical coherence tomography for high-resolution imaging of human gastrointestinal tract malignancies. *J Gastroenterol* 2000; **35**: 87-92
12 **Kobayashi K**, Izatt JA, Kulkarni MD, Willis J, Sivak MV Jr. High-resolution cross-sectional imaging of the gastrointestinal tract using optical coherence tomography: preliminary results. *Gastrointest Endosc* 1998; **47**: 515-523
13 **Sergeev AM**, Gelikonov VM, Gelikonov GV, Feldchtein FI, Kuranov RV, Gladkova ND, Shakhova NM, Snopova LB, Shakhov AV, Kuznetzova LA, Denisenko AN, Pochinko VV, Chumakov YP, Streltzova OS. In vivo endoscopic OCT imaging of precancer and cancer states of human mucosa. *Opt Express* 1997; **1**: 432-440
14 **Testoni PA**, Mangiavillano B, Albarello L, Arcidiacono PG, Mariani A, Masci E, Doglioni C. Optical coherence tomography to detect epithelial lesions of the main pancreatic duct: an Ex Vivo study. *Am J Gastroenterol* 2005; **100**: 2777-2783
15 **Jaeckle S**, Gladkova N, Feldchtein F, Terentieva A, Brand B, Gelikonov G, Gelikonov V, Sergeev A, Fritscher-Ravens A, Freund J, Seitz U, Soehendra S, Schrodern N. In vivo endoscopic optical coherence tomography of the human gastrointestinal tract--toward optical biopsy. *Endoscopy* 2000; **32**: 743-749
16 **Cilesiz I**, Fockens P, Kerindongo R, Faber D, Tytgat G, Ten Kate F, Van Leeuwen T. Comparative optical coherence tomography imaging of human esophagus: how accurate is localization of the muscularis mucosae? *Gastrointest Endosc* 2002; **56**: 852-857
17 **Poneros JM**. Diagnosis of Barrett's esophagus using optical coherence tomography. *Gastrointest Endosc Clin N Am* 2004; **14**: 573-588
18 **Hsiung PL**, Pantanowitz L, Aguirre AD, Chen Y, Phatak D, Ko TH, Bourquin S, Schnitt SJ, Raza S, Connolly JL, Mashi-

mo H, Fujimoto JG. Ultrahigh-resolution and 3-dimensional optical coherence tomography ex vivo imaging of the large and small intestines. *Gastrointest Endosc* 2005; **62**: 561-574

19 **Masci E**, Mangiavillano B, Albarello L, Mariani A, Doglioni C, Testoni PA. Optical coherence tomography in the diagnosis of coeliac disease: a preliminary report. *Gut* 2006; **55**: 579

20 **Masci E**, Mangiavillano B, Albarello L, Mariani A, Doglioni C, Testoni PA. Pilot study on the correlation of optical coherence tomography with histology in celiac disease and normal subjects. *J Gastroenterol Hepatol* 2007; **22**: 2256-2260

21 **Shen B**, Zuccaro G Jr, Gramlich TL, Gladkova N, Trolli P, Kareta M, Delaney CP, Connor JT, Lashner BA, Bevins CL, Feldchtein F, Remzi FH, Bambrick ML, Fazio VW. In vivo colonoscopic optical coherence tomography for transmural inflammation in inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2004; **2**: 1080-1087

22 **Tearney GJ**, Brezinski ME, Southern JF, Bouma BE, Boppart SA, Fujimoto JG. Optical biopsy in human pancreatobiliary tissue using optical coherence tomography. *Dig Dis Sci* 1998; **43**: 1193-1199

23 **Testoni PA**, Mariani A, Mangiavillano B, Albarello L, Arcidiacono PG, Masci E, Doglioni C. Main pancreatic duct, common bile duct and sphincter of Oddi structure visualized by optical coherence tomography: An ex vivo study compared with histology. *Dig Liver Dis* 2006; **38**: 409-414

24 **Testoni PA**, Mangiavillano B, Albarello L, Mariani A, Arcidiacono PG, Masci E, Doglioni C. Optical coherence tomography compared with histology of the main pancreatic duct structure in normal and pathological conditions: an 'ex vivo study'. *Dig Liver Dis* 2006; **38**: 688-695

25 **Singh P**, Chak A, Willis JE, Rollins A, Sivak MV Jr. In vivo optical coherence tomography imaging of the pancreatic and biliary ductal system. *Gastrointest Endosc* 2005; **62**: 970-974

26 **Testoni PA**, Mariani A, Mangiavillano B, Arcidiacono PG, Di Pietro S, Masci E. Intraductal optical coherence tomography for investigating main pancreatic duct strictures. *Am J Gastroenterol* 2007; **102**: 269-274

27 **Poneros JM**, Tearney GJ, Shiskov M, Kelsey PB, Lauwers GY, Nishioka NS, Bouma BE. Optical coherence tomography of the biliary tree during ERCP. *Gastrointest Endosc* 2002; **55**: 84-88

28 **Seitz U**, Freund J, Jaeckle S, Feldchtein F, Bohnacker S, Thonke F, Gladkova N, Brand B, Schroder S, Soehendra N. First in vivo optical coherence tomography in the human bile duct. *Endoscopy* 2001; **33**: 1018-1021

29 **Jaeckle S**, Gladkova N, Feldchtein F, Terentieva A, Brand B, Gelikonov G, Gelikonov V, Sergeev A, Fritscher-Ravens A, Freund J, Seitz U, Schroder S, Soehendra N. In vivo endoscopic optical coherence tomography of esophagitis, Barrett's esophagus, and adenocarcinoma of the esophagus. *Endoscopy* 2000; **32**: 750-755

30 **Faruqi SA**, Arantes V, Bhutani MS. Barrett's esophagus: current and future role of endosonography and optical coherence tomography. *Dis Esophagus* 2004; **17**: 118-123

31 **Li XD**, Boppart SA, Van Dam J, Mashimo H, Mutinga M, Drexler W, Klein M, Pitris C, Krinsky ML, Brezinski ME, Fujimoto JG. Optical coherence tomography: advanced technology for the endoscopic imaging of Barrett's esophagus. *Endoscopy* 2000; **32**: 921-930

32 **Chen Y**, Aguirre AD, Hsiung PL, Desai S, Herz PR, Pedrosa M, Huang Q, Figueiredo M, Huang SW, Koski A, Schmitt JM, Fujimoto JG, Mashimo H. Ultrahigh resolution optical coherence tomography of Barrett's esophagus: preliminary descriptive clinical study correlating images with histology. *Endoscopy* 2007; **39**: 599-605

33 **Isenberg G**, Sivak MV Jr, Chak A, Wong RC, Willis JE, Wolf B, Rowland DY, Das A, Rollins A. Accuracy of endoscopic optical coherence tomography in the detection of dysplasia in Barrett's esophagus: a prospective, double-blinded study. *Gastrointest Endosc* 2005; **62**: 825-831

34 **Poneros J**. Optical coherence tomography and the detection of dysplasia in Barrett's esophagus. *Gastrointest Endosc* 2005; **62**: 832-833

35 **Evans JA**, Nishioka NS. The use of optical coherence tomography in screening and surveillance of Barrett's esophagus. *Clin Gastroenterol Hepatol* 2005; **3**: S8-S11

36 **Evans JA**, Poneros JM, Bouma BE, Bressner J, Halpern EF, Shishkov M, Lauwers GY, Mino-Kenudson M, Nishioka NS, Tearney GJ. Optical coherence tomography to identify intramucosal carcinoma and high-grade dysplasia in Barrett's esophagus. *Clin Gastroenterol Hepatol* 2006; **4**: 38-43

37 **Anandasabapathy S**. Endoscopic imaging: emerging optical techniques for the detection of colorectal neoplasia. *Curr Opin Gastroenterol* 2008; **24**: 64-69

38 **Westphal V**, Rollins AM, Willis J, Sivak MV, Izatt JA. Correlation of endoscopic optical coherence tomography with histology in the lower-GI tract. *Gastrointest Endosc* 2005; **61**: 537-546

39 **Pfau PR**, Sivak MV Jr, Chak A, Kinnard M, Wong RC, Isenberg GA, Izatt JA, Rollins A, Westphal V. Criteria for the diagnosis of dysplasia by endoscopic optical coherence tomography. *Gastrointest Endosc* 2003; **58**: 196-202

40 **Mangiavillano B**, Mariani A A, Petrone MC. An intrapancreatic cholangiocarcinoma detected with optical coherence tomography during endoscopic retrograde cholangiopancreatography. *Clin Gastroenterol Hepatol* 2008; **6**: A30

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REVIEW

Role of probiotics, prebiotics and synbiotics in chemoprevention for colorectal cancer

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Abstract

Colorectal cancer is the third most common form of cancer. Current treatments are all associated with a high risk of complications and a low success rate. Recently, synbiotics have been proposed as a new preventive and therapeutic option. There is no direct experimental evidence for cancer suppression in humans as a result of the consumption of pro-, pre- or synbiotics. However, there is a wealth of evidence emerging from laboratory studies. The mechanisms by which pro-, pre- and synbiotics may inhibit colon cancer are now beginning to be understood and will be addressed in the present review.

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Key words: Probiotics; Prebiotics; Synbiotics; Colorectal cancer; Treatment; Prevention

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INTRODUCTION

Over the past years yogurt and the lactic acid producing bacteria that it contains, have received much attention as potential cancer prevention agents in the diet. Prebiotics, such as non-digestible oligosaccharides, appear to have similar effects, while some studies indicate that combinations of pro and probiotics (synbiotics) are more effective.

Colorectal cancer (CRC) is the third most prevalent form of cancer in men and women, with a 5-year survival rate of 63%, decreasing to 10% in patients with metastatic disease[1]. More than 80% of colorectal neoplasms occur sporadically, arising from adenomatous polyps *via* the long-term accumulation of mutations in genes including *APC*, *K-ras* and *TP53*[2]. Mortality and incidence of CRC is the third only to that of prostate and lung cancer in men, breast and lung cancer in women and has shown little sign of decreasing in the last 20-30 years. Diet makes an important contribution to CRC risk[3], implying that the risks of CRC are potentially reducible. Evidence also supports the view that the colonic microflora are involved in the etiology of CRC[3]. This has led to an intense interest in factors that can modulate the gut microflora and their metabolism, such as probiotics and prebiotics.

The original definition of a probiotic was "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"[4]. Recent definitions are more general, omitting the aspect of intestinal balance. The most acceptable definition is "a living microorganism which upon ingestion in certain numbers, exert health benefits beyond inherent general nutrition"[5]. A probiotic should be non-pathogenic and non-toxic and also resistant to low pH and bile salts to improve its chances of survival in the gastrointestinal tract[6]. Most probiotics are members of two genera of lactic acid bacteria (LAB), Lactobacillus and Bifidobacterium, but Saccharomyces and Enterococcus are also used. The list of beneficial

effects attributed to probiotics bacteria is extensive and includes alleviation of lactose-intolerance symptoms, serum cholesterol reduction, alleviating constipation, prevention of drug-induced colitis, while they also demonstrate efficacy in a number of conditions including ulcerative colitis, pouchitis, radiation colitis, atopic eczema and diarrhea.

A prebiotic, as defined by Gibson and Roberfroid, is "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon that have the potential to improve host health"[7]. A number of poorly digested carbohydrates fall into the category of prebiotics, including certain fibers and resistant starches, but the most widely described prebiotics are non-digestible oligosaccharides. Combinations of probiotics and prebiotics can result in additive or synergistic effects on gastrointestinal function. The term synbiotic has been proposed for such combinations. A synbiotic has been defined as "a mixture of prebiotics and probiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare" (Gibson *et al*[7], 1995).

MECHANISMS BY WHICH PROBIOTIC BACTERIA MAY INHIBIT COLON CANCER

There is accumulating evidence describing the ability of probiotic strains to prevent CRC. Some epidemiological studies have indicated that consumption of large quantities of fermented milk products containing lactobacillus or bifidobacteria are associated with a lower incidence of colon cancer[8], although, other studies have suggested that consumption of fermented dairy products imparts little, or no, protection[9]. The mechanisms by which probiotics may inhibit colon cancer are not yet fully characterized. However, there is evidence for: Alteration of the metabolic activities of intestinal microflora, alteration of physicochemical conditions in the colon, binding of potential carcinogens, short chain fatty acid production, production of anti-tumorigenic or anti-mutagenic compounds, elevating the hosts' immune response and altering the hosts' physiology.

Alteration of the metabolic activities of intestinal microflora

The bacterial enzyme β-glucuronidase has the ability to hydrolyse many glucuronides. Many foreign compounds are detoxified by glucuronide formation in the liver. In that way, many carcinogenic aglycones are liberated in the intestinal lumen. Several more bacterial enzymes have been implicated in the carcinogenic process, releasing carcinogens in the intestinal tract. Lactic acid bacteria reduced the specific activities of fecal enzymes in human volunteer studies[10]. Goldin and Gorbach also studied the effect of feeding L.acidophilus strains NCFM and N-2 on the activity of three bacterial enzymes (β-glucuronidase, nitroreductase and azoreductase) in 21 healthy volunteers[11]. Both strains had similar effects and caused a significant decline in the specific activity of the three enzymes in all subjects after 10 d of feeding. A reversal of the effect was observed within 10-30 d of stopping Lactobacillus feeding; suggesting that continuous consumption of these bacteria was necessary to maintain the effect. Human studies have demonstrated that the capacity for probiotics to decrease the activity of bacterial enzymes is strain specific. To this end, *L. plantarum* 299V, *L. rhamnosus* DR20 and *L. acidophilus* A1 were unable to decrease β-glucuronidase activity in healthy subjects[12,13,14], while *L. casei Shirota* and *L. acidophilus* significantly decreased enzymatic activity[15,16]. Reports published to date do not always find reductions in the same enzymes, although findings with β-glucuronidase and nitroteductase are most consistently positive. However, we do not know how or whether a reduction in these enzyme activities affect cancer rates in man.

Alteration of physicochemical conditions in the colon

It has been suggested that large bowel cancer could be influenced directly by reducing intestinal pH[17], thereby preventing the growth of putrefactive bacteria. In rats given inulin containing diets with or without *B. longum*, an increase in caecal weight and β-glucosidase and a decrease in caecal pH were observed[18], though some other studies did not detect a significant change in intestinal pH[19].

One hypothesis regarding colon carcinogenesis involves a cytotoxic effect on the colonic epithelium, exerted by bile acids in the aqueous phase of faeces, followed by an increased proliferation of cells in the intestine[20]. Dietary fat has also been considered a risk factor for colon cancer. This phenomenon may be mediated by increased levels of secondary bile acids in the colon, produced by the action of bacterial 7a-dehydroxylase on primary bile acids. It has been demonstrated that a 6-wk administration of *L. acsidophilus* fermented milk supplements to colon cancer patients resulted in lower concentrations of soluble bile acids in faeces[21]. In another study, patients with colonic adenomas participated in a 3-mo study, where *L. acidophilus* was administered together with *B. bifidum*[22]. During this period, the faecal pH was reduced significantly, and patients having a higher proliferative activity in the upper colonic crypts than that calculated for subjects at low risk for colon cancer showed a significant decrease after therapy with the lactic acid bacteria.

Binding and degrading potential carcinogens

Mutagenic compounds, commonly found in the western meat-rich diet, can be bound to the intestinal and lactic acid bacteria *in vitro* and binding has been found to be correlated well with the reduction in the

mutagenicity observed after exposure to the bacterial strains. In a study, the ability of 22 strains of intestinal bacteria to bind the mutagenic pyrolyzates was investigated and compared their ability to that of some dietary fibres[23]. Some indoles, including 3-amino-1-methyl-5H-pyrido (4, 3-β) indole (Trp-P-2) were effectively bound to all gram-positive and some gram-negative bacterial cells, maize bran, and apple pulp and soybean fiber. The mutagenicity of Trp-P-2 for *Salmonella typhimurium* TA98 in the presence of S9 mix was inhibited by the addition of *L. casei* to the reaction mixture, indicating that bound Trp-P-2 did not cause mutation under the assay conditions. A more recent study demonstrated a reduced uptake of Trp-P-2 and its metabolites in various tissues of mice supplemented with dietary lactic acid bacteria[24]. In addition to that, the consumption of lactobacilli by human volunteers has been shown to reduce the mutagenicity of urine and feces associated with the ingestion of carcinogens in cooked meat[25]. It is possible that the lactic acid bacteria supplements are influencing the uptake and excretion of mutagens by simply binding them in the intestine. Lactobacilli have also been shown to degrade nitrosamines[26]. Nitrosamines have been shown to be carcinogenic in animal models and these compounds have been detected in human faeces.

Short chain fatty acid (SCFA) production

The production of SCFAs, such as butyrate, is one key mechanism by which probiotics and prebiotics may impart beneficial effects. Butyrate has been shown to inhibit cancer cell proliferation and promote apoptosis *in vitro*[27]. Butyrate administration in animal models of CRC has produced varying results[28]. Laminar delivery of butyrate reduced aberrant crypt foci (ACF) by 45% compared to untreated rats[29], while other studies have shown butyrate to be ineffective. The bacterial strain *Butyrivibrio fibrisolvens* MDT-1 has been investigated in the context of CRC treatment as it produces high amounts of butyrate[30]. In a mouse model of colon cancer, administration of MDT-1 led to a significant decrease in ACF, and the number of mice with an increased proportion of ACF was also reduced, indicating an inhibited progression of tumour development. MDT-1 also reduced β-glucuronidase activity and increased the immune response, indicated by an increase in NK cell numbers. Similar effects have been observed in the propionate and acetate producing probiotic, *Propionibacterium acidipropionici*[31]. It has been suggested that short chain fatty acid delivery *via* probiotic ingestion may be an exciting new treatment option for CRC[32].

Production of anti-tumorigenic or anti-mutagenic compounds

It has been suggested that lactic acid bacteria or a soluble compound produced by the bacteria may interact directly with tumor cells in culture and inhibit their growth[33]. In a study, lactic acid bacteria significantly reduced the growth and viability of the human colon cancer cell line HT-29 in culture and dipeptyl peptidase Ⅳ and brush-border enzymes were increased[34], suggesting that these cells might have entered a differentiation process. In another study, milk fermented with *B. infantis*, *B. bifidum*, *B. animalis*, *L. acidophilus* and *L. paracasei* inhibited the growth of the MCF7 breast cancer cell line, with their anti-proliferative effect not being related to the presence of the bacteria[35]. On these grounds, the presence of a soluble compounds produced by lactic acid bacteria during milk fermentation has been suggested.

Elevation of the host's immune response

One explanation for tumor suppression by probiotics is the enhancement of the host's immune response. In 1985 it was suggested by Sekine *et al*[36] that *B. infantis* stimulates the host-mediated response, leading to tumor suppression or regression. There are many studies that suggest that lactic acid bacteria play an important role and function in the host's immunoprotective system by increasing various mechanisms to have an anti-tumor effect. *L. casei Shirota* has been shown to have anti-tumor and anti-metastatic effects on transplantable tumor cells, to suppress chemically induced carcinogenesis in rodents and to induce the production of several cytokines, such as interferon-γ, IL-1β and TNF-α which resulted in the inhibition of tumor growth and the increased survival of tumor bearing mice[37]. Similar results have been reported recently for strains of *L. acidophilus SNUL*, *L. casei* YIT9029 and *B. longum* HY8001[38]. Sun *et al*[39] demonstrated *in vivo* that peptidoglycan from a lactobacillus species was able to dose-dependently reduce the growth of CT26 colon cancer cells in BALB/c mice *via* an increased level of apoptosis. Interestingly, peptidoglycan had no effect on tumor cell apoptosis *in vitro*, implying that the *in vivo* anti-tumorigenic effect may have been mediated by the immune response. In addition to that, recent studies have shown probiotics to be effective against Caco-2 colonic adenocarcinoma[40], but also against a breast cancer cell line[41], suggesting that probiotic therapeutic interventions may not necessarily be restricted to cancers affecting the gastrointestinal system.

Effects on the host's physiology

The ileal mucosa as well as the colonic mucosa has the capacity to absorb mutagenic compounds from the intestinal lumen, which are then passed into the bloodstream. Lactic acid bacteria have been shown to increase colonic NADPH-cytochrome P-450 reductase activity[42] and glutathione S-transferase levels[43] and to reduce hepatic uridine diphosphoglucuronyl-transferase activity[19], enzymes which are involved in the metabolism of carcinogens in rats.

PREBIOTICS AND COLORECTAL CANCER

Prebiotics have also been linked to the reduction of CRC. Friedenreich *et al*[44] concluded in a meta-analysis that the consumption of over 27 g of fiber per day

resulted in a 50% reduction in CRC compared to consumption of less than 11 g. Inulin-type fructans present in foods such as garlic, onion, artichoke and asparagus have been demonstrated to elevate the levels of bifidobacteria and to increase SCFA concentrations in the intestinal lumen. Inulin and oligofructose have been demonstrated to reduce the severity of 1,2-dimethylhydrazine induced colon cancer in rats[45]. A further study demonstrated the capacity for the prebiotic resistant starch type-3 Novelose 330, to reduce the incidence of colon carcinogenesis *via* induced apoptosis of damaged cells in rats[46]. This effect was attributed to the increased production of butyrate. In another study, the consumption of modified arabinoxylan rice bran was able to enhance the activity of NK cells and the binding of NK cells to tumor cells[47]. This demonstrates the ability of prebiotics to enhance the hosts' immune response.

SYNBIOTICS AND COLORECTAL CANCER

The combinations of pro- and prebiotics have a synergistic effect, greater than that of either the pro- or prebiotic administered individually. Rowland *et al*[48] reported that the combination of inulin and *B. longum* was more successful at decreasing azoxymethane-induced ACF than either treatment alone. Another study demonstrated that the consumption of *B. lactis* and resistant starch was able to increase the apoptotic response to azoxymethane in rats, and this was suggested to be due to the resistant starch acting as a metabolic substrate to provide optimal activity of the probiotic species[49]. Roller *et al*[50], demonstrated that synbiotic treatment prevented azoxymethane-induced suppression of NK-cell activity in Peyer's patches, an effect not observed in the individual pro- and prebiotic treatments. These studies suggest that synbiotics may have a role in CRC treatment.

CONCLUSION

Overall, studies *in vitro* systems and in a wide range of animal models provide considerable evidence that probiotics, prebiotics and synbiotics exert anti-neoplastic effects. Their consumption may be beneficial in preventing the onset of cancer, but also in the treatment of existing tumors. However, evidence from human studies is still limited. Many researchers have pointed out the need for carefully designed human clinical trials. Furthermore, research is required to identify the probiotic, prebiotic or synbiotic combination that will be more effective for humans. It is very likely that there will not be an ideal treatment for all cases, but the treatment will depend on the individuals' unique intestinal flora composition. New options are given through the genetic manipulation of probiotics, designed to act as a delivery system for anti-neoplastic factors in the colon. Although this field of study is promising and exciting, this enthusiasm should be tempered by the fact that we are likely many years away from determining how to use these agents and their ultimate role may remain quite limited. However, it is safe to conclude that pro-, pre- and synbiotics hold great potential as a new strategy for the prevention and treatment of colorectal cancer.

REFERENCES

1 **Goldberg RM**. Advances in the treatment of metastatic colorectal cancer. *Oncologist* 2005; **10** Suppl 3: 40-48
2 **Huycke MM**, Gaskins HR. Commensal bacteria, redox stress, and colorectal cancer: mechanisms and models. *Exp Biol Med* (Maywood) 2004; **229**: 586-597
3 **Rafter J**. The effects of probiotics on colon cancer development. *Nutr Res Reviews* 2004; **17**: 277-284
4 **Fuller R**. Probiotics in man and animals. *J Appl Bacteriol* 1989; **66**: 365-378
5 **Guarner F**, Schaafsma GJ. Probiotics. *Int J Food Microbiol* 1998; **39**: 237-238
6 **Fuller R**. Probiotics in human medicine. *Gut* 1991; **32**: 439-442
7 **Gibson GR**, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* 1995; **125**: 1401-1412
8 **Shahani KM**, Ayebo AD. Role of dietary lactobacilli in gastrointestinal microecology. *Am J Clin Nutr* 1980; **33**: 2448-2457
9 **Kampman E**, Goldbohm RA, van den Brandt PA, van't Veer P. Fermented dairy products, calcium, and colorectal cancer in The Netherlands Cohort Study. *Cancer Res* 1994; **54**: 3186-3190
10 **Goldin BR**, Gorbach SL. Alterations of the intestinal microflora by diet, oral antibiotics, and Lactobacillus: decreased production of free amines from aromatic nitro compounds, azo dyes, and glucuronides. *J Natl Cancer Inst* 1984; **73**: 689-695
11 **Goldin BR**, Gorbach SL. The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. *Am J Clin Nutr* 1984; **39**: 756-761
12 **Goossens D**, Jonkers D, Russel M, Stobberingh E, Van Den Bogaard A, StockbrUgger R. The effect of Lactobacillus plantarum 299v on the bacterial composition and metabolic activity in faeces of healthy volunteers: a placebo-controlled study on the onset and duration of effects. *Aliment Pharmacol Ther* 2003; **18**: 495-505
13 **Tannock GW**, Munro K, Harmsen HJ, Welling GW, Smart J, Gopal PK. Analysis of the fecal microflora of human subjects consuming a probiotic product containing Lactobacillus rhamnosus DR20. *Appl Environ Microbiol* 2000; **66**: 2578-2588
14 **Marteau P**, Pochart P, Flourie B, Pellier P, Santos L, Desjeux JF, Rambaud JC. Effect of chronic ingestion of a fermented dairy product containing Lactobacillus acidophilus and Bifidobacterium bifidum on metabolic activities of the colonic flora in humans. *Am J Clin Nutr* 1990; **52**: 685-688
15 **Goldin BR**, Swenson L, Dwyer J, Sexton M, Gorbach SL. Effect of diet and Lactobacillus acidophilus supplements on human fecal bacterial enzymes. *J Natl Cancer Inst* 1980; **64**: 255-261
16 **Spanhaak S**, Havenaar R, Schaafsma G. The effect of consumption of milk fermented by Lactobacillus casei strain Shirota on the intestinal microflora and immune parameters in humans. *Eur J Clin Nutr* 1998; **52**: 899-907
17 **Modler GW**, McKellar RC, Yaguchi M. Bifidobacteria and bifidogenic factors. *J Inst Can Sci Technol Ailment* 1990; **23**: 29-41
18 **Rowland IR**, Rumney CJ, Coutts JT, Lievense LC. Effect of Bifidobacterium longum and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. *Carcinogenesis* 1998; **19**: 281-285
19 **Abdelali H**, Cassand P, Soussotte V, Daubeze M, Bouley C, Narbonne JF. Effect of dairy products on initiation of precursor lesions of colon cancer in rats. *Nutr Cancer* 1995;

24: 121-132
20 **Bruce WR**. Recent hypotheses for the origin of colon cancer. *Cancer Res* 1987; **47**: 4237-4242
21 **Lidbeck A**, Geltner-Allinger U, Orrhage KM, Ottova L, Brismar B, Gustafson J, Rafter JJ, Nord CE. Impact of L.acidophilus supplements on the faecal microflora and soluble faecal bile acids in colon cancer patients. *Microb Ecology in Health and Disease* 1991; **4**: 81-88
22 **Biasco G**, Paganelli GM, Brandi G, Brillanti S, Lami F, Callegari C, Gizzi G. Effect of lactobacillus acidophilus and bifidobacterium bifidum on rectal cell kinetics and fecal pH. *Ital J Gastroenterol* 1991; **23**: 142
23 **Morotomi M**, Mutai M. In vitro binding of potent mutagenic pyrolysates to intestinal bacteria. *J Natl Cancer Inst* 1986; **77**: 195-201
24 **Orrhage KM**, Annas A, Nord CE, Brittebo EB, Rafter JJ. Effects of lactic acid bacteria on the uptake and distribution of the food mutagen Trp-P-2 in mice. *Scand J Gastroenterol* 2002; **37**: 215-221
25 **Hayatsu H**, Hayatsu T. Suppressing effect of Lactobacillus casei administration on the urinary mutagenicity arising from ingestion of fried ground beef in the human. *Cancer Lett* 1993; **73**: 173-179
26 **Rowland IR**, Grasso P. Degradation of N-nitrosamines by intestinal bacteria. *Appl Microbiol* 1975; **29**: 7-12
27 **Pool-Zobel BL**. Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data. *Br J Nutr* 2005; **93** Suppl 1: S73-S90
28 **Sengupta S**, Muir JG, Gibson PR. Does butyrate protect from colorectal cancer? *J Gastroenterol Hepatol* 2006; **21**: 209-218
29 **Wong CS**, Sengupta S, Tjandra JJ, Gibson PR. The influence of specific luminar factors on the colonic epithelium: high-dose butyrate and physical changes suppress early carcinogenesis events in rats. *Dis Colon Rectum* 2005; **48**: 549-559
30 **Ohkawara S**, Furuya H, Nagashima K, Asanuma N, Hino T. Oral administration of butyrivibrio fibrisolvens, a butyrate-producing bacterium, decreases the formation of aberrant crypt foci in the colon and rectum of mice. *J Nutr* 2005; **135**: 2878-2883
31 **Jan G**, Belzacq AS, Haouzi D, Rouault A, Metivier D, Kroemer G, Brenner C. Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. *Cell Death Differ* 2002; **9**: 179-88
32 **Geier MS**, Butler RN, Howarth GS. Probiotics, prebiotics and synbiotics: a role in chemoprevention for colorectal cancer? *Cancer Biol Ther* 2006; **5**: 1265-1269
33 **Reddy GV**, Friend BA, Shahani KM, Farmer RE. Antitumour activity of yogurt components. *J Food Prot* 1983; **46**: 8-11
34 **Baricault L**, Denariaz G, Houri JJ, Bouley C, Sapin C, Trugnan G. Use of HT-29, a cultured human colon cancer cell line, to study the effect of fermented milks on colon cancer cell growth and differentiation. *Carcinogenesis* 1995; **16**: 245-252
35 **Biffi A**, Coradini D, Larsen R, Riva L, Di Fronzo G. Antiproliferative effect of fermented milk on the growth of a human breast cancer cell line. *Nutr Cancer* 1997; **28**: 93-99
36 **Sekine K**, Toida T, Saito M, Kuboyama M, Kawashima T, Hashimoto Y. A new morphologically characterized cell wall preparation (whole peptidoglycan) from Bifidobacterium infantis with a higher efficacy on the regression of an established tumor in mice. *Cancer Res* 1985; **45**: 1300-1307
37 **Matsuzaki T**. Immunomodulation by treatment with Lactobacillus casei strain Shirota. *Int J Food Microbiol* 1998; **41**: 133-140
38 **Lee JW**, Shin JG, Kim EH, Kang HE, Yim IB, Kim JY, Joo HG, Woo HJ. Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum. *J Vet Sci* 2004; **5**: 41-48
39 **Sun J**, Shi YH, Le GW, Ma XY. Distinct immune response induced by peptidoglycan derived from Lactobacillus sp. *World J Gastroenterol* 2005; **11**: 6330-6337
40 **Ghoneum M**, Hamilton J, Brown J, Gollapudi S. Human squamous cell carcinoma of the tongue and colon undergoes apoptosis upon phagocytosis of Saccharomyces cerevisiae, the baker's yeast, in vitro. *Anticancer Res* 2005; **25**: 981-989
41 **Ghoneum M**, Gollapudi S. Induction of apoptosis in breast cancer cells by Saccharomyces cerevisiae, the baker's yeast, in vitro. *Anticancer Res* 2004; **24**: 1455-1463
42 **Pool-Zobel BL**, Neudecker C, Domizlaff I, Ji S, Schillinger U, Rumney C, Moretti M, Vilarini I, Scassellati-Sforzolini R, Rowland I. Lactobacillus- and bifidobacterium-mediated antigenotoxicity in the colon of rats. *Nutr Cancer* 1996; **26**: 365-380
43 **Challa A**, Rao DR, Chawan CB, Shackelford L. Bifidobacterium longum and lactulose suppress azoxymethane-induced colonic aberrant crypt foci in rats. *Carcinogenesis* 1997; **18**: 517-521
44 **Friedenreich CM**, Brant RF, Riboli E. Influence of methodologic factors in a pooled analysis of 13 case-control studies of colorectal cancer and dietary fiber. *Epidemiology* 1994; **5**: 66-79
45 **Hughes R**, Rowland IR. Stimulation of apoptosis by two prebiotic chicory fructans in the rat colon. *Carcinogenesis* 2001; **22**: 43-47
46 **Bauer-Marinovic M**, Florian S, Muller-Schmehl K, Glatt H, Jacobasch G. Dietary resistant starch type 3 prevents tumor induction by 1,2-dimethylhydrazine and alters proliferation, apoptosis and dedifferentiation in rat colon. *Carcinogenesis* 2006; **27**: 1849-1859
47 **Ghoneum M**, Abedi S. Enhancement of natural killer cell activity of aged mice by modified arabinoxylan rice bran (MGN-3/Biobran). *J Pharm Pharmacol* 2004; **56**: 1581-1588
48 **Rowland IR**, Rumney CJ, Coutts JT, Lievense LC. Effect of Bifidobacterium longum and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. *Carcinogenesis* 1998; **19**: 281-285
49 **Le Leu RK**, Brown IL, Hu Y, Bird AR, Jackson M, Esterman A, Young GP. A synbiotic combination of resistant starch and Bifidobacterium lactis facilitates apoptotic deletion of carcinogen-damaged cells in rat colon. *J Nutr* 2005; **135**: 996-1001
50 **Roller M**, Pietro Femia A, Caderni G, Rechkemmer G, Watzl B. Intestinal immunity of rats with colon cancer is modulated by oligofructose-enriched inulin combined with Lactobacillus rhamnosus and Bifidobacterium lactis. *Br J Nutr* 2004; **92**: 931-938

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REVIEW

Cholangiocarcinoma: A compact review of the literature

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Abstract

Cholangiocarcinoma (CC) is a devastating cancer arising from biliary epithelia. Unfortunately, the incidence of this disease is increasing in Western countries. These tumors progress insidiously, and liver failure, biliary sepsis, malnutrition and cancer cachexia are general modes of death associated with this disease. To date, no established therapy for advanced disease has been established or validated. However, our knowledge in tumor biology is increasing dramatically and new drugs are under investigation for treatment of this notorious tumor. In clinical practice, there are better diagnostic tools in use to facilitate an earlier diagnosis of CC, at least in those patients with known risk factors. CC is resectable for cure in only a small percentage of patients. Preoperative staging for vascular and biliary extension of CC is very important in this tumor. Laparoscopy and recently endosonography seem to protect against unnecessary laparotomies in these patients. During the last 15 years, aggressive surgical approaches, including combined liver resections and vascular reconstructive surgical expertise, have improved survival in patients with CC. Surgery is contraindicated in CC cases having primary sclerosing cholangitis (PSC). Although CC was previously considered a contraindication to liver transplantation, new cautious protocols, including neo-adjuvant chemoradiation therapies and staging procedures before the transplantation, have made it possible to achieve long-term survival after liver transplantation in this disease. New ablative therapies with photodynamic therapy, intraductal high-intensity ultrasonography and chemotherapy-impregnated plastic biliary endoprosthesis are important steps in the palliative management of extrahepatic CCs. Radiofrequency and chemo-embolization methods are also applicable for intra-hepatic CCs as palliative modes of treatment. We need more prospective randomized controlled trials to evaluate the role of the new emerging therapies for CC patients.

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Key words: Cholangiocarcinoma; Primary sclerosing cholangitis; Radiofrequency ablation; Chemoembolization

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INTRODUCTION

Cholangiocarcinoma (CC) is a malignant epithelial tumor of the biliary tree that accounts for approximately 10% to 15% of all hepatobiliary malignancies. CC mostly arises from the extra-hepatic biliary tree (50%-60% hilar CC, "Klatskin" tumors), spreads slowly and infiltrates periductal tissues. Hilar lesions are further subdivided based on location as indicated by Bismuth-Corlette classification (Figure 1). This form of CC characteristically presents with signs of ductal and vascular obliteration. Biliary tract sepsis, liver failure and/or cancer cachexia and malnutrition are the most important causes of death associated with these tumors[1]. The intra-hepatic form of CC appears as a mass lesion in the liver, which is mostly confused with metastatic tumor. These tumors usually progress insidiously, are difficult to diagnose and have grave prognosis. Unfortunately, the treatment alternatives are few. Effective surgery and other non-surgical treatment modalities for these patients with CC often fail due to characteristically late clinical presentation of this tumor.

Although our current understanding of tumor biology has far exceeded our past knowledge, the overall survival (including the resected patients) over the past 30 years is poor, with less than 5% of cases surviving for five years[1].

EPIDEMIOLOGY

Generally, CC is the second most common primary liver cancer after hepatocellular carcinoma (HCC) in most parts of the world. However, in some populations where HCC is uncommon, such as in Danish women, the prevalence of CC surpasses that of HCC[2]. Presentation is usually in the seventh decade in patients with CC, and it demonstrates slight male preponderance[3,4]. Fortunately, this tumor is a relatively rare kind of malignancy. In autopsy series, its prevalence is reported as around 0.01%-0.5%[5]. It represents 3% of all gastrointestinal system (GIS) cancers[6]. Of concern, some reports indicate that the incidence and mortality of intra-hepatic CC are increasing worldwide[7-9], while those of extra-hepatic CC are decreasing[7,8,10]. However, the increase in intra-hepatic CC is higher than the rate of decline in extra-hepatic CC. Whether this represents a real increase in this tumor or whether it can be attributed to better detection rates or changes in classification strategies is debatable. However, no increase has been noted in early stage or smaller-sized intra-hepatic CCs, perhaps indicating that these tumors are not better detected at present[11]. Thus, the underlying reason for the increasing incidence of intra-hepatic CC remains ambiguous.

RISK FACTORS AND PATHOPHYSIOLOGY

There are well-recognized risk factors for this tumor, such as congenital biliary anomalies, primary sclerosing cholangitis (PSC), hepatolithiasis, parasitic infections, chronic typhoid carriage, bile duct adenoma, biliary papillomatosis, drug exposure and genetic risks[12,13]. Chronic biliary inflammation is the common denominator in these conditions. An inflammatory milieu is believed to dysregulate or change the expression patterns of growth factors, pro-inflammatory cytokines and their receptors. Cytokines produced by cholangiocytes and activated macrophages can modulate gene expression and lead to activation of carcinogen metabolism. For example, interleukin (IL) -6 is a potent mitogen for cholangiocytes[14]. This cytokine can also induce nitric oxide (NO) synthase expression in cholangiocytes. NO can also directly injure the cellular DNA. Consumption of cellular detoxification and dysregulation of DNA repair and apoptosis are final steps of biliary carcinogenesis[5]. Bile acids also have been shown to activate inducible cyclo-oxygenase 2 and an anti-apoptotic molecule, myeloid cell leukemia protein 1 in cholangiocytes[15]. Thus, an inflammatory milieu and toxic bile constituents act together to promote carcinogenesis in the biliary tree. Histomorphological aspects in biliary carcinogenesis indicate that the intestinal metaplasia-dysplasia-carcinoma sequence can also be valid for CC[5].

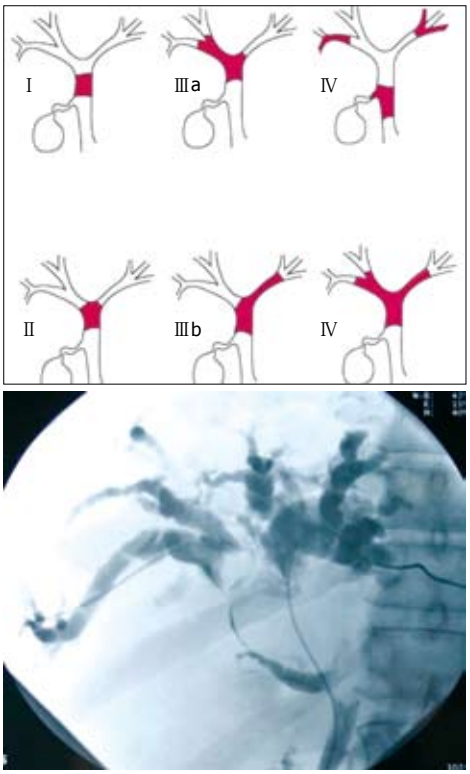

Figure 1 Hilar lesions are further subdivided based on location as indicated by Bismuth-Corlette classification. A: Bismuth-Corlette classification of hilar CC. Type Ⅰ involves the common hepatic duct, distal to the bifurcation of the biliary tree. Type Ⅱ affects the bifurcation. Type Ⅲa affects the right hepatic duct in addition to the bifurcation. Type IIIb affects the left hepatic duct in addition to the bifurcation. Type Ⅳ involves the bifurcation and both right and left hepatic ducts or indicates multifocal CC; B: Endoscopic retrograde cholangiography reveals hilar cholangiocarcinoma (Klatskin type 2) in one of our cases.

Congenital biliary cysts and pancreaticobiliary malfunction together have been obviously linked to biliary carcinogenesis, as both were present in 90% of cases in a reported clinical series[16]. Mixture of bile and pancreatic fluid due to impaired function of the Oddi sphincter induces chronic inflammation in the biliary tree with possible activation of carcinogenesis cascades.

PSC is one of the most common causes of CC in the Western world. As much as 42% of patients with PSC were reported to have CC in autopsy series[17]. Interestingly, the duration of PSC is not a risk factor for developing CC. Chronic inflammation and increased proliferation of biliary epithelium, along with increased production of endogenous mutagens in the bile, seemed to be related to carcinogenesis associated with PSC. Moreover, k-ras mutations were known to be common both in colon and biliary tree cancers in association with PSC[17].

Parasites such as *Opisthorchis viverrini* and *Clonorchis sinensis* have both direct strong carcinogenic effects and increase the susceptibility of cholangiocytes to endogenous and exogenous carcinogens. This is *via* chronic irritation and increased cellular turnover[18].

Hepatolithiasis leads to chronic proliferative cholangitis near to stone-bearing ducts and it has been reported in around 17.5% of patients with CC in some East Asian countries[19].

Chronic typhoid carriage and chronic cholangitis are representatives of chronic inflammatory conditions in the biliary tree and these conditions are accepted risk factors for developing CC.

Environmental toxins such as dioxin, and vinyl chloride, are known to be responsible for some cases of CC[20,21]. Thorotrast, which was used as a radiocontrast agent in the 1930s, is a potent carcinogenic agent for CC[22]. Nitrosamines, either taken exogenously or with tobacco use, or endogenously by nitrosation of nitrogenous compounds *via* NO, are potent carcinogens for biliary cancer[23,24].

Biliary papillomatosis, although a benign disease, has a moderately high malignant transformation rate[25]. Bile duct adenomas also carry appreciable risk for CC development[26].

Genetic polymorphisms in CYP1A2 and glutathione-S-transferase omega 1 and 2 have been related with this tumor[27]. These polymorphisms are believed to influence environmentally toxic substances, such as asbestos or dioxin.

A follow-up study of more than 1000 patients indicated late development of CC after biliary enteric diversion operations for benign biliary diseases[28,29].

Indeed, the majority of CC cases do not have these classic risk factors. Recently, a report from the United States included chronic viral hepatitis, cirrhosis and alcohol use among the risk factors for development of CC in elderly patients[30].

Obesity, moreover, was implicated as a risk factor for CC in a Korean study[1].

ROLE OF CLINICS AND LABORATORY TESTS IN DIAGNOSTIC EVALUATION

Extra-hepatic CC presents with classic signs of cholestasis including jaundice, dark urine, pale stools, pruritus, malaise and weight loss. Laboratory investigations reveal increased alkaline phosphatase, gamma-glutamyl transpeptidase and bilirubin. Prolonged obstruction of the main bile ducts can cause increased prothrombin time and a reduction in fat soluble vitamins. As the disease advances further, albumin, hemoglobin and lactate dehydrogenase can decrease. A glycoprotein tumor marker, CA 19-9, can be found elevated in 85% of such cases. A value of > 100 U/mL in PSC patients has a sensitivity of 89% and specificity of 86% for the diagnosis of CC. CC should not be diagnosed only on the basis of elevated CA 19-9. However, in patients without PSC, the sensitivity of CA 19-9 > 100 U/mL is 53%[31,32]. CEA is elevated in 30% of cases, while CA 125 is elevated in 40%-50% of patients with CC. CEA and CA 125 are also non-specific markers. A high index of suspicion is necessary to diagnose perihilar and extra-hepatic CCs because of other possible alternatives, including benign strictures, metastatic lymph nodes and/or gall bladder cancer. This is also true for PSC patients having dominant strictures. The confirmation of CC in these patients is quite challenging. Nevertheless, recent advances, especially in cytodiagnostic techniques, have contributed to establishing a correct diagnosis in nearly 80% of such cases.

Intra-hepatic CCs present mostly with non-specific symptoms, such as abdominal pain, weight loss, malaise and decreased appetite. Occasionally, an incidental abdominal mass detected during physical examination or radiologic evaluation is the single finding. Mildly elevated alkaline phosphatase and normal bilirubin levels are noticed on laboratory testing. CA 19-9 can also be found increased. These tumors are generally confused with metastatic adenocarcinomas. Indeed, a liver mass with adenocarcinoma histology, without an obvious primary source, should be seriously considered as intra-hepatic CC. A needle biopsy of the dominant liver mass is a straightforward diagnostic approach in these patients. Exclusion of another primary source can usually be accomplished by systemic physical examinations, chest X-ray, and tomography of the abdomen and pelvis.

Ultrasound is usually the first choice during investigation to visualize the location and extent of disease. Sonography can classify intra-hepatic CC as mass lesions. Contrast enhanced helical computerized tomography (CT) is very sensitive for detecting intra-hepatic CC larger than 1 cm. CT can also locate the site of obstruction and the presence of lymphadenopathy[33,34]. CT angiography can also detect vascular encasement[35]. Helical CT is only 60% correct in determining resectability. Although the experience with CT cholangiography is limited, this technique has been reported to be superior to conventional spiral CT examination. Ninety-four percent diagnostic accuracy has been noted for the diagnosis of malignant biliary lesions with CT cholangiography[36]. Magnetic resonance imaging (MRI) with MR Cholangiopancreatography (MRCP) has mostly replaced CT in diagnosis and staging evaluation of CC[37-39]. MR investigations can detect the site and extent of tumor involvement in the absence of PSC. MR angiography can show vascular involvement in these cases[40]. Thus, MRI studies have the advantage of showing vascular anatomy, cross-sectional imaging of the liver and cholangiography with a single technique and may exemplify an optimal imaging technique for this disease. However, in one study, MR cholangiography was reported to under-stage malignant hilar strictures in as high as 20% of patients[41]. Endosonography (EUS) is the most recent addition to the list of imaging modalities in diagnosis and staging of CCs[42]. EUS-guided fine needle aspiration (FNA) of hilar lymph nodes can be very important for staging procedure in CCs. EUS with FNA can diagnose Klatskin cases with 89% diagnostic accuracy even in the presence of negative brush cytology[43]. This technique does not induce contamination of the biliary tree, which can easily occur with endoscopic retrograde cholangiography (ERC). However, the hazard with this technique has been indicated as peritoneal tumor seeding[43]. Though a normal positron emission tomography (PET) scan with (18F)-fluorodeoxyglucose (FDG) can not exclude cancer and false-positives are highly common due to inflammatory conditions, PET may be useful for detecting metastatic disease[44]. FDG-PET/CT combination in detecting

Table 1 Memorial Sloan Kettering T stage for hilar CC

Stage	Criteria
T1	Tumor involving biliary confluence ± unilateral extension to second-order biliary radicals
T2	T1 ± ipsilateral portal vein involvement ± ipsilateral hepatic lobar atrophy
T3	Tumor involving biliary confluence + bilateral extension to second-order biliary radicals; or unilateral extension to second-order biliary radicals with contralateral portal vein involvement; or unilateral extension to second-order radicals with contralateral hepatic lobar atrophy; or main or bilateral portal vein involvement

Klatskin tumors was highly recommended in such patients[45].

Other methods to depict the biliary tree are ERC and percutaneous transhepatic cholangiography. These techniques allow visualization of a very detailed topography of the biliary tree. ERC is one of the main tools in the diagnosis of CCs. It can easily detect a stenosing tumor along the bile ducts. Unfortunately, both techniques carry a risk of bacterial cholangitis, a rather common complication after ERC. Using these techniques, brush cytology can provide cell samples to analyze and diagnose the biliary tract malignancy. Unfortunately, brush cytology has been reported to carry a very low diagnostic yield, ranging from 9%-24%. This was reported to be independent of the quantity of the specimen cellularity[46]. New technologies, such as the use of fluorescence *in situ* hybridization and digital image analysis methods, were reported to be more sensitive than the routine cytology. In one study, these technologies doubled the diagnostic accuracy of brush cytology[47]. The combined use of cholangioscopy improved the diagnostic yield of ERC for CCs. Cholangioscopy can detect the tumor vessel and improve diagnostic potential of direct cholangiographic examination[48]. Intraductal ultrasonography has also been reported to increase diagnostic accuracy of direct cholangiography[49].

OTHER POSSIBLE DIAGNOSES

Most patients with hilar stenosis and jaundice have CC. On the other hand, 10%-15% of cases had alternative diagnoses, including gall bladder carcinoma, Mirizzi syndrome and benign focal stenosis. The thickened and irregular gall bladder wall, infiltration to the right portal vein origin and liver segments 4 and 5, and occlusion of the common hepatic and cystic ducts suggest the presence of gall bladder cancer. Mirizzi syndrome results from periductal inflammation and fibrosis due to a large gall stone impacted at the neck of the gall bladder. Benign focal strictures involving the hilar region are uncommon. Other causes of the strictures affecting the whole biliary tree and benign in nature are postoperative strictures, chronic infectious cholangitis due to gall stones and flukes, ischemic cholangiopathy, chemoradiotherapy, vasculitis, human immunodeficiency virus cholangiopathy, infections such as tuberculosis and histoplasmosis, bile duct varices, papillary stenosis, and sphincter of Oddi dysfunction[50]. These strictures, though benign in nature, can masquerade malignancy on ERC as they may give a "pseudocholangiocarcinoma sign", which is sometimes difficult to differentiate from a true sign of CC. This sign was first reported by some authors in a patient with bile duct varices due to portal vein thrombosis and portal cavernomatous transformation[51].

STAGING AND TREATMENT MODALITIES

Resection and/or liver transplantation are the only curative options for CC. Accurate preoperative staging will determine the treatment approach in these patients. Although a pathologic staging system has been developed for ductal CC, it has a limited value in clinically assessing extra-hepatic CC. TNM classification does not correlate with resectability in patients with extra-hepatic CC. Conversely, the Memorial Sloan-Kettering staging system evaluates the biliary and vascular involvement of these tumors and clearly correlates with resectability and survival (Table 1). Indeed, clinical staging has three important points in presurgical evaluation of these cases. The first is the determination of proximal and distal extent of the disease. The second and third goals are to assess vascular involvement and the presence of metastasis, which can be done by Doppler ultrasound or MRI, CT and EUS examinations. FDG-PET scanning changes the surgical management in a third of patients, with an overall sensitivity for metastasis in 65%. FDG-PET has high false positivity in PSC patients and patients with biliary stents[52]. Laparoscopy for staging improved overall accuracy in choosing the optimal management formula in these cases. In one study, laparoscopy prevented unnecessary laparotomies in 42% of cases[53].

Surgery is the most suitable option for patients with intra-hepatic CC. With curative surgery, three-year survival rates have been reported to be approximately 40%-60%[54]. Surgery performed with curative intents is the best option for hilar and other extra-hepatic ductal CC cases without PSC. Surgery in patients with positive margins show no better results than with palliative therapies[54]. To obtain tumor-free margins, partial liver resections are often necessary. The liver resection has a great impact on obtaining negative margins in patients undergoing a potentially curative resection for hilar CC (Table 2)[55-60]. A combined data from United States and European experiences proved that five-year survival is higher in patients undergoing liver resection than in those not[61]. An analysis of recent surgical series indicated that five-year survival data is around 40% (Table 3)[62-66]. Most surgeons advocate caudate lobe resections, as the drainage of hilar biliary structures is directly

Table 2 Summary of some studies indicating potential influence of liver resection on tumor margin-negative resections and 5-year survival data on hilar CC (1986-1999)

	Resection	Liver resection	Operative mortality	Margin- (%)	5-yr survival	
					Margin-	Margin+
Kosuge *et al* 1999[55]	65	80	9	52	56	13
Burke *et al* 1998[56]	30	73	6	83	40	0
Miyazaka *et al* 1998[57]	76	86	13	71	26	0
Nagino *et al* 1998[58]	138	90	9	76	21	-
Klempnauer *et al* 1996[59]	151	78	10	76	31	5
Nimura *et al* 1986[60]	100	91	10	55	35	0

Table 3 Summary of some recent studies indicating 1-, 3- and 5-yr survival data on margin-negative tumor resections of hilar CC (2000-2005)

	Resections	1-yr survival (margin-)	3-yr survival (margin-)	5-yr survival (margin-)
Jarnagin *et al* 2005[65]	25	100	80	45
	81	85	45	30
Silva *et al* 2005[64]	19	83	58	41
Kondo *et al* 2004[63]		-	-	40
Neuhaus *et al* 2003[62]	133	70	42	36
Tsao *et al* 2000[66]	25	-	-	43

into the caudate lobe bile ducts. In cases with involvement of the distal common bile duct, pancreatoduodenectomy is performed additionally to obtain negative margins. In patients with tumor-free margins after surgery, five-year survival is around 20%-40% and operative mortality 10%[54]. The presence of one of the following indicates unresectability: Bilateral involvement of secondary biliary radicals, invasion of the main portal vein proximal to its bifurcation, unilateral hepatic lobar atrophy together with invasion of contralateral portal vein and/or contralateral secondary biliary radicals, and metastatic involvement of N2 lymph nodes, liver, lung or peritoneum. Indeed, the invasion of the main portal vein may not be an absolute contraindication to surgery as surgeons can prefer to extend their surgical expertise by portal vein resection and its reconstruction in such cases. Though the significance of biliary drainage before surgery is controversial, preoperative biliary drainage of the obstructed lobe *via* percutaneous or endoscopic methods in hilar CCs is generally not indicated. Postoperative morbidity in such cases mostly arises from the introduction of bacteria into the biliary tree *via* preoperatively placed stents. Usually, drainage of the contralateral lobe, free of tumor, is preferred by most surgeons if the involved liver lobe is to be resected and there is deep jaundice. Some authors have shown that lack of intestinal bile delays liver regeneration associated with cyclin E-associated kinase inactivation after surgery[67].

In patients with PSC and CCs, surgery has the worst results. Five-year postoperative survival in these cases has been reported as low as 10%[17,68]. Postoperative recurrent cholangitis attacks, intolerance of partial hepatectomy, due to concomitant advanced fibrosis and high risks of *de nova* CC elsewhere in the biliary tree, are the main obstacles precluding surgery in PSC cases with CCs[69,70]. Thus, liver transplantation can be an alternative option in these cases.

Previously, CC was considered a contraindication for liver transplantation. However, three centers from the United States reported long-term survival following liver transplantation[71-73]. The Mayo Clinic reports five-year survival rates of more than 80% for patients with TNM stage 1 and 2 disease after liver transplantation[72]. The liver transplant protocols in these cases include preoperative chemo-irradiation therapy and exploratory laparotomy. Thus, longer survival expectations for CC cases can be possible with these new fastidious liver transplantation protocols. A recent study compared two treatment modalities, liver transplantation with neoadjuvant protocols and resection surgery, in extrahepatic CC cases. Five-year survival rates were 82% for 38 patients who underwent liver transplantation and 21% for 26 cases who were resected[74]. Adjuvant treatment modalities such as external beam and intraluminal radiation therapies showed no benefit in two separate reports from Johns Hopkins[75,76]. There is currently no role for adjuvant chemotherapy. However, another study revealed positive effects of radiation therapy on survival in histologically positive tumor margin[76]. Similarly, one study demonstrated higher resectability in patients given neoadjuvant radiation therapy before exploration[76]. However, these studies were non-randomized and most consisted of small patient groups.

In patients with unresectable disease, the initial approach is to provide the patient with supportive care and, if necessary, to plan some form of biliary drainage. Palliative therapies provide less than 18 mo of survival. Intractable pruritus, cholangitis, and need for intraluminal radiotherapy and chemotherapy make it necessary to decompress the biliary tree. The patients with unresectable hilar tumors may not be suitable for an endoscopic approach, due to high failure rates and subsequent cholangitis with this technique. However, there are new metal stent designs for the biliary tree that can provide high success rates of endoscopic insertion without complications. For example, a newly designed Y-shaped metal stent with central wide-open mesh provided 80% technical success in bilateral stent insertion for advanced hilar CCs[77]. Percutaneous biliary drainage and subsequent placement of a self-expandable metallic stent can also be easily and successfully applied in patients with hilar tumors. The patency rates of metallic endoprosthesis at the hilus is approximately 6 mo, which is significantly lower than that reported for similar stents placed in the

distal bile duct. A small pilot study investigated the role of a new percutaneous drainage tube coated with carboplatin[78]. The carboplatin-coated tube continuously released a fixed amount of carboplatin for 4 wk in five patients. Partial response was reported in three (60%) of the cases. Operative segment Ⅲ bypass provides excellent biliary drainage and is less prone to occlusion than metal stents. In one study, the one-year patency of segment 3 bypass was reported to be 80%[79].

Palliative external radiation therapy and percutaneous intraluminal iridium-192 for patients with unresectable locally advanced tumors, but without evidence of widespread disease, did not improve survival compared with biliary decompression[75,80-84]. In a group of 12 patients treated with this regimen, the median survival was around 14.5 mo[80]. Another report indicated improved survival in irradiated compared to non-irradiated patients. However, both groups had less than one-year survival[75]. A group of authors reported a beneficial effect of radiotherapy only in patients with aneuploid Klatskin tumors[85]. Radiation therapy is clearly not indicated in widespread disease. Successful ablation of intra-hepatic CC cases with radiofrequency ablative therapy was reported previously[86]. Photodynamic therapy is another palliative approach. This therapy is accomplished by the systemic administration of a photosensitizer that accumulates in the malignant cells. Red laser light-induced photoactivation at the time of ERC destroys the malignant cells. This therapy facilitates biliary decompression, and pilot studies have suggested a survival benefit and improvement in cholestasis, performance status and quality of life with this approach[87-90]. Another palliative treatment option is endoscopic administration of high-intensity ultrasound to induce coagulative necrosis of tumoral tissue. Local tumor destruction, with a high-intensity ultrasound probe during ERC, has been reported to induce complete regression of extra-hepatic CC in a pilot study[91]. For mass-forming tumors, transarterial chemoembolization or radiofrequency ablation may be useful. However, these therapies have been tested in small patient groups. Results with chemotherapy appear to be disappointing. Gemcitabine appears to be the most effective single agent[92]. Systemic chemotherapy combined with regional chemoembolization was proven feasible in a small group of patients[93]. New promising drugs are under investigation for their anti-tumoral effects on CCs. A very recent *in vitro* study clearly indicated a high apoptotic effect of proteasome inhibitors on CC cell lines[94].

CONCLUSION

CC is a devastating tumor with a high mortality rate. Its incidence is increasing and there is no new proven medical treatment modality. It is notorious as being difficult to diagnose as well as treat. Strategies are needed to detect these tumors at an early stage to apply radical curative therapy modalities. EUS-guided FNA is the most promising approach in this respect. Liver transplantation protocols must be supported, as necessitated by the recent reports of success using these protocols. Investigations of neoadjuvant and adjuvant treatment alternatives should continue and new *in vitro* effective anti-tumoral agents should be investigated in *in vivo* studies.

REFERENCES

1 **Oh SW**, Yoon YS, Shin SA. Effects of excess weight on cancer incidences depending on cancer sites and histologic findings among men: Korea National Health Insurance Corporation Study. *J Clin Oncol* 2005; **23**: 4742-4754
2 **Parkin DM**, Muir CS, Whelan SL, Gao YT, Ferlay S, Powel J, editors. Cancer Incidence in Five Continents. Lyon: International Agency for Research on Cancer, 1992
3 **Shaib Y**, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 115-125
4 **Thompson R**, Strautnieks S. BSEP: function and role in progressive familial intrahepatic cholestasis. *Semin Liver Dis* 2001; **21**: 545-550
5 **Holzinger F**, Z'graggen K, Buchler MW. Mechanisms of biliary carcinogenesis: a pathogenetic multi-stage cascade towards cholangiocarcinoma. *Ann Oncol* 1999; **10** Suppl 4: 122-126
6 **Vauthey JN**, Blumgart LH. Recent advances in the management of cholangiocarcinoma. *Semin Liver Dis* 1994; **14**: 109-114
7 **Khan SA**, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 2002; **37**: 806-813
8 **Patel T**. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer* 2002; **2**: 10
9 **Taylor-Robinson SD**, Toledano MB, Arora S, Keegan TJ, Hargreaves S, Beck A, Khan SA, Elliott P, Thomas HC. Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968-1998. *Gut* 2001; **48**: 816-820
10 **Patel T**. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001; **33**: 1353-1357
11 **Shaib YH**, Davila JA, McGlynn K, El-Serag HB. Rising incidence of intrahepatic cholangiocarcinoma in the United States: a true increase? *J Hepatol* 2004; **40**: 472-477
12 **Lazaridis KN**, Gores GJ. Cholangiocarcinoma. *Gastroenterology* 2005; **128**: 1655-1667
13 **Khan SA**, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. *Lancet* 2005; **366**: 1303-1314
14 **Goydos JS**, Brumfield AM, Frezza E, Booth A, Lotze MT, Carty SE. Marked elevation of serum interleukin-6 in patients with cholangiocarcinoma: validation of utility as a clinical marker. *Ann Surg* 1998; **227**: 398-404
15 **Yoon JH**, Werneburg NW, Higuchi H, Canbay AE, Kaufmann SH, Akgul C, Edwards SW, Gores GJ. Bile acids inhibit Mcl-1 protein turnover via an epidermal growth factor receptor/Raf-1-dependent mechanism. *Cancer Res* 2002; **62**: 6500-6505
16 **Iwai N**, Yanagihara J, Tokiwa K, Shimotake T, Nakamura K. Congenital choledochal dilatation with emphasis on pathophysiology of the biliary tract. *Ann Surg* 1992; **215**: 27-30
17 **Rosen CB**, Nagorney DM. Cholangiocarcinoma complicating primary sclerosing cholangitis. *Semin Liver Dis* 1991; **11**: 26-30
18 **Thamavit W**, Tiwawech D, Moore MA, Ito N, Shirai T. Equivocal evidence of complete carcinogenicity after repeated infection of Syrian hamsters with Opisthorchis viverrini. *Toxicol Pathol* 1996; **24**: 493-497
19 **Sugihara S**, Kojiro M. Pathology of cholangiocarcinoma. In: Okuda K, Ishak KG, editors. Neoplasms of the Liver. Tokyo: Springer, 1987: l43
20 **Walker NJ**, Crockett PW, Nyska A, Brix AE, Jokinen

MP, Sells DM, Hailey JR, Easterling M, Haseman JK, Yin M, Wyde ME, Bucher JR, Portier CJ. Dose-additive carcinogenicity of a defined mixture of "dioxin-like compounds". *Environ Health Perspect* 2005; **113**: 43-48

21 **Bond GG**, McLaren EA, Sabel FL, Bodner KM, Lipps TE, Cook RR. Liver and biliary tract cancer among chemical workers. *Am J Ind Med* 1990; **18**: 19-24

22 **Rubel LR**, Ishak KG. Thorotrast-associated cholangiocarcinoma: an epidemiologic and clinicopathologic study. *Cancer* 1982; **50**: 1408-1415

23 **Ishimura N**, Bronk SF, Gores GJ. Inducible nitric oxide synthase upregulates cyclooxygenase-2 in mouse cholangiocytes promoting cell growth. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G88-G95

24 **Jaiswal M, LaRusso NF**, Burgart LJ, Gores GJ. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. *Cancer Res* 2000; **60**: 184-190

25 **Cox H**, Ma M, Bridges R, Debru E, Bathe O, Sutherland F, Dixon E. Well differentiated intrahepatic cholangiocarcinoma in the setting of biliary papillomatosis: a case report and review of the literature. *Can J Gastroenterol* 2005; **19**: 731-733

26 **Fairchild R**, Reese J, Solomon H, Garvin P, Esterl R. Biliary cystadenoma: a case report and review of the literature. *Mo Med* 1993; **90**: 656-657

27 **Prawan A**, Kukongviriyapan V, Tassaneeyakul W, Pairojkul C, Bhudhisawasdi V. Association between genetic polymorphisms of CYP1A2, arylamine N-acetyltransferase 1 and 2 and susceptibility to cholangiocarcinoma. *Eur J Cancer Prev* 2005; **14**: 245-250

28 **Tocchi A**, Mazzoni G, Liotta G, Lepre L, Cassini D, Miccini M. Late development of bile duct cancer in patients who had biliary-enteric drainage for benign disease: a follow-up study of more than 1,000 patients. *Ann Surg* 2001; **234**: 210-214

29 **Bettschart V**, Clayton RA, Parks RW, Garden OJ, Bellamy CO. Cholangiocarcinoma arising after biliary-enteric drainage procedures for benign disease. *Gut* 2002; **51**: 128-129

30 **Carriaga MT**, Henson DE. Liver, gallbladder, extrahepatic bile ducts, and pancreas. *Cancer* 1995; **75**: 171-190

31 **Nichols JC**, Gores GJ, LaRusso NF, Wiesner RH, Nagorney DM, Ritts RE Jr. Diagnostic role of serum CA 19-9 for cholangiocarcinoma in patients with primary sclerosing cholangitis. *Mayo Clin Proc* 1993; **68**: 874-879

32 **Patel AH**, Harnois DM, Klee GG, LaRusso NF, Gores GJ. The utility of CA 19-9 in the diagnoses of cholangiocarcinoma in patients without primary sclerosing cholangitis. *Am J Gastroenterol* 2000; **95**: 204-207

33 **Teefey SA**, Baron RL, Rohrmann CA, Shuman WP, Freeny PC. Sclerosing cholangitis: CT findings. *Radiology* 1988; **169**: 635-639

34 **Zhang Y**, Uchida M, Abe T, Nishimura H, Hayabuchi N, Nakashima Y. Intrahepatic peripheral cholangiocarcinoma: comparison of dynamic CT and dynamic MRI. *J Comput Assist Tomogr* 1999; **23**: 670-677

35 **Tillich M**, Mischinger HJ, Preisegger KH, Rabl H, Szolar DH. Multiphasic helical CT in diagnosis and staging of hilar cholangiocarcinoma. *AJR Am J Roentgenol* 1998; **171**: 651-658

36 **Ahmetoglu A**, Kosucu P, Kul S, Dinc H, Sari A, Arslan M, Alhan E, Gumele HR. MDCT cholangiography with volume rendering for the assessment of patients with biliary obstruction. *AJR Am J Roentgenol* 2004; **183**: 1327-1332

37 **Angulo P**, Pearce DH, Johnson CD, Henry JJ, LaRusso NF, Petersen BT, Lindor KD. Magnetic resonance cholangiography in patients with biliary disease: its role in primary sclerosing cholangitis. *J Hepatol* 2000; **33**: 520-527

38 **Oberholzer K**, Lohse AW, Mildenberger P, Grebe P, Schadeck T, Bantelmann M, Thelen M. [Diagnosis of primary sclerosing cholangitis: prospective comparison of MR cholangiography with endoscopic retrograde cholangiography] *Rofo* 1998; **169**: 622-626

39 **Textor HJ**, Flacke S, Pauleit D, Keller E, Neubrand M, Terjung B, Gieseke J, Scheurlen C, Sauerbruch T, Schild HH. Three-dimensional magnetic resonance cholangiopancreatography with respiratory triggering in the diagnosis of primary sclerosing cholangitis: comparison with endoscopic retrograde cholangiography. *Endoscopy* 2002; **34**: 984-990

40 **Lee MG**, Park KB, Shin YM, Yoon HK, Sung KB, Kim MH, Lee SG, Kang EM. Preoperative evaluation of hilar cholangiocarcinoma with contrast-enhanced three-dimensional fast imaging with steady-state precession magnetic resonance angiography: comparison with intraarterial digital subtraction angiography. *World J Surg* 2003; **27**: 278-283

41 **Zidi SH**, Prat F, Le Guen O, Rondeau Y, Pelletier G. Performance characteristics of magnetic resonance cholangiography in the staging of malignant hilar strictures. *Gut* 2000; **46**: 103-106

42 **Fritscher-Ravens A**, Broering DC, Sriram PV, Topalidis T, Jaeckle S, Thonke F, Soehendra N. EUS-guided fine-needle aspiration cytodiagnosis of hilar cholangiocarcinoma: a case series. *Gastrointest Endosc* 2000; **52**: 534-540

43 **Fritscher-Ravens A**, Broering DC, Knoefel WT, Rogiers X, Swain P, Thonke F, Bobrowski C, Topalidis T, Soehendra N. EUS-guided fine-needle aspiration of suspected hilar cholangiocarcinoma in potentially operable patients with negative brush cytology. *Am J Gastroenterol* 2004; **99**: 45-51

44 **Fritscher-Ravens A**, Bohuslavizki KH, Broering DC, Jenicke L, Schafer H, Buchert R, Rogiers X, Clausen M. FDG PET in the diagnosis of hilar cholangiocarcinoma. *Nucl Med Commun* 2001; **22**: 1277-1285

45 **Reinhardt MJ**, Strunk H, Gerhardt T, Roedel R, Jaeger U, Bucerius J, Sauerbruch T, Biersack HJ, Dumoulin FL. Detection of Klatskin's tumor in extrahepatic bile duct strictures using delayed 18F-FDG PET/CT: preliminary results for 22 patient studies. *J Nucl Med* 2005; **46**: 1158-1163

46 **Baron TH**, Harewood GC, Rumalla A, Pochron NL, Stadheim LM, Gores GJ, Therneau TM, De Groen PC, Sebo TJ, Salomao DR, Kipp BR. A prospective comparison of digital image analysis and routine cytology for the identification of malignancy in biliary tract strictures. *Clin Gastroenterol Hepatol* 2004; **2**: 214-219

47 **Rumalla A**, Baron TH, Leontovich O, Burgart LJ, Yacavone RF, Therneau TM, de Groen PC, Sebo TJ. Improved diagnostic yield of endoscopic biliary brush cytology by digital image analysis. *Mayo Clin Proc* 2001; **76**: 29-33

48 **Kim HJ**, Kim MH, Lee SK, Yoo KS, Seo DW, Min YI. Tumor vessel: a valuable cholangioscopic clue of malignant biliary stricture. *Gastrointest Endosc* 2000; **52**: 635-638

49 **Domagk D**, Wessling J, Reimer P, Hertel L, Poremba C, Senninger N, Heinecke A, Domschke W, Menzel J. Endoscopic retrograde cholangiopancreatography, intraductal ultrasonography, and magnetic resonance cholangiopancreatography in bile duct strictures: a prospective comparison of imaging diagnostics with histopathological correlation. *Am J Gastroenterol* 2004; **99**: 1684-1689

50 **Judah JR**, Draganov PV. Endoscopic therapy of benign biliary strictures. *World J Gastroenterol* 2007; **13**: 3531-3539

51 **Bayraktar Y**, Balkanci F, Ozenc A, Arslan S, Koseoglu T, Ozdemir A, Uzunalimoglu B, Telatar H, Gurakar A, Van Thiel DH. The "pseudo-cholangiocarcinoma sign" in patients with cavernous transformation of the portal vein and its effect on the serum alkaline phosphatase and bilirubin levels. *Am J Gastroenterol* 1995; **90**: 2015-2019

52 **Anderson CD**, Rice MH, Pinson CW, Chapman WC, Chari RS, Delbeke D. Fluorodeoxyglucose PET imaging in the evaluation of gallbladder carcinoma and cholangiocarcinoma. *J Gastrointest Surg* 2004; **8**: 90-97

53 **Connor S**, Barron E, Wigmore SJ, Madhavan KK, Parks RW, Garden OJ. The utility of laparoscopic assessment in the preoperative staging of suspected hilar cholangiocarcinoma. *J Gastrointest Surg* 2005; **9**: 476-480

54 **Jarnagin WR**, Fong Y, DeMatteo RP, Gonen M, Burke EC, Bodniewicz BS J, Youssef BA M, Klimstra D, Blumgart LH. Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. *Ann Surg* 2001; **234**: 507-517; discussion 517-519

55 **Kosuge T**, Yamamoto J, Shimada K, Yamasaki S, Makuuchi M. Improved surgical results for hilar cholangiocarcinoma with procedures including major hepatic resection. *Ann Surg* 1999; **230**: 663-671

56 **Burke EC**, Jarnagin WR, Hochwald SN, Pisters PW, Fong Y, Blumgart LH. Hilar Cholangiocarcinoma: patterns of spread, the importance of hepatic resection for curative operation, and a presurgical clinical staging system. *Ann Surg* 1998; **228**: 385-394

57 **Miyazaki M**, Ito H, Nakagawa K, Ambiru S, Shimizu H, Shimizu Y, Kato A, Nakamura S, Omoto H, Nakajima N, Kimura F, Suwa T. Aggressive surgical approaches to hilar cholangiocarcinoma: hepatic or local resection? *Surgery* 1998; **123**: 131-136

58 **Nagino M**, Nimura Y, Kamiya J, Kanai M, Uesaka K, Hayakawa N, Yamamoto H, Kondo S, Nishio H. Segmental liver resections for hilar cholangiocarcinoma. *Hepatogastroenterology* 1998; **45**: 7-13

59 **Klempnauer J**, Ridder GJ, Werner M, Weimann A, Pichlmayr R. What constitutes long-term survival after surgery for hilar cholangiocarcinoma? *Cancer* 1997; **79**: 26-34

60 **Nimura Y**, Hayakawa N, Kamiya J, Kondo S, Shionoya S. Hepatic segmentectomy with caudate lobe resection for bile duct carcinoma of the hepatic hilus. *World J Surg* 1990; **14**: 535-543; discussion 544

61 **Saldinger PF**, Blumgart LH. Resection of hilar cholangiocarcinoma--a European and United States experience. *J Hepatobiliary Pancreat Surg* 2000; **7**: 111-114

62 **Neuhaus P**, Jonas S, Settmacher U, Thelen A, Benckert C, Lopez-Hanninen E, Hintze RE. Surgical management of proximal bile duct cancer: extended right lobe resection increases resectability and radicality. *Langenbecks Arch Surg* 2003; **388**: 194-200

63 **Kondo S**, Hirano S, Ambo Y, Tanaka E, Okushiba S, Morikawa T, Katoh H. Forty consecutive resections of hilar cholangiocarcinoma with no postoperative mortality and no positive ductal margins: results of a prospective study. *Ann Surg* 2004; **240**: 95-101

64 **Silva MA**, Tekin K, Aytekin F, Bramhall SR, Buckels JA, Mirza DF. Surgery for hilar cholangiocarcinoma; a 10 year experience of a tertiary referral centre in the UK. *Eur J Surg Oncol* 2005; **31**: 533-539

65 **Jarnagin WR**, Bowne W, Klimstra DS, Ben-Porat L, Roggin K, Cymes K, Fong Y, DeMatteo RP, D'Angelica M, Koea J, Blumgart LH. Papillary phenotype confers improved survival after resection of hilar cholangiocarcinoma. *Ann Surg* 2005; **241**: 703-712; discussion 712-714

66 **Tsao JI**, Nimura Y, Kamiya J, Hayakawa N, Kondo S, Nagino M, Miyachi M, Kanai M, Uesaka K, Oda K, Rossi RL, Braasch JW, Dugan JM. Management of hilar cholangiocarcinoma: comparison of an American and a Japanese experience. *Ann Surg* 2000; **232**: 166-174

67 **Ueda J**, Chijiiwa K, Nakano K, Zhao G, Tanaka M. Lack of intestinal bile results in delayed liver regeneration of normal rat liver after hepatectomy accompanied by impaired cyclin E-associated kinase activity. *Surgery* 2002; **131**: 564-573

68 **Boberg KM**, Bergquist A, Mitchell S, Pares A, Rosina F, Broome U, Chapman R, Fausa O, Egeland T, Rocca G, Schrumpf E. Cholangiocarcinoma in primary sclerosing cholangitis: risk factors and clinical presentation. *Scand J Gastroenterol* 2002; **37**: 1205-1211

69 **Fleming KA**, Boberg KM, Glaumann H, Bergquist A, Smith D, Clausen OP. Biliary dysplasia as a marker of cholangiocarcinoma in primary sclerosing cholangitis. *J Hepatol* 2001; **34**: 360-365

70 **Chalasani N**, Baluyut A, Ismail A, Zaman A, Sood G, Ghalib R, McCashland TM, Reddy KR, Zervos X, Anbari MA, Hoen H. Cholangiocarcinoma in patients with primary sclerosing cholangitis: a multicenter case-control study. *Hepatology* 2000; **31**: 7-11

71 **Sudan D**, DeRoover A, Chinnakotla S, Fox I, Shaw B Jr, McCashland T, Sorrell M, Tempero M, Langnas A. Radiochemotherapy and transplantation allow long-term survival for nonresectable hilar cholangiocarcinoma. *Am J Transplant* 2002; **2**: 774-779

72 **De Vreede I**, Steers JL, Burch PA, Rosen CB, Gunderson LL, Haddock MG, Burgart L, Gores GJ. Prolonged disease-free survival after orthotopic liver transplantation plus adjuvant chemoirradiation for cholangiocarcinoma. *Liver Transpl* 2000; **6**: 309-316

73 **Shimoda M**, Farmer DG, Colquhoun SD, Rosove M, Ghobrial RM, Yersiz H, Chen P, Busuttil RW. Liver transplantation for cholangiocellular carcinoma: analysis of a single-center experience and review of the literature. *Liver Transpl* 2001; **7**: 1023-1033

74 **Rea DJ**, Heimbach JK, Rosen CB, Haddock MG, Alberts SR, Kremers WK, Gores GJ, Nagorney DM. Liver transplantation with neoadjuvant chemoradiation is more effective than resection for hilar cholangiocarcinoma. *Ann Surg* 2005; **242**: 451-458; discussion 458-461

75 **Cameron JL**, Pitt HA, Zinner MJ, Kaufman SL, Coleman J. Management of proximal cholangiocarcinomas by surgical resection and radiotherapy. *Am J Surg* 1990; **159**: 91-97; discussion 97-98

76 **Pitt HA**, Nakeeb A, Abrams RA, Coleman J, Piantadosi S, Yeo CJ, Lillemore KD, Cameron JL. Perihilar cholangiocarcinoma. Postoperative radiotherapy does not improve survival. *Ann Surg* 1995; **221**: 788-797; discussion 797-798

77 **Lee JH**, Kang DH, Kim JY, Lee SM, Kim do H, Park CW, Cho HS, Kim GH, Kim TO, Heo J, Song GA, Cho M, Kim S, Kim CW, Lee JW. Endoscopic bilateral metal stent placement for advanced hilar cholangiocarcinoma: a pilot study of a newly designed Y stent. *Gastrointest Endosc* 2007; **66**: 364-369

78 **Mezawa S**, Homma H, Sato T, Doi T, Miyanishi K, Takada K, Kukitsu T, Murase K, Yoshizaki N, Takahashi M, Sakamaki S, Niitsu Y. A study of carboplatin-coated tube for the unresectable cholangiocarcinoma. *Hepatology* 2000; **32**: 916-923

79 **Jarnagin WR**, Burke E, Powers C, Fong Y, Blumgart LH. Intrahepatic biliary enteric bypass provides effective palliation in selected patients with malignant obstruction at the hepatic duct confluence. *Am J Surg* 1998; **175**: 453-460

80 **Kuvshinoff BW**, Armstrong JG, Fong Y, Schupak K, Getradjman G, Heffernan N, Blumgart LH. Palliation of irresectable hilar cholangiocarcinoma with biliary drainage and radiotherapy. *Br J Surg* 1995; **82**: 1522-1525

81 **Bowling TE**, Galbraith SM, Hatfield AR, Solano J, Spittle MF. A retrospective comparison of endoscopic stenting alone with stenting and radiotherapy in non-resectable cholangiocarcinoma. *Gut* 1996; **39**: 852-855

82 **Vallis KA**, Benjamin IS, Munro AJ, Adam A, Foster CS, Williamson RC, Kerr GR, Price P. External beam and intraluminal radiotherapy for locally advanced bile duct cancer: role and tolerability. *Radiother Oncol* 1996; **41**: 61-66

83 **Vatanasapt V**, Uttaravichien T, Mairiang EO, Pairojkul C, Chartbanchachai W, Haswell-Elkins M. Cholangiocarcinoma in north-east Thailand. *Lancet* 1990; **335**: 116-117

84 **Nagano H**, Sasaki Y, Imaoka S, Masutani S, Ohashi I, Ishikawa O, Oohigashi H, Yasuda T, Furukawa H, Fukuda I. [Intraarterial and intraportal chemotherapy combined with decollateralization for cholangiocellular carcinoma and metastatic liver cancer] *Gan To Kagaku Ryoho* 1990; **17**: 1758-1762

85 **Sato Y**, van Gulik TM, Bosma A, Lygidakis NJ, Koyama K, van der Heyde MN. Prognostic significance of tumor DNA content in carcinoma of the hepatic duct confluence. *Surgery* 1994; **115**: 488-494

86 **Zgodzinski W**, Espat NJ. Radiofrequency ablation for incidentally identified primary intrahepatic cholangio-

carcinoma. *World J Gastroenterol* 2005; **11**: 5239-5240

87 **Wiedmann M**, Berr F, Schiefke I, Witzigmann H, Kohlhaw K, Mossner J, Caca K. Photodynamic therapy in patients with non-resectable hilar cholangiocarcinoma: 5-year follow-up of a prospective phase II study. *Gastrointest Endosc* 2004; **60**: 68-75

88 **Rumalla A**, Baron TH, Wang KK, Gores GJ, Stadheim LM, de Groen PC. Endoscopic application of photodynamic therapy for cholangiocarcinoma. *Gastrointest Endosc* 2001; **53**: 500-504

89 **Ortner MA**, Liebetruth J, Schreiber S, Hanft M, Wruck U, Fusco V, Muller JM, Hortnagl H, Lochs H. Photodynamic therapy of nonresectable cholangiocarcinoma. *Gastroenterology* 1998; **114**: 536-542

90 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodes J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430

91 **Prat F**, Lafon C, De Lima DM, Theilliere Y, Fritsch J, Pelletier G, Buffet C, Cathignol D. Endoscopic treatment of cholangiocarcinoma and carcinoma of the duodenal papilla by intraductal high-intensity US: Results of a pilot study. *Gastrointest Endosc* 2002; **56**: 909-915

92 **Kubicka S**, Rudolph KL, Tietze MK, Lorenz M, Manns M. Phase II study of systemic gemcitabine chemotherapy for advanced unresectable hepatobiliary carcinomas. *Hepatogastroenterology* 2001; **48**: 783-789

93 **Kirchhoff T**, Zender L, Merkesdal S, Frericks B, Malek N, Bleck J, Kubicka S, Baus S, Chavan A, Manns MP, Galanski M. Initial experience from a combination of systemic and regional chemotherapy in the treatment of patients with nonresectable cholangiocellular carcinoma in the liver. *World J Gastroenterol* 2005; **11**: 1091-1095

94 **Ustundag Y**, Bronk SF, Gores GJ. Proteasome inhibition-induces endoplasmic reticulum dysfunction and cell death of human cholangiocarcinoma cells. *World J Gastroenterol* 2007; **13**: 851-857

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TOPIC HIGHLIGHT

Giuseppe Montalto, Professor, *Series Editor*

Antiviral therapy in hepatitis C virus cirrhotic patients in compensated and decompensated condition

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Abstract

The main goals of treating cirrhotic patients with antiviral therapy are to attain sustained viral clearance (SVR), halt disease progression, and prevent re-infection of the liver graft. However, while the medical need is great, the use of interferon and ribavirin might expose these patients to severe treated-related side effects as a large proportion of them have pre-existing hematological cytopenias. We have reviewed potential benefits and risks associated with antiviral drugs in patients with liver cirrhosis, due to hepatitis C virus (HCV) infection. In cases presenting with bridging fibrosis or cirrhosis, current regimens of antiviral therapy have attained a 44%-48% rate of SVR. In cirrhotic patients with portal hypertension, the SVR rate was 22% overall, 12.5% in patients with genotype 1, and 66.7% in those with genotypes 2 and 3 following therapy with low doses of either Peg-IFN alpha-2b and of ribavirin. In patients with decompensated cirrhosis, full dosages of Peg-IFN alpha-2b and of ribavirin produced a SVR rate of 35% overall, 16% in patients with genotype 1 and 4, and 59% in those with genotype 2 and 3. Use of hematological cytokines will either ensure full course of treatment to be accomplished with and prevent development of treatment-associated side effects. Major benefits after HCV eradication were partial recovery of liver metabolic activity, prevention of hepatitis C recurrence after transplantation, and removal of some patients from the waiting list for liver transplant. Several observations highlighted that therapy is inadvisable for individuals with poor hepatic reserve (Child-Pugh-Turcotte score $\geqslant$ 10). Although SVR rates are low in decompensated cirrhotics due to hepatitis C, these patients have the most to gain as successful antiviral therapy is potentially lifesaving.

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Key words: Hepatitis C virus; Cirrhosis; Peg-interferon; Ribavirin; Therapy

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INTRODUCTION

Extensive fibrotic deposition in advanced hepatitis C virus (HCV) liver disease is the histopathological hallmark of a chronic necro-inflammatory process that involves parenchymal cells. Progressive derangement of normal liver architecture correlates with a reduction in liver synthetic function and brings to its late clinical expressions of decompensation, such as intractable ascites, hepatic synthetic failure, encephalopathy, jaundice, variceal bleeding, and hepatocellular carcinoma. Once cirrhosis is present, the process is generally considered as irreversible and predisposing to high mortality risk with a survival rate of 50% at 5 years[1]. A mathematical model of the natural history of chronic hepatitis C projected number of cases with cirrhosis to increase by more than 50% by 2010[2]. As a result, there will be a dramatic increase in the number of cases with complications of liver failure throughout the next three decades.

Once decompensation complicates liver cirrhosis, liver transplantation is the only successful therapeutic option. However, the limited number of organ donors as well as impairment in age-related cardiovascular, renal, and pulmonary functions, renders this option unlikely for the majority of patients. In addition, age over 65 years is commonly considered as an exclusion criterion

to enlist patients for liver transplant. Exploring new therapeutic options to offer patients with HCV end-stage liver cirrhosis is a critical need, as cirrhotics who are never listed for liver transplant could still potentially benefit from non-surgical therapy.

In advanced liver cirrhosis, antiviral therapy is currently not recommended despite the fact that theoretical benefits of treating HCV-related patients with or without decompensated cirrhosis would be an improvement in liver histology, reversal of established cirrhosis, and prevention of life-threatening complications. Despite this, a large proportion of these patients have pre-existing neutropenia, thrombocytopenia, and anemia that will tend to worsen with the use of interferon and ribavirin, exposing them to potentially severe side effects. We will review this topic with the intent to highlight potential benefits and risks associated with antiviral therapy in patients with compensated and decompensated cirrhosis due to HCV infection.

HCV CIRRHOSIS IN COMPENSATED PHASE

Compensated cirrhosis is defined by the absence of clinical complications and presence of both preserved hepatic synthetic function and adequate bone marrow reserve. Post-hoc analyses of patients with bridging fibrosis or compensated cirrhosis included in two international, registrative trials on the efficacy of Peg-IFN and ribavirin, produced a 43% rate of sustained viral clearance (SVR) by 48 wk administration of once weekly Peg-IFN alpha 2a 180 μg plus ribavirin 1000-1200 mg/d[3]: a similar rate of 44% was seen in those treated with Peg-IFN alpha-2b 1.5 μg/kg weekly plus ribavirin 800 mg/d for 48 wk[4]. From previous trials, data on cirrhotic patients could not be retrieved separately from those with bridging fibrosis, so that exact figures of SVR rate in cirrhotics remain to be determined. Moreover, cirrhotics enrolled in these studies were commonly in a Child-Pugh-Turcotte (CPT) class A, had as a rule a compensated disease, and slightly abnormal hematological parameters; moreover, no information was usually given on the degree of portal hypertension.

The first study proving the benefits of antiviral therapy in cirrhotics with signs of portal hypertension, a subset of patients with more advanced cirrhosis than the cohort of cirrhotics enrolled in registrative trials, was the one published by Di Marco *et al*[5]. In the study, a branch of 51 cirrhotics received 1 μg/kg per week of Pegylated-interferon alpha-2b plus oral ribavirin at a fixed dose of 800 mg/d for 52 wk. By intention-to-treat analysis, a sustained virologic response (SVR) was achieved by 11 patients (21.6%), with a therapeutical efficacy poorest in those infected with genotype 1 and 4 (6 of 45, 13.3%) than in those with genotypes 2 and 3 (5 of 6, 83.3%). All responders achieved negative HCV-RNA during the first 12 wk of treatment, and no subject still positive at this time evaluation became negative later despite continued treatment. The median WBC count decreased from baseline, particularly during the first two months of treatment. Five patients stopped PEG-IFN due to neutrophil counts $< 0.75 \times 10^3$/dL, but none of them developed infections. Cumulative incidence of events was significantly higher in patients without an SVR: disease deterioration occurred only in 6% of patients with SVR as compared to 38% of non-responders. This study established the effectiveness of antiviral therapy in the subgroup of patients with severe portal hypertension, although it must be emphasized that entry criteria for the trial excluded patients with clinical, biochemical, or hematological decompensation of the liver disease.

Safety of combination therapy in cirrhotics is a major concern. Bone marrow suppression by administration of both standard or Peg-IFN-alpha leads to significant decrease in all 3 lineages of the hematopoietic system, whereas anemia through haemolysis is more a sequela of ribavirin therapy[6]. Absolute neutrophil and lymphocyte counts typically decrease by 30% to 50% of baseline values during therapy[7]. In a recent analysis of patients treated at a single referral center, where basal neutropenia was not an exclusion criterion, therapy was safely accomplished despite decreases in neutrophils below the usual levels that lead to dose reduction or drug interruption[8]. No patients who developed infections had a pre-existing neutropenia (below 1500 cells/mL), and none developed neutropenia of less than 750 cells/mL at any point during treatment. Although the use of interferon in cirrhotics with neutropenia is usually not recommended, there is no evidence, up to now, of a significantly higher risk of severe infections or death correlated with interferon therapy in cirrhotics, a concern supported by the studies of Heathcote and Di Marco, where none of the cirrhotics with interferon-induced neutropenia of less than 750 cells/mL developed serious infection or sepsis[4,5].

It is currently recommended that development of leucopenia during antiviral therapy, an event that develops in 15%-20% of cases[9], to be managed by modification or withdrawal of drugs. This recommendation may potentially affect achieving an SVR, being the therapeutic outcome dependent on both dose and duration of currently administered drugs[10]. Hematopoietic cytokines, such as granulocyte colony-stimulating factor (G-CSF), at the weekly dosage of 300 μg, can reverse neutropenia, enabling patients to remain on full dosage and duration of therapy[11,12]. However, G-CSF is prescribed less frequently than erythropoietin, likely for two reasons: First, ribavirin-induced anaemia is more common than IFN-induced neutropenia and, secondly, practitioners are probably less responsive to neutropenia than to anaemia being the latter more often symptomatic than neutropenia. As regards to chronic hepatitis C, no official guidelines have been set for the use of G-CSF, probably because of the absence of firm data of the benefit of the cytokine administration in increasing SVR while reducing infections.

Treatment of HCV-infected patients induces hemoglobin drop in the majority of patients[13]. The anemia is of "mixed" type: Ribavirin induces a dose-dependent hemolytic anaemia, whereas IFN alpha suppresses eryth-

Table 1 Summary features of trials of antiviral therapy in HCV-infected cirrhotics with signs of hepatic decompensation

Author	Yr	No. of patients	Type of IFN	Dosage	RBV	Dosage per day	Length of therapy	Genotype 1 (%)	CPT score	MELD score	Decompensated (%)	Overall SVR *n* (%)	Genotypes 1 and 4 SVR *n* (%)	Genotypes 2 and 3 SVR *n* (%)
Crippin *et al*[36]	2002	15	IFN α2b	(1) 1 MU (2) 3 MU 3 times/wk	(1) Yes/ No	800 mg	Mean time: 1.95 mo (range 0.25 to 5 mo)	73	11.9 ± 1.2	No data	100	0	0	0
Thomas *et al*[35]	2003	20	IFN α2b	5 MU/d	No		Until the day of transplantation (14 ± 2.5 mo)	67	10 ± 0.5	13.0 ± 2.5	100	4 (20)	2 (10)	2 (100)
Forns *et al*[34]	2003	30	IFN α2b	3 MU/d	Yes	800 mg	12 wk	83	[1]	No data	43	6 (20)	3 (12)	3 (60)
Everson *et al*[32]	2003	124	IFN α2b	Increasing doses until standard dose	Yes	Increasing doses until standard dose	6 mo for genotype 2 and 3 and 12 mo for genotypes 1 and 4	70	7.4 ± 2.3	11.0 ± 3.7	63	30 (24)	11 (13)	19 (50)
Iacobellis *et al*[33]	2007	66	PEG-IFN α2b	1 μg/kg per wk	Yes	800 or 1000 mg	6 mo	65.2	8 ± 1.2	14.2 ± 2.7	100	13 (19.7)	3 (7)	10 (43.5)

[1]Child A 15 (50%), B 13 (43%), C 2 (7%).

roid progenitor cell and red blood cell production[7]. The mean maximal hemoglobin decrease, reached within the first 12 wk of therapy, is a relevant point as a reduction in ribavirin dosage in the initial 12 wk of therapy below 80% of the starting dose lowers the chance of early virologic response (EVR)[14] and compromise treatment success[15,16]. The management of anemia recommends ribavirin dose reduction untill 600 mg/d if hemoglobin decreases to < 10 g/dL in a patient without cardiac risk factors, and discontinuation of ribavirin if hemoglobin becomes < 8.5 g/dL[17]. A decrease in hemoglobin levels is normally accompanied by an increase in the serum erythropoietin (sEPO) level, an endogenous hormone that acts in the bone marrow to increase the number of erythroid progenitor cells[18], which will ultimately normalize the hemoglobin level[19]. In HCV-infected patients treated with PEG-IFN/RBV, although increased levels of sEPO, the hemoglobin level did not return to normal, suggesting that the physiological increase in sEPO is not sufficient to fully compensate for the degree of anemia. Administration of recombinant human erythropoietin at a dosage of 40 to 60 U once weekly, may constitute an alternative: 88% of patients receiving erythropoietin *versus* 60% of controls could maintain the assigned ribavirin doses[20].

Several lines of evidence have supported the hypothesis that the fibrotic component of cirrhosis is a reversible process[21-26]. SVR is the "sine qua non" to pursue histological benefits[27] and has been associated with a mean reduction in fibrosis score of -0.88 ± 0.08 U/year at 3 years of follow up[22]. Resolution of cirrhosis, defined as a decrease of the fibrotic score from 4 to 1 by Knodell index, was observed in 9 of 109 cirrhotic patients (8.2%), with a delay between pre- and post-therapy biopsies of 4.0 ± 2.3 years[21]. The most striking result of a post hoc analysis[25] of 4 major clinical trials involving 3010 patients randomized to various treatment regimens was the reversal of fibrosis at different extent after therapy in 75 out of 153 patients with cirrhosis (49%). These observations provide evidence for major beneficial effects of antiviral therapy on cumulative probabilities of disease progression, development of HCC, and death or liver transplantation in patients attaining an SVR[28].

HCV CIRRHOSIS IN DECOMPENSATED PHASE

Antiviral therapy is commonly deferred in cirrhotics with signs of liver decompensation, due to even more compelling concerns over treatment-induced side effects. Along with the progression of liver disease, a reduction in the capability to remove endotoxin and bacteria from the bloodstream, due an acquired immunodeficiency state, is observed in these patients[29]. However, the majority of cirrhotics with HCV infection have reasonably stable hepatic function after a successful treatment of a decompensated event and, therefore, might be suitable candidates for antiviral therapy. It seems conceivable that tolerance of antiviral therapy in this particular setting of patients might be extremely poor due to their advanced age, as adherence to combination therapy is negatively influenced by increasing patient's age[30]. In addition, impaired age-related cardiovascular and pulmonary functions may reduce tolerance of ribavirin-induced anemia, while impaired renal function by increasing blood levels of ribavirin (primarily cleared by kidney) may worsen anemia. Finally, insulin resistance secondary to HCV infection may further impair response to combination therapy[31].

Several reports on antiviral therapy in HCV-infected cirrhotic patients who sustained one or more episodes of liver decompensation are available in the literature[32-36] (Table 1): The results indicate that these patients might tolerate current antiviral regimens. However, unstandardized dosages of antiviral drugs that have been administered for a variable and different treatment length may have underestimated and differentiated outcomes and virologic response rates in previous reports. After reviewing existing experience, the International Liver Transplantation Society Expert

Table 2 Suggested guidelines for the use of interferon-based therapy in patients with cirrhosis (International liver transplantation society expert panel, 2003)

Consider treatment	CTP score	MELD score
Strongly consider	⩽ 7	⩽ 18
Possibly consider	8-11	18-25
No, avoid treatment	> 11	> 25

Panel issued guidelines, in 2003, for the use of interferon-based therapy in patients with cirrhosis, strongly considering therapy in those with a CTP score ⩽ 7, and possibly in those with a score of 8 to 11 (Table 2). In the low-accelerating dosage regimen, starting doses were 1.5 MU thrice weekly for conventional interferon, 0.5 μg/kg per week for Peg-IFN alpha 2b, or 90 μg/wk for Peg-IFN alpha 2a, given alone or in combination with a ribavirin dose of 400 mg/dL[32]. Dose adjustments for each of the two drugs were made every 2 wk as tolerated to achieve optimal effective doses. Using an initially low, accelerating regimen of non-pegylated interferon plus ribavirin, 39% of 102 patients experienced clearance of HCV-RNA on treatment, and 21% attained an SVR, 11% with genotype 1, and 50% with genotypes 2 and 3[36]. Moreover, of patients with SVR, none relapsed after liver transplantation[37]. By including Peg-IFNs, further improvement in efficacy can be expected. In our initial investigation, Peg-IFN alpha 2b (1.0 μg/kg) plus standard dose of ribavirin were administered for a short treatment duration (24 wk for all genotypes) to decompensated cirrhotic patients[33]: The overall SVR rate attained with this suboptimal regimen of therapy amounted to 19.7% (13 of 66 treated cases), higher efficacy (43.5% of SVR) being found in genotype 2 and 3 infection than in genotype 1 and 4 (7%). Therapy was tolerated by patients, at a remarkable exception of individuals with very advanced liver disease (CTP score > 10) who experienced severe life-threatening side effects.

Based on our initial investigation, we went further to determine whether currently recommended dosages of Peg-IFN and ribavirin for the standard length of treatment could be safely tolerated in decompensated cirrhotic patients. An ongoing protocol has been set up at our institution where all cirrhotic patients with a CTP score ⩽ 9 and a decompensated event that abated with common management are offered therapy with Peg-IFN alpha-2b (1.5 μg/kg) and ribavirin (800-1000 mg for genotypes 2 and 3, and 1000-1200 mg for genotypes 1 and 4) for the recommended treatment duration (48 and 24 wk for genotype 1 and non-1, respectively) (submitted, unpublished data). In this program, at the end of the 24 wk follow-up period off therapy, 35% of our end-staged cirrhotics cleared the HCV infection, 16% of patients with genotype 1 and 4, and 59% of patients with genotype 2 and 3. Almost 60% of patients tolerated full dosage and duration of treatment, whilst 18 (19.1%) patients discontinued treatment and among these 4 developed severe infections.

All previous reports have outlighted the feasibility of antiviral treatment of patients with a decompensated cirrhosis, allowed to further refine selection of these patients (treatment unsafe for CTP ⩾ 10), and established the relative safety of current schedules of treatment, providing that administration of cytokines could maintain safe levels of hemoglobin (> 10 g/dL) and of neutrophils (> 750/dL). Scant data are available on the impact of therapy on "long term" disease progression, avoidance of transplantation, and improvement of life expectancy. These hard clinical end points are particularly applicable to patients with advanced disease, as liver function is more likely to deteriorate within a few years in these subjects. Achieving HCV clearance has been clearly correlated with improved liver function, as apparent from significant reductions in CTP and MELD scores after treatment[33]. A standardized mortality rate analysis reported a lower liver-related mortality among cirrhotics with SVR (0.6: CI: 0.0-3.1) than in untreated patients[26]. Further benefits after HCV eradication and partial recovery of liver metabolic activity were no more allograft failure secondary to recurrence of viral infection[34], and eventually long term removal of those patients who cleared HCV-RNA from the waiting list for liver transplant. We have reported a significant improvement in overall and event-free survivals as well as in clinical status and laboratory profile of patients who eventually cleared HCV after treatment with Peg-IFN alpha-2b and ribavirin administered for 24 wk. During the follow-up, the total number of decompensated events was significantly higher in controls and non-responders as compared with patients who achieved a SVR.

CONCLUSION

The main goals of treating cirrhotic patients are to attain SVR, halt disease progression, and prevent re-infection of the liver graft. Antiviral therapy for patients with chronic hepatitis C and an advanced stage of compensated or decompensated liver cirrhosis, is evolving: If left untreated, cirrhosis due to chronic HCV infection, is associated with decreased survival, whereas current data from existing trials suggest a reduction in the complication for those with an SVR. As in patients with milder liver disease, standard schedules of treatment may be efficacious particularly for those harboring HCV genotype 2 and 3 infection, in which HCV-RNA is rendered negative during treatment in more than half of individuals. Conversely, the risk-benefit ratio of treating patients with genotype 1 infection remains to be defined. Liberal use of hematopoietic cytokines will either enable the recipient to tolerate full dosage and course of treatment and prevent development of treatment-associated infections. Therapy is inadvisable for individuals with poor hepatic reserve. Although response rates appear to be lower in cirrhotic patients with and without complications of liver disease, successful antiviral therapy is potentially lifesaving.

REFERENCES

1 **Fattovich G**, Giustina G, Degos F, Diodati G, Tremolada F, Nevens F, Almasio P, Solinas A, Brouwer JT, Thomas H,

Realdi G, Corrocher R, Schalm SW. Effectiveness of interferon alfa on incidence of hepatocellular carcinoma and decompensation in cirrhosis type C. European Concerted Action on Viral Hepatitis (EUROHEP). *J Hepatol* 1997; **27**: 201-205
2 **Davis G**L, Albright JE, Cook SF, Rosenberg DM. Projecting future complications of chronic hepatitis C in the United States. *Liver Transpl* 2003; **9**: 331-338
3 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
4 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
5 **Di Marco V**, Almasio PL, Ferraro D, Calvaruso V, Alaimo G, Peralta S, Di Stefano R, Craxi A. Peg-interferon alone or combined with ribavirin in HCV cirrhosis with portal hypertension: a randomized controlled trial. *J Hepatol* 2007; **47**: 484-491
6 **Yoshida H**, Arakawa Y, Sata M, Nishiguchi S, Yano M, Fujiyama S, Yamada G, Yokosuka O, Shiratori Y, Omata M. Interferon therapy prolonged life expectancy among chronic hepatitis C patients. *Gastroenterology* 2002; **123**: 483-491
7 **Peck-Radosavljevic M**, Wichlas M, Homoncik-Kraml M, Kreil A, Hofer H, Jessner W, Gangl A, Ferenci P. Rapid suppression of hematopoiesis by standard or pegylated interferon-alpha. *Gastroenterology* 2002; **123**: 141-151
8 **Soza A**, Everhart JE, Ghany MG, Doo E, Heller T, Promrat K, Park Y, Liang TJ, Hoofnagle JH. Neutropenia during combination therapy of interferon alfa and ribavirin for chronic hepatitis C. *Hepatology* 2002; **36**: 1273-1279
9 **Almasio PL**, Venezia G, Craxi A. The impact of antiviral therapy on the course of chronic HCV infection. A systematic review. *Panminerva Med* 2003; **45**: 175-182
10 **Juarez-Navarro A**, Vera-de-Leon L, Navarro JM, Chirino-Sprung R, Diaz-Hernandez M, Casillas-Davila L, Dehesa-Violante M. Incidence and severity of infections according to the development of neutropenia during combined therapy with pegylated interferon-alpha2a plus ribavirin in chronic hepatitis C infection. *Methods Find Exp Clin Pharmacol* 2005; **27**: 317-322
11 **Manfredi R**, Sabbatani S. Multiple, repeated filgrastim treatment cycles to recover severe, recurring pegylated interferon-related neutropenia. *Hepatogastroenterology* 2007; **54**: 4
12 **Sharvadze L**, Gochitashvili N, Tophuria A, Bolokadze N, Tsertsvadze T. IFN/RBV treatment induced neutropenia and its correction with neupogen in patients with hepatitis C. *Georgian Med News* 2007; 52-55
13 **Sulkowski MS**, Wasserman R, Brooks L, Ball L, Gish R. Changes in haemoglobin during interferon alpha-2b plus ribavirin combination therapy for chronic hepatitis C virus infection. *J Viral Hepat* 2004; **11**: 243-250
14 **Davis GL**, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645-652
15 **McHutchison JG**, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, Dienstag J, Lee WM, Mak C, Garaud JJ, Albrecht JK. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061-1069
16 **Hadziyannis SJ**, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355
17 **Spivak JL**. The blood in systemic disorders. *Lancet* 2000; **355**: 1707-1712
18 **Barosi G**. Inadequate erythropoietin response to anemia: definition and clinical relevance. *Ann Hematol* 1994; **68**: 215-223
19 **McHutchison JG**, Manns MP, Brown RS Jr, Reddy KR, Shiffman ML, Wong JB. Strategies for managing anemia in hepatitis C patients undergoing antiviral therapy. *Am J Gastroenterol* 2007; **102**: 880-889
20 **Afdhal NH**, Dieterich DT, Pockros PJ, Schiff ER, Shiffman ML, Sulkowski MS, Wright T, Younossi Z, Goon BL, Tang KL, Bowers PJ. Epoetin alfa maintains ribavirin dose in HCV-infected patients: a prospective, double-blind, randomized controlled study. *Gastroenterology* 2004; **126**: 1302-1311
21 **Pol S**, Carnot F, Nalpas B, Lagneau JL, Fontaine H, Serpaggi J, Serfaty L, Bedossa P, Brechot C. Reversibility of hepatitis C virus-related cirrhosis. *Hum Pathol* 2004; **35**: 107-112
22 **Shiratori Y**, Imazeki F, Moriyama M, Yano M, Arakawa Y, Yokosuka O, Kuroki T, Nishiguchi S, Sata M, Yamada G, Fujiyama S, Yoshida H, Omata M. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann Intern Med* 2000; **132**: 517-524
23 **Dufour JF**, DeLellis R, Kaplan MM. Regression of hepatic fibrosis in hepatitis C with long-term interferon treatment. *Dig Dis Sci* 1998; **43**: 2573-2576
24 **Poynard T**, McHutchison J, Manns M, Trepo C, Lindsay K, Goodman Z, Ling MH, Albrecht J. Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. *Gastroenterology* 2002; **122**: 1303-1313
25 **Benvegnu L**, Chemello L, Noventa F, Fattovich G, Pontisso P, Alberti A. Retrospective analysis of the effect of interferon therapy on the clinical outcome of patients with viral cirrhosis. *Cancer* 1998; **83**: 901-909
26 **Yoshida H**, Arakawa Y, Sata M, Nishiguchi S, Yano M, Fujiyama S, Yamada G, Yokosuka O, Shiratori Y, Omata M. Interferon therapy prolonged life expectancy among chronic hepatitis C patients. *Gastroenterology* 2002; **123**: 483-491
27 **Heathcote EJ**, Shiffman ML, Cooksley WG, Dusheiko GM, Lee SS, Balart L, Reindollar R, Reddy RK, Wright TL, Lin A, Hoffman J, De Pamphilis J. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. *N Engl J Med* 2000; **343**: 1673-1680
28 **Bruno S**, Stroffolini T, Colombo M, Bollani S, Benvegnu L, Mazzella G, Ascione A, Santantonio T, Piccinino F, Andreone P, Mangia A, Gaeta GB, Persico M, Fagiuoli S, Almasio PL. Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. *Hepatology* 2007; **45**: 579-587
29 **Jacob AI**, Goldberg PK, Bloom N, Degenshein GA, Kozinn PJ. Endotoxin and bacteria in portal blood. *Gastroenterology* 1977; **72**: 1268-1270
30 **Iwasaki Y**, Ikeda H, Araki Y, Osawa T, Kita K, Ando M, Shimoe T, Takaguchi K, Hashimoto N, Kobatake T, Tomita M, Kawaguchi M, Kobashi H, Sakaguchi K, Shiratori Y. Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. *Hepatology* 2006; **43**: 54-63
31 **Romero-Gomez M**, Del Mar Viloria M, Andrade RJ, Salmeron J, Diago M, Fernandez-Rodriguez CM, Corpas R, Cruz M, Grande L, Vazquez L, Munoz-De-Rueda P, Lopez-Serrano P, Gila A, Gutierrez ML, Perez C, Ruiz-Extremera A, Suarez E, Castillo J. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology* 2005; **128**: 636-641
32 **Everson GT**, Trotter J, Forman L, Kugelmas M, Halprin A, Fey B, Ray C. Treatment of advanced hepatitis C with a low accelerating dosage regimen of antiviral therapy. *Hepatology* 2005; **42**: 255-262
33 **Iacobellis A**, Siciliano M, Perri F, Annicchiarico BE, Leandro G, Caruso N, Accadia L, Bombardieri G, Andriulli A.

Peginterferon alfa-2b and ribavirin in patients with hepatitis C virus and decompensated cirrhosis: a controlled study. *J Hepatol* 2007; **46**: 206-212

34 **Forns X**, Garcia-Retortillo M, Serrano T, Feliu A, Suarez F, de la Mata M, Garcia-Valdecasas JC, Navasa M, Rimola A, Rodes J. Antiviral therapy of patients with decompensated cirrhosis to prevent recurrence of hepatitis C after liver transplantation. *J Hepatol* 2003; **39**: 389-396

35 **Thomas RM**, Brems JJ, Guzman-Hartman G, Yong S, Cavaliere P, Van Thiel DH. Infection with chronic hepatitis C virus and liver transplantation: a role for interferon therapy before transplantation. *Liver Transpl* 2003; **9**: 905-915

36 **Crippin JS**, McCashland T, Terrault N, Sheiner P, Charlton MR. A pilot study of the tolerability and efficacy of antiviral therapy in hepatitis C virus-infected patients awaiting liver transplantation. *Liver Transpl* 2002; **8**: 350-355

37 **Everson GT**. Treatment of patients with hepatitis C virus on the waiting list. *Liver Transpl* 2003; **9**: S90-S94

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Adiponectin deficiency enhances colorectal carcinogenesis and liver tumor formation induced by azoxymethane in mice

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Abstract

AIM: To investigate the causal relationship between hypoadiponectinemia and colorectal carcinogenesis in *in vivo* experimental model, and to determine the contribution of adiponectin deficiency to colorectal cancer development and proliferation.

METHODS: We examined the influence of adiponectin deficiency on colorectal carcinogenesis induced by the administration of azoxymethane (AOM) (7.5 mg/kg, intraperitoneal injection once a week for 8 wk), by using adiponectin-knockout (KO) mice.

RESULTS: At 53 wk after the first AOM treatment, KO mice developed larger and histologically more progressive colorectal tumors with greater frequency compared with wild-type (WT) mice, although the tumor incidence was not different between WT and KO mice. KO mice showed increased cell proliferation of colorectal tumor cells, which correlated with the expression levels of cyclooxygenase-2 (COX-2) in the colorectal tumors. In addition, KO mice showed higher incidence and frequency of liver tumors after AOM treatment. Thirteen percent of WT mice developed liver tumors, and these WT mice had only a single tumor. In contrast, 50% of KO mice developed liver tumors, and 58% of these KO mice had multiple tumors.

CONCLUSION: Adiponectin deficiency enhances colorectal carcinogenesis and liver tumor formation induced by AOM in mice. This study strongly suggests that hypoadiponectinemia could be involved in the pathogenesis for colorectal cancer and liver tumor in human subjects.

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Key words: Adiponectin; Colorectal carcinogenesis; Azoxymethane; Cell proliferation; Cyclooxygenase-2; Liver tumor formation

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INTRODUCTION

Obesity is a global medical problem because of its association with the development of various disorders such as diabetes, cardiovascular diseases, and several

cancers[1,2]. Recent studies have indicated that obesity is a significant risk factor for colorectal cancer[3], but the relationship between obesity and colorectal carcinogenesis is poorly understood at the molecular level.

Adipose tissue is currently recognized as an important endocrine organ that secretes various bioactive substances, conceptualized as adipokines or adipocytokines[1,2,4,5]. We and others identified adiponectin as an adipose-specific secretory factor[6-8]. Adiponectin exists abundantly in the circulation[9], and its plasma levels are reduced in obesity, type-2 diabetes, and coronary artery disease[9-11]. Recently, adiponectin has attracted much attention because of its potential role in the development and progression of various obesity-related malignancies[12]. Several clinical studies have shown that plasma adiponectin level is inversely associated with the risk of obesity-related cancers, including uterine, breast, gastric, and prostate cancers[13-16]. It has been reported that treatment with recombinant adiponectin significantly suppresses the growth of several types of cancer cells in cultured cells and/or in xenograft models by inhibiting cell proliferation[17-20].

Recent clinical studies have shown that low plasma adiponectin levels is an independent risk factor for colorectal cancer and its precursory adenoma[21-23]. Moreover, colorectal adenomas in patients with low plasma adiponectin levels tend to be larger and histologically more progressive[23]. However, the causal relationship between low plasma adiponectin levels and colorectal carcinogenesis has not been fully elucidated in *in vivo* experimental model.

Azoxymethane (AOM) is a well-characterized colon carcinogen, and AOM-induced colorectal cancer in rodents is similar to human colorectal cancer with respect to morphology, proliferation characteristics and involvement of gene mutation[24,25]. The present study was designed to explore the mechanisms of hypoadiponectinemia and colorectal carcinogenesis. For this purpose, we used AOM to induce colorectal cancer in adiponectin-knockout (KO) mice.

MATERIALS AND METHODS

Mice and experimental procedures

The animal care and use procedures were approved by the Animal Care Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. The generation of KO mice has been described previously[26]. We mated wild-type (WT) littermate mice produced by backcrossing to the C57BL/6J strain for five generations and used their offspring as WT controls in this study. Mice were maintained on a 12-h light/dark cycle with free access to drinking water and a standard diet. We injected 10-wk-old male mice with AOM (Sigma Chemical Co., St. Louis, MO) at a dose of 7.5 mg/kg body weight intraperitoneally once a week for 8 wk, and control mice received equal volume of saline injection (WT + saline, $n = 9$; WT + AOM, $n = 23$; KO + saline, $n = 13$; and KO + AOM, $n = 24$). The mice were sacrificed 53 wk after the first AOM injection, and the colons and small intestines were removed immediately. The harvested specimens were opened longitudinally, and the number and size of tumors were recorded. Using calipers, we measured the length (L), width (W), and depth (D) of each intestinal tumor and calculated the tumor volume using the formula $V = L \times W \times D \times \pi/6$, as described previously[27]. We also noted the development of liver tumors.

Histopathology and immunohistochemistry

Tumors were fixed in 10% buffered formalin, embedded in paraffin blocks, and sectioned at 3.0-μm thickness. Some sections of the colon tumors were subjected to hematoxylin and eosin (HE) staining for histopathology, and others were used for immunohistochemistry. proliferating cell nuclear antigen (PCNA) was visualized by staining with rat anti-mouse PCNA monoclonal antibody (Dakocytomation, Glostrup, Denmark). To determine the PCNA labeling index, we selected five representative PCNA-positive fields in each section, counted more than 200 tumor cells in each field, and then calculated the percentage of PCNA-positive cells. cyclooxygenase-2 (COX-2) was visualized by staining with rabbit anti-mouse COX-2 polyclonal antibody (Cayman, Ann Arbor, MI). We observed immunoreactive COX-2 expression in the periluminal stromal cells and epithelium of the colorectal tumors. Therefore, we evaluated COX-2 expression; both the intensity of immunoreactivity and the percentages of positively-stained areas in relation to the circumference of the tumor. We graded COX-2 expression of each immunostained section on a 0 to 4+ scale; no immunoreactivity (0), weak immunoreactivity and 1% to 25% positive regions (1+), mild immunoreactivity and 26% to 50% positive regions (2+), moderate immunoreactivity and 51% to 75% positive regions (3+), strong immunoreactivity and 76% to 100% positive regions (4+). We regarded a case displaying very weak immunoreactivity, or less than 1% positive regions, as negative. Sections of the liver tumors were subjected to HE staining for histopathology.

Statistical analysis

Results are expressed as mean ± SE. Statistical analyses of data were performed using the Student's t-test, the chi-square test, Fisher's exact probability test, Wilcoxon rank sum test, or the Spearman's rank correlation. Statistical significance was defined as $P < 0.05$.

RESULTS

Enhanced colorectal carcinogenesis induced by AOM in KO mice

We treated WT and KO mice with AOM at a dose of 7.5 mg/kg or with saline vehicle once a week for 8 wk. At 53 wk after the first AOM treatment, the mice were sacrificed to evaluate the development of colorectal tumors. Body weight changes did not differ between WT and KO

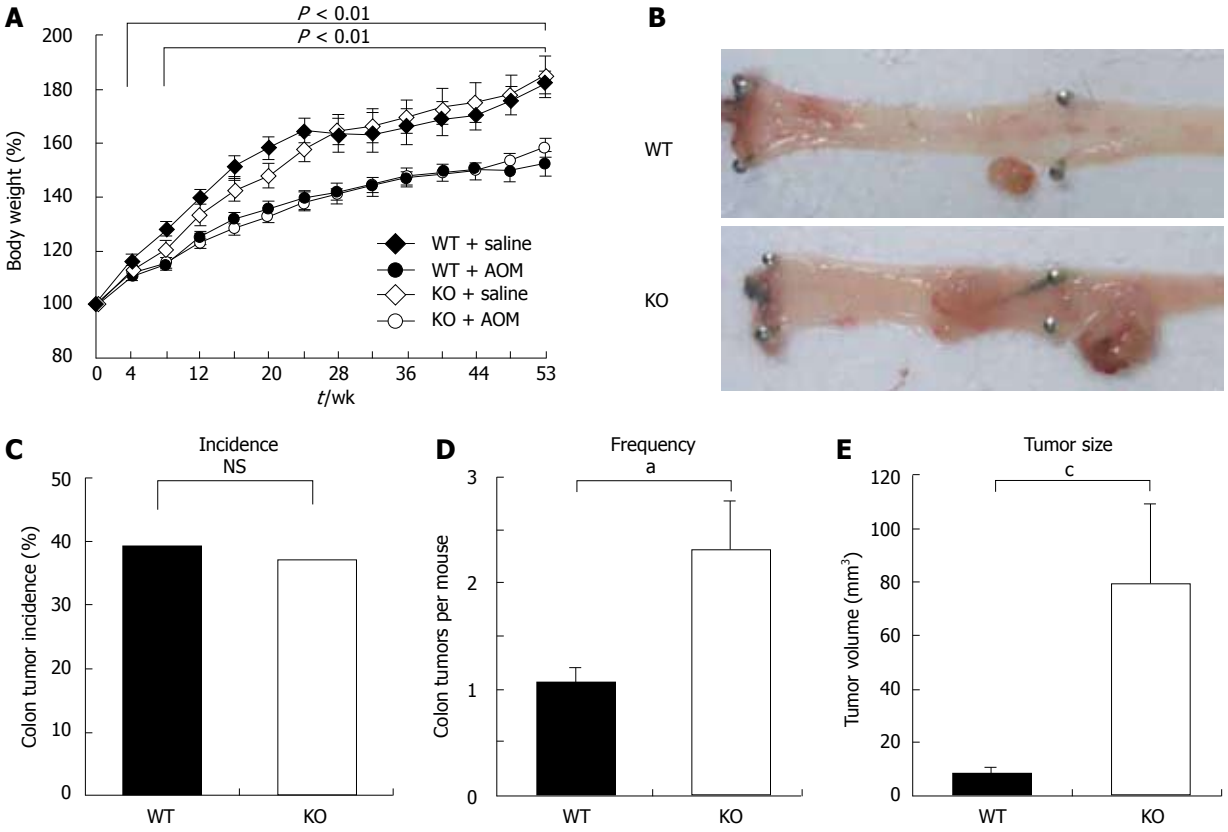

Figure 1 Enhanced AOM-induced colorectal carcinogenesis in KO mice. A: Changes in percentage of body weight. $P < 0.01$, between WT + saline ($n = 9$) and WT + AOM ($n = 23$). $P < 0.001$, between KO + saline ($n = 13$) and KO + AOM ($n = 24$); B: Representative pictures of the colorectal tumors arising in WT and KO mice after AOM treatment; C: Tumor incidence is expressed as the ratio of mice with tumor/total number of mice (NS: Not statistically significant; χ^2 test); D: Tumor frequency. WT, $n = 9$; KO, $n = 9$ ($^aP < 0.05$, Student's *t*-test); E: Tumor size. WT, $n = 9$; KO, $n = 19$ ($^cP < 0.05$, Student's *t*-test). Results are presented as mean ± SE.

mice after AOM treatment, although AOM treatment resulted in a significant reduction in body weight gain of both WT and KO mice (16.4% *vs* 14.4% reduction at sacrifice, Figure 1A). We observed no colorectal tumors in either WT or KO mice treated with only saline (data not shown). Figure 1B shows representative pictures of the colorectal tumors arising in WT and KO mice treated with AOM. There was no difference in the incidence of colorectal tumor formation between WT and KO mice (39.1% *vs* 37.5%, Figure 1C). However, the number of colorectal tumors per mouse was significantly greater in KO mice (2.33 ± 0.47, range: 1-4) than in WT mice (1.11 ± 0.11, 1-2) ($P < 0.05$; Figure 1D), and the average volume of colorectal tumors was markedly larger in KO mice (79.2 ± 29.2 mm^3) than in WT mice (9.02 ± 1.46 mm^3) ($P < 0.05$; Figure 1E). Histological analysis revealed that KO mice developed more progressive tumors in the colon than WT mice (Figure 2). We observed one adenoma (1 of 9, 11%), three carcinomas *in situ*, as assessed by the findings of high-grade dysplasia (3 of 9, 33%) and five adenocarcinomas (5 of 9, 56%) of the colorectal tumors arising in WT mice (Figure 2A). In contrast, of the colorectal tumors in KO mice examined, all tumors (14 of 14) were classified as adenocarcinomas (2 well- and 12 moderately-differentiated adenocarcinomas) (Figure 2B). These findings suggest that adiponectin deficiency enhances AOM-induced colorectal carcinogenesis. One KO mouse developed a small intestinal tumor, classified as a moderately-differentiated adenocarcinoma, in the duodenum (data not shown), whereas none of WT mice developed small intestinal tumors.

High expression of PCNA and COX-2 in colorectal tumors of KO mice

To characterize the influence of adiponectin deficiency on colorectal tumor growth, we evaluated cell proliferation of colorectal tumor cells using the PCNA labeling index assessed by immunohistochemistry. Figure 3A shows representative sections of PCNA-labeled nuclei in colorectal tumors from WT and KO mice. Expression of PCNA was identified by cell nuclei that stained brown to PCNA. For quantitative analysis, we determined the PCNA labeling index by calculating the percentage of PCNA-positive cells. The PCNA-labeling index of KO mice (68.5% ± 2.3%) was greater than that of WT mice (44.8% ± 6.3%) ($P < 0.01$; Figure 3B), suggesting that adiponectin deficiency increased cell proliferation of colorectal tumor cells. To investigate the mechanisms by which adiponectin deficiency increased cell proliferation, we first examined the expression of cyclin-dependent kinase inhibitors (CDKIs), $p21^{CIP}$ and $p27^{KIP}$, in colorectal tumors by immunohistochemistry. However, WT and KO mice showed absence or only focal positivity of both proteins in the colorectal tumors, and there was no significant difference (data not shown).

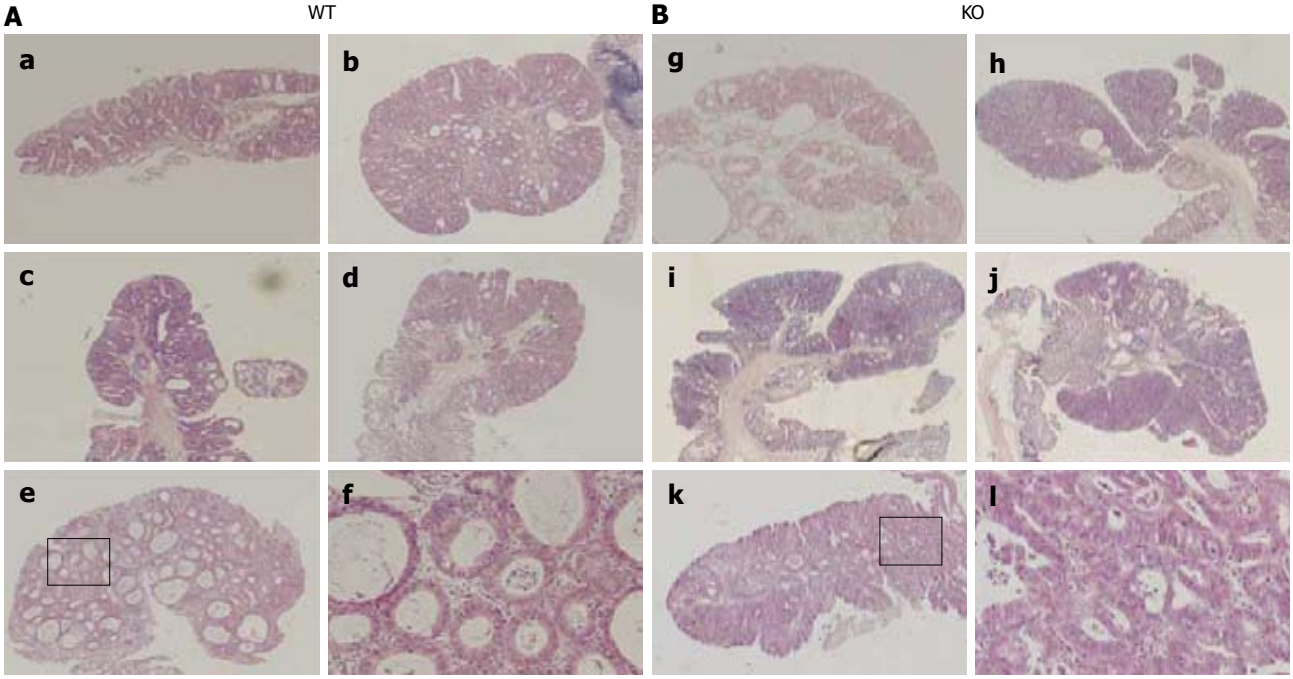

Figure 2 Histological analysis of colorectal tumors induced by AOM. Representative HE-stained sections of colon tumors in WT mice (A): a: Adenoma; b, c, e: Carcinomas *in situ*; d: Adenocarcinoma; f: Boxed area in e is shown at a higher magnification. Representative HE-stained sections of colorectal tumors in KO mice (B). All tumors in KO mice showed features of adenocarcinoma: l: Boxed area in k is shown at a higher magnification. Original magnification: × 20 for b, c, d, h, i, j; × 40 for a, e, g, k; and × 200 for f and l.

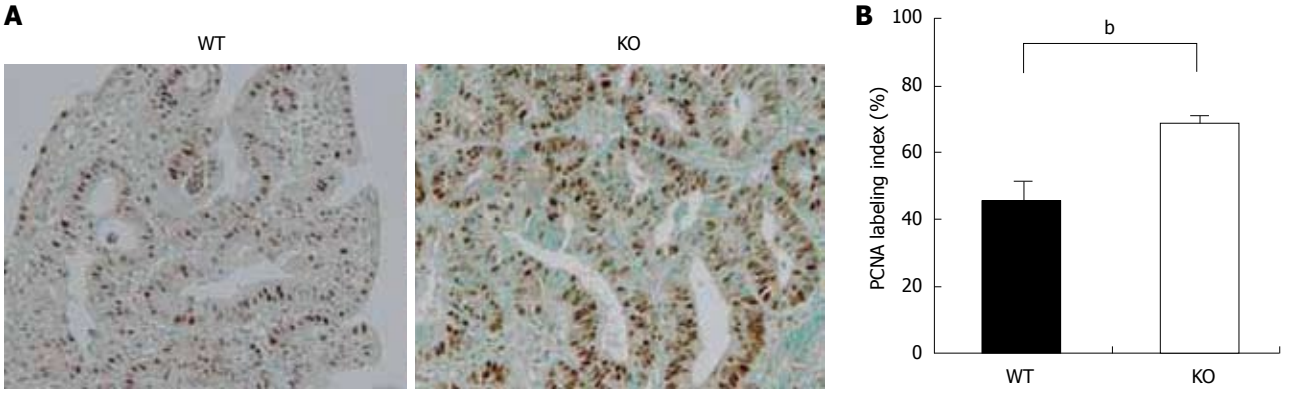

Figure 3 PCNA overexpression in colorectal tumor cells of KO mice. Immunohistochemical staining for PCNA in colorectal tumors using rat anti-mouse PCNA monoclonal antibody. Cells with strongly stained nuclei were considered positive for PCNA. A: Representative section of a colorectal tumor in a WT mouse and a KO mouse (× 200); B: PCNA labeling index in colorectal tumors. WT mice (n = 9), KO mice (n = 11) ($^bP < 0.01$, Student's t-test). Results are mean ± SE.

Next, we examined the expression of COX-2, a well-established pathogenic factor in colorectal carcinogenesis[28,29], by immunohistochemistry. Figure 4 depicts representative sections stained for COX-2. We found no COX-2 positive staining in normal colonic tissues of WT and KO mice. In the colorectal tumor tissues, we observed immunoreactive COX-2 expression in the epithelium and periluminal stromal cells, predominantly in the myofibroblasts. We detected very weak COX-2 staining in 22% (2 of 9) and only local COX-2 staining in 56% (5 of 9) of the colorectal tumors in WT mice (Figure 4A), whereas colorectal tumors in KO mice showed higher levels of COX-2 staining (Figure 4B). In 36% (4 of 11) of the colorectal tumors in KO mice, we detected marked COX-2 staining almost all over the surface of the tumor. Statistical analysis revealed that the expression of COX-2 in colorectal tumors of KO mice was higher than in WT mice (Figure 4C, $P < 0.05$; Wilcoxon rank sum test). Moreover, we found a significant correlation ($r = 0.89$, $P < 0.001$; Spearman's rank correlation) between the PCNA-labeling index and COX-2 expression level (Figure 4D). These results suggest that adiponectin deficiency may promote proliferation of colorectal tumor cells, which may be associated with overexpression of COX-2 in the periluminal stromal cells.

Enhanced liver tumor formation induced by AOM in KO mice

AOM is primarily metabolized by the liver and induces DNA damage in both the colon and the liver[30]. While AOM can induce tumor formation in the liver[31,32], tu-

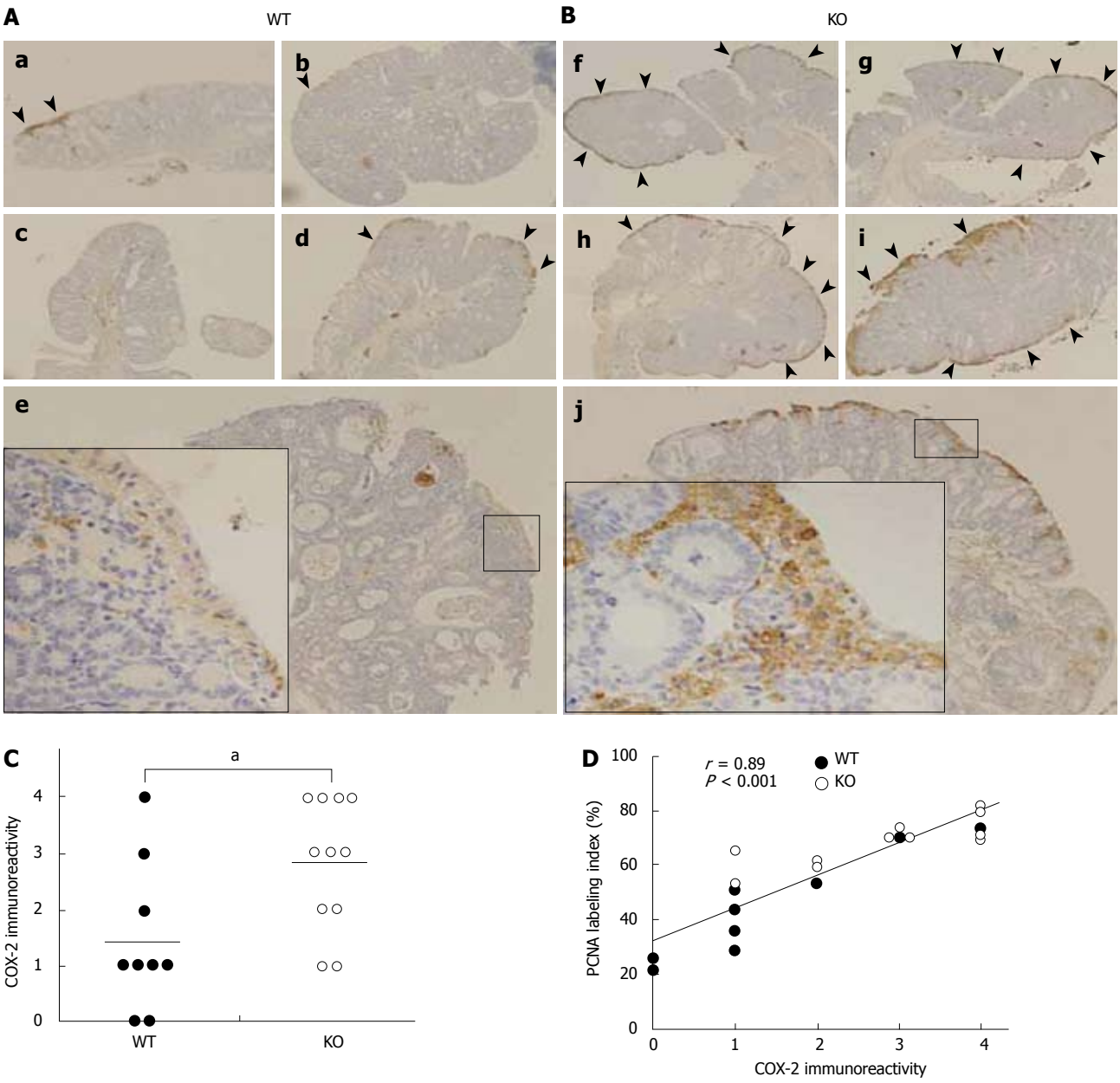

Figure 4 COX-2 overexpression in colorectal tumors of KO mice. Immunohistochemical staining for COX-2 in colorectal tumors using rabbit anti-mouse COX-2 polyclonal antibody. Immunoreactive COX-2 expression is observed in the epithelium and periluminal stromal cells of the tumors (Arrowheads). A: Representative sections of colorectal tumors in WT mice: e: Boxed area is shown at a higher magnification; B: Representative sections of colorectal tumors in KO mice: j: Boxed area is shown at a higher magnification. Original magnification: × 20 for b, c, d, f, g, h; × 40 for a, e, i, j; × 200 for the boxed area in e and j. C: Statistical analysis of immunoreactive COX-2 expression in colorectal tumors. COX-2 expression was higher in colorectal tumors of KO mice (n = 11) compared with WT mice (n = 9; $^{a}P < 0.05$, Wilcoxon rank sum test). Horizontal lines: mean value of COX-2 immunoreactivity in colorectal tumors of WT or KO mice. D: Significant correlation between COX-2 immunoreactivity and PCNA-labeling index in colorectal tumors (r = 0.89, $P < 0.001$, Spearman's rank correlation). WT mice (n = 9), KO mice (n = 11).

mors are reported to be formed almost exclusively in the colon[30]. Consistent with the previous observation[32], the incidence of liver tumor formation after AOM treatment was only 13% (3 of 23) in WT mice (Figure 5A), and all three WT mice had only a single tumor (Figure 5B). In contrast, the incidence of liver tumor formation was 50% (12 of 24) in KO mice (Figure 5A); 5 mice with a single tumor (42%), 2 mice with two tumors (16%), and 5 mice with more than three tumors (42%) (Figure 5B). Figure 5C and D show representative HE-stained sections of the liver tumors in KO mice. The majority of the liver tumors were identified as hepatocellular neoplastic nodules (Figure 5C), and one was a hepatocellular carcinoma (Figure 5D). We also observed that WT mice developed 2 hepatocellular neoplastic nodules and one hepatocellular carcinoma (data not shown). These results suggest that adiponectin deficiency may promote AOM-induced liver tumor formation.

DISCUSSION

Recent epidemiological studies have shown an inverse association between plasma adiponectin levels and the risk of colorectal cancer and its precursor adenoma[21-23]. In the present study, KO mice developed larger and more advanced colorectal tumors compared with WT

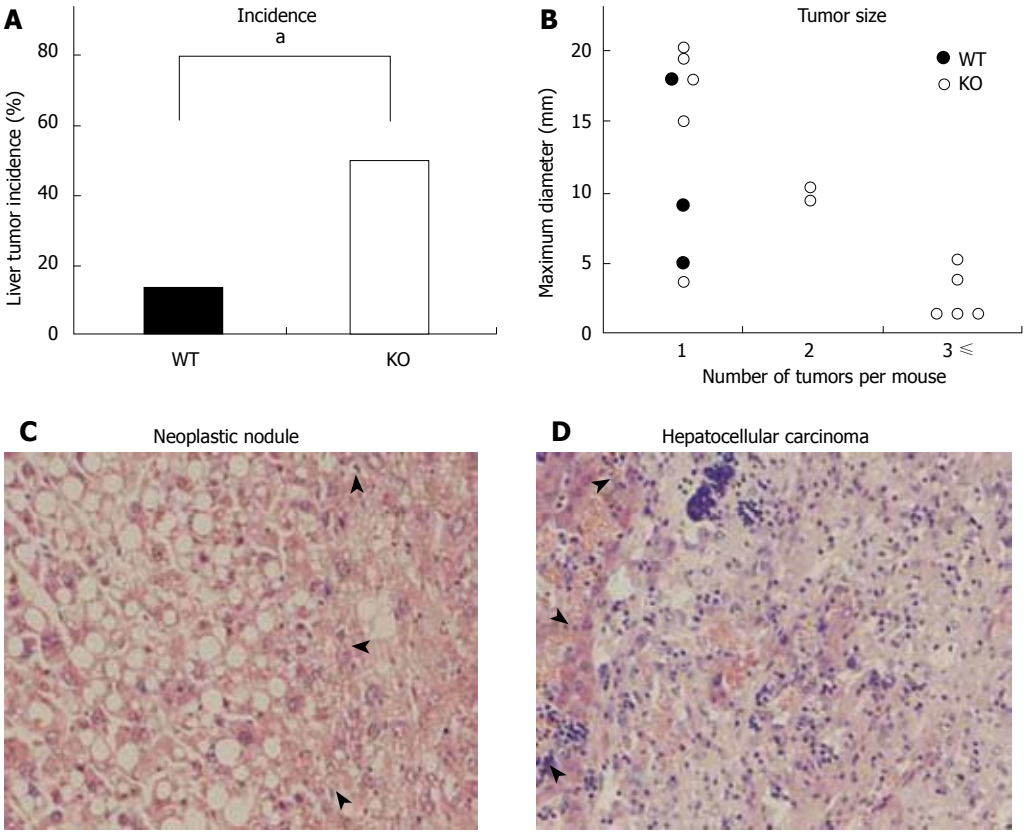

Figure 5 Enhanced AOM-induced liver tumor formations in KO mice. A: Tumor incidence is expressed as the ratio of mice with tumor/total number of mice ($^aP < 0.05$; Fisher's exact probability test); B: Tumor size is evaluated by its maximum diameter. Mice were divided into three groups according to the number of tumors per mouse. Maximum diameter of the tumors is plotted individually in each group. WT mice ($n = 3$), KO mice ($n = 12$). C and D: Representative HE-stained sections liver tumors in KO mice (× 200). Arrowheads indicate liver tumors. C: Neoplastic nodule; D: Hepatocellular carcinoma.

mice, although there was no difference in the tumor incidence between WT and KO mice. These findings suggest that adiponectin deficiency may influence colorectal tumor growth. Indeed, we found that cell proliferation of the colorectal tumor cells was significantly increased in KO mice compared with WT mice, as evaluated by the PCNA labeling index. The anti-proliferative effect of adiponectin on several types of cancer cells has been well documented in cultured cells and/or xenograft models[17-20]. Here we examined whether adiponectin deficiency directly influences colorectal tumor cells. AMP-activated protein kinase (AMPK), which is traditionally thought of as a regulator of cellular energy balance[33], has been reported to suppress cell proliferation in a variety of cell types including several cancer cells[34,35]. These effects of AMPK may be mediated partly through the cell cycle regulation by augmenting the p53-p21 axis[34,35]. Since adiponectin is known to stimulate AMPK[36], the molecular pathways regulated by AMPK could be the potential mechanisms through which adiponectin regulates carcinogenesis[12]. However, immunohistochemical analysis of the CDKIs such as $p21^{CIP}$ and $p27^{KIP}$, as downstream targets of AMPK, showed a negative or only focal immunopositivity of these CDKIs in colorectal tumors of both WT and KO mice, and there was no significant difference between the two strains (data not shown). Further studies are required to elucidate the involvement of this AMPK-CDKIs pathway in the anti-proliferative effect of endogenous adiponectin on colorectal tumors.

Next, to investigate other mechanisms by which adiponectin deficiency promoted colorectal tumor growth in KO mice, we determined the levels of COX-2 expression in the colorectal tumor tissues by immunohistochemistry. COX-2, the inducible enzyme involved in prostaglandins production, plays a crucial role in colorectal carcinogenesis[28,29]. It has been shown that inactivation of COX-2, by disruption of the COX-2 gene or the use of selective COX-2 inhibitors, markedly reduces intestinal tumor formation in the *Apc*$^{\Delta 716}$ mice[29]. Treatment with selective COX-2 inhibitors also suppressed AOM-induced colorectal carcinogenesis in rodents[37]. Moreover, clinical trials of selective COX-2 inhibitors reported a successful reduction in intestinal tumors of patients with familial adenomatous polyposis[38] and sporadic colorectal adenomas[39,40]. Here, we found that adiponectin deficiency enhanced COX-2 expression in the epithelium and periluminal stromal cells of the colorectal tumors, predominantly in the myofibroblasts. We also found that adiponectin deficiency promoted tumor cell proliferation in proportion to COX-2 expression levels. Intestinal myofibroblasts, a family of α-smooth muscle actin-positive fibroblast-like cells, play a pivotal role in the carcinogenic process[41]. The tumor cell-derived cytokines such as transforming growth factor-β provoke the transdifferentiation of fibroblasts

into myofibroblasts, which promote adjacent tumor cell proliferation through paracrine secretion of various mediators including cytokines, chemokines, growth factors, and extracellular matrix molecules[41]. Moreover, it has been shown that COX-2-expressing myofibroblasts synthesize and release prostaglandin E_2 into the tumor microenvironment, which is reported to promote epithelial cell proliferation[42]. Based on these reports, the influence of adiponectin deficiency on cell proliferation of colorectal tumor cells observed in this study could be explained, at least in part, by COX-2 overexpression in the stromal myofibroblasts.

KO mice treated with AOM had greater incidence and frequency of liver tumors, compared with WT mice. AOM is metabolically activated in the liver mainly by cytochrome P450 2E1 (CYP2E1)[43], and can induce tumor formation also in the liver[31]. The increased hepatic CYP2E1 level is considered as one possible mechanism that ethanol treatment may enhance DNA adduct formation by AOM metabolites and may potentiate dysplasia of the liver[44]. In a mouse model of nonalcoholic steatohepatitis induced by choline-deficient *L*-amino acid-defined diet, KO mice showed increased expression of hepatic CYP2E1, which might lead to the progression of liver tumor formation through the enhancement of oxidative stress[45]. On the basis of these reports, adiponectin deficiency might increase hepatic CYP2E1 activity, due to AOM exposure and, therefore, contribute to enhanced liver tumor formation. Further studies are required to clarify these points.

In conclusion, adiponectin deficiency in mice was directly associated with enhanced colorectal carcinogenesis and liver tumor formation induced by AOM. This is the first evidence that adiponectin deficiency a more caused severer and increased frequent carcinogenesis in *in vivo* model. Our results strongly suggest that hypoadiponectinemia could be involved in the pathogenesis for colorectal cancer and liver tumor in human subjects.

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COMMENTS

Background

Adiponectin has attracted much attention because of its potential role in the development and progression of obesity-related malignancies. It has been reported that hypoadiponectinemia is an independent risk factor for colorectal cancer and its precursory adenoma.

Research frontiers

The anti-proliferative effect of adiponectin on several types of cancer cells has been well documented in cultured cells and/or xenograft models. The molecular pathways regulated by AMP-activated protein kinase (AMPK) have been reported to be the potential mechanisms through which adiponectin regulate carcinogenesis.

Innovations and breakthroughs

This study represented the causal relationship between hypoadiponectinemia and colorectal carcinogenesis in an *in vivo* model. Adiponectin deficient (KO) mice developed more advanced colorectal tumors, and showed enhanced liver tumor formation after azoxymethane (AOM) treatment.

Applications

This study demonstrated overexpression of cyclooxygenase-2 (COX-2) in the stromal myofiroblasts of the colorectal tumors in knockout (KO) mice. COX-2 overexpression might be a potential mechanism through which adiponectin deficiency was involved in the colorectal carcinogenesis.

Terminology

Adiponectin is one of the major adipocytokines, and its plasma levels are reduced in obesity, type-2 diabetes, and coronary artery disease.

Peer review

The present paper shows highest growth and progression of AOM-induced colon cancer in KO mice, with respect to wild type (WT) control mice. The results are of certain interest. Nevertheless, some points need to be further clarified and considered in depth to increase the interest of the paper.

REFERENCES

1 **Friedman JM**. Obesity in the new millennium. *Nature* 2000; **404**: 632-634
2 **Kopelman PG**. Obesity as a medical problem. *Nature* 2000; **404**: 635-643
3 **Giovannucci E**, Michaud D. The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. *Gastroenterology* 2007; **132**: 2208-2225
4 **Spiegelman BM**, Flier JS. Obesity and the regulation of energy balance. *Cell* 2001; **104**: 531-543
5 **Shimomura I**, Funahashi T, Takahashi M, Maeda K, Kotani K, Nakamura T, Yamashita S, Miura M, Fukuda Y, Takemura K, Tokunaga K, Matsuzawa Y. Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat Med* 1996; **2**: 800-803
6 **Scherer PE**, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995; **270**: 26746-26749
7 **Maeda K**, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem Biophys Res Commun* 1996; **221**: 286-289
8 **Hu E**, Liang P, Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 1996; **271**: 10697-10703
9 **Arita Y**, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999; **257**: 79-83
10 **Hotta K**, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1595-1599
11 **Ouchi N**, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 1999; **100**: 2473-2476
12 **Kelesidis I**, Kelesidis T, Mantzoros CS. Adiponectin and cancer: a systematic review. *Br J Cancer* 2006; **94**: 1221-1225
13 **Dal Maso L**, Augustin LS, Karalis A, Talamini R, Franceschi S, Trichopoulos D, Mantzoros CS, La Vecchia C. Circulating adiponectin and endometrial cancer risk. *J Clin Endocrinol Metab* 2004; **89**: 1160-1163
14 **Mantzoros C**, Petridou E, Dessypris N, Chavelas C, Dalamaga M, Alexe DM, Papadiamantis Y, Markopoulos C, Spanos E, Chrousos G, Trichopoulos D. Adiponectin and breast cancer risk. *J Clin Endocrinol Metab* 2004; **89**: 1102-1107

15 **Ishikawa M**, Kitayama J, Kazama S, Hiramatsu T, Hatano K, Nagawa H. Plasma adiponectin and gastric cancer. *Clin Cancer Res* 2005; **11**: 466-472

16 **Goktas S**, Yilmaz MI, Caglar K, Sonmez A, Kilic S, Bedir S. Prostate cancer and adiponectin. *Urology* 2005; **65**: 1168-1172

17 **Bråkenhielm E**, Veitonmäki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, Funahashi T, Cao Y. Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci USA* 2004; **101**: 2476-2481

18 **Bub JD**, Miyazaki T, Iwamoto Y. Adiponectin as a growth inhibitor in prostate cancer cells. *Biochem Biophys Res Commun* 2006; **340**: 1158-1166

19 **Wang Y**, Lam JB, Lam KS, Liu J, Lam MC, Hoo RL, Wu D, Cooper GJ, Xu A. Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. *Cancer Res* 2006; **66**: 11462-11470

20 **Ishikawa M**, Kitayama J, Yamauchi T, Kadowaki T, Maki T, Miyato H, Yamashita H, Nagawa H. Adiponectin inhibits the growth and peritoneal metastasis of gastric cancer through its specific membrane receptors AdipoR1 and AdipoR2. *Cancer Sci* 2007; **98**: 1120-1127

21 **Wei EK**, Giovannucci E, Fuchs CS, Willett WC, Mantzoros CS. Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. *J Natl Cancer Inst* 2005; **97**: 1688-1694

22 **Ferroni P**, Palmirotta R, Spila A, Martini F, Raparelli V, Fossile E, Mariotti S, Del Monte G, Buonomo O, Roselli M, Guadagni F. Prognostic significance of adiponectin levels in non-metastatic colorectal cancer. *Anticancer Res* 2007; **27**: 483-489

23 **Otake S**, Takeda H, Suzuki Y, Fukui T, Watanabe S, Ishihama K, Saito T, Togashi H, Nakamura T, Matsuzawa Y, Kawata S. Association of visceral fat accumulation and plasma adiponectin with colorectal adenoma: evidence for participation of insulin resistance. *Clin Cancer Res* 2005; **11**: 3642-3646

24 **Shamsuddin AK**, Trump BF. Colon epithelium. II. In vivo studies of colon carcinogenesis. Light microscopic, histochemical, and ultrastructural studies of histogenesis of azoxymethane-induced colon carcinomas in Fischer 344 rats. *J Natl Cancer Inst* 1981; **66**: 389-401

25 **Shivapurkar N**, Tang Z, Ferreira A, Nasim S, Garett C, Alabaster O. Sequential analysis of K-ras mutations in aberrant crypt foci and colonic tumors induced by azoxymethane in Fischer-344 rats on high-risk diet. *Carcinogenesis* 1994; **15**: 775-778

26 **Maeda N**, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 2002; **8**: 731-737

27 **Rao CV**, Rivenson A, Katiwalla M, Kelloff GJ, Reddy BS. Chemopreventive effect of oltipraz during different stages of experimental colon carcinogenesis induced by azoxymethane in male F344 rats. *Cancer Res* 1993; **53**: 2502-2506

28 **Kargman SL**, O'Neill GP, Vickers PJ, Evans JF, Mancini JA, Jothy S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res* 1995; **55**: 2556-2559

29 **Oshima M**, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996; **87**: 803-809

30 **Papanikolaou A**, Shank RC, Delker DA, Povey A, Cooper DP, Rosenberg DW. Initial levels of azoxymethane-induced DNA methyl adducts are not predictive of tumor susceptibility in inbred mice. *Toxicol Appl Pharmacol* 1998; **150**: 196-203

31 **Ward JM**, Yamamoto RS, Brown CA. Pathology of intestinal neoplasms and other lesions in rats exposed to azoxymethane. *J Natl Cancer Inst* 1973; **51**: 1029-1039

32 **Nozaki T**, Fujihara H, Watanabe M, Tsutsumi M, Nakamoto K, Kusuoka O, Kamada N, Suzuki H, Nakagama H, Sugimura T, Masutani M. Parp-1 deficiency implicated in colon and liver tumorigenesis induced by azoxymethane. *Cancer Sci* 2003; **94**: 497-500

33 **Hardie DG**, Scott JW, Pan DA, Hudson ER. Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett* 2003; **546**: 113-120

34 **Imamura K**, Ogura T, Kishimoto A, Kaminishi M, Esumi H. Cell cycle regulation via p53 phosphorylation by a 5'-AMP activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside, in a human hepatocellular carcinoma cell line. *Biochem Biophys Res Commun* 2001; **287**: 562-567

35 **Motoshima H**, Goldstein BJ, Igata M, Araki E. AMPK and cell proliferation--AMPK as a therapeutic target for atherosclerosis and cancer. *J Physiol* 2006; **574**: 63-71

36 **Yamauchi T**, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002; **8**: 1288-1295

37 **Kawamori T**, Rao CV, Seibert K, Reddy BS. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res* 1998; **58**: 409-412

38 **Steinbach G**, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000; **342**: 1946-1952

39 **Bertagnolli MM**, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Wittes J, Corle D, Hess TM, Woloj GM, Boisserie F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB, Hawk ET. Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med* 2006; **355**: 873-884

40 **Arber N**, Eagle CJ, Spicak J, Racz I, Dite P, Hajer J, Zavoral M, Lechuga MJ, Gerletti P, Tang J, Rosenstein RB, Macdonald K, Bhadra P, Fowler R, Wittes J, Zauber AG, Solomon SD, Levin B. Celecoxib for the prevention of colorectal adenomatous polyps. *N Engl J Med* 2006; **355**: 885-895

41 **Powell DW**, Adegboyega PA, Di Mari JF, Mifflin RC. Epithelial cells and their neighbors I. Role of intestinal myofibroblasts in development, repair, and cancer. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G2-G7

42 **Sonoshita M**, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, Oshima M, Taketo MM. Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice. *Nat Med* 2001; **7**: 1048-1051

43 **Sohn OS**, Fiala ES, Requeijo SP, Weisburger JH, Gonzalez FJ. Differential effects of CYP2E1 status on the metabolic activation of the colon carcinogens azoxymethane and methylazoxymethanol. *Cancer Res* 2001; **61**: 8435-8440

44 **Hakkak R**, Korourian S, Ronis MJ, Badger TM. Effects of diet and ethanol treatment on azoxymethane-induced liver and gastrointestinal neoplasia of male rats. *Cancer Lett* 1996; **107**: 257-264

45 **Kamada Y**, Matsumoto H, Tamura S, Fukushima J, Kiso S, Fukui K, Igura T, Maeda N, Kihara S, Funahashi T, Matsuzawa Y, Shimomura I, Hayashi N. Hypoadiponectinemia accelerates hepatic tumor formation in a nonalcoholic steatohepatitis mouse model. *J Hepatol* 2007; **47**: 556-564

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Relation of atrophic gastritis with *Helicobacter pylori*-CagA$^+$ and interleukin-1 gene polymorphisms

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Abstract

AIM: To determine the association of *Helicobacter pylori* (*H pylori*) CagA$^+$ infection and pro-inflammatory polymorphisms of the genes interleukin (IL)-1RN and IL-1B with the risk of gastric atrophy and peptic ulcers in a dyspeptic population in Costa Rica, a country with high incidence and mortality of gastric cancer.

METHODS: Seven biopsy specimens, a fasting blood sample and a questionnaire concerning nutritional and sociodemographic factors were obtained from 501 consecutive patients who had undergone endoscopy for dyspeptic symptoms. A histopathological diagnosis was made. Pepsinogen concentrations were analyzed by enzyme linked immunosorbent assay (ELISA). Infection with *H pylori* CagA$^+$ was determined by serology and polymerase chain reaction (PCR). IL-1B and IL-1RN polymorphisms genotyping was performed by PCR-restriction fragment length polymorphism (PCR-RFLP) and PCR respectively.

RESULTS: In this dyspeptic population, 86% were *H pylori* positive and of these, 67.8% were positive for CagA. Atrophic antral gastritis (AAG) was associated with CagA$^+$ status [odd ratio (OR) = 4.1; $P < 0.000$] and fruit consumption (OR = 0.3; $P < 0.00$). Atrophic body gastritis (ABG) was associated with pepsinogen PGI/PGII < 3.4 (OR = 4.9; $P < 0.04$) and alcohol consumption (OR = 7.3; $P < 0.02$). Duodenal ulcer was associated with CagA$^+$ (OR = 2.9; $P < 0.04$) and smoking (OR = 2.4; $P < 0.04$). PGI < 60 μg/L as well as PGI/PGII < 3.4 were associated with CagA$^+$.

CONCLUSION: In a dyspeptic population in Costa Rica, *H pylori* CagA$^+$ is not associated with ABG, but it is a risk factor for AAG. The pro-inflammatory cytokine polymorphisms IL-1B + 3945 and IL-1RN are not associated with the atrophic lesions of this dyspeptic population.

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Key words: Atrophic gastritis; Pepsinogen; Peptic ulcers; *Helicobacter pylori*-CagA; Interleukins

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INTRODUCTION

Colonization with *Helicobacter pylori* (*H pylori*) is associated with atrophic gastritis, peptic ulcer and distal gastric cancer[1,2]. Nevertheless, many colonized individuals never develop these pathologies. Genetic characteristics of the host and the bacteria as well as environmental factors may be involved in the final clinical outcome[3]. The pathogenicity and virulence of *H pylori* increases when the infecting strain expresses the *cagA* gene that codes for a highly immunogenic protein CagA, which is a marker

for the presence of the *cag* pathogenicity island (PAI)[4,5]. Moreover, the immune response of the host is considered a key event in the pathogenic process that leads to gastric cancer. A number of studies have reported that carriers of certain alleles in genes encoding proinflammatory and anti-inflammatory cytokines exhibit a stronger inflammatory response against *H pylori* and a marked inhibition of acid secretion resulting in an increased risk of gastric cancer and its precursors, atrophic gastritis and intestinal metaplasia[6,7]. However, this association has not been found in all studied populations[8].

The development of atrophic gastritis is central in the multi-step process which leads to gastric cancer, and the risk increases with the severity and physical extension of the atrophic lesion[9,10]. Serum levels of pepsinogen I (PGI) and the ratio of PGI/PGII serum levels decrease significantly with increased extension and severity of atrophic gastritis and the presence of gastric cancer. Therefore, these parameters have been proposed as serological markers for those histopathological changes[11-13]. Costa Rica is one of the countries with the highest incidence and mortality rates of gastric cancer worldwide. The prevalence of *H pylori* associated gastritis is high from an early age[14].

Recently, we found an association between the PGI/PGII ratio and atrophic body gastritis in dyspeptic patients[15]. In the present study, the same population was analyzed for associations of atrophic body and antral gastritis and presence of peptic ulcers with *H pylori* *CagA*$^+$ infection and pro-inflammatory interleukin-1 (*IL-1*) gene polymorphisms.

MATERIALS AND METHODS

Patients

As previously described[15], between January and July, 2000, 800 consecutive patients referred to the endoscopy service at the Calderón Guardia Hospital in San José for dyspeptic symptoms were interviewed. This hospital is a tertiary hospital that, however, also provides gastroenterology service at primary and secondary levels. Patients were excluded if they were under 18 years of age, had not resided in Costa Rica for the previous two years, had received *H pylori* eradication therapy, had taken antibiotics during the 3 mo preceding endoscopy, had taken bismuth compounds at the time of endoscopy, had a history of gastric surgery, suffered from diseases associated with coagulation, or presented digestive bleeding. A total of 501 patients fulfilled the inclusion criteria and signed an informed consent form before 25 mL of fasting blood was obtained. The project had been previously approved by the ethic committees of the University of Costa Rica and the Hospital. A questionnaire with information concerning sociodemographic factors, family history, general health status, consumption of salt, coffee, alcohol, fruit, vegetables, and smoking habits was filled out[15]. Blood pressure and height and weight were recorded to calculate the body mass index.

Endoscopy procedure

Seven biopsies were obtained: Two biopsies from the middle part of the antrum were taken from the major and minor curvatures and two from the gastric body at the anterior and posterior walls of the central body for histopathological diagnosis. One biopsy from the antrum and one from the body were taken for culture and one from the antrum for rapid urease test[15].

Histological diagnosis

Sections of formaline-fixed and paraffin-embedded biopsies, stained with hematoxylin and eosin, were used for the histopathological diagnosis according to the Sydney Classification[16]. All slides were independently read by two specialists in gastric pathology (JA Ramírez in San José, Costa Rica, and A De Mascarel in Bordeaux, France). Cases with discrepant diagnoses were reviewed by both pathologists until a consensus was reached. A patient was considered to have: (1) atrophic body gastritis (ABG), if atrophy or metaplasia was present in any of the biopsies from the body; (2) atrophic antral gastritis (AAG), when atrophy was found in any of the biopsies from the antrum and not in the body; (3) non-atrophic gastritis (NAG), in the case of gastritis without atrophy, and (4) Normal mucosa, when no pathology was found in the antrum or the body and no granulocytic activity or lymphoid follicles were observed. In 22 of the patients, there was no histopathological diagnosis due to insufficient tissue in the biopsies[15].

H pylori status

Infection with *H pylori* was determined by histology, culture and serology. For histological detection, the slides were stained with toluidine blue. One biopsy from the antrum and one from the body were cultured for *H pylori*. Biopsies from the antrum and corpus were ground in brucella broth with an electric homogenizer. The suspensions were plated on two in-house media: A Wilkins Chalgren agar containing 10% human blood and antibiotics and a Columbia blood agar without antibiotics incubated for 10 d in a microaerobic atmosphere at 37℃. Standard identification was performed. Serum antibodies to *H pylori* were measured by an in-house enzyme linked immunosorbent assay (ELISA) developed in our laboratory and based on a modification of a previously described ELISA[17]. The antigen preparation and determination of cutoff points was previously reported[15]. A patient was considered positive for *H pylori* when positive by any of the three methods: culture or histology or serology and negative when none of the methods were positive.

CagA status

The *cagA* status was determined by polymerase chain reaction (PCR) on isolated strains using primers *cag*A_1-A_2 and, if negative, a second set of primers, *cag*A_3-A_4, were used[18]. Serum antibodies to CagA were measured by ELISA as described by Blaser *et al*, 1995[19]. A patient was considered positive for *H pylori* CagA$^+$ infection when positive by PCR and/or serology.

Serum pepsinogen concentrations

The concentrations of PGI and PGII in sera were

measured by an enzyme immunosorbent assay (EIA) (Eiken Chemical Company, Tokyo, Japan) according to the manufacturers' recommendations. The validation of pepsinogen levels for this population was described previously[15]. In order to detect as many patients as possible with ABG, a cut-off point that favored sensitivity was selected. The optimal cut-off point for this population had been set at a PGI/PGII ratio of 3.4, which gave a sensitivity of 91.2% and a specificity of 38.5%[15]. The predictive value for a positive sample was 11.2% and for a negative 98.1%. Pepsinogen levels could not be measured in 51 patients, because insufficient amounts of blood were obtained or because the patients did not accept being bled.

IL-1B and IL-1RN genotyping

IL-1B polymorphism analysis was performed by PCR-restriction fragment length polymorphism (PCR-RFLP). 100 ng of genomic DNA were amplified with the primers and PCR conditions previously reported[20]. The PCR products for IL-1B + 3954 were digested with the restriction enzyme *Taq*I and the allele designation for this polymorphism was the same as previously reported[21].

Genomic DNA (100 ng) was amplified using the same primers and PCR conditions as previously reported for *IL-1RN*[20]. For statistical analysis purposes and because of the low frequency of alleles 3, 4 and 5, this polymorphism was treated as biallelic by dividing alleles into short and long categories, in which the short allele has two repeats (allele 2) and long allele has more than two repeats (allele L). For 110 (22%) patients genotyping was not done because of insufficient leukocyte samples or problems in the isolation or analysis of the DNA.

Statistical analysis

χ^2 statistics were used for comparing genotype frequencies among the groups studied and to assess Hardy-Weinberg equilibrium for each of the loci studied. Allelic frequencies were assessed using Estimating Haplotype Frequencies (EH) (available at ftp://linkage.rockefeller.edu/software/eh/). Politomic logistic regression was used to compare genotypic frequencies among the groups studied here using STATA/SE 8.0 (STATA Corporation, College Station, TX).

A logistic regression model was used to calculate the odd ratios (ORs) for (dependent variable): (1) ABG compared to dyspepsia without atrophic gastritis; (2) AAG compared to dyspepsia without atrophic gastritis; (3) Gastric ulcer compared to all of the others; (4) Duodenal ulcer compared to the rest; (5) Levels of PGI < 60 μg/L compared to levels of PGI > 60 μg/L; and 6) Values of PGI/PGII < 3.4 compared to values of PGI/PGII > 3.4. The systematic independent variables were *H pylori* CagA infection, IL-1B + 3954 allele T carriers, IL-1RN allele 2 carriers, and those that had been associated with the dependent variables in a previous study of the same population: Age, sex, overweight, frequency of alcohol and fruit consumption and the PGI/PGII ratio for atrophic gastritis and age; gender and cigarette smoking for peptic ulcers[15]. The logistic regression analyses were performed including either, only *H pylori* infected individuals or, all of the participants. The association was determined as an OR at a confidence interval of 95%. Statistical significance was set at $P < 0.05$. The software STATA/SE 8.0 (STATA Corporation, College Station, TX) was used for the statistical analysis.

Table 1 Sex, age and pepsinogen levels according to histopathological diagnosis

	Sex (M/F) (*n* = 155/324)	Age (yr), mean (95% CI)	PGI (mg/L), mean (95% CI)	PGI/PGII, mean (95% CI)
Normal	9/12	38 (30.7-45.7)	39.7 (27.9-51.5)	4.3 (3.3-5.4)
NAG	104/225	45 (43.2-46.6)	53.1 (49.4-56.9)	3.3 (3.1-3.6)
AAG	26/67	50 (47.0-52.8)	60.2 (52.9-67.5)	3.3 (2.9-3.7)
ABG	16/20	53 (48.2-57.6)	36.1 (26.6-45.5)[1]	1.9 (1.5-2.4)[1]

[1]$P \leq 0.001$, ABG *vs* the rest.

RESULTS

In this study, 501 patients were included, 338 women with an average age of 46.3 years, and 163 men with an average age of 46 years. The mean age was higher in groups with the most severe pathologies (Table 1).

Pepsinogen

PGI y PGII concentrations could be measured in 450 patients. Serum concentrations of PGI as well as the PGI/PGII ratio were lower in patients with ABG as compared to other dyspeptic patients (Table 1). Dyspeptic patients with a ratio below 3.4 were at increased risk of having ABG compared to those with a ratio higher than 3.4 (Table 2).

H pylori CagA$^+$

The frequency of *H pylori* infection was 86%, out of which 67.8% were CagA$^+$, however, the prevalence varied according to the pathology. Patients with a normal histopathological diagnosis, although few, showed a lower incidence of infection with *H pylori* and *H pylori* CagA$^+$ (15.0% and 5.3%, respectively) as compared to the rest, NAG (88.9% and 55.5%), AAG (93.3% and 81.6%) or ABG (82.9% and 58.8%). The prevalence of infection with *H pylori* and *H pylori* CagA$^+$ for gastric ulcer was 100% and 52.2%, respectively, and for duodenal ulcer 98% and 85%, respectively. Among patients with PGI/PGII < 3.4, the prevalence of infection with *H pylori* was 87.7% and with *H pylori* CagA$^+$ 61.3% while that of patients with ratios > 3.4 were at 82.1% and 50.0%. *H pylori* CagA+ was associated with a PGI/PGII ratio < 3.4 and PGI < 60 μg/L (Table 3).

Polymorphisms: IL-1RN and IL-1B + 3954

Genotyping of the cytokine polymorphisms was successfully performed in 371 patients (74%). Genotypic distributions of IL-1RN and IL-1B + 3954 polymorphisms were not significantly different among the studied groups (Table 4). There was Hardy-Weinberg

Table 2 OR with 95% CI for the association between atrophic gastritis and risk factors in dyspeptic *H pylori*-status$^+$ patients[1]

	Atrophic gastritis in antrum (AAG)			Atrophic gastritis in body (ABG)		
	OR	95% CI	*P*	OR	95% CI	*P*
Age > 50 yr	1.6	0.8-3.0	0.2	2.8	1.0-7.7	0.04
Men	0.6	0.3-1.2	0.2	1.2	0.4-3.5	0.7
Overweight (BMI > 25.9)	0.7	0.3-1.2	0.2	0.8	0.3-2.0	0.6
Fruit consumption						
0-1 times/wk	1.0			1.0		
2-6 times/wk	0.4	0.2-0.8	0.01	1.4	0.4-4.6	0.6
More than 6 times/wk	0.3	0.2-0.7	0.00	0.7	0.2-2.6	0.6
Alcohol consumption						
No consumption	1.0			1.0		
Weekends or more	3.0	0.8-12	0.1	7.3	1.5-35.8	0.02
H pylori						
CagA status$^+$	4.1	1.9-9.0	0.000	1.2	0.4-3.5	0.7
IL-1B + 3954						
T carriers *vs* CC	0.6	0.3-1.2	0.16	0.9	0.3-2.4	0.8
IL-1RN						
2 carriers *vs* LL	1.4	0.8-2.6	0.3	1.3	0.5-3.5	0.6
PGI/PGII ≤ 3.4	1.4	0.7-2.8	0.3	4.9	1.1-22.5	0.04

[1]Logistic regression, AAG and ABG *vs* non-atrophic gastritis.

Table 3 OR with 95% CI for the association between PGI and PGI/PGII and risk factors in dyspeptic *H pylori*-status$^+$ patients[1]

	PGI < 60 μg/L			PGI/PGII < 3.4		
	OR	95% CI	*P*	OR	95% CI	*P*
Age > 50 yr	1.1	0.6-1.8	0.8	1.6	1.0-2.7	0.07
Men	1.6	0.9-2.7	0.09	0.7	0.4-1.2	0.2
Overweight (BMI > 25.9)	1.0	0.6-1.7	0.9	0.6	0.4-1.0	0.04
Fruit consumption						
0-1 times/wk	1.0			1.0		
2-6 times/wk	0.6	0.3-1.2	0.1	1.6	0.9-2.8	0.1
More than 6 times/wk	1.3	0.7-2.4	0.4	1.3	0.7-2.4	0.4
Alcohol consumption						
No consumption	1.0			1.0		
Weekends or more	0.2	0.05-1.1	0.07	1.8	0.5-6.3	0.3
H pylori						
CagA status$^+$	2.5	1.4-4.3	0.002	1.8	1.1-3.0	0.03
IL-1B + 3954						
T carriers *vs* CC	1.2	0.7-1.9	0.5	0.8	0.5-1.3	0.3
IL-1RN						
2 carriers *vs* LL	1.6	1.0-2.7	0.06	0.9	0.6-1.4	0.3

[1]Logistic regression, PGI < 60 μg/L *vs* PGI > 60 μg/L and PGI/PGII < 3.4 *vs* PGI/PGII > 3.4.

equilibrium for the IL-1RN locus in the groups used here as controls (dyspepsia without atrophic gastritis and all of the groups together excluding ABG). However, in the case of the IL-1B + 3954 polymorphism, there was no equilibrium for any of the control groups (data not shown). The frequency of the allele 2 for IL-1RN polymorphism was not significantly different in normal patients, compared to those with some type of gastritis (Table 4).

Logistic regression atrophic gastritis

An increased risk of ABG was observed among patients older than 50 years, those consuming alcohol and those with a PGI/PGII ratio < 3.4. Patients infected with CagA$^+$ strains of *H pylori* and those with lower consumption of fruit were at higher risk of developing AAG, (Table 2). The presence of pro-inflammatory alleles, IL-1B + 3954T and IL-RN*2, did not confer an enhanced risk of any type of atrophy (Table 2).

Table 4 Genotypic and allelic frequencies for the studied groups *n* (%)

	Normal	NAG	AAG	ABG
IL-1RN				
L*L	11/17 (65)	124/253 (49)	33/76 (43)	13/25 (52)
L*2	6/17 (35)	107/253 (42)	31/76 (41)	8/25 (32)
2*2	0/17 (0)	22/253 (9)	12/76 (16)	4/25 (16)
Allelic freq. 2	0.16	0.27	0.29	0.28
IL-1B + 3954				
CC	12/18 (66)	123/250 (49)	46/77 (60)	13/26 (50)
CT	5/18 (28)	121/250 (48)	30/77 (39)	13/26 (50)
TT	1/18 (6)	6/250 (3)	1/77 (1)	0/26 (0)
Allelic freq. T	0.22	0.26	0.20	0.24

Table 5 OR with 95% CI for the association between peptic ulcers and risk factors in dyspeptic *H pylori*-status$^+$ patients[1]

	Gastric ulcer			Duodenal ulcer		
	OR	95% CI	*P*	OR	95% CI	*P*
Age > 50 yr	3.3	1.2-9.1	0.02	0.8	0.4-1.8	0.6
Men	0.3	0.9-1.3	0.1	3.2	1.5-7.2	0.004
Present smoker	1.2	0.3-4.7	0.8	2.4	1.0-5.6	0.04
H pylori						
CagA status$^+$	0.7	0.3-1.8	0.4	2.9	1.0-7.9	0.04
IL-1B + 3954						
T carriers *vs* CC	1.2	0.5-3.2	0.7	0.9	0.4-1.9	0.8
IL-1RN						
2 carriers *vs* LL	1.4	0.5-3.6	0.5	0.7	0.3-1.4	0.3

[1]Logistic regression, Peptic ulcer *vs* no peptic ulcer.

Peptic ulcers

Duodenal ulcer was more frequent than gastric ulcer (52/24) in both genders. However, it was twice as common in males and the average age of patients with duodenal ulcer (average 46; 95% IC, 42.5-50.2) was lower than that of those with gastric ulcer (average 58; 95% CI, 52.2-63.6). Males, smokers and patients infected with *H pylori* CagA$^+$ were at increased risk of duodenal ulcer (Table 5).

DISCUSSION

While NAG associated with *H pylori* is more prevalent in industrialized countries, atrophic gastritis is more common in the developing world. The reason for this is probably a combination of effects caused by *H pylori* and other infections in early childhood, as well as other environmental factors and genetic composition[4,22]. Atrophic gastritis initiates in the antrum and may extend upwards towards the gastric body, often resulting in more severe atrophic gastritis with increased age[9]. It has been reported in sev-

eral studies that infection with *H pylori* CagA$^+$ increases the risk of atrophic gastritis and gastric cancer[4,23]. In the present study, infection with *H pylori* CagA$^+$ is associated with atrophic gastritis of the antrum, but not of the body, results which are in accordance with those reported by Oksanen *et al*, 2000[24]. Indeed, in a stomach affected by ABG, the microenvironment is more hostile for the survival and growth of *H pylori* promoting spontaneous eradication of the bacteria and a progressive decline in concentrations of serum antibodies to *H pylori*[25-27]. Atrophic gastritis has been considered as a consequence of prolonged gastritis caused by *H pylori* and the association between *H pylori infection* and gastric cancer appears stronger when the infection is recorded several years before the onset of cancer[28]. Therefore, in our study, the association of *H pylori* CagA$^+$ with ABG may be underestimated, taking into account that patients belong to a population in which *H pylori*-associated gastritis is prevalent from childhood[14]. *H pylori* and its virulence factor CagA may be involved in processes during the early stages of inflammation of the antrum that lead to extension of atrophic gastritis towards the gastric body[29].

In concordance with other studies, infection with *H pylori*-CagA$^+$ is associated with low PGI/PGII ratios[24]. Several studies have reported an association of low PGI concentrations and low PGI/PGII ratios with precancerous lesions as well as with gastric cancer[30-32]. In the study population, PGI/PGII values below 3.4 were previously shown to be associated with ABG but not with AAG[15]. The studies performed in Costa Rica to date indicate low specificities of the pepsinogen test for the detection of gastric cancer and ABG (64% and 38.5%, respectively). Nevertheless, its high negative predictive values (99.5% and 98%) could make the PG test useful to eliminate, from subsequent steps of screening programs, persons that are unlikely to develop gastric cancer[15,33].

Our results fail to demonstrate an association between AAG, ABG and pro-inflammatory polymorphisms IL-1B + 3945 or IL-1RN. The control group consisted of dyspeptic patients of which a large majority suffered from some inflammatory condition (NAG). In a previous study, performed by our group, among participants in a program at the Center for Early Detection of Gastric Cancer in Costa Rica, it was found that IL-1B + 3945T and IL-1RN*2 were associated with an increased risk of gastric cancer: OR = 3.7 $P = 0.007$ and OR = 2.9; $P = 0.03$ respectively[34]. In this study, the control groups were normal individuals as judged by X-ray (double contrast, gastric study).

The combination of IL-1B + 3954 and IL-1RN proinflammatory genotypes, or that of proinflammatory alleles and CagA, did not reveal any association with any particular pathology when included in the logistic regression analysis (data not shown).

Recent reports concerning the association of polymorphisms enhancing the expression of the gene IL-1B with atrophic gastritis, gastric cancer and peptic ulcer, diverge in their results[35]. Several studies link these polymorphisms to a reduction in acid secretion, gastric inflammation, atrophy, and gastric cancer[6,20,36-38], whereas others do not[39-42]. These contradictory results may be related to the characteristics of the studied population, the methodology used, regional differences with regard to the frequencies of pro- or anti-inflammatory polymorphisms, the prevalence and time of infection with *H pylori* and the characteristics of the infecting strain, as well as diet and other environmental factors that interact with and influence the final result of the pathological process initiated by the infection[43-45].

The results of the present study show that consumption of fruit, even in modest quantities, diminishes the risk of AAG, but has no effect on more advanced atrophy, ABG. It has been suggested that the protective properties of fruit are due to its content of antioxidants that would counteract the oxidative stress induced by *H pylori*[46].

The data presented here are in accordance with reports from other laboratories stating that CagA$^+$ individuals are at higher risk of developing duodenal ulcers[47,48]. Although patients with duodenal ulcer have reduced risk of gastric cancer, *H pylori* predisposes to both conditions[49]. These different consequences may be a result of the host response to the infection. It has been reported that an increase in acidity predisposes for duodenal ulceration whereas hypochlorhydria is associated with a higher risk of developing gastric cancer[50].

In summary, this study does not permit the conclusion that there exists no association between infection with CagA$^+$ *H pylori* and ABG, because *H pylori* is spontaneously eradicated with the severity and extension of atrophic lesions. Future epidemiologic investigations should eliminate the inherent biases of sub-detection of *H pylori* and specially *H pylori* CagA$^+$. The true relationships between *H pylori* and diseases of the upper gastrointestinal tract are highly complex. It is of crucial importance to identify the factors that direct the initial inflammatory reactions to different gastric pathologies and decipher the mechanisms involved those processes, not only for the understanding of carcinogenesis, but also for the prevention and detection of diseases related to *H pylori*.

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COMMENTS

Background

Costa Rica has a high incidence of and mortality from gastric cancer. Infection with *H pylori* and proinflammatory interleukin (IL) gene polymorphisms have been associated with precancerous gastric lesions and gastric adenocarcinoma. Atrophic gastritis may lead to the development of cancer. This study addresses the association of atrophic antral and body gastritis with infection with

H pylori-CagA+ strains and IL-1 gene polymorphisms in a dyspeptic population in an area at high risk of gastric cancer in Costa Rica.

Research frontiers

In a dyspeptic population in Costa Rica, *H pylori* CagA$^+$ is not associated with atrophic body gastritis, but it is a risk factor for atrophic antral gastritis (AAG). The pro-inflammatory cytokine polymorphisms IL-1B + 3945 and IL-1RN are not associated with the atrophic lesions.

Innovations and breakthroughs

Studies of various populations have indicated an association between gastric cancer and *H pylori*-CagA as well as proinflammatory gene variants. The etiology of gastric cancer varies among populations. The present study shows that *H pylori*-CagA is related to active AAG, but not to more advanced atrophy of the gastric body in a high-risk population. It is speculated that the bacteria may have disappeared at this stage. Furthermore, the study demonstrates no relation between two proinflammatory IL-1 gene polymorphisms and atrophy of the stomach in dyspeptic patients.

Applications

In developing countries with high incidence of *H pylori* infection and dyspepsia it is not feasible to screen large populations with endoscopy. The information generated here may be used to create a battery of markers to detect risk factors for gastric cancer from a blood sample. Screening and intervention may then be concentrated on people with a high-risk profile.

Peer review

The report is interesting. It could be useful in clinical settings. It indicated that in a dyspeptic population in Costa Rica, *H pylori* CagA$^+$ is a risk factor for AAG.

REFERENCES

1 **Graham DY**. Helicobacter pylori infection in the pathogenesis of duodenal ulcer and gastric cancer: a model. *Gastroenterology* 1997; **113**: 1983-1991

2 **Stemmermann GN**, Fenoglio-Preiser C. Gastric carcinoma distal to the cardia: a review of the epidemiological pathology of the precusors to a preventable cancer. *Pathology* 2002; **34**: 494-503

3 **Matysiak-Budnik T**, Megraud F. Helicobacter pylori infection and gastric cancer. *Eur J Cancer* 2006; **42**: 708-716

4 **Huang JQ**, Zheng GF, Sumanac K, Irvine EJ, Hunt RH. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. *Gastroenterology* 2003; **125**: 1636-1644

5 **Wu AH**, Crabtree JE, Bernstein L, Hawtin P, Cockburn M, Tseng CC, Forman D. Role of Helicobacter pylori CagA+ strains and risk of adenocarcinoma of the stomach and esophagus. *Int J Cancer* 2003; **103**: 815-821

6 **Machado JC**, Figueiredo C, Canedo P, Pharoah P, Carvalho R, Nabais S, Castro Alves C, Campos ML, Van Doorn LJ, Caldas C, Seruca R, Carneiro F, Sobrinho-Simoes M. A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. *Gastroenterology* 2003; **125**: 364-371

7 **Zabaleta J**, Camargo MC, Piazuelo MB, Fontham E, Schneider BG, Sicinschi LA, Ferrante W, Balart L, Correa P, Ochoa AC. Association of interleukin-1beta gene polymorphisms with precancerous gastric lesions in African Americans and Caucasians. *Am J Gastroenterol* 2006; **101**: 163-171

8 **Furuta T**, Shirai N, Sugimoto M. Controversy in polymorphisms of interleukin-1beta in gastric cancer risks. *J Gastroenterol* 2004; **39**: 501-503

9 **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992; **52**: 6735-6740

10 **Sipponen P**, Kekki M, Haapakoski J, Ihamaki T, Siurala M. Gastric cancer risk in chronic atrophic gastritis: statistical calculations of cross-sectional data. *Int J Cancer* 1985; **35**: 173-177

11 **Sasazuki S**, Inoue M, Iwasaki M, Otani T, Yamamoto S, Ikeda S, Hanaoka T, Tsugane S. Effect of Helicobacter pylori infection combined with CagA and pepsinogen status on gastric cancer development among Japanese men and women: a nested case-control study. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1341-1347

12 **Dinis-Ribeiro M**, Yamaki G, Miki K, Costa-Pereira A, Matsukawa M, Kurihara M. Meta-analysis on the validity of pepsinogen test for gastric carcinoma, dysplasia or chronic atrophic gastritis screening. *J Med Screen* 2004; **11**: 141-147

13 **Oishi Y**, Kiyohara Y, Kubo M, Tanaka K, Tanizaki Y, Ninomiya T, Doi Y, Shikata K, Yonemoto K, Shirota T, Matsumoto T, Iida M. The serum pepsinogen test as a predictor of gastric cancer: the Hisayama study. *Am J Epidemiol* 2006; **163**: 629-637

14 **Sierra R**, Munoz N, Pena AS, Biemond I, van Duijn W, Lamers CB, Teuchmann S, Hernandez S, Correa P. Antibodies to Helicobacter pylori and pepsinogen levels in children from Costa Rica: comparison of two areas with different risks for stomach cancer. *Cancer Epidemiol Biomarkers Prev* 1992; **1**: 449-454

15 **Sierra R**, Une C, Ramirez V, Gonzalez MI, Ramirez JA, de Mascarel A, Barahona R, Salas-Aguilar R, Paez R, Avendano G, Avalos A, Broutet N, Megraud F. Association of serum pepsinogen with atrophic body gastritis in Costa Rica. *Clin Exp Med* 2006; **6**: 72-78

16 **Price AB**. The Sydney System: histological division. *J Gastroenterol Hepatol* 1991; **6**: 209-222

17 **Perez-Perez GI**, Dworkin BM, Chodos JE, Blaser MJ. Campylobacter pylori antibodies in humans. *Ann Intern Med* 1988; **109**: 11-17

18 **Labigne A**, Lamouliatte H, Birac C, Sedallian A, Megraud F. Distribution of the cagA gene among Helicobacter pylori strains associated with peptic ulcer. *Am J Gastroenterol* 1994; **89**: 1326

19 **Blaser MJ**, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, Stemmermann GN, Nomura A. Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* 1995; **55**: 2111-2115

20 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402

21 **Zeng ZR**, Hu PJ, Hu S, Pang RP, Chen MH, Ng M, Sung JJ. Association of interleukin 1B gene polymorphism and gastric cancers in high and low prevalence regions in China. *Gut* 2003; **52**: 1684-1689

22 **Smith VC**, Genta RM. Role of Helicobacter pylori gastritis in gastric atrophy, intestinal metaplasia, and gastric neoplasia. *Microsc Res Tech* 2000; **48**: 313-320

23 **Kuipers EJ**, Perez-Perez GI, Meuwissen SG, Blaser MJ. Helicobacter pylori and atrophic gastritis: importance of the cagA status. *J Natl Cancer Inst* 1995; **87**: 1777-1780

24 **Oksanen A**, Sipponen P, Karttunen R, Miettinen A, Veijola L, Sarna S, Rautelin H. Atrophic gastritis and Helicobacter pylori infection in outpatients referred for gastroscopy. *Gut* 2000; **46**: 460-463

25 **Karnes WE Jr**, Samloff IM, Siurala M, Kekki M, Sipponen P, Kim SW, Walsh JH. Positive serum antibody and negative tissue staining for Helicobacter pylori in subjects with atrophic body gastritis. *Gastroenterology* 1991; **101**: 167-174

26 **Annibale B**, Negrini R, Caruana P, Lahner E, Grossi C, Bordi C, Delle Fave G. Two-thirds of atrophic body gastritis patients have evidence of Helicobacter pylori infection. *Helicobacter* 2001; **6**: 225-233

27 **Kokkola A**, Kosunen TU, Puolakkainen P, Sipponen P, Harkonen M, Laxen F, Virtamo J, Haapiainen R, Rautelin H. Spontaneous disappearance of Helicobacter pylori antibodies in patients with advanced atrophic corpus gastritis. *APMIS* 2003; **111**: 619-624

28 **Ekstrom AM**, Held M, Hansson LE, Engstrand L, Nyren O. Helicobacter pylori in gastric cancer established by CagA

immunoblot as a marker of past infection. *Gastroenterology* 2001; **121**: 784-791
29 **Kuipers EJ**, Sipponen P. Helicobacter pylori eradication for the prevention of gastric cancer. *Helicobacter* 2006; **11** Suppl 1: 52-57
30 **Varis K**, Sipponen P, Laxen F, Samloff IM, Huttunen JK, Taylor PR, Heinonen OP, Albanes D, Sande N, Virtamo J, Harkonen M. Implications of serum pepsinogen I in early endoscopic diagnosis of gastric cancer and dysplasia. Helsinki Gastritis Study Group. *Scand J Gastroenterol* 2000; **35**: 950-956
31 **Bodger K**, Wyatt JI, Heatley RV. Variation in serum pepsinogens with severity and topography of Helicobacter pylori-associated chronic gastritis in dyspeptic patients referred for endoscopy. *Helicobacter* 2001; **6**: 216-224
32 **Sipponen P**, Ranta P, Helske T, Kaariainen I, Maki T, Linnala A, Suovaniemi O, Alanko A, Harkonen M. Serum levels of amidated gastrin-17 and pepsinogen I in atrophic gastritis: an observational case-control study. *Scand J Gastroenterol* 2002; **37**: 785-791
33 Sierra R, Mena F, Ramirez V, Mendez E, Salazar M, Une C, Kajiwara T. Pepsinógenos séricos para detectar cáncer gástrico en Costa Rica. *Acta Bioquím Clin Latinoam* 2003; **37**: 357-362
34 **Alpizar-Alpizar W**, Perez-Perez GI, Une C, Cuenca P, Sierra R. Association of interleukin-1B and interleukin-1RN polymorphisms with gastric cancer in a high-risk population of Costa Rica. *Clin Exp Med* 2005; **5**: 169-176
35 **Perez-Perez GI**, Garza-Gonzalez E, Portal C, Olivares AZ. Role of cytokine polymorphisms in the risk of distal gastric cancer development. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 1869-1873
36 **Furuta T**, El-Omar EM, Xiao F, Shirai N, Takashima M, Sugimura H. Interleukin 1beta polymorphisms increase risk of hypochlorhydria and atrophic gastritis and reduce risk of duodenal ulcer recurrence in Japan. *Gastroenterology* 2002; **123**: 92-105
37 **Figueiredo C**, Machado JC, Pharoah P, Seruca R, Sousa S, Carvalho R, Capelinha AF, Quint W, Caldas C, van Doorn LJ, Carneiro F, Sobrinho-Simoes M. Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *J Natl Cancer Inst* 2002; **94**: 1680-1687
38 **Camargo MC**, Mera R, Correa P, Peek RM Jr, Fontham ET, Goodman KJ, Piazuelo MB, Sicinschi L, Zabaleta J, Schneider BG. Interleukin-1beta and interleukin-1 receptor antagonist gene polymorphisms and gastric cancer: a meta-analysis. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1674-1687
39 **Kato S**, Onda M, Yamada S, Matsuda N, Tokunaga A, Matsukura N. Association of the interleukin-1 beta genetic polymorphism and gastric cancer risk in Japanese. *J Gastroenterol* 2001; **36**: 696-699
40 **Lee SG**, Kim B, Choi W, Lee I, Choi J, Song K. Lack of association between pro-inflammatory genotypes of the interleukin-1 (IL-1B -31 C/+ and IL-1RN *2/*2) and gastric cancer/duodenal ulcer in Korean population. *Cytokine* 2003; **21**: 167-171
41 **Gatti LL**, Burbano RR, de Assumpcao PP, Smith Mde A, Payao SL. Interleukin-1beta polymorphisms, Helicobacter pylori infection in individuals from Northern Brazil with gastric adenocarcinoma. *Clin Exp Med* 2004; **4**: 93-98
42 **Kamangar F**, Abnet CC, Hutchinson AA, Newschaffer CJ, Helzlsouer K, Shugart YY, Pietinen P, Dawsey SM, Albanes D, Virtamo J, Taylor PR. Polymorphisms in inflammation-related genes and risk of gastric cancer (Finland). *Cancer Causes Control* 2006; **17**: 117-125
43 **Hatakeyama M**. Oncogenic mechanisms of the Helicobacter pylori CagA protein. *Nat Rev Cancer* 2004; **4**: 688-694
44 **Rebbeck TR**, Sankar P. Ethnicity, ancestry, and race in molecular epidemiologic research. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2467-2471
45 **Zabaleta J**, Schneider BG, Ryckman K, Hooper PF, Camargo MC, Piazuelo MB, Sierra RA, Fontham ET, Correa P, Williams SM, Ochoa AC. Ethnic differences in cytokine gene polymorphisms: potential implications for cancer development. *Cancer Immunol Immunother* 2008; **57**: 107-114
46 **Kobayashi M**, Tsubono Y, Sasazuki S, Sasaki S, Tsugane S. Vegetables, fruit and risk of gastric cancer in Japan: a 10-year follow-up of the JPHC Study Cohort I. *Int J Cancer* 2002; **102**: 39-44
47 **Nomura AM**, Perez-Perez GI, Lee J, Stemmermann G, Blaser MJ. Relation between Helicobacter pylori cagA status and risk of peptic ulcer disease. *Am J Epidemiol* 2002; **155**: 1054-1059
48 **Atherton JC**. The clinical relevance of strain types of Helicobacter pylori. *Gut* 1997; **40**: 701-703
49 **Hansson LE**, Nyren O, Hsing AW, Bergstrom R, Josefsson S, Chow WH, Fraumeni JF Jr, Adami HO. The risk of stomach cancer in patients with gastric or duodenal ulcer disease. *N Engl J Med* 1996; **335**: 242-249
50 **Wu MS**, Chen CJ, Lin JT. Host-environment interactions: their impact on progression from gastric inflammation to carcinogenesis and on development of new approaches to prevent and treat gastric cancer. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 1878-1882

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CLINICAL RESEARCH

Fractalkine and TGF-β1 levels reflect the severity of chronic pancreatitis in humans

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Abstract

AIM: To clarify whether serum chemokine and cytokine levels can become useful biological and functional markers to assess the severity of chronic pancreatitis (CP). This study aimed at clarifying whether serum chemokine and cytokine levels can become useful biological and functional markers to assess the severity of CP.

METHODS: Serum monocyte chemoattractant protein-1 (MCP-1), transforming growth factor beta-1 (TGF-β1), and soluble type fractalkine (s-fractalkine) concentrations were examined in patients with CP (n = 109) and healthy controls (n = 116). Severity of disease was classified in patients with CP by a staging system. Relationships between stage-specific various clinical factors and serum MCP-1, TGF-β1, and s-fractalkine levels were investigated. Furthermore, 57 patients with non-alcoholic CP were similarly evaluated in order to exclude influence of alcohol intake.

RESULTS: Patients with CP showed significant higher levels of serum TGF-β1 and s-fractalkine, but not MCP-1, compared to the controls. Serum TGF-β1 in the severe stage and s-fractalkine in the mild and the severe stage of CP significantly increased compared to those of controls. However, it was observed that both TGF-β1 and s-fractalkine levels were affected by alcohol intake. In patients with non-alcoholic CP, serum TGF-β1 showed significant increase in the moderate stage of CP, and serum s-fractalkine revealed significant increase in the early stage of CP.

CONCLUSION: It is suggested that the measurement of serum F-fractalkine is useful to diagnose early-stage CP. Moreover, the combined determination of both, s-fractalkine and TGF-β1, in human sera may be helpful in evaluating the severity status of CP.

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Key words: Chronic pancreatitis; Transforming growth factor beta-1; Soluble fractalkine; Monocyte chemoattractant protein-1

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INTRODUCTION

Chronic pancreatitis (CP) is a chronic clinical disorder characterized by irreversible damage to the pancreas, the development of histologic evidence of inflammation and fibrosis, and eventually the destruction and permanent loss of exocrine and endocrine tissue[1-4]. Furthermore, patients with long-standing CP are also at a markedly increased risk of developing pancreatic cancer[3]. Unfortunately, simple, indirect measurements of decreased pancreatic function do not show abnormality until CP is considerably advanced. Imaging or function tests may not reveal early CP, and the results of these tests do not necessarily correlate with each other[5-9]. The quest continues for useful biological and functional markers of early-stage CP. Recently, chemokines and

cytokines have been recognized to be important factors in the progression of CP[10,11]. A preliminary study by us in a small number of CP patients showed the possibility that measurement of serum soluble type fractalkine (s-fractalkine) may be useful for diagnosing early-stage of CP[7]. Thus, in the current study, using a large number of CP patients classified by severity with a staging system, we investigated whether chemokine and cytokine levels can become useful biological and functional markers of early-stage CP, focusing particularly on monocyte chemoattractant protein-1 (MCP-1), transforming growth factor beta-1 (TGF-β1), and s-fractalkine, which are supposed to be involved in chronic inflammation.

MATERIALS AND METHODS

Patients

One hundred nine patients with CP (63 males and 46 females; age range, 25-81 years; mean, 56.8 years) who fulfilled clinical diagnostic criteria for CP by the Japan Pancreas Society[12] and 116 healthy controls (69 males and 47 females; age range, 26–93 years; mean, 56.5 years) were selected for this study. Patients and healthy controls with recent inflammatory diseases, e.g. infectious diseases, chronic hepatitis, or substantial alcohol consumption were excluded. The study protocol was approved by the ethics committee at Kyushu University.

CP patients were classified according to a staging system reported previously (Table 1)[12-14]. The staging system comprises 6 parameters: exocrine pancreatic function (scored 0-3), pancreatic imaging tests by endoscopic retrograde cholangiopancreatography (ERCP; scored 0-4), glucose metabolism (scored 0-4), pain (scored 0-4), alcohol intake (scored 0-2), and complications associated with CP (scored 0-2). The CP is then subclassified, according to a total score from these 6 grading factors, into mild (total score, 0-3), moderate (total score, 4-7), and severe (total score, ⩾ 8). The number of patients with mild, moderate, or severe disease was 36 (33.0%), 49 (45.0%), and 24 (22.0%), respectively (Table 2). We analyzed whether each grading factors and severity of CP are related with serum MCP-1, TGF-β1 and s-fractalkine.

Table 1 CP staging system (partial modification of reference 12, 16, 17)

Exocrine pancreatic function (score, 0-3)
Each of the following abnormalities is scored as 1: Decreased serum level of pancreatic amylase or trypsin, abnormal bentiromide-para amino benzoic acid (BT-PABA) test result, and low fecal chymotrypsin.
0 No abnormalities in the above examination
1 Total score 1 in the above examinations
2 Total score 2 in the above examinations
3 Total score 3 in the above examinations
Pancreatography by endoscopic retrograde cholangiopancreatography (ERCP; score, 0-4)
0 Normal
1 Slightly abnormal (simple dilatation of the main pancreatic duct or localized and irregular dilation of two to three branches)
2 Mild pancreatitis (diffuse and irregular mild dilatation of the main pancreatic duct or branches, or moderate dilatation of the main pancreatic duct localized in the body and/or tail of the pancreas)
3 Moderate pancreatitis (diffuse and irregular moderate dilatation of the main pancreatic duct or branches, or advanced dilation of the main pancreatic duct localized in the body and/or tail of the pancreas)
4 Severe pancreatitis (diffuse and irregular advanced dilatation of the main pancreatic duct and branches)
Glucose metabolism (score, 0-4)
0 Normal glucose tolerance (urine glucose negative; postprandial glucose < 160 mg/dL)
1 Slightly impaired glucose tolerance (impaired glucose tolerance after oral glucose loading test; postprandial glucose, > 160 mg/dL, < 200 mg/dL)
2 Mild diabetes mellitus (urine glucose positive after meal; postprandial glucose, > 200 mg/dL; < 300mg/dL; HbA1c < 7%)
3 Moderate diabetes mellitus (postprandial glucose, > 300 mg/dL; HbA1c 7%-11%)
4 Severe diabetes mellitus (HbA1c > 11%, diabetic retinopathy, or diabetic nephropathy)
Pain (evaluated in the previous 1 yr, score 0-4)
0 No or only slight pain (requires no analgesics)
1 Mild pain (occasional pain but requires no analgesics)
2 Moderate pain (frequent pain attacks, often requires analgesics)
3 Severe (always requires analgesics)
4 Most severe (requires frequent injections of analgesics, and, often, hospitalization)
Alcohol intake (score, 0-2)
0 Less than 180 mL sake[1], not every day
1 Less than 540 mL sake, almost every day
2 More than 540 mL sake, almost every day
Complications associated with chronic pancreatitis (score, 0-2)
0 No complications such as pseudocyst and stenosis of the biliary tract
1 Complications that require no treatment
2 Complications that require treatment

Total score	Severity of chronic pancreatitis
0-3	Mild
4-7	Moderate
> 8	Severe

[1]Sake and shochu are typical Japanese alcoholic beverages, with an ethanol content of about 16% and 25%, respectively. One unit of sake, which contains 29 g of ethanol, represents one Japanese drink.

Laboratory methods

Enzyme-linked immunosorbent assays (ELISAs) were used to determine serum MCP-1, TGF-β1, and s-fractalkine concentrations. Samples were examined with commercial kits according to the manufacturers' instructions; human MCP-1, human TGF-β1 (Biosource, Camarillo, CA), and s-fractalkine (R&D systems, Minneapolis, MN). MCP-1 and TGF-β1 assays were run according to the protocol that was recommended in the kit. S-fractalkine was measured according to the protocol mentioned below. S-fractalkine was assessed using the basic components required by the manufacturer, and briefly, for the plate preparation, 4 μg/mL of mouse antibody to human fractalkine was used as the capture antibody with 96 well low-cell-binding EIA plates (Nunc, Roskilde, Denmark) in an overnight incubation at 25℃. Next, the plate was blocked with phosphate buffer saline (PBS) containing 10% fetal bovine serum (Invitrogen, Auckland, New Zealand), biotinylated mouse antibody to human fractalkine (500 μg/mL) was added after serum samples and standard, and were incubated for two hours at 25℃. Recombinant human fractalkine (R&D systems) was used as a standard. After that, the plate was incubated with streptavidin conjugated to horseradish peroxidase (HRP) for 20 min at 25℃ in the

Table 2 Number of each classification in patients with CP

Score	0	1	2	3	4	Total
Pancreatic imaging tests	0	47	33	17	12	109
Exocrine function	31	66	12	0	0	109
Glucose metabolism	63	18	13	4	11	109
Pain	65	35	7	2	0	109
Alcohol intake	57	30	22	N/A	N/A	109
Complications	90	17	2	N/A	N/A	109
Stage of severity	Mild (0-3)	Moderate (4-7)	Severe (> 8)			
(total score)	36	49	24	109		

N/A: Not applicable.

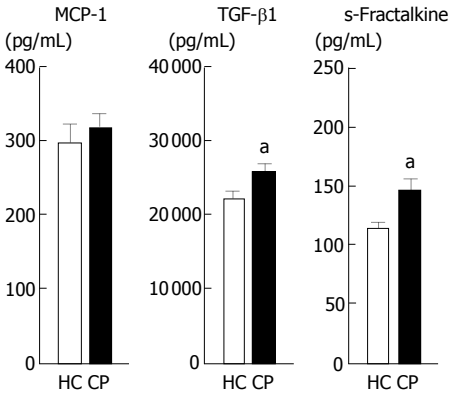

Figure 1 Serum MCP-1, TGF-β1, and s-fractalkine concentrations in patients with CP. Serum MCP-1, TGF-β1, and s-fractalkine concentrations were measured by ELISA for each pancreatic disease. Healthy control (HC, $n = 116$) and chronic pancreatitis (CP, $n = 109$). Bars represent the mean ± SD. [a]$P < 0.05$ *vs* healthy control.

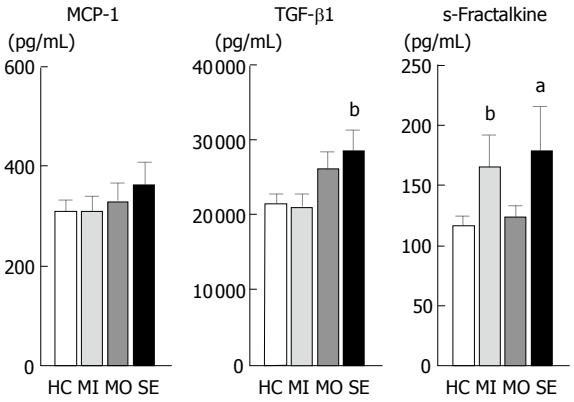

Figure 2 Serum MCP-1, TGF-β1, and s-fractalkine concentrations with regard to severity of CP. Serum MCP-1, TGF-β1, and s-fractalkine concentrations were measured by ELISA for each stage of severity. Healthy control (HC, $n = 116$), mild severity (MI, $n = 36$), moderate severity (MO, $n = 49$), and severe (SE, $n = 24$). Bars represent the mean ± SD. [a]$P < 0.05$ *vs* healthy control, [b]$P < 0.01$ *vs* healthy control.

dark. Finally, antibody complex was detected by base on the formation of the avidin-biotin complex, and the color was developed with tetramethylbenzidine (TMB) solution by incubating for 30 min at 25℃ in the dark. Absorbance was read at 450 nm/570 nm.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney *U* test. *P* values less than 0.05 were considered significant. Pearson's correlation analysis was used to calculate correlations between the data.

RESULTS

Serum MCP-1, TGF-β1, and s-fractalkine concentrations in patients with CP (Figure 1)

Serum MCP-1 levels in patients with CP were not significantly elevated. On the other hand, serum TGF-β1 levels in patients with CP were significantly higher than those of healthy controls ($P = 0.029$). Furthermore, s-fractalkine levels in CP were also significantly increased when compared to those of healthy controls ($P = 0.011$). Thus, we next analyzed whether specific grading factors as pancreatic imaging tests, exocrine function, glucose metabolism, pain, alcohol intake, or complications and severity of CP are related to serum MCP-1, TGF-β1, and s-fractalkine concentrations.

Serum MCP-1, TGF-β1, and s-fractalkine concentrations in relation to the severity stage of CP (Figure 2)

First, we examined the MCP-1, TGF-β1, and s-fractalkine levels in each stage of severity. Serum TGF-β1 levels in the severe stage of CP were significantly higher than in healthy controls ($P = 0.008$). On the contrary, serum s-fractalkine levels in the mild and in the severe stage of CP were significantly elevated compared to healthy controls ($P = 0.004$ and $P = 0.046$, respectively). However, the serum MCP-1 level didn't significantly increase for each stage of severity.

Serum MCP-1, TGF-β1, and s-fractalkine concentrations in relation to imaging test scores in patients with CP (Figure 3)

Serum of MCP-1, TGF-β1, and s-fractalkine levels were analyzed regarding imaging scores. Serum TGF-β1 levels in patients with CP revealed a score of > 3 in pancreatic imaging tests and was found to be significantly enhanced when compared to healthy controls ($P = 0.0001$). On the other hand, serum s-fractalkine levels in patients with a score of 1 and a score of $\geqslant 3$ were significantly elevated when compared to healthy controls ($P = 0.010$ and $P = 0.041$, respectively). However, no relationship was found between serum MCP-1 levels and any level of the imaging tests.

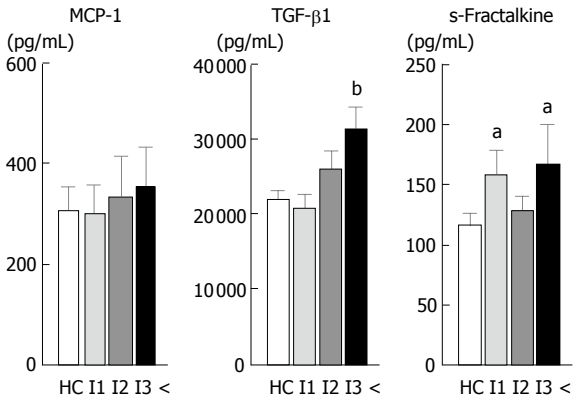

Figure 3 Serum MCP-1, TGF-β1, and s-fractalkine concentrations for each score of imaging tests in CP patients. Serum MCP-1, TGF-β1, and s-fractalkine concentrations were measured by ELISA for each score for pancreatic imaging tests. Healthy control (HC, *n* = 116), score 1 on pancreatic imaging tests (I1, *n* = 47), score 2 on pancreatic imaging tests (I2, *n* = 33) and score ≥ 3 on pancreatic imaging tests (> I3, *n* = 29). Bars represent the mean ± SD. [a]*P* < 0.05 *vs* healthy control, [b]*P* < 0.01 *vs* healthy control.

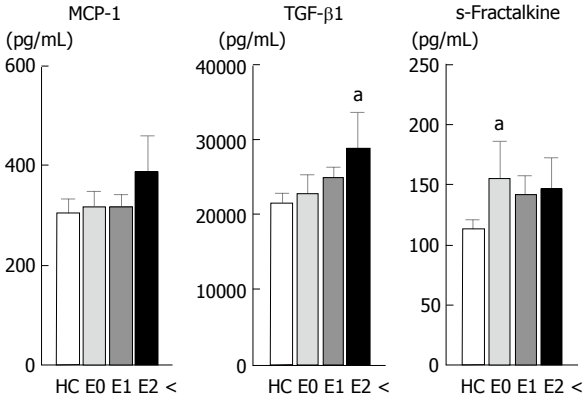

Figure 4 Serum MCP-1, TGF-β1, and s-fractalkine concentrations for each score for the exocrine function tests in CP patients. Serum MCP-1, TGF-β1, and s-fractalkine concentrations were measured by ELISA for each score for the exocrine function. Healthy control (HC, *n* = 116), score 0 for exocrine function (E0, *n* = 31), score 1 for exocrine function (E1, *n* = 66) and score > 2 for exocrine function (> E2, *n* = 12). Bars represent the mean ± SD. [a]*P* < 0.05 *vs* healthy control.

Serum MCP-1, TGF-β1, and s-fractalkine concentrations in relation to exocrine function test scores in patients with CP (Figure 4)

Serum TGF-β1 levels in patients with an exocrine function score ≥ 2 were significantly higher than in healthy controls ($P = 0.042$). In contrast, serum s-fractalkine was significantly elevated in patients with an exocrine function score of 0 compared to healthy controls ($P = 0.030$). However, no significant relationship was observed between serum MCP-1 levels and the exocrine function test.

Serum MCP-1, TGF-β1, and s-fractalkine concentrations for each score of alcohol intake in patients with CP (Figure 5)

Serum TGF-β1 and s-fractalkine levels in patients with an alcohol intake score of ≥ 1 were significantly higher than in healthy controls ($P = 0.018$ and $P = 0.010$, respectively). Serum MCP-1 levels, in contrast, appeared not to be related to alcohol consumption.

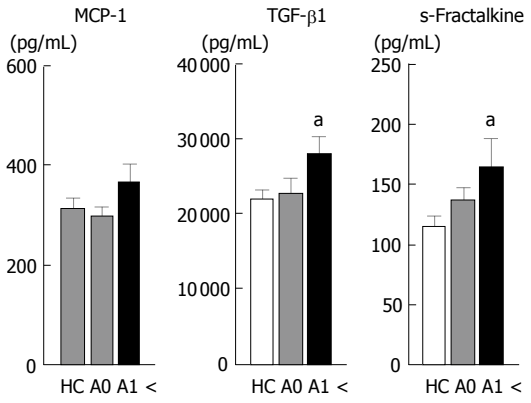

Figure 5 Serum MCP-1, TGF-β1, and s-fractalkine concentrations in relation to alcohol intake in CP patients. Serum MCP-1, TGF-β1, and s-fractalkine concentrations were measured by ELISA for each score for alcohol intake. Healthy control (HC, *n* = 116), score 0 for score of alcohol intake (A0, *n* = 57) and score > 1 for score of alcohol intake (> A1, *n* = 52). Bars represent the mean ± SD. [a]*P* < 0.05 *vs* healthy control.

Analysis of serum MCP-1, TGF-β1, and s-fractalkine concentrations on other factors in patients with CP

Finally, serum MCP-1, TGF-β1, and s-fractalkine concentrations were analyzed in relationship to other grading factors. However, we did not observe any significant relation among them. Furthermore, serum MCP-1, TGF-β1, and s-fractalkine levels did not correlate with each other. Moreover, MCP-1, TGF-β1, and s-fractalkine levels also did not correlate with age, sex, or serum pancreatic enzymes (p-amylase and lipase).

Serum TGF-β1 concentrations in non-alcoholic CP

In order to assess the influence of alcohol intake, we next focused on 57 patients with non-alcoholic CP. In the classification of severity for CP, patients with CP in the moderate stage alone showed a significant increase in serum TGF-β1 compared to healthy controls ($P = 0.039$, Figure 6A). In the classification of imaging tests and exocrine function tests, patients with CP revealing a moderate progressive stage tended to have an increase in serum TGF-β1 (Figure 6B and C).

Serum s-fractalkine concentrations in non-alcoholic CP

Similarly, we examined serum s-fractalkine in patients with non-alcoholic CP. In the classification of severity and pancreatic imaging tests, patients with CP in a mild stage alone and with a score of 1 alone showed a significant increase in serum s-fractalkine compared to healthy controls ($P = 0.026$ and $P = 0.025$, respectively; Figure 7A and B). Serum s-fractalkine didn't have a tendency for the exocrine function test (Figure 7C).

DISCUSSION

Recently, the impact of chemokines and cytokines have been recognized in the progression of chronic inflammatory diseases[15]. However, until now, there are

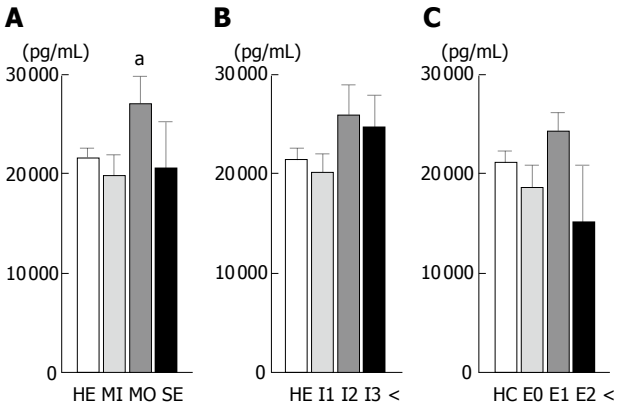

Figure 6 Serum TGF-β1 concentrations in non-alcoholic CP for each factor. Serum TGF-β1 concentrations were measured in patients with non-alcoholic CP (n = 57) by ELISA. A: Serum TGF-β1 concentrations for each stage of severity: Healthy control (HE, n = 116), mild severity (MI, n = 33), moderate severity (MO, n = 19) and severe (SE, n = 5); B: Serum TGF-β1 concentrations for each imaging test score: Healthy control (HE, n = 116), score 1 for imaging tests score (I1, n = 33), score 2 for imaging tests score (I2, n = 15) and score < 3 for imaging tests score (< I3, n = 9); C: Serum TGF-β1 concentrations for each score for exocrine function. Healthy control (HC, n = 116), score 0 for exocrine function (E0, n = 16), score 1 for exocrine function (E1, n = 37) and score > 2 for exocrine function (> E2, n = 4). Bars represent the mean ± SD. $^aP < 0.05$ *vs* healthy control.

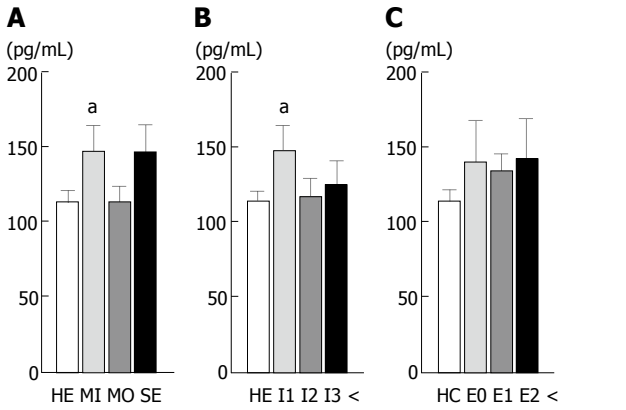

Figure 7 Serum s-fractalkine concentrations in non-alcoholic CP for each factor. Serum s-fractalkine concentrations were measured for patients with non-alcoholic CP (n = 57) by ELISA. A: Serum s-fractalkine concentrations were measured by ELISA for each stage of severity: Healthy control (HE, n = 116), mild severity (MI, n = 33), moderate severity (MO, n = 19) and severe (SE, n = 5); B: Serum s-fractalkine concentrations were measured by ELISA for each imaging tests score. Healthy control (HE, n = 116), score 1 for imaging tests score (I1, n = 33), score 2 for imaging tests score (I2, n = 15), and score < 3 for imaging tests score (< I3, n = 9); C: Serum s-fractalkine concentrations for each score of exocrine function. Healthy control (HC, n=116), score 0 for exocrine function (E0, n = 16), score 1 for exocrine function (E1, n = 37) and score > 2 for exocrine function (> E2, n = 4). Bars represent the mean ± SD. $^aP < 0.05$ *vs* healthy control.

only few reports related to the serum chemokines and cytokines levels in patients with pancreatic diseases, especially CP. Therefore, in the present study, we examined serum MCP-1, TGF-β1, and s-fractalkine concentrations that are supposed to be involved in the progression of chronic inflammatory diseases in patients with CP. In patients with CP, TGF-β1 and s-fractalkine levels, but not MCP-1 levels, were significantly increased compared to healthy controls. CP patients were classified according to severity of disease using a staging system which is based on clinical symptoms and pancreatic functions[12-14] and analyzed for the relationship with those chemokines and cytokines in order to assess the usefulness of these mediators as biological or functional markers of CP.

MCP-1, a family member of C-C chemokines, is known to play an important role in the development of the pancreatic fibrosis in CP. Previously, we reported that MCP-1 is expressed strongly in mild to moderate stages of pancreatic fibrosis in CP model rats, and suggested MCP-1 to be a pro-fibrogenic factor for CP[16,17]. Furthermore, we showed fibrosis of CP is inhibited by blocking MCP-1[18,19]. In the present study, we thus examined whether MCP-1 might be a useful marker in CP. MCP-1 levels in patients with CP, however, were not significantly higher than in healthy controls. Also in previous reports, serum MCP-1 didn't increase in patients with CP[20]. Consequently, it is thought that serum MCP-1 doesn't become a useful marker in the diagnosis of CP.

TGF-β1 is a homodimeric, multifunctional cytokine[21]. Until now, it has been generally well understood that TGF-β1 plays an important role in the development of the pancreatic fibrosis in CP[22]. TGF-β1 is thought to be expressed in pancreatic stellate cells (PSC) and acinar cells closer to pancreatic fibrosis, and to regulate the synthesis of collagen from PSC[23-25]. In our results, serum TGF-β1 levels of the patients with CP elevated significantly in the more severe stages of CP, especially in the group with more advanced scores in pancreatic imaging and exocrine function tests. These findings suggest that serum TGF-β1 levels tended to increase significantly in patients with more advanced CP. Previously, Su *et al*[26] reported the expression of TGF-β1 in pancreatic tissue in WBN/Kob rats which is considered as a CP model, and demonstrated that expression of TGF-β1 and fibronectin showed a peak at 12 wk, whereas pancreatic fibrosis peaked at 16 wk. It was concluded that TGF-β1 may trigger fibrogenesis. Furthermore, Detlefsen *et al*[27] classified the pancreatic tissue from CP patients into histological staging by an inflammatory process, and showed that TGF-β1 receptors had expressed predominantly in the early to moderate stage of pancreatic fibrosis. Given these data, we had expected that TGF-β1 levels might increase in the progressive process of pancreatic fibrosis for the present study. However, our results showed that TGF-β1 levels are elevated in patients with most advanced CP. On the other hand, interestingly, CP patients with high alcohol consumption showed a significant increase in levels of TGF-β1 (Figure 5). Concerning other organs, such as the liver and lungs, it is well known that the expression of TGF-β1 increases in the tissue of local organs from the effects of alcohol[28,29]. Therefore, in the present study, in order to clarify whether serum TGF-β1 levels might increase by TGF-β1 originated from various organs under the influence of alcohol, we focused on 57 patients with non-alcoholic CP who are not influenced by alcohol, and analyzed similarly the

relationship between TGF-β1 and each factor (Figure 6). For the classification of severity of CP, patients with CP in the moderate stage alone showed a significant increase in serum TGF-β1 compared to healthy controls. Furthermore, in the classification of imaging tests and exocrine function tests, CP patients revealing a moderate progressive stage tended to have higher serum TGF-β1 levels. These results are in accordance with previous reports[26,27]. That is to say, our results support the idea that serum TGF-β1 levels might increase because the expression of TGF-β1 was elevated in the moderate stage in which fibrosis had been proceeding in a broad range of pancreatic tissue. On the other hand, serum TGF-β1 levels might decrease in severe stages because pancreatic tissue had been already replaced with fibrosis. Taken together, it is suggested that the determination of serum TGF-β1 levels might be useful to diagnose moderate stages in patients with non-alcoholic CP.

Fractalkine/CX3CL1, a family member of CX3C chemokines, has recently been reported to be expressed as a membrane-spanning adhesion molecule that can be cleaved from the cell surface to produce a soluble chemoattractant[30-33]. The expression of fractalkine has been observed on various cells such as epithelial cells or endothelial cells of several organs. Membrane-bound fractalkine (m-fractalkine) is shed by metalloproteinase, and releases s-fractalkine[34]. M-fractalkine functions as an adhesion molecule, whereas s-fractalkine acts as a chemoattractant and recruits inflammatory cells expressing fractalkine receptors such as monocytes[33]. In inflamed local organs, such as the liver, lungs, and the kidneys, the participation of fractalkine has been recently noted[35-40]. Furthermore, increased s-fractalkine serum levels have been reported for patients with various chronic inflammatory diseases[40-44]. However, until now, there are no reports related to fractalkine in pancreatic inflammatory diseases. In the present study, we measured s-fractalkine in the serum of patients with CP. We found serum s-fractalkine levels to be significantly elevated in patients with CP. In classification of severity and pancreatic imaging tests, serum s-fractalkine levels showed significant bisferious increase in mild and severe stages. In classification of the exocrine function tests, serum s-fractalkine levels increased in mild stages alone. On the other hand, CP patients with high alcohol consumption showed a significant increase in s-fractalkine levels, similar to those of TGF-β1 (Figure 5). Since the relationship between alcohol and fractalkine was still unclear in organs, including the pancreas, we focused on 57 patients with non-alcoholic CP, and analyzed the relationship between s-fractalkine and each factors, similar to TGF-β1 (Figure 7). In classification of severity and pancreatic imaging tests, only patients with CP in the mild stage alone showed a significant increase in serum s-fractalkine compared to healthy controls. Thus, in patients with non-alcoholic CP, the measurement of serum s-fractalkine may be useful biological and functional markers to diagnose early-stage CP.

In conclusion, it is suggested that the measurement of serum TGF-β1 may be available to diagnose moderate stage of non-alcoholic CP, and that the measurement of serum s-fractalkine may be useful to diagnose early stages of non-alcoholic CP. Therefore, the measurement of a combination of TGF-β1 and s-fractalkine may be helpful to evaluate the status of CP.

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COMMENTS

Background

Chronic pancreatitis (CP) is a chronic clinical disorder characterized by irreversible damage to the pancreas. Unfortunately, simple, indirect measurements of decreased pancreatic function have not shown abnormality until CP is advanced. The quest continues for useful biological and functional markers of early-stage CP. Recently, the roles of chemokines and cytokines have been made clear in the progression of chronic inflammatory diseases. Similarly, chemokines and cytokines have been recognized as important factors in the progression of CP. However, until now, there are only few reports addressing serum chemokine and cytokine levels in patients with pancreatic diseases, especially CP.

Research frontiers

Recently, it is widely accepted that pancreatic stellate cells are responsible for the progression of pancreatic fibrosis production of an extracellular matrix, chemokines and cytokines. Especially, the expression of transforming growth factor beta-1 (TGF-β1) is, prior to pancreatic fibrosis in WBN/Kob rats, supposed to be a trigger for the fibrogenic process. Monocyte chemoattractant protein-1 (MCP-1) is related to the pancreatic fibrosis in di-n-butyl tin dichloride (DBTC)-induced rats. Next, it has been reported that soluble type fractalkine (s-fractalkine) increased in the serum of patients with various chronic inflammatory diseases: Atopic dermatitis, the nervous system, lupus erythematosus, rheumatoid vasculitis, and pityriasis rosea. Therefore, the purpose is to investigate whether the determination of serum MCP-1, TGF-β1, and s-fractalkine concentration can become a useful biological and functional marker of CP using large number of CP patients classified by severity with a staging system.

Innovations and breakthroughs

Serum TGF-β1 levels of the patients with CP tended to increase in the patients with more advanced CP, whereas serum s-fractalkine levels showed bimodal increase in mild and severe stages. However, it was observed that both TGF-β1 and s-fractalkine levels were affected by alcohol intake. Thus, serum TGF-β1 showed significant increase in the moderate stage of CP, and serum s-fractalkine revealed significant increase in the early stage of CP, when removed alcoholic CP. Therefore, the measurement of serum TGF-β1 may be available to diagnose moderate stage of non-alcoholic CP, and that the measurement of serum s-fractalkine may be useful to diagnose early stages of non-alcoholic CP. The measurement of a combination of TGF-β1 and s-fractalkine may be helpful to evaluate the severity status of CP.

Applications

Patients with CP fulfilled clinical diagnostic criteria for CP by the Japan Pancreas Society and healthy control patients excluded with recent inflammatory diseases such as infectious diseases and chronic hepatitis, and a large scale of drinking were selected. We classified the CP patients into stages of severity by the modified staging system which consists of 6 grading factors. Then, we analyzed whether each grading factor (pancreatic imaging tests, exocrine function, glucose metabolism, pain, alcohol intake, complications) and severity of CP are related with serum MCP-1, TGF-β1 or s-fractalkine levels. Enzyme-linked immunosorbent assays (ELISA) were performed to quantify serum MCP-1, TGF-β1, and s-fractalkine concentrations.

Peer review

The conclusion of the study is that serum s-fractalkine determination is useful at the early stage of the disease. First, the classification of severity of chronic pancreatitis seems complicated since involving 6 grading factors. Apart from the clinical course and histological stage, the staging system for CP, based on

clinical symptoms and pancreatic functions has been proposed in Japan. Next, to analyze a third group, a group of "healthy" but alcoholic controls without liver or other organic disease would be interesting although they are excluded in this study. An interesting perspective of this work should be the longitudinal evolution of this maker during progression of the disease.

REFERENCES

1 **Clain JE**, Pearson RK. Diagnosis of chronic pancreatitis. Is a gold standard necessary? *Surg Clin North Am* 1999; **79**: 829-845
2 **Chari ST**, Singer MV. The problem of classification and staging of chronic pancreatitis. Proposals based on current knowledge of its natural history. *Scand J Gastroenterol* 1994; **29**: 949-960
3 **Etemad B**, Whitcomb DC. Chronic pancreatitis: diagnosis, classification, and new genetic developments. *Gastroenterology* 2001; **120**: 682-707
4 **Sarles H**. Pancreatitis. Symposium; 1963 April; Marseille, France. Basel: Karger, 1965, **7**: 1-20
5 **Nakano S**, Horigauchi Y, Takeda T, Suzuki T, Nakajima S. Comparative diagnostic value of endoscopic pancreatography and pancreatic function tests. *Scand J Gastroenterol* 1974; **9**: 383-390
6 **Heij HA**, Obertop H, van Blankenstein M, Nix GA, Westbroek DL. Comparison of endoscopic retrograde pancreatography with functional and histologic changes in chronic pancreatitis. *Acta Radiol* 1987; **28**: 289-293
7 **Ito T**. Can measurement of chemokines become useful biological and functional markers of early-stage chronic pancreatitis? *J Gastroenterol* 2007; **42 Suppl 17**: 72-77
8 **Ito T**, Otsuki M, Itoi T, Shimosegawa T, Funakoshi A, Shiratori K, Naruse S, Kuroda Y. Pancreatic diabetes in a follow-up survey of chronic pancreatitis in Japan. *J Gastroenterol* 2007; **42**: 291-297
9 **Malfertheiner P**, Büchler M. Correlation of imaging and function in chronic pancreatitis. *Radiol Clin North Am* 1989; **27**: 51-64
10 **Grady T**, Liang P, Ernst SA, Logsdon CD. Chemokine gene expression in rat pancreatic acinar cells is an early event associated with acute pancreatitis. *Gastroenterology* 1997; **113**: 1966-1975
11 **Saurer L**, Reber P, Schaffner T, Büchler MW, Buri C, Kappeler A, Walz A, Friess H, Mueller C. Differential expression of chemokines in normal pancreas and in chronic pancreatitis. *Gastroenterology* 2000; **118**: 356-367
12 **Otsuki M**. Chronic pancreatitis in Japan: epidemiology, prognosis, diagnostic criteria, and future problems. *J Gastroenterol* 2003; **38**: 315-326
13 **Hayakawa T**, Kondo T, Shibata T, Noda A, Suzuki T, Nakano S. Relationship between pancreatic exocrine function and histological changes in chronic pancreatitis. *Am J Gastroenterol* 1992; **87**: 1170-1174
14 **Hayakawa T**, Kitagawa M, Naruse S, Ishigyrro H, Mizuno N, Nakajima M. Staging of chronic pancreatitis (in Japanese). *Suizou (J Jpn Pancreas Soc)* 2001; **16**: 381-385
15 **Wynn TA**. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008; **214**: 199-210
16 **Inoue M**, Ino Y, Gibo J, Ito T, Hisano T, Arita Y, Nawata H. The role of monocyte chemoattractant protein-1 in experimental chronic pancreatitis model induced by dibutyltin dichloride in rats. *Pancreas* 2002; **25**: e64-e70
17 **Gibo J**, Ito T, Kawabe K, Hisano T, Inoue M, Fujimori N, Oono T, Arita Y, Nawata H. Camostat mesilate attenuates pancreatic fibrosis via inhibition of monocytes and pancreatic stellate cells activity. *Lab Invest* 2005; **85**: 75-89
18 **Zhao HF**, Ito T, Gibo J, Kawabe K, Oono T, Kaku T, Arita Y, Zhao QW, Usui M, Egashira K, Nawata H. Anti-monocyte chemoattractant protein 1 gene therapy attenuates experimental chronic pancreatitis induced by dibutyltin dichloride in rats. *Gut* 2005; **54**: 1759-1767
19 **Kaku T**, Oono T, Zhao H, Gibo J, Kawabe K, Ito T, Takayanagi R. IS-741 attenuates local migration of monocytes and subsequent pancreatic fibrosis in experimental chronic pancreatitis induced by dibutyltin dichloride in rats. *Pancreas* 2007; **34**: 299-309
20 **Pedersen N**, Larsen S, Seidelin JB, Nielsen OH. Alcohol modulates circulating levels of interleukin-6 and monocyte chemoattractant protein-1 in chronic pancreatitis. *Scand J Gastroenterol* 2004; **39**: 277-282
21 **Massagué J**. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990; **6**: 597-641
22 **di Mola FF**, Friess H, Martignoni ME, Di Sebastiano P, Zimmermann A, Innocenti P, Graber H, Gold LI, Korc M, Büchler MW. Connective tissue growth factor is a regulator for fibrosis in human chronic pancreatitis. *Ann Surg* 1999; **230**: 63-71
23 **Shek FW**, Benyon RC, Walker FM, McCrudden PR, Pender SL, Williams EJ, Johnson PA, Johnson CD, Bateman AC, Fine DR, Iredale JP. Expression of transforming growth factor-beta 1 by pancreatic stellate cells and its implications for matrix secretion and turnover in chronic pancreatitis. *Am J Pathol* 2002; **160**: 1787-1798
24 **Apte MV**, Wilson JS. Mechanisms of pancreatic fibrosis. *Dig Dis* 2004; **22**: 273-279
25 **Madro A**, Celiński K, Słomka M. The role of pancreatic stellate cells and cytokines in the development of chronic pancreatitis. *Med Sci Monit* 2004; **10**: RA166-RA170
26 **Su SB**, Motoo Y, Xie MJ, Miyazono K, Sawabu N. Expression of transforming growth factor-beta in spontaneous chronic pancreatitis in the WBN/Kob rat. *Dig Dis Sci* 2000; **45**: 151-159
27 **Detlefsen S**, Sipos B, Feyerabend B, Klöppel G. Fibrogenesis in alcoholic chronic pancreatitis: the role of tissue necrosis, macrophages, myofibroblasts and cytokines. *Mod Pathol* 2006; **19**: 1019-1026
28 **Crews FT**, Bechara R, Brown LA, Guidot DM, Mandrekar P, Oak S, Qin L, Szabo G, Wheeler M, Zou J. Cytokines and alcohol. *Alcohol Clin Exp Res* 2006; **30**: 720-730
29 **Apte MV**, Zima T, Dooley S, Siegmund SV, Pandol SJ, Singer MV. Signal transduction in alcohol-related diseases. *Alcohol Clin Exp Res* 2005; **29**: 1299-1309
30 **Rossi DL**, Hardiman G, Copeland NG, Gilbert DJ, Jenkins N, Zlotnik A, Bazan JF. Cloning and characterization of a new type of mouse chemokine. *Genomics* 1998; **47**: 163-170
31 **Chapman GA**, Moores K, Harrison D, Campbell CA, Stewart BR, Strijbos PJ. Fractalkine cleavage from neuronal membranes represents an acute event in the inflammatory response to excitotoxic brain damage. *J Neurosci* 2000; **20**: RC87
32 **Imai T**, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall TJ, Yoshie O. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* 1997; **91**: 521-530
33 **Ludwig A**, Berkhout T, Moores K, Groot P, Chapman G. Fractalkine is expressed by smooth muscle cells in response to IFN-gamma and TNF-alpha and is modulated by metalloproteinase activity. *J Immunol* 2002; **168**: 604-612
34 **Umehara H**, Bloom ET, Okazaki T, Nagano Y, Yoshie O, Imai T. Fractalkine in vascular biology: from basic research to clinical disease. *Arterioscler Thromb Vasc Biol* 2004; **24**: 34-40
35 **Isse K**, Harada K, Zen Y, Kamihira T, Shimoda S, Harada M, Nakanuma Y. Fractalkine and CX3CR1 are involved in the recruitment of intraepithelial lymphocytes of intrahepatic bile ducts. *Hepatology* 2005; **41**: 506-516
36 **Rimaniol AC**, Till SJ, Garcia G, Capel F, Godot V, Balabanian K, Durand-Gasselin I, Varga EM, Simonneau G, Emilie D, Durham SR, Humbert M. The CX3C chemokine fractalkine in allergic asthma and rhinitis. *J Allergy Clin Immunol* 2003; **112**: 1139-1146

37 **Ito Y**, Kawachi H, Morioka Y, Nakatsue T, Koike H, Ikezumi Y, Oyanagi A, Natori Y, Natori Y, Nakamura T, Gejyo F, Shimizu F. Fractalkine expression and the recruitment of CX3CR1+ cells in the prolonged mesangial proliferative glomerulonephritis. *Kidney Int* 2002; **61**: 2044-2057
38 **Brand S**, Hofbauer K, Dambacher J, Schnitzler F, Staudinger T, Pfennig S, Seiderer J, Tillack C, Konrad A, Göke B, Ochsenkühn T, Lohse P. Increased expression of the chemokine fractalkine in Crohn's disease and association of the fractalkine receptor T280M polymorphism with a fibrostenosing disease Phenotype. *Am J Gastroenterol* 2006; **101**: 99-106
39 **Hulshof S**, van Haastert ES, Kuipers HF, van den Elsen PJ, De Groot CJ, van der Valk P, Ravid R, Biber K. CX3CL1 and CX3CR1 expression in human brain tissue: noninflammatory control versus multiple sclerosis. *J Neuropathol Exp Neurol* 2003; **62**: 899-907
40 **Echigo T**, Hasegawa M, Shimada Y, Takehara K, Sato S. Expression of fractalkine and its receptor, CX3CR1, in atopic dermatitis: possible contribution to skin inflammation. *J Allergy Clin Immunol* 2004; **113**: 940-948
41 **Kastenbauer S**, Koedel U, Wick M, Kieseier BC, Hartung HP, Pfister HW. CSF and serum levels of soluble fractalkine (CX3CL1) in inflammatory diseases of the nervous system. *J Neuroimmunol* 2003; **137**: 210-217
42 **Yajima N**, Kasama T, Isozaki T, Odai T, Matsunawa M, Negishi M, Ide H, Kameoka Y, Hirohata S, Adachi M. Elevated levels of soluble fractalkine in active systemic lupus erythematosus: potential involvement in neuropsychiatric manifestations. *Arthritis Rheum* 2005; **52**: 1670-1675
43 **Matsunawa M**, Isozaki T, Odai T, Yajima N, Takeuchi HT, Negishi M, Ide H, Adachi M, Kasama T. Increased serum levels of soluble fractalkine (CX3CL1) correlate with disease activity in rheumatoid vasculitis. *Arthritis Rheum* 2006; **54**: 3408-3416
44 **Gangemi S**, Cannavò SP, Guarneri F, Merendino RA, Sturniolo GC, Minciullo PL, Di Pasquale G, Valenzise M, Drago F, Rebora A. The CX3C-chemokine fractalkine (CX3CL1) is detectable in serum of patients affected by active pityriasis rosea. *J Eur Acad Dermatol Venereol* 2006; **20**: 1366-1367

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BASIC RESEARCH

Effects of electroacupuncture on cardiac and gastric activities in acute myocardial ischemia rats

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Abstract

AIM: To observe the effect of electroacupuncture (EA) of "Neiguan" (PC6) and "Gongsun" (SP4) on pathological changes of the heart and stomach in rats with acute myocardial ischemia (AMI), and to explore its underlying mechanism.

METHODS: Fifty Wistar rats were randomized into control, model, PC6, SP4 and PC6 + SP4 groups (n = 8 each group). An AMI model was established by occlusion of the descending anterior branch (DAB) of the left coronary artery. ECG-ST of cervico-thoracic lead and electrogastrogram (EGG) were recorded. EA was applied to PC6, SP4 and PC6 + SP4 groups, respectively. At the end of experiments, the rats were transcardically perfused with 4% paraformaldehyde, and the heart base myocardium, gastric antrum and duodenum tissues were sampled, sectioned and stained with a reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase histochemical method for displaying nitric oxide synthase (NOS) activity.

RESULTS: After AMI, ECG-ST values elevated. After EA, the elevated ECG-ST values at 20 min in PC6 group, at 30 min in PC6 + SP4 and SP4 groups had no significant differences in comparison with their respective basal values before AMI. Following AMI, the amplitude and frequency of slow waves of EGG decreased remarkably ($P < 0.05$). At 30 min after EA, the mean amplitude and frequency of slow waves of EGG in the three EA groups had no marked differences compared with their individual basal levels and those in the control group. After AMI, the mean integral grey values of NOS-positive product in myocardium, gastric antrum and duodenum tissues in the model group increased remarkably in comparison with the control group, while those in three EA groups were lower than those in the model group. No significant differences were found in ECG-ST and EGG improvement among the three EA groups. However, EA of PC6 had a better effect on ECG-ST and EA of PC4 had a better effect on EGG, respectively.

CONCLUSION: EA of PC6, SP4 and PC6 + SP4 can significantly promote the recovery of cardiac and gastric electrical activities after AMI, and up-regulate NOS expression in myocardium, gastric antrum and duodenum tissues.

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Key words: Electroacupuncture; Myocardial ischemia; ECG-ST; Electrogastrogram; Heart base; Gastric antrum; Duodenum; Nitric oxide synthase expression

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INTRODUCTION

It has been well documented that acupuncture of Neiguan (PC6) can effectively improve symptoms of angina, palpitation, *etc* and the left cardiac function in coronary heart disease (CHD) patients[1-4]. Experimental studies in rabbits, rats, and cats also showed that acupuncture improves myocardial ischemia (MI), hemodynamics, microcirculation and energy metabolism of ischemic heart, decreases susceptibility to ventricular

tachycardia, and protects myocardiocytes from injury[5-12]. PC6 combined with other acupoints often functions well in relieving pregnancy or MI-induced vomiting, alimentary canal ulceration, abdominal pain, *etc* and regulating secretion activities of various hormones in the gastrointestinal tract[13-15]. PC6 combined with Gongsun (SP4) can improve electrocardiogram (ECG) of CHD patients[16], and lessen gastrointestinal reactions in cancer patients undergoing chemotherapy[17,18]. It was reported that after injection of choleratoxin B coupled horseradish peroxidase (HRP) in PC6 and simultaneous electroacupuncture (EA) stimulation of SP4, the number of the labeled neurons in C6-C8 spinal ganglia and dorsal horns increases significantly and the distribution segments are expended[19], displaying a synergistic action. However, majority of these studies only explored the effect of acupuncture of a single acupoint on one internal organ and fewer studies have been conducted to explore its effect of one or more acupoints on two-visceral activities. For this reason, the present study was designed to observe the characteristics and regularities of EA of PC6 and/or SP4 in regulating the cardiac and gastric functional activities in acute myocardial ischemia (AMI) rats, and to analyze its underlying mechanisms.

MATERIALS AND METHODS

Animals and grouping

Forty male Wistar rats (weighing 250-320 g), supplied by the Experimental Animal Center of Chinese Academy of Medical Sciences, were used in the present study. All the rats fasted for about 24 h before each experiment (with free access to water) and were randomized into control, model, PC6, SP4 and PC6 + SP4 groups (n = 8 in each group). All procedures were approved by the Administrative Committee of Laboratory Animal Care and Use of Chinese Academy of Medical Sciences.

Main instruments and reagents

Instruments used in this study included multi-channel physiological recorder (RM-6000 Nihon Kohden, Japan), PowerLab data acquisition system (Australia, AD/ instruments), mini-animal respirator (Jiangwan Type-Ⅱ), HANS EA apparatus (LH202H), DRB-2D electrical blanket, cryostat freezing microtome (Leica, Leitz1720), light microscope (OLYMPUS), image analyzer (MSP UV-VIS 2000, USA).

Reagents used in this study were a reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), nitroblue tetrazolium (NBT, Sigma), paraformaldehyde (Sigma).

Experimental protocols

Surgical operation: After anesthesia with a mixture solution (0.5 mL/100 g, ip) of 1.5% chloralose (5 mg/100 g) and 25% urethane (42 mg/100 g), the rat was fixed on an animal table for performing trachea cannula, thoracotomy between the 3rd and 4th intercostal spaces on the left side of the chest along the sternum, and laparotomy below the xiphoid-process.

Establishment of AMI model: After turning on artificial respirator (60 cycles/min), a surgical suture (gauge-0) was put through the myocardium beneath the root part of the descending anterior branch (DAB) of the left coronary artery for inducing AMI.

Electrogastrogram (EGG) recording electrode resettlement: For EGG recording, a pair of stainless steel electrodes was implanted beneath the subserosa about 0.5 cm apart from the pylorus of gastric antrum and a reference electrode was placed subcutaneously in the adjoining incision.

Electrocardiogram (ECG) recording electrode resettlement: For recording ECG of the cervico-thorax lead, two stainless steel electrodes were placed beneath the skin close and left to the xiphoid-process, and the neck back, respectively, and the reference electrode was placed beneath the skin of the right hindlimb.

EA: Bilateral PC6 and SP4 were located as previously described[20]. Two acupuncture needles (Gauge-28, 0.5 cm) were separately inserted into each acupoint, and connected to the HANS EA apparatus to stimulate the acupoint for 30 min with parameters of 2/15 Hz and 1-3 mA (increased gradually).

Procedure of management: After settlement of all the electrodes, ECG and EGG were recorded using polygraph, Power-Lab/8S-Chart 6.0 and computer before and during occlusion of DAB (30 min) and reperfusion (AMI/R, 30 min). EA was given just before the occlusion.

Sampling: After physiological experiments were finished, the animals were subjected to transcardial perfusion with normal saline (300 mL), and 4% polyoxymethylene in a phosphate buffer (4℃, 400 mL, 0.1 mol/L, pH 7.4), respectively. The left-atria tissue of the cardiac base (between the aorta and the pulmonary artery, containing nerve plexus controlling cardiac functional activities), gastric antrum and annectant duodenum tissues were taken and equilibrated in 20% sucrose overnight, and stored at 4℃.

Staining and observation of tissue samples: Tissue samples were cut into 20-μm thick sections and mounted onto gel-treated glass slides. The sections were washed twice with PBS and incubated to demonstrate NADPH-diaphorase activity in the following medium: 0.1 mol/L phosphate buffer solution (pH 7.4, 10 mL) containing 5 mg of NBT, 10 mg of beta-NADPH, and 0.1 mol/L Tris-HCL for 30 min at 37℃. The sections were then incubated in 0.1 mol/L PBS (pH 7.4) containing 0.2% Triton X-100 (1.0 mL) for 120 min, dehydrated in a graded series of alcohol, and sealed with neutral gum.

Under a light microscope, cells containing NADPH-

diaphorase, a marker for the presence of inducible nitric oxide synthase (NOS), were observed and the integral grey value was detected using an image analyzer.

Statistical analysis

Data were expressed as mean ± SD and analyzed with one-way ANOVA using SPSS11.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of EA on ischemic ECG-ST

After occlusion of DAB, ECG-ST elevated significantly in model, PC6, SP4, PC6 + SP4 groups ($P < 0.05$, Figure 1A). Following AMI/R, ECG-ST elevated in the model group and declined in the three EA groups (PC6, SP4, PC6 + SP4). The ECG-ST values were significantly lower in PC6, SP4 and PC6 + SP4 groups than those in the model group at 10, 20 and 30 min after EA ($P < 0.05$). No significant difference was found among the three EA groups ($P > 0.05$). The ECG-ST values were still markedly higher in the SP4 and model groups than those in control group 30 min after EA ($P < 0.05$), and no significant difference was found between the PC6 and control groups or between the PC6 + SP4 and control groups.

Effect of EA on the amplitude of slow waves of EGG

After AMI, the slow wave amplitude of EGG declined remarkably ($P < 0.05$, Figure 1B), the slow wave amplitude of EGG in the model group decreased further, while those in the PC6, SP4 and PC6 + SP4 groups had an apparent recovery. The slow wave amplitude of the model group was significantly lower than that in the control group after AMI and AMI/R ($P < 0.05$). The slow wave amplitudes of the SP4, PC6 and PC6 + SP4 groups were comparable after AMI/R, and significantly higher in the SP4 group than that in the control group ($P < 0.05$). The effect of EA of SP4 was slightly stronger on increasing the slow wave amplitude of EGG.

Effect of EA on the frequency of slow waves of EGG

Following AMI, the frequency of slow waves of ischemic EGG decreased significantly in comparison with the basal values in each group ($P < 0.05$, Figure 1C). After AMI/R, the mean frequency of slow waves of ischemic EGG in the model group decreased further, and was still markedly lower than its basal value 30 min post-EA ($P < 0.05$). The mean frequencies of slow waves were significantly higher in the three EA groups than that in model group after AMI/R ($P < 0.05$). At 20 min after EA, the mean frequency of slow waves of ischemic EGG in the SP4 group was not significantly different from that in the control group ($P > 0.05$). At 10 min after AMI/R and EA, the mean frequencies of slow waves of ischemic EGG in the PC6 + SP4 and PC6 groups were obviously higher than that in the model group ($P < 0.05$, Figure 1C) and did not return to their basal values 30 min after EA

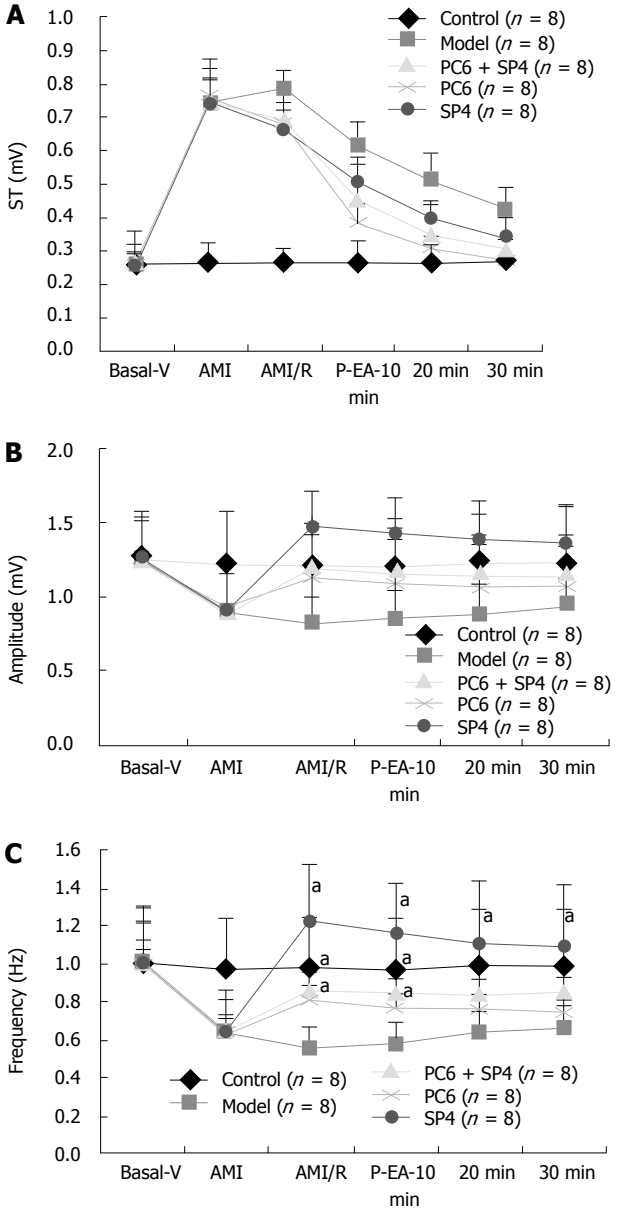

Figure 1 Changes in rabbit ECG-ST (mV, A), amplitude (mV, B) and frequency (pulses/s, C) of slow waves of electrogastrogram (EGG) in control, model, PC6, SP4 and PC6 + SP4 groups ($n = 8$ in each group) at different courses after acute myocardial ischemia and reperfusion (AMI/R). [a]$P < 0.05$ *vs* model group; Basal-V: Basal value; P-EA: Post-EA.

($P < 0.05$). The effect of EA of SP4 was slightly better on increasing the frequency of slow waves of ischemic EGG.

Effect of EA on the frequency of fast waves of ischemic EGG

During experiments, burst (fast) of waves ischemic of EGG appeared only in some animals, particularly after AMI/R. We randomly chose three rats in each group and analyzed the effect of EA (Table 1). The frequency of fast waves of ischemic EGG increased significantly from the beginning of AMI/R after EA in the SP4 group, while the frequencies of fast waves of ischemic EGG in the model, PC6 and PC6 + SP4 groups were close to that in the control group.

Table 1 Changes in mean frequency of EGG fast waves in rabbits at different time-courses after AMI/R and EA (mean ± SD, pulses/s, *n* = 3/group)

Groups	Basal values	AMI	AMI/R	P-EA 10 min	P-EA-20	P-EA-30 min
Control	1.90 ± 1.91	1.84 ± 1.82	1.89 ± 1.87	1.84 ± 1.82	1.90 ± 1.84	1.85 ± 1.83
Model	1.98 ± 0.93	0.91 ± 0.10	0.93 ± 0.91	1.05 ± 0.09	1.53 ± 0.61	1.76 ± 1.00
PC6	3.48 ± 1.52	1.92 ± 2.17	3.81 ± 3.12	3.47 ± 4.31	2.65 ± 2.83	2.10 ± 2.10
SP4	2.01 ± 0.38	1.26 ± 0.25	4.04 ± 1.19	3.89 ± 0.98	3.58 ± 0.93	2.93 ± 0.36
PC6 + SP4	1.45 ± 1.21	0.68 ± 0.85	1.61 ± 1.19	1.52 ± 1.18	1.30 ± 0.95	1.19 ± 0.90

Table 2 Mean integral grey values of NADPH-diaphorase /NOS expression in myocardium, gastric antrum and duodenum in rabbits (mean ± SD)

Groups	Cases	Myocardium	Gastric antrum	Duodenum
Control	4	122.92 ± 22.40	168.21 ± 58.30	127.01 ± 46.26
Model	4	212.93 ± 22.30	218.40 ± 23.30	190.71 ± 33.91
PC6	4	143.41 ± 38.01	171.11 ± 37.11	178.53 ± 40.05
SP4	4	191.63 ± 16.20	163.35 ± 24.85	140.86 ± 60.84
PC6 + SP4	4	184.72 ± 30.51	140.86 ± 60.84	178.09 ± 51.26

Effect of EA on the NOS activity of myocardium, gastric antrum and duodenum muscle

NADPH-diaphorase staining of myocardium, gastric antrum and duodenum (Figure 2A-C) showed that NADPH-diaphorase/NOS-expressed positive fibers, neurons and their processes had a purple/blue color, which was denser in the control group, moderate in the three EA groups and lightest in the model group.

In myocardium tissue (Figure 2A), NOS positive reaction product (in nerve fibers) presented a string-of-beads-like distribution (dark blue color) on the wall of larger blood vessels along the longitudinal axis of the smooth muscle fibers, and was denser in the control group, moderate in the PC6 group, milder in the PC6 + SP4 and SP4 groups, and lightest in the model group. In gastric antrum tissue (Figure 2B), most NOS-expressed neurons were round and oval in shape. Fewer irregular polygons and their cytoplasm were uniformly dark blue and their nuclei were relatively lighter in color. NOS-positive reaction product was denser in the control group, moderate in the SP4 group, milder in the PC6 and PC6 + SP4 groups, lightest in the model group. In the duodenum tissue (Figure 2C), the shape and color of NOS-expressed positive neurons in the control, model, PC6, PC6 + SP4 and SP4 groups were similar to those in the gastric antrum.

The integral grey values for myocardium, gastric antrum and duodenum increased in the model and PC6, PC6 + SP4 and SP4 groups compared with the control group after AMI/R (Table 2). Following EA, the integral grey values for myocardium and duodenum in the PC6 and SP4 group were closer to that in the control group, suggesting that EA could suppress AMI/R- induced down-regulation of NOS.

DISCUSSION

In the light of the theory of Chinese medicine, PC6 and SP4 are two convergent acupoints of the eight Meridians. PC6, the Luo-point of the Pericardium Meridian, is often used to treat palpitation, chest distress and thoracalgia in the upper energizer, gastralgia and vomiting of the middle energizer, and irregular menstruation of the lower energizer, *etc*. SP4, the Luo-point of the Spleen Meridian of Foot-Taiyin, is frequently employed to treat gastralgia, vomiting, abdominal pain, diarrhea, *etc*. These two acupoints, used in combination, can function well in relieving disorders of the heart and gastrointestinal tract, but related experimental investigations are fewer, in spite of being frequently used in clinic practice.

Results of the present study show that ECG-ST elevated significantly after occlusion of DAB, meaning occurrence of AMI. After release of the ligature (AMI/R), ECG-ST in the model group elevated further, while decreased significantly in the PC6, SP4 and PC6 + SP4 groups. At 20 min after EA in the PC6 group, and 30 min in the PC6 + SP4 group after EA, ECG-ST values returned to their basal levels before occlusion of DAB, and those in the SP4 and model groups were still higher than their respective basal levels. No significant differences were found in ECG-ST values at different time courses among the three EA groups, indicating that EA of PC6, SP4 and PC6 + SP4 can effectively promote the recovery of ischemic myocardial injury. Unfortunately, no synergistic action of EA of PC6 + SP4 on AMI was found in the present study. The results of EA of PC6 and PC6 + SP4 are similar to the reported findings[5,7,8,16].

After AMI, the cardiac blood-pumping function was weakened, leading to decrease in cardiac output and in perfusion volume in many organs, and disturbance of blood circulation, thus an abnormal change occurred in EGG.

The results of this study show that after AMI, the amplitude and frequency of slow waves of EGG decreased considerably, and decreased further in the model group, and did not return to their pre-AMI levels at 30 min after ligation of DAB, suggesting a remarkable suppression of the basal electrical activity of the gastric smooth muscle due to insufficient blood supply. In the three EA groups, both the amplitude and frequency of slow waves of EGG increased after EA stimulation. The amplitude of slow waves of EGG in the three EA groups returned to their pre-AMI basal levels at 30 min after EA. No significant difference was found in the amplitude of slow waves of EGG among the three EA groups. Twenty minutes after EA, the mean frequency in the SP4 group was not markedly different from that in the control group. Ten minutes

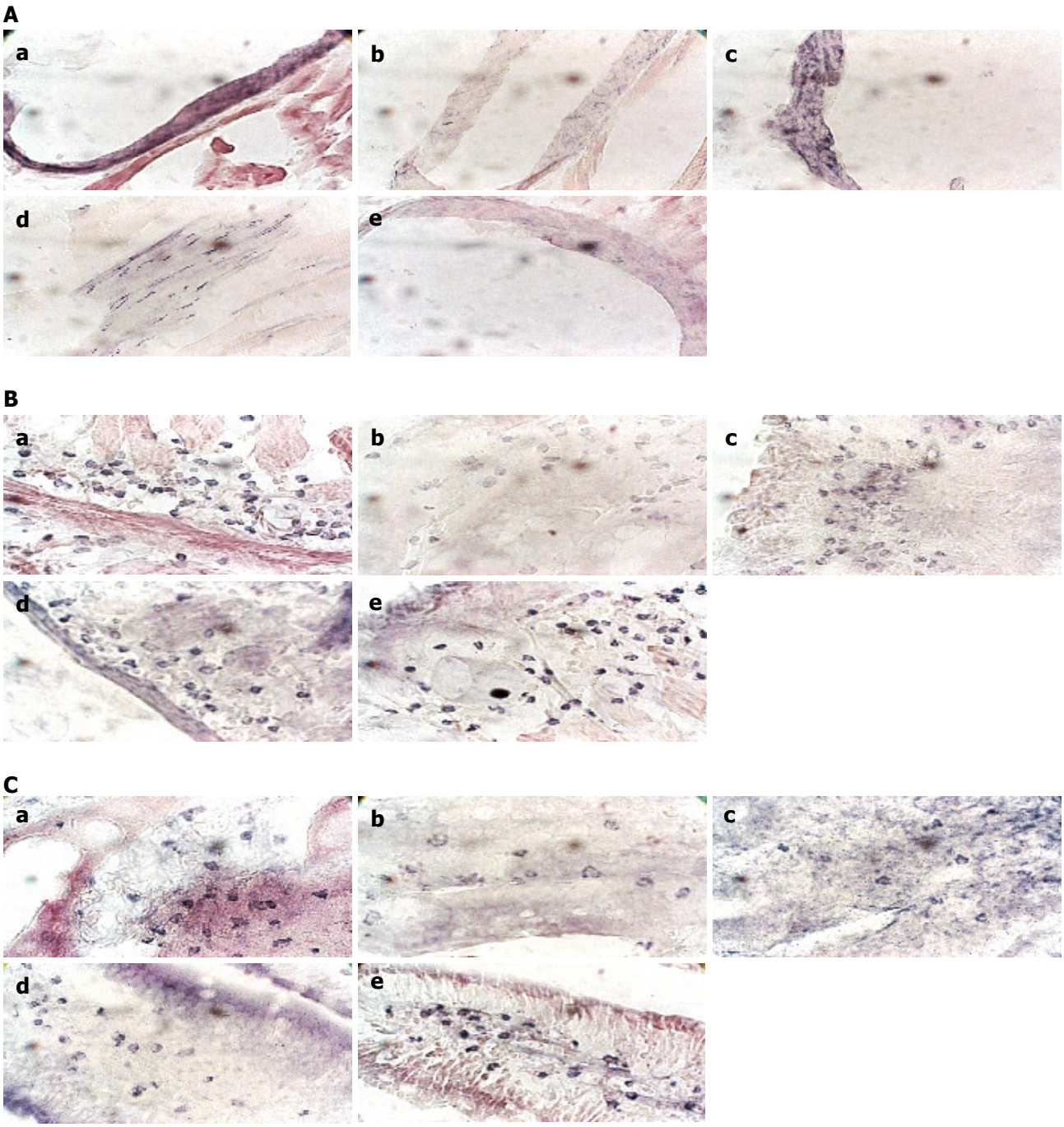

Figure 2 Photographs showing NOS expression in the myocardium of the rabbit heart base (left atrium, A), gastric antrum (B) and duodenum (C) in control (a), model (b), PC6 (c), PC6 + SP4 (d) and SP4 (e) groups. A: NOS positive reaction product (in nerve fibers) presented string-of-beads-like distribution (dark blue color) on the wall of larger blood vessels along the longitudinal axis of the smooth muscle fibers. NOS-positive product was denser in control group (a), moderate in PC6 group (c), milder in PC6 + SP4 (d) and SP4 (e) groups, lightest in model group (b); B: NOS positive reaction neurons distributing in the mucous layer and underlayer of the rabbit gastric antrum; C: NOS positive reaction neurons distributing in the mucous layer and underlayer of the rabbit duodenum tissues. Most of them were round and oval, and fewer irregular polygon, and their kytoplasm was uniformly dark blue and their nuclei were relatively lighter in color. NOS-positive reaction product was denser in control group (a), moderate in SP4 group (e), milder in PC6 (c) and PC6 + SP4 (d) groups, lightest in model group (b). (X 400).

after AMI/R and EA, the mean frequency in PC6 + SP4 and PC6 groups was obviously higher than that in the model group and did not return to their basal values 30 min after EA, indicating that the effect of EA of SP4 on the frequency of slow waves of EGG is stronger. It has been demonstrated that EA of PC6 and SP4 can ameliorate symptoms of abdominal pain, vomiting, *etc*, and the ratio of the main frequency of EGG between pre- and post-meal in functional dyspepsia patients[13,14,21,22].

It has been well-documented that the effect of acupuncture on regulating cardiac and gastrointestinal activities involves the nervous system, endocrine system, and multi-targets[1,4,12,14]. Improved microcirculation and appropriate amount of NO production may be one of the important factors. NO, a micromolecule gas, is

synthesized from *l*-arginine under the catalysis of NO synthase, and can activate guanylate cyclase of the cells and exert biological effects *via* the resultant cGMP. Continuous basal release of NO is very important for maintaining stable diastole state and base tension of the coronary artery and regulating blood pressure and myocardial blood perfusion[23]. Myocardial ischemia decreases myocardial NO concentration and NOS expression[24]. In our experiments, the blue positive reaction product in the myocardium, gastric antrum and duodenum was the densest in the control group, moderate in the three EA groups, and the lightest in the model group. The integral grey values for myocardium in the PC6 group, gastric duodenum in the PC6 + SP4 group were relatively denser, suggesting that NOS expression decreases apparently after AMI, while increases after EA, which may contribute to its effect on improving cardiac and gastric electrical activities in AMI/R rats. Our results are basically identical to other reported findings[25-29]. For example, Li *et al*[25] observed that EA of PC6 could up-regulate plasma NO and blood platelet α granule membrane protein-140 contents and reduce myocardial injury in myocardial ischemia rabbits. Luo *et al*[26] reported that acupuncture of PC6, SP4, *etc*, could relieve symptoms of peptic ulcer and elevate plasma NO level. In rats with alcohol-induced mucous membrane injury, EA of Zusanli (ST36) plus PC6 or SP4, or simple PC6, could effectively raise the contents of NO and epidemic growth factor in gastric mucosa tissue, and promote the synthesis and release of NO, thus protecting gastric mucosa from injury[27-29]. These results suggest that NO is able to mediate the effects of EA on improving cardiac and gastrointestinal functional activities. Our study did not show a synergistic action of EA of both PC6 and SP4 as reported[30].

In conclusion, EA of PC6 and SP4 can exert similar effects on cardiac and gastric activities. EA of one acupoint may simultaneously regulate the activities of multiple viscerae. Hence, in clinical practice, PC6 and SP4 used in combination are applicable to the treatment of both cardiac and gastric disorders.

COMMENTS

Background

Neiguan (PC6) and Gongsun (SP4), two convergent acupoints of the eight Meridians, are indicated for disorders of the stomach, heart and chest. In fact, the two acupoints are frequently used in combination to treat gastrointestinal disorders as nausea, vomiting, *etc*, in clinical practice. When used individually, PC6 mainly functions well in regulating problems of the heart, while SP4 is chiefly indicated for gastralgia, acute and chronic enteritis, vomiting, menstrual disorder, *etc*. The present study observed the effect of electroacupuncture (EA) of PC6 and/or SP4 on the activities of the heart and gastrointestine under pathological state (acute myocardial ischemia) from electrophysiological and histochemical aspects so as to analyze their action regularities.

Research frontiers

The correlation between acupoints and internal organs is a key theoretical basis of Chinese acupuncturology and acupuncture in clinical practice. The present study was undertaken to observe the relative specificity of EA of acupoints in regulating activities of the corresponding internal organs. In addition, joint application of two or more acupoints may have a synthetic effect or a counter action under certain circumstances. Since the related regularities and the underlying mechanism remain unknown, the present study observed the effects of EA of PC6 and /or SP4 on activities of the heart and gastrointestine simultaneously.

Innovations and breakthroughs

Fewer experimental studies have been conducted to observe the effect of EA of one or two acupoints on two internal organs at the same time. The present study demonstrated that EA of PC6, SP4 or PC6 + SP4 on myocardial ischemia-reperfusion (MI-R)-induced elevation of ECG-ST and decreased mean amplitude and frequency of slow waves of electrogastrogram (EGG), as well as reduced nitric oxide synthase (NOS) expression in the myocardium, gastric antrum and duodenum were all corrected significantly sooner or later. Moreover, EA of PC6 and SP4 could improve ECG-ST, and regulate NOS activity of myocardium and mucous membrane of duodenum.

Applications

The results of the present study provided certain new experimental evidence for treating disorders of the heart and gastrointestine by acupuncture of PC6 and SP4 and verified the theory of Chinese acupuncturology about the relatively specific effects of acupoints on their corresponding internal organs.

Terminology

According to Chinese medicine theory, there are 12 regular meridians distributed regularly in the human body, each meridian pertains to a certain Zang- or Fu-organ (internal organ). When a certain acupoint is stimulated with an acupuncture needle, the corresponding internal organ can be regulated effectively in functional activities by way of meridian, particularly under pathological state. However, the regulatied effect of the stimulated acupoint on the internal organ is definitely not absolute, some related organs may be influenced at the same time. Thus, when mentioning the acupoint specificity, it mainly refers to its relative specificity in function.

Peer review

The paper describes the characteristics, values and significance of electroacupuncture, as well as the effects of electroacupuncture on cardiac and gastric activities of rats with ischemia, which may be of some values for clinicians in their clinical practice.

REFERENCES

1 **Tian YF**. Overview of researches on the protective effects of acupuncture of Neiguan (PC6) on heart. *Zhenjiu Linchuang Zazhi* 1999; **15**: 50-52
2 **Meng J**. The effects of acupuncture in treatment of coronary heart diseases. *J Tradit Chin Med* 2004; **24**: 16-19
3 **Xu FH**, Wang JM. Clinical observation on the treatment of intractable angina with combined acupuncture and herbal medicines. *Zhongguo Zhenjiu* 2005; **25**: 89-91
4 **CAO QS**. Zhongyi Jingluo Xiandai Yanjiu. In: Hu XL. Researches on the correlation between acupoints and Zangfu-organs and their connection pathways. Beijing: People's Medical Publishing House, 1990: 210-247
5 **Liu JL**, Cao QS, Luo MF, Wen S, Liu JL, Cui RL. Researches on the mechanism of electroacupuncture of acupoints of the Pericardium Meridian in improving acute myocardial ischemia. *Zhenci Yanjiu* 1999; **24**: 282-287
6 **Luo MF**, Wang P, Wang ZY, Liu JL, Chen SP. Influence of electroacupuncture of Neiguan (PC6) on myofibrilla, mitochondria and blood platelet in myocardial ischemic region. *Zhenci Yanjiu* 2001; **26**: 119-121
7 **Tian YF**, Yan J, Lin YP, Yi SX, Chang XR, Liu JH. Effect of electroacupuncture of Neiguan (PC6) on myocardial endothelin and ultrastructure after ischemia-reperfusion. *Zhongguo Zhenjiu* 2002; **22**: 547-550
8 **Li YW**, Chen DF, Chang JS, Zhou JH. Research on the mechanism of regulatory action of acupuncture of Neiguan (PC6) on ischemic heart. *Guangzhou Zhongyiyao Daxue Xuebao* 2002; **19**: 108-111
9 **Lujan HL**, Kramer VJ, DiCarlo SE. Electroacupuncture decreases the susceptibility to ventricular tachycardia in conscious rats by reducing cardiac metabolic demand. *Am J Physiol Heart Circ Physiol* 2007; **292**: H2550-H2555
10 **Gao J**, Fu W, Jin Z, Yu X. A preliminary study on the cardioprotection of acupuncture pretreatment in rats with ischemia and reperfusion: involvement of cardiac beta-

adrenoceptors. *J Physiol Sci* 2006; **56**: 275-279
11 **Tsou MT**, Huang CH, Chiu JH. Electroacupuncture on PC6 (Neiguan) attenuates ischemia/reperfusion injury in rat hearts. *Am J Chin Med* 2004; **32**: 951-965
12 **Tjen-A-Looi SC**, Li P, Longhurst JC. Prolonged inhibition of rostral ventral lateral medullary premotor sympathetic neurons by electroacupuncture in cats. *Auton Neurosci* 2003; **106**: 119-131
13 **Ezzo J**, Streitberger K, Schneider A. Cochrane systematic reviews examine P6 acupuncture-point stimulation for nausea and vomiting. *J Altern Complement Med* 2006; **12**: 489-495
14 **Wang SB**, Liu JL. Clinical application of Neiguan (PC6) in the treatment of disorders of the digestive system-the related research progress. *World J Acu-Moxi* 2004; **14**: 1-9
15 **Dent HE**, Dewhurst NG, Mills SY, Willoughby M. Continuous PC6 wristband acupressure for relief of nausea and vomiting associated with acute myocardial infarction: a partially randomised, placebo-controlled trial. *Complement Ther Med* 2003; **11**: 72-77
16 **Cai GW**, Liang SZ, Huang XJ. Influence of electroacupuncture of Neiguan (PC6) and Gongsun (SP4) on ECG-ST of the standard lead II in coronary heart disease patients. *Zhongguo Zhenjiu* 1994; **14**: 7-8
17 **Zhou L**, Hu H, Li QW. Clinical observation on the therapeutic effect of acupuncture of Gongsun (SP4), Neiguan (PC6), etc for prevention and treatment of chemotherapy-induced vomiting. *Shandong Zhongyi Zazhi* 2006; **25**: 392-393
18 **Gardani G**, Cerrone R, Biella C, Galbiati G, Proserpio E, Casiraghi M, Arnoffi J, Meregalli M, Trabattoni P, Dapretto E, Giani L, Messina G, Lissoni P. A progress study of 100 cancer patients treated by acupressure for chemotherapy-induced vomiting after failure with the pharmacological approach. *Minerva Med* 2007; **98**: 665-668
19 **Lin JW**, Chen YG, Cai DF. Neuro-anatomical research on the combined application of Neiguan (PC6) and Gongsun (SP4). *Shanghai Zhenjiu Zazhi* 1999; **18**: 25-28
20 **Li ZR**. Shiyan Zhenjiuxue. 1st ed. Acupuncture and points of common experimental animals. Beijing: China Press of Traditional Chinese Medicine, 2003: 314-330
21 **Li HJ**, Li GP. Observation on the therapeutic effect of acupuncture treatment of functional dyspepsia. *Zhongguo Zhenjiu* 2004; **24**: 88-90
22 **Xu GX**, Liu YB. Clinical research on acupuncture treatment of functional dyspepsia. *Xiandai Zhongxiyi Jiehe Zazhi* 2005; **14**: 3076-3077
23 **Kelm M**, Schrader J. Control of coronary vascular tone by nitric oxide. *Circ Res* 1990; **66**: 1561-1575
24 **Wu WK**, Yang SY, Liu YA. Dynamic changes of nitric oxide metabolism during myocardial ischemia. *Zhongguo Bingli Shengli Zazhi* 2002; **18**: 1258-1261
25 **Li Q**, Wu XP. Effect of electroacupuncture of Neiguan (PC6) on active substances nitric oxide and blood platelet in acute myocardial ischemia rabbits. *Hubei Zhongyixueyuan Xuebao* 2006; **8**: 3-5
26 **Luo HO**, Tang Y, Pu Y, Huang ZL, Lan Q, Hu LX. Effect of acupuncture of different acupoint combinations on plasma nitric oxide in digestive ulcer patients. *Anhui Zhongyi Linchuang Zazhi* 2003; **15**: 16-17
27 **Wang L**, Peng CX, Zhou GP, Deng CQ. Effect of electroacupuncture of Zusanli (ST36) combined with Neiguan (PC6) or Gongsun (SP4) on nitric oxide and epidermicgrowth factor in gastric mucosal injury rats. *Zhongyiyao Xuekan* 2006; **24**: 2051-2052
28 **Niu WX**, He GD, Liu H, Qin XY. Effects and probable mechanisms of electroacupuncture at the Zusanli point on upper gastrointestinal motility in rabbits. *J Gastroenterol Hepatol* 2007; **22**: 1683-1689
29 **Li XP**, Yan J, Yi SX, Chang XR, Lin YP, Yang ZB, Huang A, Hu R. Effect of electroacupunture on gastric mucosal intestinal trefoil factor gene expression of stress-induced gastric mucosal injury in rats. *World J Gastroenterol* 2006; **12**: 1962-1965
30 **Yan P**, Ji LX, Hao CY, Yan LP, Yang EL. Effect of different acupoint recipes on gastric mucosal anatomical structure in acute gastric mucosal injury rats. *Zhongguo Zhenjiu* 2003; **23**: 217-219

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RAPID COMMUNICATION

Routine rectal retroflexion during colonoscopy has a low yield for neoplasia

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Abstract

AIM: To investigate the value of retroflexion in detecting neoplasia in the distal rectum.

METHODS: This was a prospective observational study performed in an academic endoscopy unit. Consecutive patients undergoing colonoscopy had careful forward viewing of the distal rectum by retroflexion. Of 1502 procedures, 1076 (72%) procedures were performed with a 140° angle of view colonoscope and 426 (28%) were performed with a 170° angle of view colonoscope. The outcome measurement was the yield of neoplasia in the distal rectum detected by forward viewing *vs* retroflexion.

RESULTS: A total of 1502 patients, including 767 (51%) females and 735 (49%) males, with mean age of 58.8 ± 12.5 years were enrolled. Retroflexion was successful in 1411 (93.9%) patients, unsuccessful or not performed because the rectum appeared narrow in 91 (6.1%). Forty patients had a polyp detected in the distal rectal mucosa. Thirty-three were visible in both the forward and retroflexed view (25 hyperplastic, 8 adenomatous). Seven polyps were visualized only by retroflexion (6 hyperplastic sessile polyps, one 4 mm sessile tubular adenoma). There was no significant difference in information added by retroflexion with 140° *vs* 170° angle of view instrument.

CONCLUSION: To our knowledge, this is the largest reported evaluation of retroflexion in the rectum. Routine rectal retroflexion did not detect clinically important neoplasia after a careful forward examination of the rectum to the dentate line. Since retroflexion has risks and may cause discomfort, the use of routine retroflexion should be at the discretion of the endoscopist.

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Key words: Colonoscopy; Colorectal polyps; Retroflexion; Rectum

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INTRODUCTION

Retroflexion is a commonly performed maneuver during colonoscopy[1], and has been strongly endorsed as an essential component of colonoscopy by experts, and specifically for the purpose of detecting neoplasia[1]. However, whether retroflexion substantially or significantly increases the detection of neoplasia during sigmoidoscopy and colonoscopy is controversial[2-5]. Among the four previously published reports of retroflexion, two failed to detect a single adenoma in retroflexion[2,3] and two detected lesions of such substantial size that the care with which the forward viewing of the distal rectum was performed is suspect[4,5]. Further, rectal retroflexion may be uncomfortable for patients and may result in perforation[6-9]. Retroflexion can be a useful therapeutic maneuver in the colon or rectum for polypectomy[10,11] and in the rectum for treatment of hemorrhoids[12,13]. However, two prospective studies found that anoscopy was superior to retroflexion for detection of hemorrhoids[14,15], and experts have opined that anoscopy provides more clinically useful information about hemorrhoids than can be detected by retroflexion[16]. Regardless of the contribution of retroflexion to the detection of non-neoplastic disease, the apparent mandate for performance of retroflexion during colonoscopy is the detection of neoplasia[1].

Given that the literature on the value of rectal

retroflexion during routine procedures is mixed[2-5], we prospectively assessed the detection of neoplasia during rectal retroflexion in 1502 consecutive patients.

MATERIALS AND METHODS

As part of our continuous quality improvement program, one of us (Douglas Kevin Rex) prospectively recorded the findings of retroflexion during colonoscopy from June 20, 2005 to August 15, 2006. Patients with poorly prepared colons, known history of rectal cancer or rectal resection, active Crohn's disease or ulcerative colitis, and patients referred for resection of known rectal polyps or for other therapeutic interventions in the rectum were excluded. Permission to review the data for publication was granted by the Institutional Review Board at Indiana University/Purdue University Indianapolis. The colonoscope used was prospectively recorded with regard to its angle of view (140º or 170º). Colonoscopes without cap-fitting were used as they were available and not otherwise selected.

The rectum was initially examined on forward view with withdrawal of the colonoscope to the dentate line with reflection of the tip in all directions using torque and up-down and right-left deviation as possible and appropriate. The endoscope was reinserted and retroflexed using the method of Grobe *et al*[2]. Manual rotation of the instrument was performed to inspect in a circumferential manner the anorectal area. The maneuver was considered successful if a complete 360º visualization of the distal rectum was obtained. Cases where retroflexion was successful, unsuccessful, or not attempted were documented. Whether polyps in the distal rectum were visible on forward or retroflexed views or both was prospectively recorded.

RESULTS

There were 1502 eligible patients, including 767 (51%) females and 735 (49%) males, with the mean age of 58.8 ± 12.5 years (range 17-95 years). Retroflexion was successfully performed in 1411 (93.9%) patients, unsuccessful in 1 patient because of a narrow rectum, and not attempted in 90 (6.0%) because the endoscopist judged that the rectum was too narrow. One thousand and seventy six (72%) procedures were performed with a 140º angle of view colonoscope while the rest 426 (28%) were performed with a 170º angle of view colonoscope.

We found that 40 of 1411 (2.8%) patients had a polyp detected in the distal rectum, of which 33 were visible in both the forward and retroflexed view (25 hyperplastic, 8 adenomatous), and 7 were visible only during retroflexion (6 hyperplastic and 1 adenomatous). Of the 8 adenomas in the distal rectum detected on both the forward and retroflexed view, the mean size was 7.3 mm (range 3-15 mm), 3 were pedunculated, 5 were sessile, 7 were tubular and 1 was tubulovillous. Each adenoma was detected in a separate patient and none had high-grade dysplasia. The adenoma detected only on retroflexion was a 4 mm tubular adenoma with low-grade dysplasia. Thus, 1 of 1411 patients had an adenoma detected only by retroflexion and none had an advanced adenoma or cancer.

Using 140º angle of view colonoscopes, 6 of 1076 (0.56%) patients had a polyp detected only by retroflexion, including the patient with the adenoma. Using 170º angle of view instruments, 1 of 426 patients (0.23%) had a polyp detected only by retroflexion (P = 0.41; Chi-square test).

DISCUSSION

In this prospective series, we found that routine performance of retroflexion during colonoscopy added little to the diagnostic yield of neoplasia. Importantly, retroflexion was performed only following a detailed forward-viewing examination of the entire rectum to the dentate line with a concerted attempt to view the entire rectal wall, including the distal rectum, on forward view. We also perform a careful digital rectal examination prior to colonoscope insertion, and none of the distal rectal polyps reported here were appreciated by palpation. Given that perforation has been reported to result from retroflexion[6-9], and that we are anecdotally aware of medical-legal actions against endoscopists for perforations occurring from retroflexion, we believe our data support a perspective that retroflexion during lower endoscopy is not mandated and must be left to the discretion of the endoscopist.

Previous studies have drawn varied conclusions with regard to the value of routine retroflexion during lower bowel endoscopy. Cutler and Pop[3] reported no adenomas detected only by retroflexion in 453 patients and questioned the value of routine retroflexion. Grobe *et al*[2] deemed retroflexion valuable in 75 patients, but did not document a single adenoma detected only by retroflexion. Hanson *et al*[4] detected four adenomas in 526 patients that were visible only on rectal retroflexion, and one was a 15 mm tubulovillous adenoma. Varadarajulu *et al*[5] reported the highest yield of routine retroflexion. Among 590 patients (91% male), 6 had adenomas detected only on retroflexion, of which all were > 1 cm in size and 2 were pedunculated. They stated that 50% of the distal rectal lesions were visible only on retroflexion. For unclear reasons, we did not encounter this prevalence of advanced pathology in the distal rectum either with forward or retroflexed views. Although the polyps we encountered were generally smaller, we could still visualize a high percentage (83%) on forward view. Thus, perhaps the value of routine retroflexion could be greater than we encountered when performed in different populations or if a different technique from the one that we used was employed to perform the forward examination that precedes retroflexion.

From an imaging perspective, the goal in exposing colorectal mucosa during colonoscopy is to expose and examine all of it. Based upon the senior authors' experience, knowledge of rectal anatomy, and logic, the need for retroflexion to complete exposure of the rectal mucosa is likely to vary based on each patients' unique

rectal anatomy. Thus, in some patients with narrow rectum, the rectal walls are visible circumferentially in a continuous fashion as they extend proximally when viewed from the internal anal verge in the forward view. Logically, retroflexion has little to add in such patients. In patients with a larger rectum, the posterior rectal wall may not be visible continuously when the instrument is looking forward from the anal verge. In this instance, retroflexion is logically more likely to add information, to be easier to perform, safer to perform, and more comfortable for the patient. In the senior authors' experience, the distal rectal walls are more likely to be continuously visible when viewed in the forward view from the anal verge using a 170º angle of view colonoscopy, as compared to a 140º angle of view instrument. In this study, the fraction of patients with a polyp detected only on retroflexion was lower with the 170º angle of view instrument compared to the 140º instrument, but the difference was not significant. Additional study of the impact of 170º angle of view instruments on the value of routine rectal retroflexion seems warranted.

Limitations of the current study include the relatively low yield of neoplasia in the distal rectum. However, the endoscopist of this study reported the highest adenoma detection rates in the literature[17], so there is no reason to believe that the study population had a low prevalence of adenomas. Indeed, the low prevalence of distal rectal adenomas may reflect the progressive shift of neoplasia toward the proximal colon in recent decades. A second limitation is that the procedures were performed by a single endoscopist. Endoscopists with less effective forward viewing technique could logically expect a higher yield from retroflexion.

Retroflexion in the rectum is unquestionably a useful diagnostic or therapeutic maneuver during colonoscopy in some patients and should be mastered by all colonoscopists. Retroflexion may provide useful information about benign anal disease and can provide a critical advantage for completion of polypectomy in some cases. Our study, however, supports the conclusion of Cutler that routine rectal retroflexion adds little to the detection of rectal neoplasia after careful forward viewing. Given the low yield of retroflexion when careful forward viewing has been completed, as well as the discomfort and risk of retroflexion, performance of retroflexion in routine cases should be at the discretion of the endoscopist. Anecdotally, we recommend that the appropriateness of retroflexion be considered after reaching the anal verge in the forward view and assessing whether the rectal walls are continuously visible as they extend proximally, as well as the size and anatomy of the patient's rectum.

COMMENTS

Background

Some experts believe that retroflexion or performance of a U-turn must be performed routinely during colonoscopy to rule out neoplasia in the distal rectum However, the available literature is mixed with regard to the value of routine retroflexion.

Applications

The study found that routine performance of retroflexion has a very low yield for neoplasia. Since retroflexion has a low but real risk, the results suggest that retroflexion is not routinely required to achieve a highly effective colonoscopy.

Terminology

Rectal retroflexion refers to a maneuver during colonoscopy in which the instrument tip is deflected in U-turn so that the imaging system is directed backward, allowing clear visualization of the distal rectum.

Peer review

Every year many patients experience complications associated with the practice of retroflexion. Prospectively collected data on whether or not retroflexion is essential is very much needed.

REFERENCES

1 **Waye JD**. What constitutes a total colonoscopy? *Am J Gastroenterol* 1999; **94**: 1429-1430
2 **Grobe JL**, Kozarek RA, Sanowski RA. Colonoscopic retroflexion in the evaluation of rectal disease. *Am J Gastroenterol* 1982; **77**: 856-858
3 **Cutler AF**, Pop A. Fifteen years later: colonoscopic retroflexion revisited. *Am J Gastroenterol* 1999; **94**: 1537-1538
4 **Hanson JM**, Atkin WS, Cunliffe WJ, Browell DA, Griffith CD, Varma JS, Plusa SM. Rectal retroflexion: an essential part of lower gastrointestinal endoscopic examination. *Dis Colon Rectum* 2001; **44**: 1706-1708
5 **Varadarajulu S**, Ramsey WH. Utility of retroflexion in lower gastrointestinal endoscopy. *J Clin Gastroenterol* 2001; **32**: 235-237
6 **Chu Q**, Petros JG. Extraperitoneal rectal perforation due to retroflexion fiberoptic proctoscopy. *Am Surg* 1999; **65**: 81-85
7 **Fu K**, Ikematsu H, Sugito M, Sano Y, Kato S, Kuroki Y, Ishikawa T, Kaji Y. Iatrogenic perforation of the colon following retroflexion maneuver. *Endoscopy* 2007; **39** Suppl 1: E175
8 **Ahlawat SK**, Charabaty A, Benjamin S. Rectal perforation caused by retroflexion maneuver during colonoscopy: closure with endoscopic clips. *Gastrointest Endosc* 2008; **67**: 771-773
9 **Thornton SC**, Hirshorn SA, Bradway M, Levien D. Anoscopy vs. retroflexion for evaluation of the anal canal. *Dis Colon Rectum* 2002; **45**: 1120-1121; author reply 1121
10 **Rex DK**, Khashab M. Colonoscopic polypectomy in retroflexion. *Gastrointest Endosc* 2006; **63**: 144-148
11 **Pishvaian AC**, Al-Kawas FH. Retroflexion in the colon: a useful and safe technique in the evaluation and resection of sessile polyps during colonoscopy. *Am J Gastroenterol* 2006; **101**: 1479-1483
12 **Fukuda A**, Kajiyama T, Arakawa H, Kishimoto H, Someda H, Sakai M, Tsunekawa S, Chiba T. Retroflexed endoscopic multiple band ligation of symptomatic internal hemorrhoids. *Gastrointest Endosc* 2004; **59**: 380-384
13 **Berkelhammer C**, Moosvi SB. Retroflexed endoscopic band ligation of bleeding internal hemorrhoids. *Gastrointest Endosc* 2002; **55**: 532-537
14 **Kelly SM**, Sanowski RA, Foutch PG, Bellapravalu S, Haynes WC. A prospective comparison of anoscopy and fiberendoscopy in detecting anal lesions. *J Clin Gastroenterol* 1986; **8**: 658-660
15 **Harish K**, Harikumar R, Sunilkumar K, Thomas V. Videoanoscopy: useful technique in the evaluation of hemorrhoids. *J Gastroenterol Hepatol* 2008; **23**: e312-e317
16 **Fernández-Bermejo M**, Maté-Jim nez J. Evaluation of hemorrhoids with the retroflexed fiberoptic colonoscope. *Gastrointest Endosc* 1999; **50**: 305-306
17 **Rex DK**, Helbig CC. High yields of small and flat adenomas with high-definition colonoscopes using either white light or narrow band imaging. *Gastroenterology* 2007; **133**: 42-47

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RAPID COMMUNICATION

Origin of and therapeutic approach to cardiac syndrome X: Results of the proton pump inhibitor therapy for angina-like lingering pain trial (PITFALL trial)

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Abstract

AIM: To investigate the frequency of gastroenterological diseases in the etiology and the efficacy of proton pump inhibitors (PPIs) in the treatment of cardiac syndrome X (CSX) as a subform of non-cardiac chest pain (NCCP).

METHODS: We investigated 114 patients with CSX using symptom questionnaires. A subgroup of these patients were investigated regarding upper gastrointestinal disorders (GIs) and treated with PPI. Patients not willing to participate in investigation and treatment served as control group.

RESULTS: Thirty-six patients denied any residual symptoms and were not further evaluated. After informed consent in 27 of the remaining 78 patients, we determined the prevalence of disorders of the upper GI tract and quantified the effect of treatment with pantoprazole. We found a high prevalence of gastroenterological pathologies (26/27 patients, 97%) with gastritis, gastroesophageal reflux disease (GERD) and acid reflux as the most common associated disorders. If treated according to the study protocol, these patients showed a significant improvement in the symptom score. Patients treated by primary care physicians, not according to the study protocol had a minor response to treatment (n = 19, -43%), while patients not treated at all (n = 26) had no improvement of symptoms (-0%).

CONCLUSION: Disorders of the upper GI tract are a frequent origin of CSX in a German population and can be treated with pantoprazole if given for a longer period.

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Key words: Non-cardiac chest pain; Gastroesophageal reflux disease; Proton pump inhibitor; Cardiac syndrome X

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INTRODUCTION

Cardiac syndrome X (CSX) is a specific subform of non-cardiac chest pain (NCCP), defined as recurrent episodes of substernal chest pain or discomfort (exercise-related or not) with pathologic electrocardiographic (ECG) changes, but without significant cardiac abnormalities[1]. As relatively specific symptom complex involving ECG abnormalities, it often leads to cardiac catheterization after non-invasive cardiac diagnostic procedures, including treadmill exercise. Several studies indicate that about 15%-20% of all invasive cardiac diagnostic

procedures can not detect any significant coronary abnormalities, reliably ruling out cardiac origin of the symptoms, supporting the assumption that the prevalence of NCCP (including CSX) is approximately 25% in the general population[2]. Several studies have contributed in elucidation of the origin and possible therapeutic approaches of NCCP with partially quite heterogenous results[2]. However, there are very limited data for the subgroup of CSX patients.

Interestingly, many patients with NCCP are not forwarded to gastroenterologists for further diagnostic work-up after exclusion of cardiac origin for chest pain[3]. Thus, they remain with their symptoms, which may cause significant economic and psychological burden because of frequent work absenteeism, frequent consultation of healthcare providers and reduced quality of life[4]. NCCP represents an important determinant of resource utilization and health care costs[5]. A defined further diagnostic work-up and targeted therapy of NCCP, therefore, is not only useful for patients, but also for the society as a whole.

Previous studies have shown that NCCP originates mostly from GERD and other gastrointestinal diseases, including motility disorders, often combined with visceral hypersensitivity or abnormal cerebral processing of visceral stimuli[6]. Musculoskeletal diseases may be a minor origin of NCCP. Additionally, psychological abnormalities play an important role in causing or modulating NCCP[7]. Published mechanistic hypotheses involve the presence of an esophagocardiac reflex, leading to coronary spasms when the distal esophageal mucosa is exposed to hypochloric acid[8]. This mechanism is also called "linked angina" and would serve as an explanation for NCCP in patients with clear pathologic ECG signs despite normal coronary angiography, which has frequently been termed as CSX[9,10].

Treatment studies of general NCCP focus on proton pump inhibitors (PPIs), and there are clear data that omeprazole, rabeprazole and lansoprazole are effective in symptom relieve of NCCP[10].

To investigate prevalence of disorders of the upper gastrointestinal tract and treatment in CSX, we designed a study enrolling patients with abnormal treadmill exercise results, but angiographic exclusion of coronary abnormalities. Firstly, these patients were investigated thoroughly regarding gastrointestinal disorders (GIs). In a second step, they were included into a therapeutic trial with pantoprazole, initially intended as a randomized, double-blind study, to test the efficacy of the drug in this subgroup of NCCP.

MATERIALS AND METHODS

Inclusion and exclusion criteria

Patients at least 18 years of age with recurrent chest pain, a pathological treadmill exercise and a normal coronary angiogram fulfilled the inclusion criteria of the study. Patients were excluded from the study if they had medications lowering the pressure of the lower esophageal sphincter (calcium antagonists, nitrates), were already on acid-suppressive drugs during cardiologic work-up (PPI or histamine blockers) or had clearly identifiable and established musculoskeletal pain in the thoracic spine or the chest. The study protocol was approved by the Local Ethics Committee.

Table 1 Mean age and proportion of female patients in all patient groups

Patient group	Age (yr, mean ± SD)	Female gender (%)
Study protocol group	59 ± 10	56
External PPI group	63 ± 8	63
Control group	62 ± 9	73

Patients

In the period between May, 2003 and November, 2004, all patients with chest pain, a pathological treadmill exercise and with a normal coronary angiogram were identified in the Cardiology Center of Aachen University, Germany. All patients were contacted by phone and, after testing the eligibility, were asked whether they still suffered from chest pain. Patients without any symptoms immediately after the angiography and without any specific medication were considered to have chest pain at least modulated by psychological diseases and were excluded from further questioning. Patients with ongoing chest pain, but treated with PPI after the normal angiography by their primary care physicians (PPI test approach[10]), were included into an external PPI group. Patients with ongoing chest pain and no specific medication were asked to join the study.

In total, 78 patients were eligible for the study, but only 72 agreed to participate in data collection. Twenty-seven patients gave written informed consent to participate in the PITFALL study protocol including diagnostic work up and therapy with pantoprazole. The initial study design is depicted in Figure 1A. The planned study randomization arms A and B were closed shortly after beginning of the study, due to low patient recruitment. Ninety seven percent (26/27) of the study patients had to be included into study arm C because of pathological findings during the diagnostic work-up. In order to establish an external control group, we included patients with ongoing chest pain who were not willing to participate in the diagnostic and therapeutic study in another group. These patients and the patients in the external PPI group received questionnaires regarding symptoms and treatment at study entry and 8 wk after study entry. The adapted study design is shown in Figure 1B. Characteristics of study patients from each group are given in Table 1.

Quantitation of symptoms

All patients (study group, both external groups) ranked the symptoms using a visual analogue scale (VAS) ranging from 0 to 10 each for intensity, duration and frequency of chest pain. Characteristics of each scale are given in Table 2. The three parameters were added (maximum score: 30 points) and used as symptom score

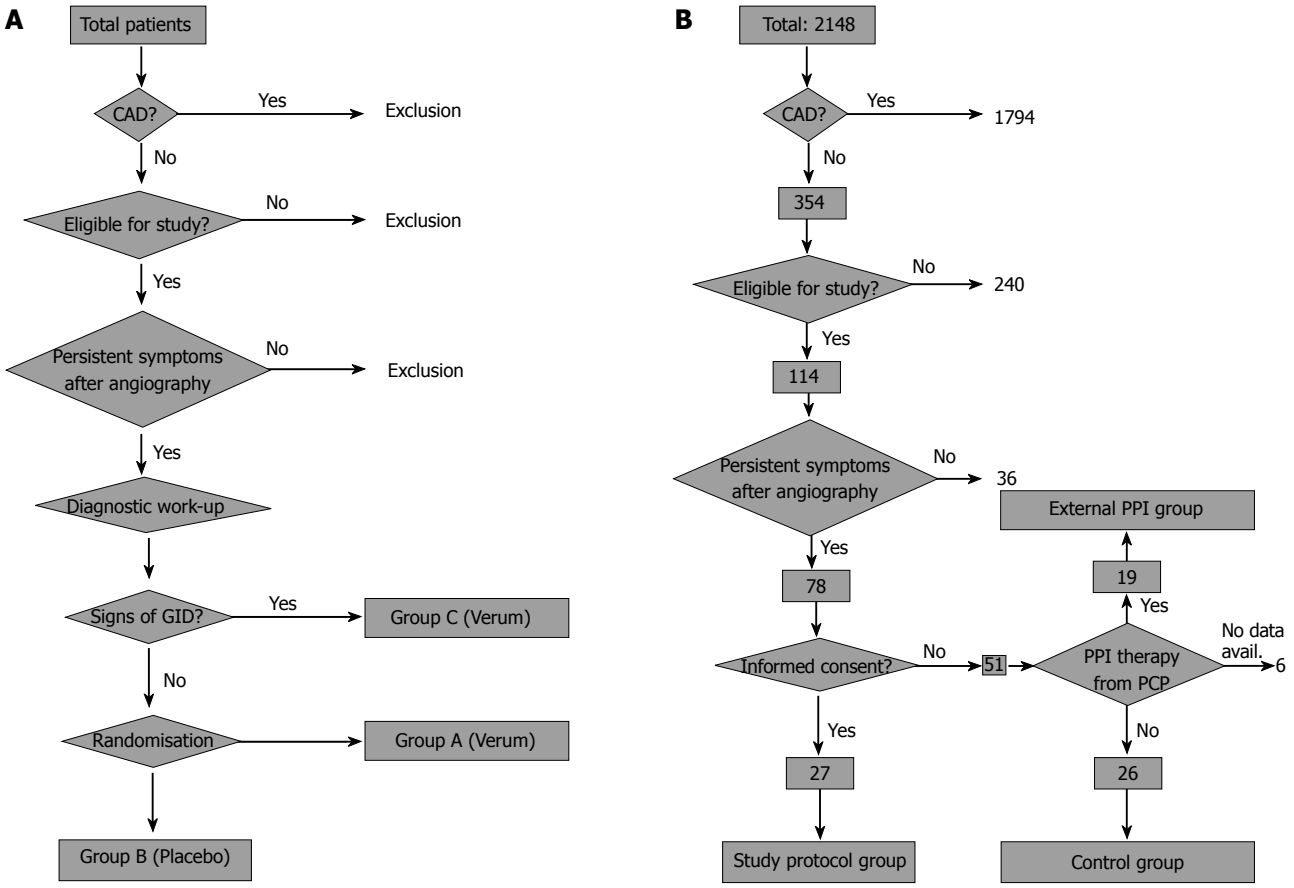

Figure 1 Study design. A: Original study design with 3 patient groups. Randomization was planned after the diagnostic procedures in order to randomize only patients without overt signs of gastrointestinal disease. After recruitment of the first 10 patients only for patient group C, the other two patient groups were closed and the study design was adapted as depicted in B (CAD: Coronary artery disease; GID: Gastrointestinal disease); B: Adapted study design without randomization. To compare the results of per protocol treatment to patients treated in a non-standardized fashion or untreated patients, an external PPI group and a control group were built by recruiting patients who were treated by their primary care physicians (external PPI group, 19 patients) or were not willing to undergo treatment at all (control group, 26 patients). These patients were not matched to the study protocol group and most of them did not receive diagnostic procedure regarding gastrointestinal diseases (CAD: Coronary artery disease; PCP: Primary care physician).

Table 2 Definitions of ranks in visual analogue scale (characteristics of symptoms)

Grade	Frequency	Duration	Intensity
10	Permanent	Permanent	
9	Several times daily	Almost whole day	
8	Daily	More than 6 h	
7	Almost daily	More than 2 h	
6	Several times weekly	About 1 h	Grading by patients
5	Up to 3 times weekly	10-30 min	on a scale from 0 to 10
4	Once weekly	5-10 min	
3	Several times in 1 mo	1-5 min	
2	Up to 3 times in 1 mo	Less than 1 min	
1	Once per month	A few seconds	
0	Never or very seldom	Never	

Patients were asked for symptoms and were helped to grade the frequency, duration and intensity using the characteristics below.

(VAS total score). These four different VAS scores were evaluated before and after therapy (in the external placebo group in a time difference of 8 wk). Patients in both external groups were also asked regarding their co-medication during the 8-wk period.

Diagnostic work-up

All patients in the study group received gastroscopy, 24-h esophageal pH-monitoring and concurrent impedancometry and manometry (CIM) in the gastroenterological endoscopy unit of Aachen University Medical Center. The latter procedure (CIM) was conducted using a custom-made catheter consisting of 11 impedance segments (each 2 cm long) and 4 semiconductor pressure transducers. The solid-state pressure transducers are located between the impedance channels 1-2, 4-5, 7-8 and 10-11 with an intertransducer distance of 6 cm. The configuration of the catheter allows a simultaneous registration of bolus transport and esophageal peristalsis (details in reference by Nguyen *et al*[11]). The catheter was passed through the nose into the esophagus with the most distal pressure sensor placed at the level of the lower esophageal sphincter.

If the diagnosis of acid-related disorders such as gastritis, ulcer disease or GERD was established, the patients were enrolled into study arm C. This led to closure of study arms A and B as described above.

Diagnostic findings from patients in the external groups were not used for study purposes.

Therapeutic study protocol

Patients in study arm C received pantoprazole 40 mg bid for 8 wk. After this period, treatment strategy was

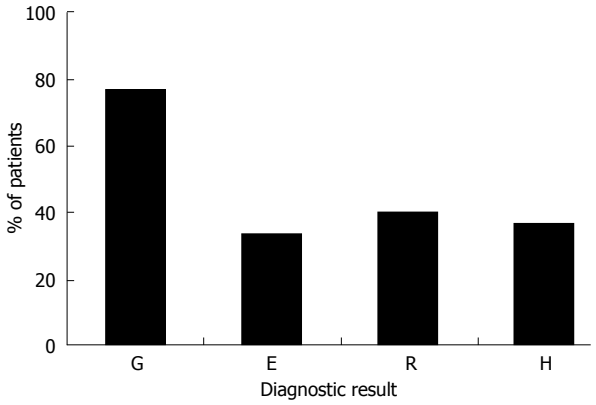

Figure 2 Percentage of study protocol patients with gastritis (G, partially erosive or ulcerative), esophagitis (E), reflux as diagnosed by pH monitoring (R) and/or hiatal hernia (H). Multiple diagnoses in a patient were possible so that the total percentage is above 100.

open and depending on symptoms. If tested positive for *H pylori*, eradication treatment was initiated. Intolerance of pantoprazole led to prescription of omeprazole 20 mg bid. After completion of the therapeutic study arm, patients gave information about their symptoms using the same VAS scores as described above and most patients underwent control investigations of gastroscopy and 24-h esophageal pH-monitoring.

Statistical analysis

Categorical data were summarized as absolute and corresponding relative frequencies. Observed VAS scores were condensed by minimum, maximum, median and corresponding interquartile range, separately for the two different time points (before therapy, after therapy) in each of the three study groups. Parallel boxplots were used for graphical comparison of the obtained VAS scores between the time points before and after therapy within each study group.

In each group, paired Wilcoxon tests were conducted for comparison of the different VAS scores before and after therapy. A global significance level of $\alpha = 5\%$ was chosen. Only the results (*P*-values) of the tests carried out with respect to the VAS total scores were interpreted in a confirmatory fashion, while results for the scores of intensity, duration and frequency were meant solely exploratively. Thus, $P \leqslant 0.05$ was considered statistically significant. All statistical analyses were performed with the statistical analysis software package R (http://www.R-project.org).

RESULTS

Large subgroup of patients without symptoms after angiography

Only patients with normal coronary angiography performed for chest pain, with abnormal treadmill exercise and without any further diagnostic procedures or therapy after angiography were included into the study. In telephone interviews, 114 eligible patients were asked for persisting symptoms following the diagnostic procedure. Thirty-six (31%) of the 114 interviewed patients without further therapy denied any residual symptoms. It is likely that a substantial subset of these patients had chest pain due to psychosomatic reasons. These patients were excluded from further investigations of this study.

Further stratification of study population

Only 27 of the 78 patients meeting the inclusion criteria of the study group agreed to join the diagnostic and therapeutic study according to the PITFALL study protocol. Of the remaining 51 patients, 19 discussed the possibility of gastrointestinal diseases with their primary care physicians and received diagnostic work-up and therapy from them, mostly in the form of a PPI test. They agreed to give information about their symptoms during and after the primary care physician-guided therapy. The gained information was included in the study and the patients were grouped as "external PPI group". The prescribed therapy did not follow the study protocol in most cases regarding dose and length of treatment (data not shown) and involved prescription of several different proton-pump inhibitors, as well as different length and follow-up of therapy.

Twenty-six of 51 patients denied any diagnostic procedure and therapy and explained that they were not interested in any further work-up of persisting symptoms. These patients, however, agreed to give information about their symptoms in certain intervals and were grouped as "external control group"; the gained information (i.e. different VAS scores at different time points) of these patients was included into statistical evaluation of the study as well. Age and gender distribution of all patient groups are given in Table 1.

Six of 51 patients refused any cooperation or data collection and were excluded from further questioning.

Prevalence of GIs in NCCP patients

Fifty-seven percent of the study patients had signs of acid reflux consistent with GERD (De Meester Score > 14, pH time < 4 more than 5% and/or gastroscopic evidence of esophagitis). Relevant gastritis with or without erosive changes of the mucosa was present in 78% of the patients (histologically proven), from which 27% had no signs of GERD. *H pylori* was detected in 11% and eradicated using the French triple therapy with amoxicillin and clarithromycin. In total, 97% (26/27) patients showed a relevant acid-related disorder (GERD or histologically proven gastritis) of the upper gastrointestinal tract (Figure 2).

Symptomatic improvement in the study protocol group

Results of the total symptom score before and after PPI therapy are given in Figure 3A. The study patients experienced a statistically significant improvement in symptoms after 8 wk of PPI therapy in doubled standard dose ($P < 0.0001$). If analyzed in an explorative fashion for all subscores, this improvement turned out

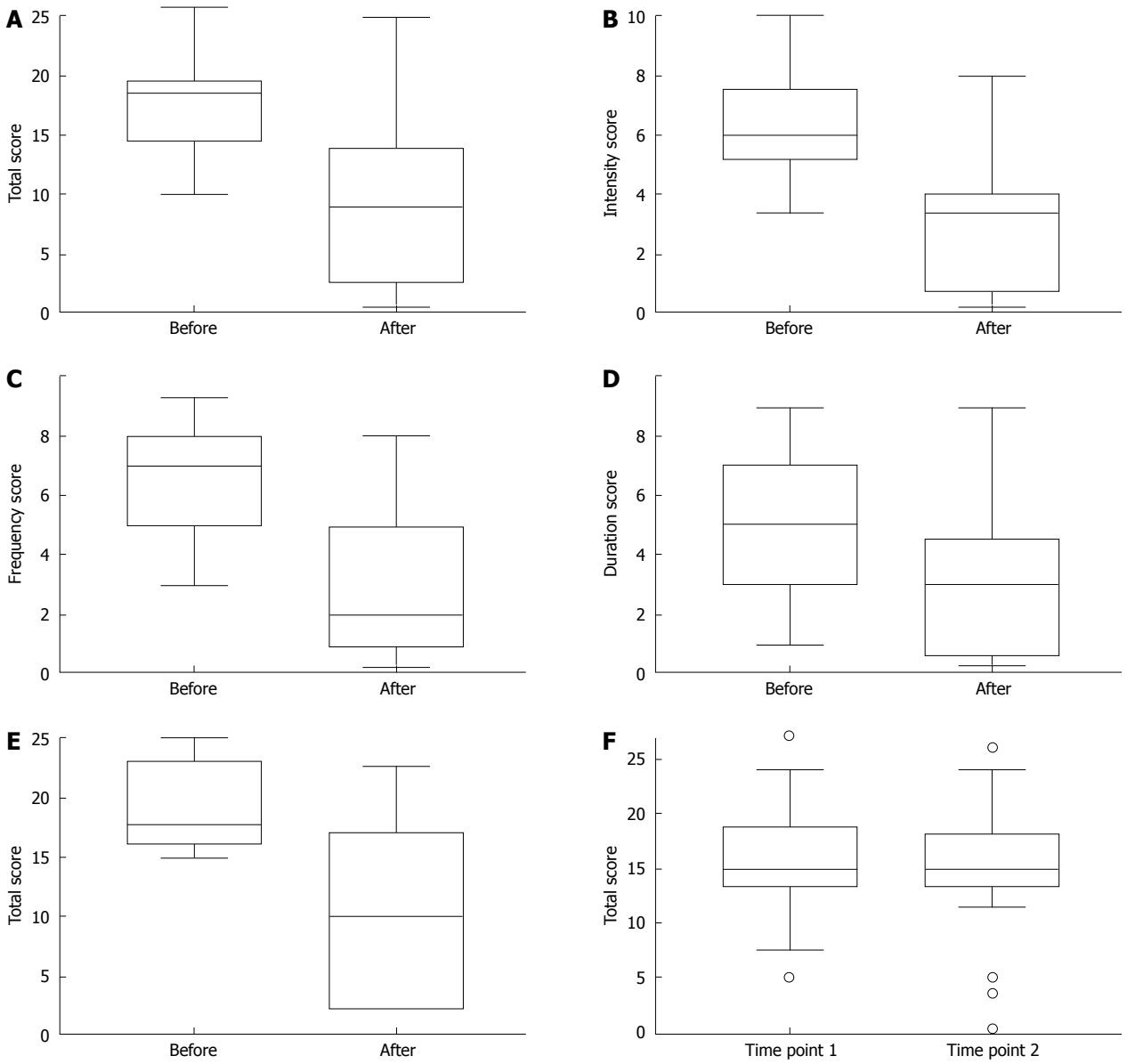

Figure 3 Results of the different group by graphical representation (parallel boxplot). A: Total score; B: Intensity score; C: Frequency score; D: Duration score. The three symptom scores (B-D) are before and after treatment with pantoprazole in the study protocol group. There was statistically significant difference between the scores before and after therapy ($P < 0.001$ for all four scores). E: Graphical representation (parallel boxplot) of the total score before and after treatment in the external PPI group. The total score after treatment was significantly different compared to the total score before treatment ($P = 0.0011$); F: Graphical representation (parallel boxplot) of the total score before and after an observation period of 8 wk in the control group. There was no significant difference between the scores before and after the observation period ($P = 0.2448$).

to be valid for all three quantities (intensity, frequency and duration of symptoms, Figure 3B-D, $P < 0.001$ for all quantities).

Symptomatic improvement in the external PPI group

Similar to the study group, patients in the external PPI group reported statistically significant improvement in symptoms after a variable PPI therapy ($P = 0.0011$).

The improvement was not as large as in the study group (Figure 3E). Explorative analysis of the subscores showed a benefit for all three symptom quantities (similar to the study group, data not shown).

No symptomatic improvement in the external control group

In contrast to the first two groups, we observed no improvement of the total VAS score for the patients in the external control group. Scores at start and 8 wk later without any specific treatment were not statistically significant different ($P = 0.2448$, Figure 3F).

Identification of specific risk factors for gastrointestinal causes of NCCP or treatment success

In the original study protocol, a multivariate analysis was planned to identify specific risk factors for stratifying NCCP patients with gastrointestinal causes. Furthermore, we wanted to identify specific parameters to predict the extent of treatment success in the study group. Due to the changed study design and the low patient volume in all groups, especially in the study protocol therapy group, we did not conduct such data analysis.

DISCUSSION

Patients with a special subgroup of NCCP, the so-called CSX, were investigated in the present study. This syndrome is defined as recurrent chest pain with pathological electrocardiography, but normal coronary angiography. Given the results of pathophysiological studies and the common efferent and afferent nerve supply of the upper gastrointestinal tract and the heart[8,9], our results confirm that symptoms of CSX patients are almost invariably caused by acid-related disorders of the upper gastrointestinal tract. The described symptoms consequently could be treated very well using the doubled standard dose of pantoprazole.

Interestingly, there is a large group of patients with CSX, which presents with much improved or absent symptoms after coronary angiography. This seems to indicate that many patients probably exhibit stress-induced symptoms of psychosomatic character. Recent studies show a prevalence of psychological abnormalities between 17% and 43% in NCCP patients[7], which is in line with our results (31%) in patients with CSX. Additionally, stress can provoke gastrointestinal acid-related disorders as well. However, many patients with psychosomatic origin of NCCP still have persistent symptoms after cardiac diagnostic procedures and they often do not respond to PPI therapy. In our study protocol group, 2/27 patients (7%, but 30% in the external PPI group) showed no or only little benefit of PPI therapy (defined as improvement < 10%), so that these patients may have psychosomatic disorders as well. Very recently, it was shown that hypnosis might contribute to symptom improvement in this patient group[12].

The referral rate of patients with chest pain, after exclusion of cardiac abnormalities, was low in our study. Only 8 of 78 (10%) patients were already in diagnostic work-up for or in treatment of GIs. This result is in agreement with previous studies indicating a low interest or strain in further diagnostic work-up after cardiologic examination[2]. Whether this low referral rate is due to the patients or their primary care physicians is not completely clear and deserves further studies. Nevertheless, this finding clearly demonstrates the need for a closer follow-up of NCCP patients in every day practice.

All but one patient in the treatment group had evidence of upper gastrointestinal tract disease of different degrees. The most important diagnostic tool in this setting was gastroscopy, followed by 24-h esophageal pH-monitoring. Interestingly, we could not find one relevant motility disorder using a highly sensitive method with measurement of impedance and motility in the esophagus (CIM). This indicates that motility disorders are rare in causing the investigated subform of NCCP/CSX. Though dysmotility has often been demonstrated in NCCP[13], its impact on chest pain is unclear. There is no specific treatment for dysmotility and PPI therapy has no influence on the motility disorder. Data observing an effect of PPI therapy on chest pain associated with motility disorders[14] showed only a very minor effect on the respective motility disorder itself. These data indicate that dysmotility only is a facet or associated symptom of NCCP, not the main origin[15].

Interestingly, a large subgroup of patients presented with different degrees of histologically proven gastritis. These patients did not complain about epigastric pain, but exhibited chest pain. GERD has been the central investigated acid-related disorder in causing NCCP[6]. Gastritis may play a more important role in the etiology of CSX or has been underestimated up till now for causing NCCP. It is also possible that gastritis is, in our study, simply an expression for esophageal acid reflux, which could not be detected by 24-h esophageal pH-monitoring. It has been shown that extending the recording time of pH measurement to 48 h increases the sensitivity of the procedure[16]. Further studies with larger patient groups and improved pH measurement are necessary to solve this issue.

Treatment with pantoprazole, in the treatment group, led to a significant improvement in the symptom score. This supports our assumption that the symptoms were caused by the diagnosed GIs including gastritis, and indicates the value of the tested substance in treating NCCP patients. Seven of 27 patients were completely asymptomatic after 8 wk of treatment with pantoprazole and 54% of the patients showed an improvement of at least 50% in the symptom score. In the external PPI group, the improvement of symptoms was lower, mostly owing to shorter treatment periods and lower dosages of PPI. This clearly indicates that treatment of gastrointestinal NCCP causes requires a clear protocol with initial high doses (double the standard dose per day) and long treatment periods. Several recent studies, especially those using a "test therapy approach", treated for only 7 d which may have led to underestimation of the PPI benefit[10]. In the external control group, we observed no improvement in the symptom score. This is in line with our expectations and underlines, though we were not able to randomize these patients, the treatment effect of pantoprazole in our study group.

Unfortunately, we were not able to identify any significant predictor (other than the diagnosis of an acid-related disorder) for treatment response in our patient group. Additionally, our sample size was too small to identify predictors for NCCP before cardiac catheterization in order to reduce normal coronary angiographic results and induce a PPI test treatment in such patients which may benefit from this approach. It has been shown in the past that the "PPI-test" is cost-effective in terms of avoiding the traditional invasive gastroenterological diagnostic strategy[17]. However, we consider an approach to identify patients with NCCP before they receive invasive cardiological diagnostic procedures much more important, because these diagnostic procedures are more expensive and more prone to side effects than gastroscopy and 24-h esophageal pH-monitoring.

In conclusion, we have shown that in patients with CSX, acid-related disorders are frequent and respond very well to a long-term therapy with pantoprazole.

Motility disorders obviously are extremely rare, as shown by a very sensitive diagnostic tool (CIM) in our study. The rate of gastroenterological referral after exclusion of cardiac abnormalities is still too low. It is useful to conduct additional studies with more patients, which should lead to criteria to identify patients having a benefit from a PPI therapy before coronary angiography is done.

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COMMENTS

Background

Non-cardiac chest pain (NCCP) and its subform cardiac syndrome X (CSX) have a high incidence in Western countries and represent a significant health burden.

Research frontiers

In this study, authors investigated, for the first time, the pathogenesis and possible treatment of the specific NCCP subform CSX in a German population.

Innovations and breakthroughs

The authors established that gastrointestinal disorder (GI)-tract-associated disorders in a high percentage are responsible for CSX and that the symptoms can be treated by proton pump inhibitor (PPI).

Applications

The results of this study are important for primary care physicians, cardiologists and gastroenterologists in the medical care of chest pain patients.

Terminology

NCCP denotes angina-like typical chest pain which could not be linked to the heart by specific investigations (including angiography). CSX is a subform of NCCP and means that these patients showed typical electrocardiographic (ECG) abnormalities despite a normal angiogram.

Peer review

This is an interesting paper. It investigated the frequency of gastroenterological diseases in the etiology and the efficacy of PPIs in the treatment of CSX as a subform of NCCP. It provided useful information to the readers.

REFERENCES

1 **Kachintorn U**. How do we define non-cardiac chest pain? *J Gastroenterol Hepatol* 2005; **20** Suppl: S2-S5
2 **Fass R**, Dickman R. Non-cardiac chest pain: an update. *Neurogastroenterol Motil* 2006; **18**: 408-417
3 **Wong WM**, Beeler J, Risner-Adler S, Habib S, Bautista J, Fass R. Attitudes and referral patterns of primary care physicians when evaluating subjects with noncardiac chest pain--a national survey. *Dig Dis Sci* 2005; **50**: 656-661
4 **Eslick GD**, Talley NJ. Non-cardiac chest pain: predictors of health care seeking, the types of health care professional consulted, work absenteeism and interruption of daily activities. *Aliment Pharmacol Ther* 2004; **20**: 909-915
5 **Eslick GD**. Noncardiac chest pain: epidemiology, natural history, health care seeking, and quality of life. *Gastroenterol Clin North Am* 2004; **33**: 1-23
6 **Van Handel D**, Fass R. The pathophysiology of non-cardiac chest pain. *J Gastroenterol Hepatol* 2005; **20** Suppl: S6-S13
7 **Aziz Q**. Acid sensors in the gut: a taste of things to come. *Eur J Gastroenterol Hepatol* 2001; **13**: 885-888
8 **Chauhan A**, Petch MC, Schofield PM. Cardio-oesophageal reflex in humans as a mechanism for "linked angina". *Eur Heart J* 1996; **17**: 407-413
9 **Kaski JC**. Pathophysiology and management of patients with chest pain and normal coronary arteriograms (cardiac syndrome X). *Circulation* 2004; **109**: 568-572
10 **Wong WM**. Use of proton pump inhibitor as a diagnostic test in NCCP. *J Gastroenterol Hepatol* 2005; **20** Suppl: S14-S17
11 **Nguyen HN**, Silny J, Matern S. Multiple intraluminal electrical impedancometry for recording of upper gastrointestinal motility: current results and further implications. *Am J Gastroenterol* 1999; **94**: 306-317
12 **Jones H**, Cooper P, Miller V, Brooks N, Whorwell PJ. Treatment of non-cardiac chest pain: a controlled trial of hypnotherapy. *Gut* 2006; **55**: 1403-1408
13 **Dekel R**, Pearson T, Wendel C, De Garmo P, Fennerty MB, Fass R. Assessment of oesophageal motor function in patients with dysphagia or chest pain - the Clinical Outcomes Research Initiative experience. *Aliment Pharmacol Ther* 2003; **18**: 1083-1089
14 **Achem SR**, Kolts BE, Wears R, Burton L, Richter JE. Chest pain associated with nutcracker esophagus: a preliminary study of the role of gastroesophageal reflux. *Am J Gastroenterol* 1993; **88**: 187-192
15 **DiMarino AJ Jr**, Allen ML, Lynn RB, Zamani S. Clinical value of esophageal motility testing. *Dig Dis* 1998; **16**: 198-204
16 **Prakash C**, Clouse RE. Value of extended recording time with wireless pH monitoring in evaluating gastroesophageal reflux disease. *Clin Gastroenterol Hepatol* 2005; **3**: 329-334
17 **Fass R**, Fennerty MB, Ofman JJ, Gralnek IM, Johnson C, Camargo E, Sampliner RE. The clinical and economic value of a short course of omeprazole in patients with noncardiac chest pain. *Gastroenterology* 1998; **115**: 42-49

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RAPID COMMUNICATION

Comparison of the liver stiffness measurement by transient elastography with the liver biopsy

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Abstract

AIM: To compare the liver stiffness (LS) measurement by transient elastography (TE) to the liver biopsy (LB)-considered the "gold standard" in the evaluation of patients with chronic hepatitis C.

METHODS: During a period of 12 mo, we evaluated 199 consecutive patients with chronic hepatitis due to hepatitis C virus (HCV), in which LB and LS assessments (by means of TE) were performed during the same session.

RESULTS: Out of 199 patients, a valid measurement of the LS could not be obtained in 8. The mean value of LS in the cohort of 191 valid measurements was 8.45 ± 4.96 kPa, ranging from 2.3 to 38 kPa. The mean value of LS in patients with significant fibrosis at biopsy (161 patients with F ⩾ 2 according to Metavir) was 9.02 ± 5.15 kPa, significantly higher than in patients with no or mild fibrosis (30 patients with F < 2 Metavir): 5.39 ± 1.81 kPa (P < 0.0001). For a cut-off value of 6.8 kPa, the LS had a PPV of 98%, a NPV of 30.1%, a sensitivity of 59.6% and a specificity of 93.3% for the presence of significant fibrosis (at least F2 Metavir), with a diagnostic performance of 77.3% (AUROC 0.773). Using this cut-off value, we reached the best discrimination between absence of fibrosis/mild fibrosis (F < 2 Metavir) and the presence of moderate to severe fibrosis (F ⩾ 2 Metavir).

CONCLUSION: In patients with chronic hepatitis due to HCV, a cut-off value of 6.8 kPa measured by TE can differentiate between significant fibrosis and absent or mild fibrosis, with a PPV of 98%, a NPV of 30.1%, a sensitivity of 59.6%, a specificity of 93.3%, and a diagnostic performance of 77.3%.

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Key words: Liver stiffness; Transient elastography; Liver biopsy; Chronic C hepatitis; FibroScan

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INTRODUCTION

In the evolution of chronic viral and non-viral hepatitis, liver fibrosis is a very important factor associated with prognosis. Hence, it is necessary to evaluate precisely the severity of fibrosis in those patients, in order to perform a correct staging and, eventually, to take a decision regarding the treatment.

Currently, the biopsy examination of the liver is considered the optimal method to evaluate changes in fibrosis over time[1]. Nevertheless, the liver biopsy (LB) has its shortcomings: the intra- and interobserver variability[2,3], the sampling variability (as proven in a study by Ratziu *et al*[4]), and, last but not least, the fact that LB is an invasive method, with morbidity and mortality greater than zero.

Based on these premises, noninvasive methods for the evaluation of liver fibrosis have been developed during the last few years in order to replace the LB. The most promising noninvasive methods are, currently, the FibroTest-ActiTest[5] and the transient elastography (TE) measured with the FibroScan device[6,7].

TE is an ultrasound-based method. By using an ultrasound transducer probe mounted on the axis of a vibrator, the transmission of low-frequency vibrations from the right intercostal space creates an elastic shear wave that propagates into the liver. A pulse-echo ultrasound acquisition is then used to detect the velocity of wave propagation. This velocity is proportional to the liver stiffness, with faster wave progression occurring through stiffer tissues. Measurement of liver stiffness (LS) is then performed and measured in kPa[6].

The FibroScan assessment of LS was validated as method of evaluation in chronic hepatitis C. Also, there are some articles that proved the value of this method in other chronic hepatopathies, like chronic hepatitis due to the hepatitis B virus (HBV) infection, hemochromatosis, primary billiary cirrhosis or non-alcoholic steato-hepatitis[8-11].

MATERIALS AND METHODS

During a 12 mo period (June, 2007 to June, 2008), all patients with chronic hepatitis C from the Departments of Gastroenterology and Hepatology and Infectious Diseases of Timisoara were investigated in the same session by means of two methods: LS measurement by means of TE and LB, in order to compare the value of that noninvasive method for the assessment of liver fibrosis. Patients with proven liver cirrhosis (ascites, jaundice, esophageal varices) were excluded from the study, as were patients who had another cause of chronic hepatitis, apart from hepatitis C virus (HCV) (HBV, alcoholic hepatitis, *etc*).

The LS was evaluated by means of TE with a FibroScan device (Echosens-Paris, France) by three experienced physicians. Measurements were performed in the right lobe of the liver through the intercostal spaces while the patients were lying in dorsal decubitus position with the right arm in maximal abduction. The tip of the transducer was covered with coupling gel and placed on the skin, between the ribs, aiming at the right lobe of the liver. The operator, assisted by ultrasound A-mode images provided by the system, located a portion of the liver that was at least 6 cm thick, free of large vascular structures. Once the area of measurement had been located, the operator pressed the probe button to begin an acquisition. Acquisitions that did not have a correct vibration shape or a correct follow-up of the vibration propagation were automatically rejected by the software.

Ten successful acquisitions were performed on each patient. The success rate was calculated as the ratio of the number of successful acquisitions over the total number of acquisitions. The median value of the 10 valid measurements was then calculated by the device and considered to be the value of LS in that patient.

Only patients in which LS measurements had a success rate of at least 60%, with interquartile range (IQR) of all validated measurements less than 30% of the median values (IQR < 30%), were considered for further analysis.

We performed echoguided LB, with Menghini type modified needles, 1.4 and 1.6 mm in diameter. Only LB fragments of at least 2 cm, including at least 8 portal tracts were considered adequate for pathological interpretation. The LB was assessed according to the Metavir score by a senior pathologist. Fibrosis was staged on a 0-4 scale: F0-no fibrosis; F1-portal fibrosis without septa; F2-portal fibrosis and few septa extending into lobules; F3-numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4-cirrhosis

The statistical analysis was performed using Microsoft Excel and GraphPad Prism programs. For the statistical study of quantitative variables, the mean and standard variation were calculated. The diagnostic performance of LS measurements was assessed by using receiver operating characteristics (ROCs) curves. Connected with any cutoff value is the probability of a true positive (sensitivity, Se) and the probability of a true negative (specificity, Sp). The ROC curve is a plot of Se *vs* 1-Sp for all possible cutoff values. The most commonly used accuracy index is the area under the ROC curve, when values close to 1.0 indicate high diagnostic accuracy. ROC curves were thus built for the detection of significant fibrosis (F $\geqslant$ 2 Metavir) and severe fibrosis (F $\geqslant$ 3 Metavir). Optimal cutoff values for LS measurements were chosen to maximize the sum of Se and Sp.

RESULTS

The study group included 199 patients with chronic hepatitis C, 138 women and 61 men, with a mean age of 49.79 ± 10.98 years. In 8 cases we could not obtain valid measurements, as we did in the remaining 191 patients (96%).

The mean value of LS in the subgroup of 191 patients with valid measurements was 8.45 ± 4.96 kPa, ranging from 2.3 to 38 kPa.

We divided patients according to the degree of fibrosis, i.e. into a subgroup with significant fibrosis (F $\geqslant$ 2 Metavir, patients who should receive antiviral therapy) and another one with no or mild fibrosis (F < 2 Metavir, in which antiviral treatment is currently not recommended).

The mean value of LS in patients with significant fibrosis (161 patients with F $\geqslant$ 2 Metavir) was 9.02 ± 5.15 kPa, significantly higher than in patients with no or mild fibrosis (30 patients with F < 2 Metavir): 5.39 ± 1.81 kPa ($P < 0.0001$) (Figure 1A).

The values of LS in various subgroups of patients, divided according to fibrosis stage, were (Figure 1B): 5.27 ± 0.83 kPa in 4 patients with F0; 5.41 ± 1.92 kPa in 26 cases with F1; 7.18 ± 2.62 kPa in 97 patients with F2; 9.75 ± 4.63 kPa in 45 cases with F3; and 16.68 ± 8.10 kPa in 19 cases with F4.

The statistical significance of the differences between the LS in these subgroups was: F0 *vs* F1 $P = 0.8081$; F0 *vs* F2 $P = 0.1117$; F0 *vs* F3 $P < 0.0001$; F0 *vs* F4 $P < 0.0001$; F1 *vs* F2 $P = 0.0048$; F1 *vs* F3 $P < 0.0001$; F1 *vs* F4 $P < 0.0001$; F2 *vs* F3 $P = 0.0011$; F2 *vs* F4 $P < 0.0001$; F3 *vs* F4 $P = 0.0002$.

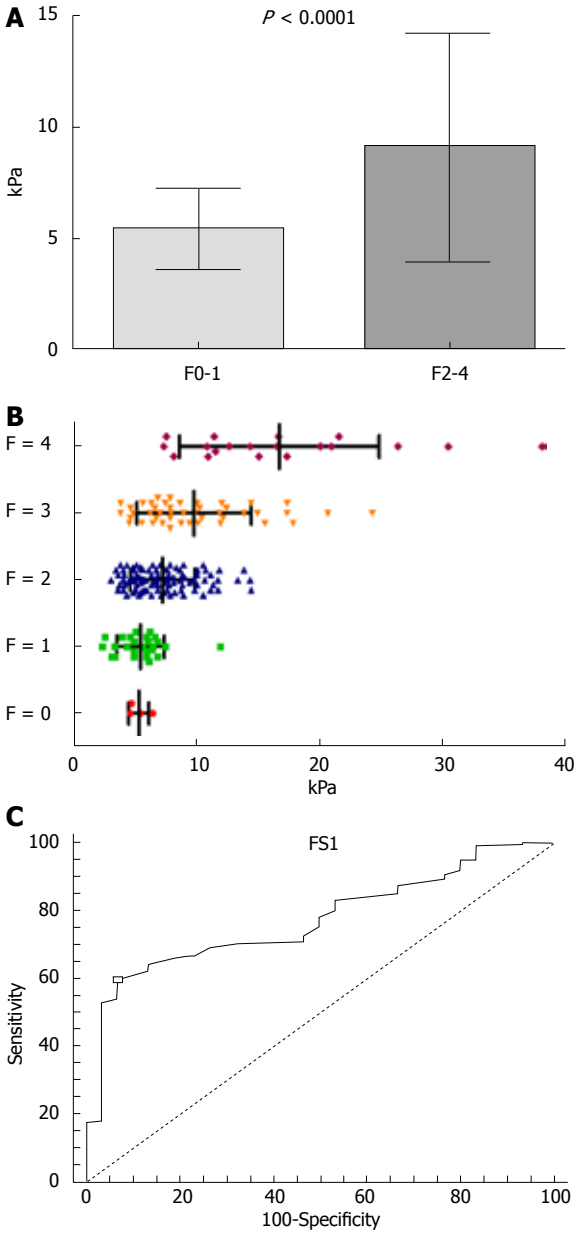

Figure 1 The mean value of LS and AUROC for LS predictive value. A: Mean value of LS in patients with significant fibrosis (F ⩾ 2 Metavir) as compared to those with no or mild fibrosis (F < 2 Metavir); B: Mean value of LS according to fibrosis; C: The AUROC for LS predictive value of the presence of significant fibrosis (at least F2 Metavir).

The statistical analysis of these subgroups showed that there were no significant differences between the mean values of LS in the F0 *vs* F1 subgroup (so that the cases with mild and no fibrosis cannot be differentiated by means of FibroScan evaluation of LS). The comparison of the mean values of LS between the other subgroups showed significant (S) or very significant (ES) differences.

The mean value of LS in patients with severe fibrosis (64 patients with F ⩾ 3 Metavir) was 11.81 ± 6.62 kPa, significantly higher than in patients with at most moderate fibrosis (127 patients with F < 3 Metavir) 6.76 ± 2.56 kPa ($P < 0.0001$).

We tried to establish the value of LS measured by means of FibroScan, which best predicts the presence of significant fibrosis (F ⩾ 2 Metavir) in patients with chronic hepatitis C.

For a cut-off value of 6.8 kPa, the LS had a PPV of 98%, a NPV of 30.1%, a sensitivity of 59.6%, a specificity of 93.3% for the presence of significant fibrosis (at least F2 Metavir), and a diagnostic performance of 77.3% (AUROC 0.773) (Figure 1C).

For a cut-off value of 7.2 kPa, the PPV was 97.8%, the NPV was 28.6%, the sensitivity was 56.5% and the specificity was 93.3% for the presence of significant fibrosis (at least F2 Metavir).

For a cut-off of value of 7.5 kPa, the PPV was 98.8%, the NPV was 27.4%, the sensitivity was 52.2% and the specificity was 96.7%.

DISCUSSION

We compared our results with data from the literature (Table 1). In a multicentric study coordinated by Beaugrand[12] on 494 patients chronically infected with HCV, who were evaluated in the same session by means of percutaneous LB and valid FibroScan, a significant correlation was found ($r = 0.57$, $P < 0.0001$) between the LS and histological fibrosis, with AUROC [confidence interval (CI) 95%] 0.84, 0.93 and 0.96 for F ⩾ 2, F ⩾ 3 and F = 4, respectively. This study tried to establish cut-off values in order to differentiate among various histological stages. Thus, a cut-off value of 7.5 kPa differentiates F01/F234 with a sensitivity of 67%, a specificity of 87%, a PPV of 86% and a NPV of 68%, with 76% diagnostic accuracy.

In a study performed by Foucher *et al*[13] that compared LB with LS evaluation by means of FibroScan in 354 patients with chronic hepatitis C, the cut-off value was 7.2 kPa, for moderate and severe fibrosis (F234), 12.5 kPa for F34 and 17.6 kPa for cirrhosis.

In a Korean study[14], the cut-off values were 7.3 kPa for F ⩾ 2, 8.8 kPa for F ⩾ 3 and 15.1 kPa for F = 4. In a study performed in the Netherlands[15], using a cut-off value of 7.1 kPa, 88% of the patients who did not have significant fibrosis (F < 2 Metavir) were correctly identified.

In a study performed in Romania[16], on 324 consecutive patients chronically infected with HCV, evaluated both by TE and LB in the same session, the LS values were strongly correlated with fibrosis ($r = 0.759$, $P < 0.0005$), but also with steatosis ($r = 0.255$, $P < 0.005$), necroinflammatory activity ($r = 0.378$, $P < 0.0005$) and hepatic iron deposition ($r = 0.143$, $P = 0.03$). The univariable regression analysis of this study demonstrated that fibrosis ($R^2 = 0.610$, $P < 0.0005$), activity ($R^2 = 0.145$, $P < 0.0005$) and steatosis ($R^2 = 0.037$, $P < 0.002$) were correlated with LS. In multiple regression analysis, all three variable independently influenced LS: fibrosis ($P < 0.0005$), activity ($P = 0.039$) and steatosis ($P = 0.025$). The conclusions of this study showed that fibrosis is the main predictor of LS, but also that the latter bis influenced by activity and steatosis.

In a study performed by Coco and co-workers[17], the value of LS was evaluated considering the level of

Table 1 Cut-off values for significant fibrosis (at least F2 Metavir) in various studies

Study	No. of cases	Cut-off value for F2 (kPa)
Ziol *et al*[12]	494	7.5
Foucher *et al*[13]	354	7.2
Kim *et al*[14]	47	7.3
Berends *et al*[15]	24	7.1
Lupşor *et al*[16]	324	7.4
Present study	199	6.8

aminotransferases. The LS dynamics profiles paralleled those of ALT, increasing 1.3 to 3 fold during ALT flares. This study showed that the LS remained unchanged in patients with stable biochemical activity.

All these studies, coming from different regions of the world, completed previous studies from France[18-22], demonstrating the value of non-invasive, FibroScan evaluation of LS for the fibrosis assessment in chronic hepatitis C. This method is not accurate enough to differentiate among contiguous stages of fibrosis (especially 0, 1 and 2), but is sensitive enough to differentiate between the absence and/or mild fibrosis from significant fibrosis, an essential step for the decision regarding treatment. In the future, we shall still find exactly whether histological activity, steatosis or biological activity (ALT) have an important role in the assessment of LS by means of FibroScan.

Considering the data presented from our study, the cut-off value of 6.8 kPa is the most accurate for the discrimination between absence or mild fibrosis (F < 2 Metavir) and the presence of moderate and severe fibrosis (F ⩾ 2 Metavir), thus allowing us to select the patients with chronic hepatitis C who should be treated (knowing that patients with F < 2 Metavir are not candidates for treatment, since they have mild disease). In our study, using a cut-off value of 6.8 kPa, the sum of sensitivity and specificity was the highest.

Based on our results, we may use the FibroScan to evaluate LS in patients with chronic hepatitis C prior therapy. By using a cut-off value of 6.8 kPa, we can differentiate patients who needed to be treated (F ⩾ 2 Metavir) from those who, according to most current guidelines, should not receive treatment (F < 2 Metavir).

The cut-off value of 6.8 kPa in our study had a PPV of 98%, meaning that we can identify quite accurately the patients who should be treated (F ⩾ 2 Metavir). For patients with values of LS smaller than 6.8 kPa, we think appropriate to perform LB, because the NVP is low (30.1%), and otherwise we could miss patients with significant fibrosis using only TE. In our cohort of 191 patients with valid measurements of LS, using a cut-off value of 6.8 kPa, we could avoid LB in 160 patients who had a LS greater than 6.8 kPa and who could directly receive treatment, without LB. On the other hand, a LB should have been performed only in 31 patients (a 83.8% reduction in the number of LB performed in our Department during this same interval of time).

Considering the data from the literature and looking to our own, we could use the TE evaluation of LS in patients with chronic hepatitis C in order to decide about therapy. All studies show that, by using a cut-off value of 6.8-7.5 kPa, we could identify accurately enough the patients who need to be treated (F ⩾ 2 Metavir) *vs* those who should not be treated (F < 2 Metavir), and this without performing a LB[13-15]. The PPV of 98% obtained in our study for a cut-off value of 6.8 kPa suggests that we can quite accurately identify patients who must be treated (F ⩾ 2 Metavir). For patients with LS smaller than 6.8 kPa, we think appropriate to perform a LB, because the NPV is low (30.1%) and therefore we may miss patients with significant fibrosis if only based on LS measurement.

CONCLUSION

The FibroScan evaluation of LS is a method in which valid measurements can be obtained in the great majority of scanned patients (96% of the cases). In patients with chronic hepatitis C, when compared to the LB considered to be the "gold standard", LS measurement by means of TE can differentiate between significant fibrosis and absent or mild fibrosis. We found that a cut-off value of 6.8 kPa is the one that best differentiates absence or mild fibrosis (F < 2 Metavir) from significant fibrosis (F ⩾ 2 Metavir), with PPV of 98%, NPV of 30.1%, sensitivity of 59.6%, specificity of 93.3% and a diagnostic performance of 77.3%. In our cohort of patients, if the cut-off value of 6.8 kPa had been used for the presence of significant fibrosis, more than 80% of the LB would have been avoided.

Considering all these facts, the FibroScan evaluation of LS is a useful non-invasive method for the evaluation of patients with chronic hepatitis C in clinical practice, and can replace in many cases the LB for the decision of therapy.

COMMENTS

Background

In the evolution of chronic viral and non-viral hepatitis, liver fibrosis is a very important factor associated with prognosis. Hence, it is necessary to evaluate precisely the severity of fibrosis in those patients, in order to perform a correct staging and, eventually, to take a decision regarding the treatment. Fibrosis can be assessed through invasive [liver biopsy (LB)] and non-invasive methods such as transient elastography (TE) assessment of liver stiffness (LS), developed in the last few years in order to replace LB.

Research frontiers

The aim of the study was to assess the value of LS measurement by TE as compared to the LB-considered to be the "gold standard", in the evaluation of patients with chronic hepatitis C.

Innovations and breakthroughs

This study concluded that LS measurement by TE can predict the presence of significant fibrosis (at least F2 Metavir, that requires treatment) in patients with chronic hepatitis C, confirming previously published studies.

Applications

Transient evaluation of LS could replace in many cases the LB for the decision of therapy in patients with chronic hepatitis C, if the values of LS are higher than the established cut-off values for significant fibrosis (6.8-7.5 kPa according to various studies). The LB should be performed only if the LS is lower than the cut-off, in order to avoid "missing" patients with significant fibrosis.

Terminology

TE is an ultrasound-based method that assesses the LS as a marker of fibrosis.

By using an ultrasound transducer probe mounted on the axis of a vibrator, the transmission of low-frequency vibrations from the right intercostal space creates an elastic shear wave that propagates into the liver. A pulse-echo ultrasound acquisition then is used to detect the velocity of wave propagation. This velocity is proportional to the tissue stiffness, with faster wave progression occurring through stiffer material. The stiffer the liver, the higher the degree of fibrosis.

Peer review

This paper investigated the usefulness of elastography in assessing the stage of biopsy-proven fibrosis in patients with chronic hepatitis C. This is a straightforward study and the authors concluded that this new technique can predict the presence of severe fibrosis that require treatment.

REFERENCES

1 **McHutchison J**, Poynard T, Afdhal N. Fibrosis as an end point for clinical trials in liver disease: a report of the international fibrosis group. *Clin Gastroenterol Hepatol* 2006; **4**: 1214-1220

2 **Regev A**, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, Feng ZZ, Reddy KR, Schiff ER. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am J Gastroenterol* 2002; **97**: 2614-2618

3 **Persico M**, Palmentieri B, Vecchione R, Torella R, de SI. Diagnosis of chronic liver disease: reproducibility and validation of liver biopsy. *Am J Gastroenterol* 2002; **97**: 491-492

4 **Ratziu V**, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, Grimaldi A, Capron F, Poynard T. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005; **128**: 1898-1906

5 **Munteanu M**. Non-invasive biomarkers FibroTest-ActiTest for replacing invasive liver biopsy: the need for change and action. *J Gastrointestin Liver Dis* 2007; **16**: 173-174

6 **Talwalkar JA**, Kurtz DM, Schoenleber SJ, West CP, Montori VM. Ultrasound-based transient elastography for the detection of hepatic fibrosis: systematic review and meta-analysis. *Clin Gastroenterol Hepatol* 2007; **5**: 1214-1220

7 **Rockey DC**. Noninvasive assessment of liver fibrosis and portal hypertension with transient elastography. *Gastroenterology* 2008; **134**: 8-14

8 **Ogawa E**, Furusyo N, Toyoda K, Takeoka H, Otaguro S, Hamada M, Murata M, Sawayama Y, Hayashi J. Transient elastography for patients with chronic hepatitis B and C virus infection: Non-invasive, quantitative assessment of liver fibrosis. *Hepatol Res* 2007; **37**: 1002-1010

9 **Adhoute X**, Foucher J, Laharie D, Terrebonne E, Vergniol J, Castéra L, Lovato B, Chanteloup E, Merrouche W, Couzigou P, de Lédinghen V. Diagnosis of liver fibrosis using FibroScan and other noninvasive methods in patients with hemochromatosis: a prospective study. *Gastroenterol Clin Biol* 2008; **32**: 180-187

10 **Yoneda M**, Yoneda M, Mawatari H, Fujita K, Endo H, Iida H, Nozaki Y, Yonemitsu K, Higurashi T, Takahashi H, Kobayashi N, Kirikoshi H, Abe Y, Inamori M, Kubota K, Saito S, Tamano M, Hiraishi H, Maeyama S, Yamaguchi N, Togo S, Nakajima A. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with nonalcoholic fatty liver disease (NAFLD). *Dig Liver Dis* 2008; **40**: 371-378

11 **Corpechot C**, El Naggar A, Poujol-Robert A, Ziol M, Wendum D, Chazouillères O, de Lédinghen V, Dhumeaux D, Marcellin P, Beaugrand M, Poupon R. Assessment of biliary fibrosis by transient elastography in patients with PBC and PSC. *Hepatology* 2006; **43**: 1118-1124

12 **Ziol M**, Marcellin P, Douvin C, de Ledinghen V, Poupon R, Beaugrand M. Liver stiffness cut off values in HCV patients: validation and comparison in an independent population. *Hepatology* 2006; **44**: Suppl 1: 269A

13 **Foucher J**, Chanteloup E, Vergniol J, Castéra L, Le Bail B, Adhoute X, Bertet J, Couzigou P, de Lédinghen V. Diagnosis of cirrhosis by transient elastography (FibroScan): a prospective study. *Gut* 2006; **55**: 403-408

14 **Kim KM**, Choi WB, Park SH, Yu E, Lee SG, Lim YS, Lee HC, Chung YH, Lee YS, Suh DJ. Diagnosis of hepatic steatosis and fibrosis by transient elastography in asymptomatic healthy individuals: a prospective study of living related potential liver donors. *J Gastroenterol* 2007; **42**: 382-388

15 **Berends MA**, Snoek J, de Jong EM, Van Krieken JH, de Knegt RJ, van Oijen MG, van de Kerkhof PC, Drenth JP. Biochemical and biophysical assessment of MTX-induced liver fibrosis in psoriasis patients: Fibrotest predicts the presence and Fibroscan predicts the absence of significant liver fibrosis. *Liver Int* 2007; **27**: 639-645

16 **Lupşor M**, Badea R, Stefănescu H, Grigorescu M, Sparchez Z, Serban A, Branda H, Iancu S, Maniu A. Analysis of histopathological changes that influence liver stiffness in chronic hepatitis C. Results from a cohort of 324 patients. *J Gastrointestin Liver Dis* 2008; **17**: 155-163

17 **Coco B**, Oliveri F, Maina AM, Ciccorossi P, Sacco R, Colombatto P, Bonino F, Brunetto MR. Transient elastography: a new surrogate marker of liver fibrosis influenced by major changes of transaminases. *J Viral Hepat* 2007; **14**: 360-369

18 **Sandrin L**, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, Beaugrand M, Palau R. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705-1713

19 **Castera L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Lédinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350

20 **Castera L**, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. *J Hepatol* 2008; **48**: 835-847

21 **Ziol M**, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Ledinghen V, Marcellin P, Dhumeaux D, Trinchet JC, Beaugrand M. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48-54

22 **Kettaneh A**, Marcellin P, Douvin C, Poupon R, Ziol M, Beaugrand M, de Ledinghen V. Features associated with success rate and performance of FibroScan measurements for the diagnosis of cirrhosis in HCV patients: a prospective study of 935 patients. *J Hepatol* 2007; **46**: 628-634

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RAPID COMMUNICATION

Myenteric neurons and intestinal mucosa of diabetic rats after ascorbic acid supplementation

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Abstract

AIM: To investigate the effect of ascorbic acid (AA) dietary supplementation on myenteric neurons and epithelial cell proliferation of the jejunum of adult rats with chronic diabetes mellitus.

METHODS: Thirty rats at 90 d of age were divided into three groups: Non-diabetic, diabetic and diabetic treated with AA (DA) (1 g/L). After 120 d of treatment with AA the animals were killed. The myenteric neurons were stained for myosin-V and analyzed quantitatively in an area of 11.2 mm²/animal. We further measured the cellular area of 500 neurons per group. We also determined the metaphasic index (MI) of the jejunum mucosa layer of about 2500 cells in the intestinal crypts, as well as the dimensions of 30 villi and 30 crypts/animal. The data area was analyzed using the Olympus BX40 microscope.

RESULTS: There was an increase of 14% in the neuronal density (792.6 ± 46.52 *vs* 680.6 ± 30.27) and 4.4% in the cellular area (303.4 ± 5.19 *vs* 291.1 ± 6.0) respectively of the diabetic group treated with AA when compared to control diabetic animals. There were no significant differences in MI parameters, villi height or crypt depths among the groups.

CONCLUSION: Supplementation with AA in the diabetic animal promoted moderate neuroprotection. There was no observation of alteration of the cellular proliferation of the jejunum mucosa layer of rats with chronic diabetes mellitus with or without supplementation with AA.

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Key words: Ascorbic acid; Diabetes mellitus; Intestinal mucosa layer; Myenteric neurons; Myosin-V

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INTRODUCTION

The disease diabetes mellitus is characterized by abnormally high plasma glucose concentrations. Chronic hyperglycemia and the associated metabolic abnormalities are responsible for many disease complications, including damage to the blood vessels, eyes, kidneys and nervous system[1].

In diabetes mellitus, the mucosa of the small intestine undergoes morphological changes[2,3]. This impairs passage of food along the intestine, secretion of enteric juices and the absorption of the digestion products, all of which depend on the integrity of the epithelial lining. Intestinal epithelium is characterized by fast cellular renewal with continuous proliferation of stem cells inside Lieberkühn crypts, cellular migration along the crypt-villi axis, cellular differentiation, polarization, apical apoptosis and luminal loss[4].

It has been shown that the autonomic nervous system has a role in intestinal epithelium renewal, which can be assessed through the elimination of the parasympathetic[5], sympathetic[6] or myenteric innervation[7]. The enteric nervous system is a division

of the autonomic nervous system in the gastrointestinal tract; it can mediate independent reflexes (motility, absorption and secretion) and is made up of sensory neurons, interneurons and interconnected motor neurons[8]. Diabetes mellitus causes a decrease in enteric innervation with a consequent onset of the diabetic neuropathy[9-11].

Several theories have been proposed to explain the neuropathy in diabetes mellitus. They include: impaired metabolism of fatty acids, reduction in the blood supply to the nerves, advanced products of non enzymatic glycation, oxidative stress, inadequate trophic support and the activation of the polyol pathway[12,13]. Aldose reductase converts glucose excess into sorbitol[14,15] which is responsible for edema, neuronal lesions[14,16,17]. The sorbitol concentration can be reduced in the diabetes mellitus through the use of ascorbic acid (AA), an inhibitor of the enzyme aldose reductase[18,19].

Another factor that can be related to the paper neuroprotector of the AA is the antioxidant activity exercised by this vitamin, being of some importance in the diabetes mellitus seen that in this pathology the oxidative stress is intensified and the defense antioxidants are diminished. In this way, our aim was to verify the effect of supplementation with AA on the myosin-V immunoreactive myenteric neurons and on the intestinal mucosa of the jejunum of diabetic rats.

MATERIALS AND METHODS

Animal procedure

Albino male rats (*Rattus norvegicus*), Wistar strain weighing about 300 g and aged 90 d were used in this study. The rats were submitted to a 14-h-fast and then injected with streptozotocin (35 mg/kg of body weight) dissolved in citrate buffer (10 mmol/L, pH 4.5) in the penial vein to induce diabetes mellitus. The onset of diabetes mellitus was verified by the glycosuria and polyuria.

Thirty rats, divided into three groups of ten animals each, were used as follows: Non diabetic rats (ND), diabetic rats (D) and diabetic treated with AA (DA). The AA was given orally for 16 wk (from the 90 d of age) in water (1 g/L prepared daily)[20]. The rats were kept in individual cages with a photoperiod of 12 h (6:00 am-6:00 pm) and at room temperature (RT) (24 ± 2℃) with water and food (Nuvital® lab chow) *ad libitum*.

At 210 d of age, the rats were anesthetized intraperitoneally with sodium thiopental-Thionembutal® (40 mg/kg of body weight). The blood was collected by heart puncture for measuring the levels of glycated hemoglobin[21], glucose[22] and AA[23]. The animals used in this study were treated under the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethics Committee in Animal Experimentation of the State University of Maringá.

Myosin-V neuronal staining[24]

Fifteen animals were perfused with 1 mL/body weight of saline solution followed by perfusion with 1 mL/g per body weight of fixation solution containing sodium periodate (10 mmol/L), lysine (75 mmol/L), paraformaldehyde (1%) in phosphate buffer (PB) (37 mmol/L, pH 7.4)[25]. Immediately after perfusion, each treated fragment of jejunum was removed, rinsed with saline solution, flushed with fixative solution and tied in its extremities (balloons). Thirty minutes after immersion in fixative solution, the jejunum was opened and left in this solution for an additional 30 min. Subsequently, the segments were opened along the mesenteric border and dehydrated in alcohols (50%, 70%, 80%, 90%, 95% and 100%), remained in each solution 10 min, cleared in xylol (10 min), rehydrated back through the ethanol 100%, 95%, 90%, 80%, 70%, and stored in 70% ethanol. Afterwards, the segments were dissected under stereomicroscopy with trans-illumination, through the removal of the mucosa and submucosa layer, obtaining muscular layer whole mounts. These mixtures were washed four times in phosphate buffered saline (PBS) (0.1 mol/L, pH 7.4) and blocked for 2 h with PBS containing bovine serum albumin (2%), goat serum (2%) and Triton X-100 (0.5%) at room temperature. Later, the segments were incubated in eppendorfs in a solution containing 0.86 μg/mL of the myosin-V primary antibody (extracted from rabbits) (1:750) diluted in PBS, BSA (1%), Triton X-100 (0.1%) and goat serum (2%) at room temperature under agitation (48:00). After incubation, the tissues were rinsed twice in PBS (0.1 mol/L), Triton X-100 (0.1%) and twice in a solution of PBS (0.1 mol/L) + Tween-20 (0.05%). Then, the tissues were incubated in peroxidase-conjugated secondary antibodies (1 μg/mL) (1:1000) at room temperature under shaking (24 h). Finally, they were rinsed four times for 15 min in PBS (0.1 mol/L) + Tween-20 (0.05%). The staining with peroxidase-conjugated antibody was developed by incubation with 0.75 mg/mL diaminebenzidine and 0.03% of H_2O_2 in water (1 mL) and PBS 0.1 mol/L (1 mL) for 15 min at room temperature under shaking. Samples were mounted in glycerol gel, containing glycerol (50%), gelatin in water (0.07 g/mL) and phenol (2 μL/mL). The negative control was performed with the omission of the primary antibody.

Quantitative and morphometric analyses of myosin-V stained myenteric neurons

Quantitative analysis: Measurements were made in the intermediate area of the jejunum intestinal circumference (60º-120º; 240º-300º), considering the mesenteric intersection as 0º[26]. Myenteric neurons were counted in 50 random fields, under a light microscope (Olympus BX40) with a 40× lens. The area of each microscope field was 0.224 mm^2.

Morphometric analysis: The images were taken with a high-resolution camera, transmitted to a microcomputer and recorded to a compact disk. The area (μm^2) of 100 cell bodies of the jejunum, in a total of 500 neurons per group, was measured through an image analysis system,

the Image-Pro-Plus 4.0. The neurons were distributed in intervals of 100 μm^2.

Morphometric analysis of the intestinal mucosa: An additional 15 animals were injected with vincristine, a blocking agent of the mitotic fuse, at a dose of 1 mg/kg of body weight, in the penial vein 2 h before segment collection.

The jejunum was collected, open along the mesenteric border and washed with saline solution, fixed in buffered paraformaldehyde (10%) for 6 h. After fixation, they were dehydrated and immersed in resin 2-hydroxyethyl-methacrylate. Later, 2 μm transverse semi-serial sections were made (a section for each five discarded), using a Leica RM 2145 microtome, with a glass razor. The resin sections were stained by HE.

Metaphasic index (MI): MI shows the number of metaphasic cells/total number of cells (%) that was obtained from the interphasic and metaphasic epithelial cells in the crypts of the jejunum. Twenty five thousand cells per animal were quantified under a light microscope.

Villi and crypts: We measured the height of 30 villi and the depth of 30 crypts per animal, in a total of 900 measurements (300 measurements/treatment), under a light microscope with an ocular micrometer. The heights of the villi were measured from the crypt-villus junction to its apex. The depth of the crypts was measured considering the extension between the crypt-villus junctions to its base.

Chemical products

AA, serum albumin bovine (BSA), diaminebenzidine (DAB), streptozotocin (STZ), Tween-20, paraformaldehyde and Triton X-100 were obtained at Sigma Chemical Company, USA); Vincristine sulfate (Eli Lilly of Brazil, Brazil); Historesin kit (Leica); secondary antibody conjugated with peroxidase serum anti-rabbit IgG (Pierce, Rockford, USES). The polyclonal antibody anti myosin-V was characterized by Espreafico *et al*[27].

Statistical analysis

The data were submitted to analysis of variance (ANOVA) and the test of Tukey as a post-test to compare the means. Since the areas of the cell bodies of the neurons did not have an even distribution, we used the Kruskal-Wallis test to compare the means. The analyses were accomplished with the prism 3.0 software. The data are shown as mean ± SE as an indicator of the observation number (*n*). The level of significance was $P < 0.05$.

RESULTS

Streptozotocin promoted the onset of the diabetic syndrome with accentuated hyperglycemia. The diabetic rats and the diabetic-treated with AA had similar levels of glycated hemoglobin ($P > 0.05$). There was a reduction of 48.7% in the plasmatic level of AA in the diabetic animals when compared to the non-diabetics ($P < 0.05$). The supplementation in group DA increased their level of AA in 25.7% when compared to the non-diabetics ($P > 0.05$), and 61.9% when compared to the diabetic group ($P > 0.05$) (Table 1).

Table 1 GLI, GHb and AA for animals with 210 d old in groups, mean ± SE (*n* = 10)

Treated group	GLI (mg/dL)	GHb (%)	AA (μg/mL)
ND	129 ± 3.9	4.1 ± 0.3	24.58 ± 5.5
D	466.4 ± 24.6	8.1 ± 0.2	12.6 ± 1.9
DA	493.0 ± 10.1	7.9 ± 0.5	33.1 ± 2.5

Table 2 Distribution of myosin-V stained myenteric neurons, classified according to their cellular profile in intervals of 100 μm^2 in animals from groups (*n* = 5 rats per group)

Size (μm^2)	Groups		
	ND	D	DA
< 100	7	2	0
101-200	113	117	96
201-300	175	172	190
301-400	125	136	114
401-500	53	53	62
501-600	19	17	30
601-700	6	2	7
> 700	2	1	1
Total of neurons	500	500	500

Quantitative analysis of the myosin-V stained myenteric neurons

Experimental diabetes mellitus caused a reduction in the myenteric neuronal density in an area of 11.2 mm^2 in the jejunum, as shown in Figure 1A ($P < 0.001$). The AA supplementation in the diabetic animals caused a 14% increase in the density of the myosin-V stained neurons when compared to the non-treated diabetic animals ($P > 0.05$).

Cellular area of myenteric neurons stained with myosin-V

The results regarding the neuronal area means (μm^2) were 289.41 ± 5.19 for the non-diabetic group, 290.1 ± 6.02 for the diabetic group and 303.4 ± 5.19 for group DA (Figure 1B). The means of the cellular areas were similar for the three studied groups ($P > 0.05$). The 201-300 μm^2 interval was the predominant cellular area for all groups (Table 2).

Morphometric analysis of the intestinal mucosa

The intestinal mucosa, assessed through the determination of the MI and measurements of the height of the villus and depth of crypts, did not show statistically significant differences among the groups.

MI of the diabetic group was 1.5% higher when compared with the non-diabetic group ($P > 0.05$). The MI obtained for group DA was 1.77% smaller than the one observed in the diabetic group ($P > 0.05$). The quantification data are presented in Figure 1C.

The villus height (μm) of the diabetic animals was 7% inferior in relation to the non-diabetic group. The

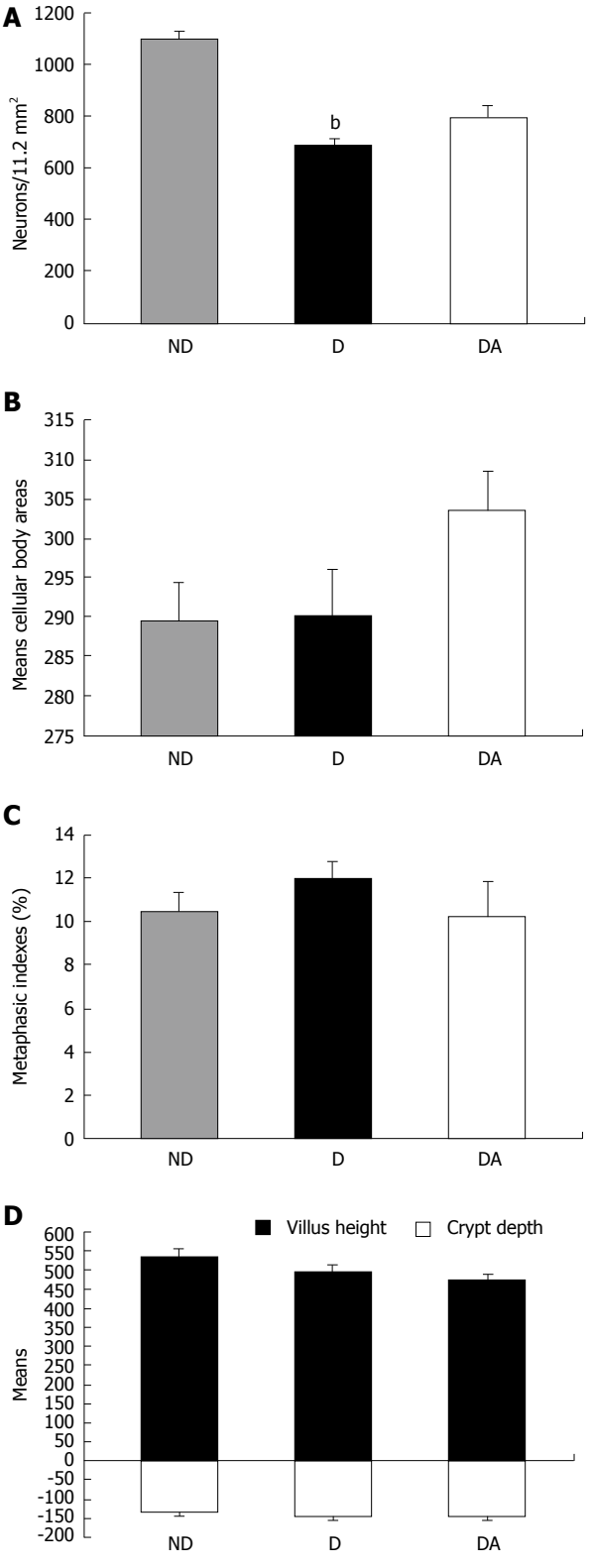

Figure 1 Quantitative and morphometric neurons meyenteric stained of myosin-V analyses. A: Number of myenteric neurons myosin-V stained quantified in 11.2 mm^2 in the jejunum of rats from groups: ND, D and DA, mean ± SE (*n* = 5). $^bP < 0.001$, *vs* the corresponding values in group ND; B: Means (μm^2) of neuronal area of myosin-V-stained myenteric neurons in animals from groups: ND, D and DA, mean ± SE (*n* = 5). There were no significant differences when comparing the three groups by Kruskal-Wallis test; C: IMs (%) of animals from groups: ND, D and DA, mean ± SE (*n* = 5). There were no significative differences when comparing the three groups by test of Tukey; D: Villus height (μm) and crypt depth (μm) in the jejunal mucosa of animals from groups: ND, D and DA, mean ± SE (*n* = 5). There were no significant differences when comparing the three groups by test of Tukey.

animals from the DA group had villi with lower heights than the non-diabetic and diabetic groups (11% and 4% lower, respectively) ($P > 0.05$).

The crypts of the diabetic group were 10% deeper than the crypts observed in the non-diabetic group. The animals from group DA showed a 17% reduction in the depth of their crypts when compared to the diabetic group, and an 8% reduction when compared to the non-diabetic group ($P > 0.05$). The data are shown in Figure 1D.

DISCUSSION

We observed the onset of diabetes mellitus in the diabetic animals and the DA group, characterized by polyuria, polydipsia and polyphagia, thus, validating this experimental model of streptozotocin-induced diabetes mellitus. The onset occurred in the first days of the induction and is associated with the elevation of glucose levels observed by the glucose oxidase and glycated hemoglobin test.

We also verified a sharp reduction of the plasma level of AA due to the diabetes mellitus, in agreement with Young *et al*[28], Garg *et al*[29] and Lindsay *et al*[30]. This reduction might be associated with the increased consumption of the AA due to the increase of the oxidative stress caused by the diabetes mellitus[20,31]. The supplementation with AA allowed the rats from the DA group an increase of 61.9% in their plasmatic level when compared to the diabetic rats, maintaining the glycemia and typical state of diabetes mellitus for these animals.

In order to examine the possible neuroprotector action of the AA in the population of myenteric neurons, we used the immunohistochemical technique for staining the neurons that had myosin-V, since these neurons have a high concentration of this protein, similar to the central nervous system[24].

The loss of neurons suffered by the diabetic animals (680.6 ± 30.27 neurons/11.2 mm^2, group D), resulting in the pathology, was 37.9% in relation to the non diabetic rats (1097 ± 29.79 neurons/11.2 mm^2) ($P < 0.001$) while in the DA group (792.6 ± 46.52 neurons/11.2 mm^2) the loss of neurons was 27.7% in relation to the same group. Treatment of the DA group with AA, resulted in an upper number of myosin-V neurons at 14% in relation to group D ($P > 0.05$). The AA acts by reducing the activity of the aldose reductase. It is the first rate-limiting enzyme of the polyol pathway[32]. Under euglycemic conditions, aldose reductase plays a minor role in glucose metabolism; however, during diabetes, its contribution is significantly enhanced[33,34] leading to a conversion of excess glucose to sorbitol in insulin independent tissues like myenteric neurons. The hyperglycemia, increases sorbitol accumulation and osmotic stress because of the slower oxidation of sorbitol to fructose by sorbitol dehydrogenase. The importance of the AA is also related to its antioxidant activity; therefore, in diabetes mellitus there is an increase of the oxidative stress and a reduction of the levels of antioxidants, endogenous

and exogenous[35]. Cotter *et al*[36], using vitamin C (150 mg/kg per day), demonstrated a 36% prevention in the reduction of the speed of motor nervous conduction provoked by the DM ($P < 0.001$). According to the author, the levels of glycemia observed in experimental diabetes and the consequent increase of free radicals, are excessively higher than the relatively controlled observed one in patients with diabetes. This would explain the statistically significant neuroprotection exerted for the vitamin, which was not observed in our animals of group DA ($P > 0.05$). Due to the fact of a differential effect of streptozotocin-diabetes on different regions of the rat intestine[9], our research group carried out experiments in different intestinal segments. Our work developed in the Department of Morphophysiological Sciences of the State University of Maringá, Paraná, Brasil in whole mounts of diabetic rats supplemented with AA (1 g/L). Zanoni *et al*[37] and Zanoni *et al*[10] verified a reduction of the area of the cellular body from enteric neurons VIP-immunoreactives and NADPH-d positives of the ileum, respectively. Also it was verified 74.3% and 33.4% of neuroprotection in NADH-d myenteric neurons of the duodenum[38] and of the proximal colon[39], respectively. This percentage supports the neuroprotection in the number of myenteric neurons in animals of group DA, when compared to group D.

When analyzing the results obtained through the mean cellular area, we verified there were no significant differences when the three groups were compared. When considering specifically the diabetic condition with the non-diabetic group, we noticed some divergences in our results. Hernandes *et al*[7] and Fregonesi *et al*[40] observed an increase in the cellular area. They attributed as a possible cause the increase on the sorbitol concentration that would lead to changes of the intracellular osmolality, increasing it and resulting in edema and neuronal lesion. Similar to our results, Zanoni *et al*[37] also did not observe differences when they compared the cellular area of the myosin-v-stained myenteric neurons of the ileum of diabetic rats with the acid ascorbic diabetic-treated rats. On the other hand, VIP-ergic neurons of the ileum showed an increase in their cellular area in the diabetic rats and the supplementation with AA prevented this increase[37]. We believe that AA supplementation has an effect on the neuronal population. However, this happens in a specific way, depending on the subpopulation studied: for example, it is evident that it affords neuroprotection Vip-ergic neurons.

The onset of the experimental diabetes mellitus in the jejunum of rats did not cause alterations in the MI or in the morphometry of the intestinal mucosa (villi height and depth of crypts) of our animals. The literature data reveal that the small intestine of the rat responds to the experimental with hyperplasia and hypertrophy of the mucosa[41,42] and an increase of the thickness of the submucosa layer and total wall[42]. These results cannot be considered contradictory to ours, since these authors maintained acute diabetes. They also agree partially with the data of Zoubi *et al*[3], who did not detect alterations in the morphometry of the intestinal crypts in the small intestine of rats after 84 d of inducing the diabetes mellitus. However, they observed an increase in the mitotic index.

As the intrinsic enteric nervous system mediates signals of the autonomic nervous system that regulate the dynamics of the intestinal epithelium carrying out an inhibitory control about the same one. Zucoloto *et al*[43] and Hernandes *et al*[44] carried out denervation of the myenteric plexus with benzalkonium chloride in rats. Zucoloto *et al*[43] did not observe differences in the cellular growth of crypts after 5 mo and Hernandes *et al*[44] observed an increase in cellular proliferation of the mucosa 15 d after denervation, but not after 23 d. We believe that the results found for the cellular proliferation of the intestinal mucosa may be related to an adaptation of the mucosa layer to the chronic pathogenesis of diabetes mellitus (if we consider the long period, 120 d, of maintaining diabetes mellitus).

Despite maintaining the morphometry of the mucosa layer, we presume that the diabetes mellitus did not lead to functional damage of the jejunum revealing an important mechanism of intestinal adaptation to chronic diabetes mellitus. On the other hand, the absence of morphometric variations on the intestinal mucosa of the diabetic rats supplemented with AA is explained by the effect of this antioxidant on the myenteric neurons being too little to promote significant changes.

The conclusion was that supplementation with AA in the diabetic animal promoted a moderate neuroprotection. Alteration of the cellular proliferation of the jejunum mucosa layer of rats was not observed, with chronic diabetes mellitus with or without supplementation with AA.

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COMMENTS

Background

Diabetes mellitus (DM) is caused by inadequacy of insulin production in the pancreas, when this organ either totally stops producing insulin or does not produce enough of the hormone. When badly controlled, the glucose level increases abnormally and causes harm, such as dysfunction and failure of several organs, especially nerves, kidneys, eyes, heart and blood vessels. In DM, the concentration of free radicals and/or the oxidative stress increases in an uncontrolled way, causing lesions on the neurons. The oxidative stress in DM is intensified due to the reduction of antioxidant levels, an increase of non-enzymatic glycation, and also frequent inflammations. Drugs that reduce the oxidative stress may have a relevant role in the treatment of the neurological complications of DM. Ascorbic acid (AA) is one of these substances, and it seems to be a promising one, not in the sense of curing diabetes, but of contributing to the maintenance of better conditions of neural conduction.

Research frontiers

The repercussions of the DM on the longevity and quality of life of individuals

in this condition, and their economic implications affect the productive potential and overburden the health system, has acted as motivation for many searches that seek to assess substances which might collaborate in the reduction of degenerative processes arising from DM. Results with AA have been promising, not to cure diabetes, but at least contribute to maintenance of better neural conditions, and reduction of oxidative stress common to this pathology.

Innovations and breakthroughs

Due to the fact of a differential effect of streptozotocin-diabetes on different regions of the rat intestine, our group carried out experiments in different intestinal segment in whole mounts of jejunum of diabetic rats supplemented with AA (1 g/L) and found that supplementation with AA in the diabetic animal promoted moderate neuroprotection.

Applications

The authors understand that the confirmation of the real benefits of AA will be consolidated or discarded based on new research, but will also be of some importance to the disclosure of reports of cases where this product was administered to treat patients, thus contributing to publication of a history containing comments on its use.

Peer review

The present study shows the myenteric neurons and intestinal mucosa of diabetic rats after AA supplementation. This present study is an interesting trial.

REFERENCES

1 **Silverthorn DU**. Fisiologia integrada. Fisiologia humana. Uma abordagem integrada. 2ed. Barueri (SP): Manole, 2003: 657

2 **Lorenz-Meyer H**, Thiel F, Menge H, Gottesbüren H, Riecken EO. Structural and functional studies on the transformation of the intestinal mucosa in rats with experimental diabetes. *Res Exp Med* (Berl) 1977; **170**: 89-99

3 **Zoubi SA**, Williams MD, Mayhew TM, Sparrow RA. Number and ultrastructure of epithelial cells in crypts and villi along the streptozotocin-diabetic small intestine: a quantitative study on the effects of insulin and aldose reductase inhibition. *Virchows Arch* 1995; **427**: 187-193

4 **Zbar AP**, Simopoulos C, Karayiannakis AJ. Cadherins: an integral role in inflammatory bowel disease and mucosal restitution. *J Gastroenterol* 2004; **39**: 413-421

5 **Musso F**, Lachat JJ, Cruz AR, Goncalves RP. Effect of denervation on the mitotic index of the intestinal epithelium of the rat. *Cell Tissue Res* 1975; **163**: 395-402

6 **Holle GE**, Granat T, Reiser SB, Holle F. Effects of superior mesenteric and coeliac ganglionectomy on the small intestinal mucosa in the Hanford mini pig. I. Histological and enzyme-histochemical study. *J Auton Nerv Syst* 1989; **26**: 135-145

7 **Hernandes L**, Bazotte RB, Gama P, Miranda-Neto MH. Streptozotocin-induced diabetes duration is important to determine changes in the number and basophily of myenteric neurons. *Arq Neuropsiquiatr* 2000; **58**: 1035-1039

8 **Wade PR**. Aging and neural control of the GI tract. I. Age-related changes in the enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G489-G495

9 **Belai A**, Lincoln J, Milner P, Burnstock G. Differential effect of streptozotocin-induced diabetes on the innervation of the ileum and distal colon. *Gastroenterology* 1991; **100**: 1024-1032

10 **Zanoni JN**, Buttow NC, Bazotte RB, Miranda Neto MH. Evaluation of the population of NADPH-diaphorase-stained and myosin-V myenteric neurons in the ileum of chronically streptozotocin-diabetic rats treated with ascorbic acid. *Auton Neurosci* 2003; **104**: 32-38

11 **Shotton HR**, Broadbent S, Lincoln J. Prevention and partial reversal of diabetes-induced changes in enteric nerves of the rat ileum by combined treatment with alpha-lipoic acid and evening primrose oil. *Auton Neurosci* 2004; **111**: 57-65

12 **Cameron NE**, Cotter MA, Maxfield EK. Anti-oxidant treatment prevents the development of peripheral nerve dysfunction in streptozotocin-diabetic rats. *Diabetologia* 1993; **36**: 299-304

13 **Stevens MJ**, Lattimer SA, Kamijo M, Van Huysen C, Sima AA, Greene DA. Osmotically-induced nerve taurine depletion and the compatible osmolyte hypothesis in experimental diabetic neuropathy in the rat. *Diabetologia* 1993; **36**: 608-614

14 **Greene DA**, Lattimer SA. Impaired rat sciatic nerve sodium-potassium adenosine triphosphatase in acute streptozocin diabetes and its correction by dietary myo-inositol supplementation. *J Clin Invest* 1983; **72**: 1058-1063

15 **Feldman EL**, Stevens MJ, Greene DA. Pathogenesis of diabetic neuropathy. *Clin Neurosci* 1997; **4**: 365-370

16 **Hosking DJ**, Bennett T, Hampton JR. Diabetic autonomic neuropathy. *Diabetes* 1978; **27**: 1043-1055

17 **Silva CB**, Teixeira MJ. Neuropatia diabética. *Rev Med* 1999; **78**: 150-162

18 **Will JC**, Byers T. Does diabetes mellitus increase the requirement for vitamin C? *Nutr Rev* 1996; **54**: 193-202

19 **Cunningham JJ**. Micronutrients as nutriceutical interventions in diabetes mellitus. *J Am Coll Nutr* 1998; **17**: 7-10

20 **Young IS**, Torney JJ, Trimble ER. The effect of ascorbate supplementation on oxidative stress in the streptozotocin diabetic rat. *Free Radic Biol Med* 1992; **13**: 41-46

21 **Koenig RJ**, Peterson CM, Jones RL, Saudek C, Lehrman M, Cerami A. Correlation of glucose regulation and hemoglobin AIc in diabetes mellitus. *N Engl J Med* 1976; **295**: 417-420

22 **Bergmeyer HU**, Bernet E. Determination of glucose with glucose-oxidase and peroxidase. Methods of enzymatic analysis. 2ed. New York: Academic Press, 1974: 6

23 **Henry RJ**, Cannon DC, Winkilman JW. Química cliníca: bases e técnicas. *Editorial JIMS* 1980: 10-12

24 **Drengk AC**, Kajiwara JK, Garcia SB, Carmo VS, Larson RE, Zucoloto S, Espreafico EM. Immunolocalisation of myosin-V in the enteric nervous system of the rat. *J Auton Nerv Syst* 2000; **78**: 109-112

25 **McLean IW**, Nakane PK. Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. *J Histochem Cytochem* 1974; **22**: 1077-1083

26 **Zanoni JN**, De Freitas P, Pereira RV, Dos Santos Pereira MA, De Miranda Neto MH. Effects of supplementation with ascorbic acid for a period of 120 days on the myosin-V and NADPHd positive myenteric neurons of the ileum of rats. *Anat Histol Embryol* 2005; **34**: 149-153

27 **Espreafico EM**, Cheney RE, Matteoli M, Nascimento AA, De Camilli PV, Larson RE, Mooseker MS. Primary structure and cellular localization of chicken brain myosin-V (p190), an unconventional myosin with calmodulin light chains. *J Cell Biol* 1992; **119**: 1541-1557

28 **Young IS**, Tate S, Lightbody JH, McMaster D, Trimble ER. The effects of desferrioxamine and ascorbate on oxidative stress in the streptozotocin diabetic rat. *Free Radic Biol Med* 1995; **18**: 833-840

29 **Garg MC**, Singh KP, Bansal DD. Effect of vitamin C supplementation on oxidative stress in experimental diabetes. *Indian J Exp Biol* 1997; **35**: 264-266

30 **Lindsay RM**, Jamieson NS, Walker SA, McGuigan CC, Smith W, Baird JD. Tissue ascorbic acid and polyol pathway metabolism in experimental diabetes. *Diabetologia* 1998; **41**: 516-523

31 **Sun F**, Iwaguchi K, Shudo R, Nagaki Y, Tanaka K, Ikeda K, Tokumaru S, Kojo S. Change in tissue concentrations of lipid hydroperoxides, vitamin C and vitamin E in rats with streptozotocin-induced diabetes. *Clin Sci* (Lond) 1999; **96**: 185-190

32 **Bhatnagar A**, Srivastava SK. Aldose reductase: congenial and injurious profiles of an enigmatic enzyme. *Biochem Med Metab Biol* 1992; **48**: 91-121

33 **Gabbay KH**. The sorbitol pathway and the complications of diabetes. *N Engl J Med* 1973; **288**: 831-836

34 **Ghahary A**, Luo JM, Gong YW, Chakrabarti S, Sima AA, Murphy LJ. Increased renal aldose reductase activity,

immunoreactivity, and mRNA in streptozocin-induced diabetic rats. *Diabetes* 1989; **38**: 1067-1071

35 **Milani E**, Nikfar S, Khorasani R, Zamani MJ, Abdollahi M. Reduction of diabetes-induced oxidative stress by phosphodiesterase inhibitors in rats. *Comp Biochem Physiol C Toxicol Pharmacol* 2005; **140**: 251-255

36 **Cotter MA**, Love A, Watt MJ, Cameron NE, Dines KC. Effects of natural free radical scavengers on peripheral nerve and neurovascular function in diabetic rats. *Diabetologia* 1995; **38**: 1285-1294

37 **Zanoni JN**, Hernandes L, Bazotte RB, Miranda Neto MH. Terminal ileum submucous plexus: Study of the VIP-ergic neurons of diabetic rats treated with ascorbic acid. *Arq Neuropsiquiatr* 2002; **60**: 32-37

38 **Pereira MA**, Bagatin MC, Zanoni JN. Effects of the ascorbic acid supplementation on NADH-diaphorase myenteric neurons in the duodenum of diabetic rats. *Biocell* 2006; **30**: 295-300

39 **Zanoni JN**, Pereira RVF, Freitas P. Effect of the ascorbic acid treatment on the NADHd-positive myenteric neurons of diabetic rats proximal colon. *Braz Arch Biol Technol* 2007; **50**: 31-38

40 **Fregonesi CEPT**, Miranda-Neto MH, Molinari SL, Zanoni JN. Quantitative study of the myenteric plexus of the stomach of rats with streptozotocin-induced diabetes. *Arq Neuropsiquiatr* 2001; **59**: 50-53

41 **Miller DL**, Hanson W, Schedl HP, Osborne JW. Proliferation rate and transit time of mucosal cells in small intestine of the diabetic rat. *Gastroenterology* 1977; **73**: 1326-1332

42 **Zhao J**, Yang J, Gregersen H. Biomechanical and morphometric intestinal remodelling during experimental diabetes in rats. *Diabetologia* 2003; **46**: 1688-1697

43 **Zucoloto S**, Diaz JA, Oliveira JS, Muccilo G, Sales Neto VN, Kajiwara JK. Effect of chemical ablation of myenteric neurones on intestinal cell proliferation. *Cell Tissue Kinet* 1988; **21**: 213-219

44 **Hernandes L**, Zucoloto S, Alvares EP. Effect of myenteric denervation on intestinal epithelium proliferation and migration of suckling and weanling rats. *Cell Prolif* 2000; **33**: 127-138

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RAPID COMMUNICATION

Rubber band ligation for 750 cases of symptomatic hemorrhoids out of 2200 cases

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Abstract

AIM: To study the results for the treatment of symptomatic hemorrhoids using rubber band ligation (RBL) method.

METHODS: A retrospective study for 750 patients who came to the colorectal unit from June, 1998 to September, 2006, data was retrieved from archived files. RBL was performed using the Mc Gown applicator on an outpatient basis. The patients were asked to return to out-patient clinic for follow up at 2 wk, 1 mo, 6 mo and through telephone call every 6 mo for 2 years).

RESULTS: After RBL, 696 patients (92.8%) were cured with no difference in outcome for second or third degree hemorrhoids (P = 0.31). Symptomatic recurrence was detected in 11.04% after 2 years. A total of 52 patients (6.93%) had 77 complications from RBL which required no hospitalization. Complications were pain, rectal bleeding and vaso-vagal symptoms (4.13%, 4.13% and 1.33% of patients, respectively). At 1 mo there were a significant improvement in mean SF-36 scores over baseline in five items, while after 2 years there were improvement in all items over baseline, but not significant. No significant manometric changes after band ligation.

CONCLUSION: RBL is a simple, safe and effective method for treating symptomatic second and third degree hemorrhoids as an out patient procedure with significant improvement in quality of life. RBL doesn't alter ano-rectal functions.

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Key words: Piles; Rectal bleeding; Barron banding; Hemorrhoidectomy

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INTRODUCTION

Hemorrhoids are the clinical manifestation of the downward disruption of normal functional structures known as the anal cushions[1]. Hemorrhoids are considered one of the most frequent diseases of the anal region with high prevalence (nearly 50% of proctological visits in a colorectal unit)[2], involving any age and affecting both males and females equally[3,4]. They commonly occur in patients with chronic increased intra-abdominal pressure as well as in pregnancy[5].

Numerous modalities and techniques have been

developed to treat symptomatic hemorrhoids ranging from simple dietary measures and bowel habit regulation, through a number of non-operative procedures, to different techniques of excision of diseased anal cushions. The vast amount of treatment options means none are close to perfection[6]. While many non-operative procedures are effective in controlling symptoms, at least from the patients' perspective, they all share the common problem of recurrence[7]. Although, surgical hemorrhoidectomy is more definitive in symptom control, it has a reputation for being a painful procedure for a relatively benign disorder[8]. First, second and third degree hemorrhoids can be treated by non surgical methods in outpatient clinics while severe prolapsed or circumferential hemorrhoids can be treated using a variety of surgical techniques, e.g. Milligan Morgan, Longo and others[9,10].

Nonsurgical methods aim at tissue fixation (sclerotherapy, cryotherapy, photocoagulation, laser), or fixation with tissue excision [rubber band ligation (RBL)][11]. RBL is considered the most widely used procedure, and it offers the possibility to resolve hemorrhoidal disease without the need for hospitalization or anaesthesia, and with lower incidence of complications[12,13].

In this retrospective study, we analyze the effectiveness, safety, quality of life and results (early and long term) of RBL in the management of symptomatic hemorrhoids as outpatient procedure.

MATERIALS AND METHODS

This is a retrospective analysis of data of patients diagnosed with hemorrhoids who were managed by RBL at the outpatient clinic of colorectal surgery unit, Mansoura university hospitals from June, 1998 to September, 2006. Data, from 750 patients who had symptomatic hemorrhoids and treated by RBL, were retrieved from archived files of our colorectal surgery unit out of 2200 cases of hemorrhoids. The mean age was 39.13 ± 14.75 years (ranging from 15 to 90 years old). Six hundreds and twenty seven patients (82.8%) were males, while 123 patients (17.2%) were females. Sixty four patients (8%) had chronic liver disease. Supplemental information was obtained from telephone follow up.

The policy of our colorectal unit stated for the following inclusion and exclusion criteria: Patients at any age with first, second or third degree internal piles were included. While, patients with: Previous ano-rectal surgery, associated ano-rectal pathology "fissure, fistula …*etc*", fourth degree hemorrhoids and complicated piles (infection, ulceration or strangulation) were excluded.

Patients who fulfilled inclusion criteria were subjected to: Thorough history taking including the following items; age, sex, occupation and residence, presentation (bleeding, prolapse, anal pain, discharge, and pruritus). For local examination, the patient was examined when relaxed in the left lateral position and the local examination to anal region is carried out by inspection, palpation, P.R. examination, proctoscopic examination and sigmoidoscopic examination for patients above 50 years.

Ano-rectal manometery was performed using a standard low compliance water perfusion system and eight-channels catheters with pressure transducer connected to 5.5 mm manometric probe with spirally located ports at 0.5 cm interval. The protocol of performance is a stationary pull through technique with recording the functional length of the anal canal (FL), mean maximum resting pressure, mean squeeze pressure.

All patients received a cleansing rectal enema before the procedure to avoid bowel movement in first 24 h so ligatures would not be expelled. The procedure was done in left lateral position. No anaesthesia was used; no antibiotics were administered, except to patients with valvular heart diseases or chronic liver disease.

The procedure was performed through the proctoscope, which was inserted and placed about 1-2 cm. above the dentate line using K-Y gel as a lubricant. The hemorrhoidal cushion was allowed to prolapse into the lumen of proctoscope, after that it was sucked into the Mc Gown ligator. It was important that the patient experienced no pain when the cushion was sucked; if pain was experienced, the cup was placed in a more proximal position. The tissue was drawn into the drum until it was taut, and the trigger was released, expelling rubber O-ring with an inner diameter of about 1 mm around the base of the haemorrhoid. The policy of our unit is that all piles are ligated in the same session.

By the end of the procedure, each patient treated by RBL was kept in the outpatient clinic and observed for 1-2 h following the procedure, in order to detect any early complication as hemorrhage and pain. The patients were informed about the progress of the treatment (fall of the necrosed hemorrhoidal nodule). We recommended high residue diet, mild laxative to softening the stool, local anal hygiene, avoidance of straining, and information concerning early and late complications.

The patients were asked to return to out-patient clinic for follow up at 2 wk, 1 mo, 6 mo and then through telephone call every 6 mo for 2 years. Subsequent ligations were performed at 1 mo after the prior one, if the patients still had symptoms.

Results were classified as following; cure if the patient was asymptomatic after the end of treatment, improvement if the symptoms had been minimized, and as failure of the method if no improvement whatsoever occurred.

Post ligation complications include: pain, Vaso-vagal symptoms (dizziness or fainting), retention of urine, bleeding per rectum whether primary, secondary or reactionary and post ligation infection were recorded. Also, the patient was asked about continence and anal stricture during scheduled P.R examinations

Functional status was assessed with the medical outcomes study 36-item short-form general health survey (SF-36) questionnaire, version 2[14] (pre procedure,

1 mo post procedure and after 2 years). The SF 36 consists of eight multi-item dimensions, each of which generates a score of between 0 and 100; higher scores indicate higher levels of perceived health.

Statistical analysis

The statistical analysis of data was done by using SPSS (Statistical Package for Social Science) version 10 under Microsoft Windows XP. The description of data was done in form of mean ± SD for quantitative data; while frequency and proportion for qualitative data. The analysis of data was done to test the statistical significant difference between groups. For quantitative data, to compare between 2 groups Student *t*-test was used and for qualitative data χ^2 test was used. $P < 0.05$ was considered significant.

RESULTS

Our study includes 750 patients with hemorrhoidal disease with a mean age was 39.13 ± 14.75 years (ranging from 15 to 90 years old). Six hundred and twenty seven patients (82.8%) were males, while 123 patients (17.2%) were females. Male to female ratio was 5.09:1.

Demographic data, clinical presentation and severity of hemorrhoids for all patients are shown in Table 1.

In 473 patients (63.06%), one session ligation was performed while in the remaining patients, multiple hemorrhoidal ligations were performed in two sessions for 259 patients (34.53%), and three sessions for 18 patients (2.4%) with 1 mo interval.

The total of 2122 ligations in 1045 sessions were carried out, with a mean of 2.35 (± 0.49) per patient and 2.03 (± 0.54) per session.

Successful results were achieved in 696 patients (92.8%), 650 patients (86.66%) were cured after the end of treatment, whereas, 46 patients (6.13%) improvement was reported. Fifty four patients (7.2%) failed to get any benefit from RBL. There was no significant difference in the outcome of RBL between second and third degree hemorrhoids ($P = 0.31$) Table 2.

Two years after the end of treatment, 643 patients 85.73% came for follow up. Symptomatic recurrence was detected in 71 out of 643 patients (11.04%), with repeated treatment by RBL in 23 cases and additional surgical treatment was required in another 48 patients due to severe symptoms in 30 patients, associated anal fissure in 6 patients, and patient desire in 12 patients Tables 3 and 4.

Seventy seven complications from RBL were encountered in 52 patients (6.93%) as shown in Table 5. Pain was occurred in 31 patients (4.13), 22 cases (3.78%) had second degree hemorrhoids and 9 cases (5.35%) had third degree hemorrhoids with no statistical significance ($P = 0.8$) (Table 5). Patients with multiple hemorrhoidal banding, when compared with patients with single banding had great discomfort and pain (19/277, 6.85% *vs* 12/473, 2.53% respectively). Pain was treated conservatively with analgesic and warm baths in all patients and no patient forced to remove the band.

Table 1 Demographic and clinical data for patients *n* (%)

Variables	Number of patients
Age (yr)	39.13 ± 4.75 (15-90)
Sex	
Male	627 (82.8)
Female	123 (17.2)
Cirrhotic patients	64 (8.53)
Child A	23
Child B	15
Child C	6
Grade of haemorrhoid	
G2	582 (77.6)
G3	168 (22.4)
Clinical presentation	
Bleeding	612 (81.6)
Prolapse	496 (66.13)
Constipation	267 (35.6)
Pruritus	64 (8.53)
Pain	30 (4)

Table 2 Early results of RBL in 750 patients *n* (%)

Results	Total	Grade Ⅱ	Grade Ⅲ	*P*
Cured	650 (86.66)	522 (89.7)	128 (76.19)	0.31
Improvement	46 (6.13)	24 (4.12)	22 (13.09)	0.23
Failure	54 (7.2)	36 (6.18)	18 (10.7)	0.16
Total	750	582	168	

Table 3 Long term results and follow up of patients with RBL

Results	Patients	Re-examined	Asymptomatic	Symptomatic	Surgery	RBL
Cured	650	610	572	38	30	8
Improvement	46	33		33	18	15
Failure	54	-	-	-	-	-
Total	750	643	572	71	48	23

Table 4 Patients with recurrence

	No. of patients (%)
Initial grade	
Grade Ⅱ (582)	49 (8.42)
Grade Ⅲ (168)	22 (13.1)
Grade at follow up	
Grade Ⅰ	19 (26.7)
Grade Ⅱ	42 (59.15)
Grade Ⅲ	10 (14.08)
Symptoms	
Bleeding	68 (95.8)
Prolapse	50 (70.42)
Pruritus	16 (22.53)
Pain	15 (21.12)
Associated fissure	6 (8.4)
Line of treatment	
Re-banding	23 (32.4)
Surgical treatment	48 (67.6)
Severe symptoms	30
Associated fissure	6
Patient's desire	12

Mild rectal bleeding was reported in 31 cases (4.13%) and it was occurred 7-14 d after the procedure. It was treated conservatively in all cases with no need for

Table 5 Post-banding complications *n* (%)

Complication	Degree of hemorrhoids		Total	*P*
	2nd (*n* = 582)	3rd (*n* = 168)		
Pain	22 (3.78)	9 (5.35)	31 (4.13)	0.8
Bleeding	21 (2.8)	10 (5.95)	31 (4.13)	0.35
Vaso-vagal symptoms	8 (1.37)	2 (1.19)	10 (1.33)	0.23
Infection	1 (0.17)	-	1 (0.13)	-
Fistula	1 (0.17)	-	1 (0.13)	-
Fissure	2 (0.34)	1 (0.59)	3 (0.4)	0.79
Total	55 (9.45)	22 (13.09)	77 (10.26)	0.09

Table 6 Quality of life using mean SF-36 scores pre-banding and at 1 mo and 2 yr follow up

Dimension	Pre-banding	After 6 mo	*P*	At 2 yr follow up	*P*
Physical functioning (PF)	58.98 ± 2.5	63.6 ± 3.4	0.03	60.12 ± 5.2	0.08
Role limitation due to physical problem (RP)	64.8 ± 15.22	63.6 ± 11.2	0.67	63.1 ± 10.2	0.56
Body pain (BP)	60.5 ± 4.32	63.6 ± 6.5	0.04	61.4 ± 7.1	0.07
General health (GH)	74.8 ± 4.9	76.5 ± 6.7	0.54	75.8 ± 6.6	0.57
Vitality (VT)	69.3 ± 12.5	70.2 ± 11.66	0.02	69.8 ± 12.3	0.09
Social functioning (SF)	73.9 ± 6.2	75.6 ± 5.66	0.05	74.1 ± 7.5	0.07
Role limitation due to emotional problem (RE)	60.4 ± 3.2	58.5 ± 5.6	0.08	65.2 ± 4.5	0.09
Mental heath (MH)	71.9 ± 2.8	73.5 ± 2.3	0.01	73.6 ± 5.2	0.04

blood transfusion or hospitalization in any of these cases. Post banding bleeding occurred in 21 cases (2.8%) with second degree hemorrhoids and in third degree hemorrhoids only 10 cases (5.95%) complicated with mild bleeding after RBL with no statistical significance ($P = 0.35$) (Table 5).

Post-banding vaso-vagal symptoms were reported in 10 cases (1.33%). There were no cases of urine retention that necessitate catheterization, fecal incontinence did not occur after RBL in this study, also no cases were complicated by anal stenosis (Table 5).

Perianal abscess occurred in one case (0.13%) after RBL. It was drained, but 2 mo later the patient developed low anal fistula which was treated by fistulectomy. Three patients (0.4%) complicated with anal fissure after RBL.

There was no significant difference in pre banding and post banding manometric study (pre banding and post banding mean resting pressure were 87.29 ± 17.44 and 86.45 ± 15.46, respectively, $P = 0.065$) and (pre banding and post banding mean squeeze pressure were 227.82 ± 43.82 and 227.04 ± 44.12, $P = 0.193$).

The SF-36 questionnaires were completed by 730 patients pre banding, 720 patients at 1 mo and 630 patients at 2 years for assessment of quality of life. At 1 mo after banding there was greater improvement in mean score over baseline, the difference observed were significant for five dimensions in physical, social activity, vitality, freedom of pain and mental health. At 2 years after banding there were greater improvement in mean scores over baseline for all items, but only mental health showed significant difference Table 6.

DISCUSSION

The need to treat hemorrhoids is based primarily on the severity of symptoms, but the type of treatment is based on the traditional classification of hemorrhoids, which may have little to do with symptom severity[15]. The best treatment remains unanswered despite of the wide variety of treatment options in use. Safety is of paramount importance, especially when treating a benign disease such as hemorrhoids[16,17]. Although surgical hemorrhoidectomy is more definitive in symptom control, it has a reputation of having a significant postoperative pain and an extended recovery time for a relatively benign disorder[7]. Nowadays, rubber-band ligation is the most widely used procedure, and it offers the possibility to resolve hemorrhoids disease without the need for hospitalization or anaesthesia, and with a lower incidence of complications when compared to conventional surgery[15].

The success rates of the method range between 79% and 91.8%[11,16,18]. Wrobleski *et al*[19] reported that 80% of their patients improved and 69% were symptom-free at a mean follow-up of 5 years. There was no difference in success rates of RBL in 1st, 2nd and 3rd degree hemorrhoids[11]. In our study, successful results were achieved in 696 patients (92.8%), 650 patients (86.66%) were cured or presented great improvement after the end of treatment and 2 years later, 572 patients were cured out of 643 patients who attended follow-up. Johanson *et al*[20] showed that 6.6%-14% of the patients undergoing RBL will require additional treatment, due to the recurrence of symptoms. Many authors reported that recurrence rate may be as high as 68% at 4 or 5 years of follow-up and symptoms usually respond to repeated ligation, but only 10% of such patients require excisional hemorrhoidectomy[21,22]. Vassillios *et al*[11] reported that symptomatic recurrence was 11.9% (53/445) 2 years after RBL, with repeat RBL or surgery in (41/445) 9.2% cases. Bayer *et al*[23] found that 18% of their patients required one or more additional sessions of RBL while 2.1% failed to be cured by RBL and were referred for conventional hemorrhoidectomy. In our study, symptomatic recurrence was detected in 11.04% (71/643) after 2 years follow up, with repeated treatment by RBL in 23 cases while additional surgical treatment was required in 48 patients.

A review of 39 studies incorporating 8 060 patients undergoing RBL revealed post banding complications in the form of severe pain in 5.8%, hemorrhage in 1.7%, infection in 0.05% anal fissure and fistula in 0.4%[24]. Bat *et al*[16] showed that the complications rate after RBL was relatively low (4.2%), most of the complications were minor and self limiting. only 2.5% of his patients had severe complications that required hospitalization. Vassillios *et al*[11] reported that in 94 patients (18.8%), complications were occurred. In our series, seventy seven complications from RBL encountered in 52 patients (6.93%) were mostly minor and no hospitalization was needed. Many authors reported that post banding pain was frequently observed even during careful placement

above the dentate line[25]. Furthermore, pain and anal discomfort were reported to be greater, ranging from 28% to 79%, when multiple bandings were performed per session[26]. Gupta[18] found that out of 44 patients underwent RBL; seven patients (15.9%) reported pain and Oueidat and Jurjus[27] noted that out of 148 patients underwent RBL; twenty patients (13.5%) reported pain. We found that, pain occurred in 31 patients (4.13%), in all cases the pain appeared immediately or few hours after the ligation and lasted less than 2-3 d. Patients with multiple hemorrhoidal banding in one session had greater discomfort and pain. These results are in accordance with those of Vassillios *et al*[11], who reported that patients with multiple hemorrhoidal banding in a single session compared with patients with single banding had greater discomfort and pain (9.35% *vs* 1.96%). Also, Lee *et al*[28] and Gehmy *et al*[29] reported the same results. On the contrary, Hardwick *et al*[30] failed to show any relationship between the number of bands applied and the degree of pain. Moreover, Khubchandani[31], in a prospective randomized study, compared the results of single, double and triple hemorrhoidal ligation, but did not notice any difference even if they were forced to remove the elastic band in many cases in the third group.

Bleeding is a significant complication of RBL and it cannot be prevented. It is the result of the fall of the hemorrhoidal nodule and local inflammation, bleeding in our series occurred in 31 cases (4.13%). It was mild and treated conservatively in all cases without hospitalization or blood transfusion. Band ligation is safe in patients with cirrhosis and portal hypertension as reported by Vassillios *et al*[11]. Bat *et al*[16] and Bayer *et al*[23] reported that only 2.2% of his patients complicated by rectal bleeding.

Watson *et al*[32], in their study of 183 cases of band ligation, found that 41 patients (30%) of patients had vaso-vagal symptoms. Kumar *et al*[33] reported that 15.3% of their studied group (98 patients) had vaso-vagal symptoms. In our study, Post-banding vaso-vagal symptoms occurred in 10 cases (1.33%). There were no cases of urine retention that necessitate catheterization, fecal incontinence or cases complicated by anal stenosis after band ligation. This also reported by Benzoni *et al*[34] and Watson *et al*[32].

Bursics *et al*[35] reported that maximum resting pressure and squeeze pressure remained unchanged after RBL, as found in our study.

Our conclusion is that RBL is a simple, safe and effective method for treating symptomatic second and third degree hemorrhoids as an out patient procedure with significant improvement in quality of life. RBL can be used to treat grade 2 and 3 hemorrhoids with similar effectiveness. RBL doesn't alter ano-rectal functions.

COMMENTS

Background

Numerous modalities and techniques have been developed to treat symptomatic hemorrhoids ranging from simple dietary measures and bowel habit regulation, through a number of non-operative procedures, to different techniques of excision of diseased anal cushions. The vast amount of treatment options means none are close to perfection. Nonsurgical methods aim at tissue fixation (sclerotherapy, cryotherapy, photocoagulation, laser), or fixation with tissue excision [rubber band ligation (RBL)].

Research frontiers

RBL is a simple, safe and effective method for treating symptomatic second and third degree hemorrhoids as out patient procedure with significant improvement in quality of life. RBL doesn't alter ano-rectal functions.

Innovations and breakthroughs

RBL is effective method for treating symptomatic hemorrhoids as an out patient procedure with significant improvement in quality of life. RBL doesn't alter ano-rectal functions.

Applications

After RBL, 696 patients (92.8%) were cured with no difference in outcome for second or third degree hemorrhoids (P = 0.31). Symptomatic recurrence was detected in 11.04% after 2 years. A total of 52 patients (6.93%) had 77 complications from RBL which required no hospitalization. Complications were pain, rectal bleeding and vaso-vagal symptoms (4.13%, 4.13% and 1.33% of patients respectively). At 1 mo there were a significant improvement in mean SF-36 scores over baseline in five items while after 2 years there were improvement in all items over baseline but not significant. No significant manometric changes after band ligation.

Peer review

This is an interesting study of a large series of patients with RBL and their results. Authors retrospectively analyzed the effectiveness, safety, quality of life and results of RBL in the management of symptomatic hemorrhoids. The paper is well written.

REFERENCES

1 **Thomson WH**. The nature of hemorrhoids. *Br J Surg* 1975; **62**: 542-552
2 **Bernal JC**, Enguix M, Lopez Garcia J, Garcia Romero J, Trullenque Peris R. Rubber-band ligation for hemorrhoids in a colorectal unit. A prospective study. *Rev Esp Enferm Dig* 2005; **97**: 38-45
3 **Salvati EP**. Nonoperative management of hemorrhoids: evolution of the office management of hemorrhoids. *Dis Colon Rectum* 1999; **42**: 989-993
4 **Haas PA**, Haas GP, Schmaltz S, Fox TA Jr. The prevalence of hemorrhoids. *Dis Colon Rectum* 1983; **26**: 435-439
5 **Loder PB**, Kamm MA, Nicholls RJ, Phillips RK. Haemorrhoids: pathology, pathophysiology and aetiology. *Br J Surg* 1994; **81**: 946-954
6 **Smith LE**. Hemorrhoids. A review of current techniques and management. *Gastroenterol Clin North Am* 1987; **16**: 79-91
7 **MacRae HM**, McLeod RS. Comparison of hemorrhoidal treatment modalities. A meta-analysis. *Dis Colon Rectum* 1995; **38**: 687-694
8 **Cheng FC**, Shum DW, Ong GB. The treatment of second degree haemorrhoids by injection, rubber band ligation, maximal anal dilatation, and haemorrhoidectomy: a prospective clinical trial. *Aust N Z J Surg* 1981; **51**: 458-462
9 **Fazio VW**. Early promise of stapling technique for haemorrhoidectomy. *Lancet* 2000; **355**: 768-769
10 **Rowsell M**, Bello M, Hemingway DM. Circumferential mucosectomy (stapled haemorrhoidectomy) versus conventional haemorrhoidectomy: randomised controlled trial. *Lancet* 2000; **355**: 779-781
11 **Vassilios A**, Komborozos VA, Skrekas GJ, Pissiotis CA. Rubber band ligation of symptomatic internal hemorrhoids: results of 500 cases. *Dig Surg* 2000; **17**: 71-76
12 **Pezzullo A**, Palladino E. [Rubber band ligation of hemorrhoids. 5-year follow-up] *G Chir* 2000; **21**: 253-256
13 **Longman RJ**, Thomson WH. A prospective study of outcome from rubber band ligation of piles. *Colorectal Dis* 2006; **8**: 145-148
14 **Ware JE Jr**, Sherbourne CD. The MOS 36-item short-form

health survey (SF-36). I. Conceptual framework and item selection. *Med Care* 1992; **30**: 473-483
15 **Shanmugam V**, Thaha MA, Rabindranath KS, Campbell KL, Steele RJ, Loudon MA. Systematic review of randomized trials comparing rubber band ligation with excisional haemorrhoidectomy. *Br J Surg* 2005; **92**: 1481-1487
16 **Bat L**, Melzer E, Koler M, Dreznick Z, Shemesh E. Complications of rubber band ligation of symptomatic internal hemorrhoids. *Dis Colon Rectum* 1993; **36**: 287-290
17 **Bleday R**, Pena JP, Rothenberger DA, Goldberg SM, Buls JG. Symptomatic hemorrhoids: current incidence and complications of operative therapy. *Dis Colon Rectum* 1992; **35**: 477-481
18 **Gupta PJ**. Radiofrequency coagulation versus rubber band ligation in early hemorrhoids: pain versus gain. *Medicina* (Kaunas) 2004; **40**: 232-237
19 **Wrobleski DE**, Corman ML, Veidenheimer MC, Coller JA. Long-term evaluation of rubber ring ligation in hemorrhoidal disease. *Dis Colon Rectum* 1980; **23**: 478-482
20 **Johanson JF**, Rimm A. Optimal nonsurgical treatment of hemorrhoids: a comparative analysis of infrared coagulation, rubber band ligation, and injection sclerotherapy. *Am J Gastroenterol* 1992; **87**: 1600-1606
21 **Savioz D**, Roche B, Glauser T, Dobrinov A, Ludwig C, Marti MC. Rubber band ligation of hemorrhoids: relapse as a function of time. *Int J Colorectal Dis* 1998; **13**: 154-156
22 **Walker AJ**, Leicester RJ, Nicholls RJ, Mann CV. A prospective study of infrared coagulation, injection and rubber band ligation in the treatment of haemorrhoids. *Int J Colorectal Dis* 1990; **5**: 113-116
23 **Bayer I**, Myslovaty B, Picovsky BM. Rubber band ligation of hemorrhoids. Convenient and economic treatment. *J Clin Gastroenterol* 1996; **23**: 50-52
24 **Wechter DG**, Luna GK. An unusual complication of rubber band ligation of hemorrhoids. *Dis Colon Rectum* 1987; **30**: 137-140
25 **Ramzisham AR**, Sagap I, Nadeson S, Ali IM, Hasni MJ. Prospective randomized clinical trial on suction elastic band ligator versus forceps ligator in the treatment of haemorrhoids. *Asian J Surg* 2005; **28**: 241-245
26 **Pfenninger JL**. Modern treatments for internal haemorrhoids. *BMJ* 1997; **314**: 1211-1212
27 **Oueidat DM**, Jurjus AR. Management of hemorrhoids by rubber band ligation. *J Med Liban* 1994; **42**: 11-14
28 **Lee HH**, Spencer RJ, Beart RW Jr. Multiple hemorrhoidal bandings in a single session. *Dis Colon Rectum* 1994; **37**: 37-41
29 **Gehamy RA**, Weakley FL. Internal hemorrhoidectomy by elastic ligation. *Dis Colon Rectum* 1974; **17**: 347-353
30 **Hardwick RH**, Durdey P. Should rubber band ligation of haemorrhoids be performed at the initial outpatient visit? *Ann R Coll Surg Engl* 1994; **76**: 185-187
31 **Khubchandani IT**. A randomized comparison of single and multiple rubber band ligations. *Dis Colon Rectum* 1983; **26**: 705-708
32 **Watson NF**, Liptrott S, Maxwell-Armstrong CA. A prospective audit of early pain and patient satisfaction following out-patient band ligation of haemorrhoids. *Ann R Coll Surg Engl* 2006; **88**: 275-279
33 **Kumar N**, Paulvannan S, Billings PJ. Rubber band ligation of haemorrhoids in the out-patient clinic. *Ann R Coll Surg Engl* 2002; **84**: 172-174
34 **Benzoni E**, Milan E, Cerato F, Narisetti P, Bresadola V, Terrosu G. Second degree haemorrhoids: patient's satisfaction, immediate and long-term results of rubber band ligation treatment. *Minerva Chir* 2006; **61**: 119-124
35 **Bursics A**, Weltner J, Flautner LE, Morvay K. Ano-rectal physiological changes after rubber band ligation and closed haemorrhoidectomy. *Colorectal Dis* 2004; **6**: 58-61

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RAPID COMMUNICATION

Colorectal carcinoma in Lagos and Sagamu, Southwest Nigeria: A histopathological review

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Abstract

AIM: To study the frequency, gender and age distribution as well as pathological characteristics of colorectal carcinoma (CRC) in Lagos and Sagamu in SW Nigeria.

METHODS: This is a retrospective pathological review of histologically diagnosed CRC from 5 laboratories in Lagos & Sagamu. The clinical data, such as age, sex and clinical summary were extracted from demographic information. Cases of anal cancer were excluded from this study.

RESULTS: There were 420 cases (237 males and 183 females) of CRC. It peaked in the 60-69 year age group (mean: 50.7; SD: 16.2), M:F ratio 1.3:1 and 23% occurred below 40 years. The majority was well to moderately differentiated adenocarcinoma 321 (76.4%), mucinous carcinoma 45 (10.7%) and signet ring carcinoma 5 (1.2%), and more common in patients under 40 years compared to well differentiated tumors. The recto-sigmoid colon was the most common site (58.6%). About 51% and 34% of cases presented at TNM stages Ⅱ and Ⅲ, respectively.

CONCLUSION: CRC is the commonest malignant gastrointestinal (GIT) tumor most commonly located in the recto-sigmoid region. The age and sex prevalence and histopathological features concur with reports from other parts of the world.

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Key words: Colorectal carcinoma; Adenocarcinoma; Pathological staging; Histopathological characteristics

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INTRODUCTION

Colorectal carcinoma (CRC) is an important cause of cancer death worldwide, but has variable geographical distribution. In developed countries, it is among the 3 most common cancers with an estimated worldwide incidence of 570 000 new cases per annum[1]. Previous

studies have shown it to be a rare disease in Africans representing 3%-6% of all malignant tumors in most African studies[2-5]. It accounts for 10%-50% of all gastrointestinal (GIT) malignancies in Nigeria[6-8]. Incidences from various parts of the country range between 3.75-6 cases per annum in South South/South-East Nigeria[9,10], 12.5-14.4 cases per annum in the North[11,12] and 26.3 cases per annum in SW regions, respectively[13]. An 81% increase in incidence over a period of two decades was reported from Ibadan SW Nigeria[13]. Several reasons have been adduced for this increase, including an increase in hospital attendance by the populace due to an increasing awareness about cancer and change in dietary habit among others[13]. Major predisposing factors in the etiopathogenesis of CRC include the presence of pre-malignant conditions, such as familial polyposis syndrome, inflammatory bowel disease (IBD) and dietary factors, such as diet rich in refined carbohydrate, diet low in fibre content and fresh vegetables, all of which increase fecal transit time[14]. The low incidence in Africans was attributed to fiber rich diet which is common practice and rarity of the familial polyposis syndrome and IBD[2]. Recent urbanization/civilization has resulted in upsurge of confectionary food outlets in major cities resulting in many Nigerians changing their dietary habit from a fiber rich diet, which was common practice to a highly refined carbohydrate and fat diet.

Molecular studies have characterized 5 subtypes of CRC which have different etio-pathogenetic pathways that correlate with the morphologic and prognostic features[15]. These subtypes are based on DNA microsatellite instability status (MMR) and CpG island methylator phenotype[15]. Hameed, in South Africa using immunohistochemistry to detect expression of hMLH1 & hMSH2 (MMR status) in CRC, found that while 60% had normal expression of both gene products, 40% showed negative expression of either of the two genes[16]. He further observed that CRC with absence MMR (hMLH1 or hMSH2) tended to be right sided, mucinous and poorly differentiated when compared with the tumors that express the gene product. Also, Lanza *et al*, in Italy studying MMR status in CRC reported that 15.9% of CRC studied had abnormal expression of hMLH1 and hMSH2. They also observed that patients whose tumor was MMR negative had better clinical outcome particularly in Stage Ⅱ and Ⅲ tumors[17]. This advantage was more evident in patients with surgery alone than those who had adjuvant chemotherapy.

The present study is aimed at documenting the frequency pattern, age and sex distribution, as well as histopathologic characteristics of CRC in Lagos and Sagamu in SW Nigeria, as a preliminary to immunophenotypic subtyping; which we are currently carrying out in collaboration with the Department of Histopathology & Molecular Biology, Leeds General infirmary, Leeds United Kingdom.

MATERIALS AND METHODS

The paraffin embedded blocks and slides as well as pathology reports of malignant colorectal tumors collected from five laboratories (Morbid Anatomy Departments of the Lagos University Teaching Hospital 1995-2007, Olabisi Onabanjo University Teaching Hospital in Sagamu, Ogun State, as well as the three private histolopathology laboratories in Lagos State between 2002-2007 viz: The Specialist Laboratory, Histolab Diagnostics and Seramoses Laboratory) constituted the materials utilized for this study.

The slides were reviewed to confirm the diagnosis, type and grade of the tumor. The resection samples were further staged using Tumor-Node-Metastases staging system of International Union against Cancer (TNM) and Duke's staging system.

A proforma was used to extract the bio data such as age, sex, clinical symptoms and signs as well as the endoscopic findings when available from the histopathology request forms; some of the patient's case files where available in their various clinics or hospitals.

The clinical biodata and histopathological characteristics were analyzed using Microsoft Excel software and presented as tables.

RESULTS

There were a total of 420 cases of CRC with 237 males and 183 females and a male to female ratio of 1.3:1. CRC was the most common malignant GIT tumor accounting for 59% of all GIT malignancies and representing 87% of all malignant tumors of the colorectal and anal regions. It accounted for 5.8% of all the 7225 malignant lesions diagnosed from the five laboratories during the period.

The youngest patient was 10 years while the oldest was 95 years with a mean age of 50.7 (SD-16.2). CRC peaked in the 60-69 years age group (mode 60 years). About 12% (12.4%) occurred in patients 30 years and below, 23% occurred below 40 years and 16.8% above the age of 60 years.

Left-sided (distal colon) tumor 261 (62%) was more common than right-sided (proximal) ones 58 (14%). More than half of the cases were located in the recto-sigmoid region 246 cases (58.6%) followed by caecum 34 cases (9%), ascending colon 24 cases (6%), transverse 19 cases (4.5%) and descending colon 15 cases (3.6%) each. In 82 cases (19.5%), the specific site was not indicated.

Macroscopically, the right sided tumors were fungating nodular lesions with surface ulcerations while the left sided tumors were flat and infiltrating or constricting. Microscopically, the tumors were adenocarcinoma of varying grades. Majority were well-differentiated adenocarcinoma in 233 (55.5%), 88 (21%) were moderately differentiated and 34 (8%) were poorly differentiated carcinoma. Mucinous and signet ring carcinomas accounted for 45 cases (10.7%) and 5 cases (1.2%), respectively. Fifteen cases (3.6%) were anaplastic (undifferentiated) tumors (Table 1).

Mucinous carcinoma (19%) and signet ring carcinoma (3%) were more common in patients under 40 years; compared to 4% & 0.6% record in patients

Table 1 Site and gender distribution and histological types of CRC in patients 40 yr and above compared with those below 40 yr *n* (%)

	40 yr and above	Below 40 yr
Site		
Cecum	23 (7.14)	11 (11)
Ascending colon	20 (6.21)	4 (4)
Transverse colon	13 (4.04)	6 (6)
Descending colon	13 (4.04)	2 (2)
Recto sigmoid	194 (60.25)	52 (53)
Unspecified	59 (18.32)	23 (23)
Total	322 (100)	98 (100)
Histological grade		
Well differentiated adenocarcinoma	191 (59.3)	42 (43)
Moderately differentiated adenocarcinoma	63 (19.6)	25 (26)
Poorly differentiated adenocarcinoma	38 (11.8)	7 (7)
Mucinous carcinoma	15 (4.7)	19 (19)
Signet Ring carcinoma	2 (0.6)	3 (3)
Undifferentiated	13 (4)	2 (2)
Total	322 (100)	98 (100)
M:F ratio	1.3:1	1.2:1

Table 2 Pathological staging of 123 cases of CRC

Duke's stage	*n* (%)	TNM staging	*n* (%)
A	17 (14)	Stage Ⅰ	17 (14)
B	64 (52)	Stage ⅡA	54 (43)
C	41 (33)	Stage ⅡB	10 (8)
D	1 (1)	Stage ⅢA	13 (11)
		Stage ⅢB	28 (23)
		Stage Ⅳ	1 (1)

above 40 years (Table 1). On the other hand, well differentiated adenocarcinoma was more common in patients above (59%) than those below 40 years (43%). The male to female ratio is also less for younger patients. The cases less than 40 years also tended to be located in the cecum (11%) compared to older patients (7%) (Table 1).

Pathological staging was carried out for 123 cases with colectomy samples using Duke's and TNM staging systems. Of the 123 cases, 14%, 51%, 34% and 1% presented at TNM stages Ⅰ, Ⅱ, Ⅲ and Ⅳ, respectively (Table 2).

The clinical presentation was varied including abdominal pain, abdominal mass, bloody mucoid stool, change in bowel habit, weight loss, anaemia, and/or features of intestinal obstruction.

DISCUSSION

In this study, CRC accounted for 5.8% of all malignancies diagnosed in the five laboratories. This concurs with figures of 3.7%-10% that have been reported from various parts of Nigeria and Africa[2-5,18]. In Libya, it was the 2nd most common malignant tumor accounting for 10% and 9% in males and females, respectively [18]. Ohanaka & Ofoegbu in Benin, South West Nigeria reported CRC to be the 3rd most common cancer[7]. From the surgical biopsy register of Morbid Anatomy Lagos University Teaching Hospital, CRC is the third most common cancer diagnosed after breast and cervix. The annual frequency of this cancer in Lagos as documented in this study, was 32.3, a figure which is higher than 3.76 cases/annum in the South-South, 14.4 in the North-Central and 26.3 recorded in Ibadan South West Nigeria, respectively[9,11,13]. The higher number in this study could be attributed to the wider coverage of all the histopathology laboratories servicing both Lagos and Ogun states in South West, Nigeria.

CRC was the most common malignant GIT tumors, accounting for 59% of all GIT malignancies in this study. This is similar to studies from other parts of Nigeria where it was found to represent between 53%-67% of all malignant GIT tumors[3,7,8]. An earlier study from this center also recorded 56%[19]. Even in the United States of America, it was reported to be the most common GIT cancer and the second leading cause of cancer death[20].

Rising incidence has been reported from various parts of Africa which were considered low incidence areas. Iliyasu reported 81% increase in Ibadan over two decades; a 2.7 fold increase has also been reported from Kenya[13,21]. In the present study, we have also noticed an increase in the number of colorectal cases recorded over the years. Although there has been an increasing awareness resulting in an increased hospital attendance, change in dietary habit of Nigerians is a possible reason for this observation and needs to be further investigated to ascertain its relationship with CRC. A South African study has demonstrated 3-fold higher incidence of MSI-H tumors in African-American patients compared with Caucasian Americans; a difference which the authors suggested may reflect dietary differences or genetic polymorphisms that may be common in the African-American population[22].

African Americans have the highest incidence and mortality rates for colon cancer among ethnic populations in the United States, the incidence being 15% higher and the mortality 40% higher in African-Americans than in Caucasian Americans[20]. The relatively low incidence of CRC in native Africans was associated with high fiber diet. Recent workers have however, affirmed that low prevalence of CRC in black African can no longer be explained by high fiber diet because dietary patterns of Africans has changed and even protective anti-oxidants, such as Vitamins C, E, A and calcium, are low in African diet[23,24]. The most incriminating risk factors for CRC in most studies are the high intake of animal protein and fat[24,25]. O'Keefe *et al*[25] earlier showed that CRC risk is determined by interactions between the external (dietary) and internal (bacterial) environments.

The mean age in this study is 50.7 years which corroborates 44.3, 49.7, 51, and 52.3 years reported from Jos in North-Central Nigeria, Kenya, Egypt and Iran, respectively[12,21,26,27]. The age incidence of CRC in Nigeria is lower compared to developed countries;

about 10 years difference has been reported in many studies[9,28,29]. Peak age reported from Nigeria ranged between 42.9 years to 53 years with a mean of 46[11,12].

There appears to be an increasing number of CRC cases occurring in the young as 23% occurred below age 40 years while 12.4% occurred in patients 30 years and below in this study. Reports from other parts of Nigeria showed that 35%-42% of patients with CRC are below age 40 years[28,30,31]. CRC in younger age has been shown to present a diagnostic and therapeutic problem and prognosis tends to be less favorable[32]. In one study, CRC in young females was reported to have more tendencies to present with a late stage disease and anemia[33]. The reason for increasing incidence may be genetic, thus underscoring the need to study the prevalence of HNPCC associated CRC in our environment. On the other hand, it may be related to dietary factors since the young Africans tend to be more civilized and more likely take Westernized diet. In Nigeria, there has been a shift from the traditional African diet, due to upsurge in the number of fast food outlets, and it has become fashionable particularly among the youths to eat these Westernized food which are high in animal protein and fat.

Similar to previous studies, the sex prevalence is in favor of males with M:F ratio of 1.3:1. Except for the studies from Ife[28] which showed higher ratio of 2:1, other studies from Nigeria had reported ratio ranging between 1.1:1 and 1.6:1[6,9,11-13]. In the Middle East, M:F ratio of 1.1:1 was reported in Iran[27].

In terms of location within the colon, our study concurs with previous studies which have indicated that majority of CRC are located in the distal part of the colon; the rectosigmoid[8-13,28,29,32]. The reason for this may partly be due to the endoscopic practice in Nigeria in which the majority do sigmoidoscopy rather than colonoscopy. For example, in Lagos and Sagamu, only three of the eight endoscopic centers do colonoscopy. This limitation might have caused under-reporting of CRC cases, thus underscoring the need for prospective studies and provision of colonoscopy facilities as well as skilled specialists.

All the CRC cases studied were adenocarcinoma of varying grades. The present study also showed that patients < 40 years tended to have poor prognostic tumors such as mucinous and signet ring carcinoma. This concurs with the finding of Fazeli *et al*[27] in Iran who reported that 22% of patients < 40 years had poorly differentiated tumor compared to 5.9% in patients above 40 years. Mucinous carcinomas have been associated with poor prognosis with poor response to chemotherapy, tend to be located in the proximal colon and associated with micro satellite instability[22]. The majority of CRC in this study presented at TNM stages Ⅱ and Ⅲ or (Duke's Stage B and C), which concurs with most reports from Africa[6,13,21,26,27].

In conclusion, CRC in Lagos and Sagamu is the commonest malignant GIT tumor. The age and sex prevalence, as well as histopathological characteristics, are similar to findings from other parts of the world. However, very little is known about the etio-pathogenetic mechanisms. We have therefore commenced follow-up immunohistochemical study, in Collaboration with the Department of Histopathology & Pathology, St. James University Hospital, Leeds, United Kingdom, to identify the molecular subtypes with a view to ascertaining the specific pathogenetic and prognostic features.

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COMMENTS

Background

Colorectal carcinoma (CRC), the most common gastrointestinal (GIT) cancer, is an important cause of cancer death worldwide, but incidence is lower in Africans representing 3%-6% of all malignant tumors in most African studies.

Research frontiers

Differences exist between the incidence of CRC in Africans and that in developed countries; CRC carcinogenesis is related to development of genetic instability.

Innovations and breakthroughs

The age and sex prevalence, as well as histopathological characteristics of CRC in Lagos & Sagamu SW Nigeria, are similar to findings from other parts of the world. However the incidence is still low compared to Europe and America and very little is known about the immunophenotypic subtypes.

Applications

This study thus underscores the importance of epidemiological study to investigate the immunophenotypic subtypes of CRC in Nigeria respect to the role of dietary factors and genetic factors in the aetiopathogenesis.

Terminology

CRC is a malignant tumor arising from the epithelium of the large bowel; Histopathological characteristics refers to tumor characteristics as seen under the light microscope; Pathological staging means extent of tumor growth and spread based on the size of the primary tumor and extent of lymph node involvement, as well as spread to distant sites (metastasis); Familial Adenomatous Polyposis syndrome is the prototype of polyposis syndrome caused by mutation of adenomatous polyposis coli gene on chromosome 5q21 and characterized by presence of multiple polyps in the GIT tract; Immunophenotypes mean subtypes of CRC based on DNA microsatellite instability status (MMR) and cpG island methylator phenotype.

Peer review

This report on the histopathological characteristics of colorectal cancer in Lagos, Sagamu and southwest Nigeria is of interest as this is a region considered historically to be a low prevalence region for CRC and reports regarding the characteristics of CRC in the region are not great in number. The data reported here are reasonably novel and the presentation is fairly clear and concise.

REFERENCES

1 **Makinen MJ**. Colorectal serrated adenocarcinoma. *Histopathology* 2007; **50**: 131-50

2 **Williams AO**, Edington GM. Malignant disease of the colon, rectum and anal canal in Ibadan, Western Nigeria. *Dis Colon Rectum* 1967; **10**: 301-308

3 **Okobia MN**, Aligbe JU. Pattern of malignant diseases at the

University of Benin Teaching Hospital. *Trop Doct* 2005; **35**: 91-92
4 **Holcombe C**, Babayo U. The pattern of malignant disease in north east Nigeria. *Trop Geogr Med* 1991; **43**: 189-192
5 **Kenda JF**. Cancer of the large bowel in the African: a 15-year survey at Kinshasa University Hospital, Zaire. *Br J Surg* 1976; **63**: 966-968
6 **Elesha SO**, Owonikoko TK. Colorectal neoplasms: a retrospective study. *East Afr Med J* 1998; **75**: 718-723
7 **Ohanaka CE**, Ofoegbu RO. The pattern of surgical cancers in Nigeria: the Benin experience. *Trop Doct* 2002; **32**: 38-39
8 **Obafunwa JO**. Pattern of alimentary tract tumours in Plateau State: a middle belt area of Nigeria. *J Trop Med Hyg* 1990; **93**: 351-354
9 **Seleye-Fubara D**, Gbobo I. Pathological study of colorectal carcinoma in adult Nigerians: a study of 45 cases. *Niger J Med* 2005; **14**: 167-172
10 **Essiet A**, Iwatt AR. Surgical management of large bowel cancer 1983-1988, University of Calabar Teaching Hospital audit. *Cent Afr J Med* 1994; **40**: 8-13
11 **Edino ST**, Mohammed AZ, Ochicha O. Characteristics of colorectal carcinoma in Kano, Nigeria: an analysis of 50 cases. *Niger J Med* 2005; **14**: 161-166
12 **Sule AZ**, Mandong BM, Iya D. Malignant colorectal tumours: a ten year review in Jos, Nigeria. *West Afr J Med* 2001; **20**: 251-255
13 **Iliyasu Y**, Ladipo JK, Akang EE, Adebamowo CA, Ajao OG, Aghadiuno PU. A twenty-year review of malignant colorectal neoplasms at University College Hospital, Ibadan, Nigeria. *Dis Colon Rectum* 1996; **39**: 536-540
14 **Kumar V**, Abbas AK, Fausto N (Eds). Robins and Cotran Pathologic basis of disease. 7th ed. Philadelphia: Elsevier Saunders, 2005: 864-865
15 **Jass JR**. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 2007; **50**: 113-130
16 **Hameed F**, Goldberg PA, Hall P, Algar U, van Wijk R, Ramesar R. Immunohistochemistry detects mismatch repair gene defects in colorectal cancer. *Colorectal Dis* 2006; **8**: 411-417
17 **Lanza G**, Gafa R, Santini A, Maestri I, Guerzoni L, Cavazzini L. Immunohistochemical test for MLH1 and MSH2 expression predicts clinical outcome in stage II and III colorectal cancer patients. *J Clin Oncol* 2006; **24**: 2359-2367
18 **El Mistiri M**, Verdecchia A, Rashid I, El Sahli N, El Mangush M, Federico M. Cancer incidence in eastern Libya: the first report from the Benghazi Cancer Registry, 2003. *Int J Cancer* 2007; **120**: 392-397
19 **Abdulkareem FB**, Faduyile FA, Daramola AO, Rotimi O, Banjo AAF, Elesha SO, Anunobi CC, Akinde OR, Abudu EK. Malignant Gastrointestinal Tumours in South Western Nigeria: A Histopathologic Analysis of 713 Cases. *West Afr J Med* 2009; **28(3):** 173–176
20 **American Cancer Society**. Cancer Facts and Figures: Special Edition 2005. Atlanta: American Cancer Society, 2005. Available from: URL: http://www.cancer.org/downloads/STT/CAFF2005CR4PWSecured.pdf
21 **Saidi H**, Nyaim EO, Githaiga JW, Karuri D. CRC surgery trends in Kenya, 1993-2005. *World J Surg* 2008; **32**: 217-223
22 **Ashktorab H**, Smoot DT, Carethers JM, Rahmanian M, Kittles R, Vosganian G, Doura M, Nidhiry E, Naab T, Momen B, Shakhani S, Giardiello FM. High incidence of microsatellite instability in colorectal cancer from African Americans. *Clin Cancer Res* 2003; **9**: 1112-1117
23 **O'Keefe SJ**, Ndaba N, Woodward A. Relationship between nutritional status, dietary intake patterns and plasma lipoprotein concentrations in rural black South Africans. *Hum Nutr Clin Nutr* 1985; **39**: 335-341
24 **O'Keefe SJ**, Kidd M, Espitalier-Noel G, Owira P. Rarity of colon cancer in Africans is associated with low animal product consumption, not fiber. *Am J Gastroenterol* 1999; **94**: 1373-1380
25 **O'Keefe SJ**, Chung D, Mahmoud N, Sepulveda AR, Manafe M, Arch J, Adada H, van der Merwe T. Why do African Americans get more colon cancer than Native Africans? *J Nutr* 2007; **137**: 175S-182S
26 **El-Bolkainy TN**, Sakr MA, Nouh AA, El-Din NH. A comparative study of rectal and colonic carcinoma: demographic, pathologic and TNM staging analysis. *J Egypt Natl Canc Inst* 2006; **18**: 258-263
27 **Fazeli MS**, Adel MG, Lebaschi AH. Colorectal carcinoma: a retrospective, descriptive study of age, gender, subsite, stage, and differentiation in Iran from 1995 to 2001 as observed in Tehran University. *Dis Colon Rectum* 2007; **50**: 990-995
28 **Ojo OS**, Odesanmi WO, Akinola OO. The surgical pathology of colorectal carcinomas in Nigerians. *Trop Gastroenterol* 1992; **13**: 64-69
29 **Adesanya AA**, da Rocha-Afodu JT. Colorectal cancer in Lagos: a critical review of 100 cases. *Niger Postgrad Med J* 2000; **7**: 129-136
30 **Adekunle OO**, Abioye AA. Adenocarcinoma of the large bowel in Nigerians: a clinicopathologic study. *Dis Colon Rectum* 1980; **23**: 559-563
31 **Akinola DO**, Arigbabu AO. Pattern and presentation of large bowel neoplasms in Nigerians. *Cent Afr J Med* 1994; **40**: 98-102
32 **Sule AZ**, Mandong BM. Malignant colorectal tumours in patients 30 years and below: a review of 35 cases. *Cent Afr J Med* 1999; **45**: 209-212
33 **Olofinlade O**, Adeonigbagbe O, Gualtieri N, Freiman H, Ogedegbe O, Robilotti J. Colorectal carcinoma in young females. *South Med J* 2004; **97**: 231-235

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RAPID COMMUNICATION

Colonoscopic evaluation of minimal rectal bleeding in average-risk patients for colorectal cancer

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Abstract

AIM: To assess the prevalence of clinically significant lesions in patients with minimal bright red bleeding per rectum (BRBPR).

METHODS: Consecutive outpatients prospectively underwent colonoscopy at Loghman Hakim Hospital, Tehran. Minimal BRBPR was defined as small amounts of red blood after wiping or in the toilet bowl. Patients with the following alarm signs were excluded: Positive personal history of colorectal neoplasms or inflammatory bowel disease (IBD), positive first degree family history of colorectal neoplasms, history of altered bowel habits, recent significant weight loss, and presence of iron deficiency anemia. Neoplastic polyps, colorectal carcinoma, and IBD were defined as significant lesions.

RESULTS: A total of 402 patients (183 female and 219 male, aged 43.6 ± 15.7 years) were studied. Hemorrhoids (54.2%), anal fissures (14.2%) and ulcerative colitis (14.2%) were the most common lesions and colonoscopy was normal in 8.0%. Significant lesions were found in 121 (30.1%) patients, including 26 patients (6.5%) with adenocarcinoma and 30 (7.5%) with adenomatous polyps. Almost all patients with significant lesions had at least one lesion in the distal colon; an adenocarcinoma and an adenomatous polyp in the proximal colon were found in 2 patients with hemorrhoids.

CONCLUSION: Flexible sigmoidoscopy appears to be sufficient for the evaluation of average risk patients with minimal BRBPR. Rigid sigmoidoscopy may be used as an alternative in patients less than 40 years of age in settings where the former is not available. The choice of colonoscopy over flexible sigmoidoscopy in patients aged over 50 years should be individualized.

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Key words: Gastrointestinal hemorrhage; Colonoscopy; Colorectal neoplasms; Inflammatory bowel disease

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INTRODUCTION

Minimal bright red bleeding per rectum (BRBPR) is a clinical problem frequently found in adults of all ages. The problem may be even more common in younger adults because of under-reporting to physicians[1]. For example, a community-based study of 1643 adults ages 20 to 64 found that 13 percent reported blood on wiping. The prevalence of any rectal bleeding was significantly higher in younger people. Only 14 percent of those with any rectal bleeding had seen a physician for bowel problems in the prior year[2].

The etiology of bleeding is highly variable and depends upon the nature of the population studied. The etiology of minimal BRBPR is often difficult to determine because individual patients may have multiple potentially culpable lesions found at endoscopy[3]. In addition, colorectal neoplasms (mostly adenomas) have been found in 16 percent of patients who were concurrently diagnosed with an anorectal source of bleeding[4]. Benign anorectal pathologies appear to account for 90 percent or more of all episodes of minimal BRBPR[3]. The true proportion of benign etiologies may be even higher since many young people with minimal BRBPR never present for care. The appropriate evaluation of a patient presenting with minimal BRBPR must be guided by the risk of underlying serious pathology.

There are relatively few studies that have addressed issues relevant to the appropriate evaluation of patients

with minimal BRBPR and most studies have not been performed in patients with strictly minimal BRBPR. It is a source of controversy as to whether minimal BRBPR necessitates total colonoscopy as a first-line procedure or a 60 cm flexible sigmoidoscopy[5]. Some authors have recommended colonoscopy in all patients with rectal bleeding[4,6,7], while others prefer colonoscopy for patients over 50 years of age and recommend sigmoidoscopy only if a potential source of bleeding is not identified on physical examination or anoscopy/proctoscopy[3].

Medical resources are limited in developing countries and a total colonoscopy may not be easily accessible for all patients with minimal BRBPR in Iran. Our aim was to determine the type and prevalence of colonoscopic findings in patients with minimal BRBPR in order to establish which patients need total colonoscopy.

MATERIALS AND METHODS

The study was performed prospectively on consecutive out-patients undergoing colonoscopy during a three-year period (October, 2004-August, 2007) at the "open access" Unit of Gastrointestinal Endoscopy at Loghman Hakim Hospital of Shaheed Beheshti University of medical sciences, in Tehran-Iran.

Minimal BRBPR was defined as small amounts of red blood after wiping or a few drops of blood in the toilet bowl after defecation. Small amounts of blood on the surface of the stool were also considered minimal BRBPR, but red blood intermixed with stool was not. Exclusion criteria were age below 12 years, positive personal history of colorectal neoplasms or inflammatory bowel disease (IBD), positive first degree family history of colorectal neoplasms, history of altered bowel habits, recent significant weight loss, presence of iron deficiency anemia, those who had already had a colonoscopy within the previous year, and those who did not consent or refused colonoscopy. Patients less than 40 years of age who refused to participate in the study underwent flexible sigmoidoscopy according to the current recommendations[3]. These patients are excluded from the main data analysis, but their results are presented as a separate group.

All patients were interviewed and examined by a gastroenterologist. Informed written consent was obtained from each patient before interview according to the guidelines of the institute. After clinical evaluation, all patients underwent anal inspection and digital rectal examination. Regardless of any anal pathologies detected, all patients underwent total colonoscopy.

Endoscopy was performed by an expert endoscopist in patients after the ingestion of 4 to 6 liters of polyethylene glycol solution. Any abnormal lesion was biopsied and sent for histology. IBD was diagnosed based on colonoscopy features and histopathological findings. Patients with poor bowel preparation were scheduled for repeat colonoscopy and the results of a satisfactory examination are reported. Colonoscopy was supplemented with double contrast barium enema if the colon was examined to at least the hepatic flexure, but the cecum could not be reached.

Patients less than 40 years of age are referred to as 'young' patients. The part of colon, situated between the rectum and the splenic flexure, was defined as distal colon. Neoplastic polyps, colorectal carcinoma, and IBD were defined as "significant lesions".

The study was approved by the institutional review board of the Loghman Hakim research unit of Shaheed Beheshti University of Medical Sciences, according to the declaration of Helsinki. Informed written consent was obtained from each patient before interview and procedures according to the guidelines of the institute.

Quantitative variables are presented with mean ± SD. The qualitative variables are expressed with number and percent. The two groups of values were compared using the chi-square test and the Fisher's exact test, a value of $P < 0.05$ was considered statistically significant.

RESULTS

Patient population

During the study period, 402 patients with minimal BRBPR were enrolled. This study group was composed of 219 males (54.5%) and 183 females (45.5%). Their ages ranged from 13 to 86 years (mean 43.6 ± 15.7 years). Of these, 177 (44.0%) were in the young age group.

There were another 94 young patients (41 male, 53 female; aged 27.6 ± 5.8 years), who met the eligibility criteria, but did not agree to participate and undergo colonoscopy.

Endoscopic lesions

Endoscopy was performed up to the cecum in 389 patients (96.8%). There were no complications attributed to the procedure. The 13 (3.2%) incomplete examinations showed distal lesions in 11 patients and 2 normal results. All barium enemas were normal.

Endoscopic findings are presented in Table 1. Hemorrhoids, anal fissures and IBD were the most common diagnoses.

Location of lesions in patients with abnormal findings

At least one distal lesion was found in all patients with abnormal findings (370 patients), but a concomitant proximal significant lesion was found in 15 patients (4.1%). The concomitant proximal lesion was in the same diagnostic category (e.g. distal and proximal polyps) in 13 patients; a 53 year-old woman with hemorrhoids was found to have adenocarcinoma in the transverse colon and one adenomatous polyp was found in the transverse colon of a 62 year-old woman with hemorrhoids.

At least one anorectal lesion was found in 359 patients (97.0%). In patients with anorectal source of bleeding, a different distal lesion was found in 31 (8.6%). A statistically significant difference in the frequency of concomitant lesions could not be found between young and older patients (5.8% *vs* 10.2%, $P = 0.14$).

Table 1 Colonoscopic findings in 402 patients with minimal bright red bleeding per rectum at Loghman Hakim hospital by age group[1]

		Total		Age < 40 yr		Age ⩾ 40 yr		*P*
		Number	Percent	Number	Percent	Number	Percent	
Significant lesions	Carcinomas	26	6.5	4	2.3	22	9.8	0.002
	Polyps	30	7.5	8	4.5	22	9.8	0.046
	UC	57	14.2	37	20.9	20	8.9	0.001
	CD	10	2.5	5	2.8	5	2.2	0.700
Insignificant lesions	Hemorrhoids	218	54.2	62	35.0	156	69.3	7.2 e-012
	Anal fissures	57	14.2	38	21.5	19	8.4	0.000
	Diverticulosis	1	0.2	0	0.0	1	0.4	0.560
	SRUS	33	8.2	23	13.0	10	4.4	0.020
	AD	1	0.2	0	0.0	1	0.4	0.560
	Normal	32	8.0	23	13.0	9	4.0	0.001

UC: Ulcerative colitis; CD: Crohn's disease; SRUS: Solitary rectal ulcer syndrome; AD: Angiodysplasia. [1]Some patients with more than one lesion were presented in more than one diagnostic category.

Table 2 Location of significant lesions according to the reach of different diagnostic procedures[1] in patients with minimal bright red bleeding per rectum at Loghman Hakim hospital

Distance from anal verge[2]	Carcinomas	Polyps	UC	CD
Age < 40 yr				
10 cm	3/4	2/8	36/37	3/5
30 cm	4/4	8/8	37/37	5/5
60 cm	4/4	8/8	37/37	5/5
Entire colon	4/4	8/8	37/37	5/5
Age ⩾ 40 yr				
10 cm	12/22	10/22	20/20	0/5
30 cm	17/22	13/22	20/20	4/5
60 cm	21/22	21/22	20/20	5/5
Entire colon	22/22	22/22	20/20	5/5

UC: Ulcerative colitis; CD: Crohn's disease. [1]The length of evaluation was considered 10 cm for anoscopy/rectoscopy, 30 cm for rigid sigmoidoscopy and 60 cm for flexible sigmoidoscopy; [2]In patients with multiple lesions of the same type, the nearest lesion to the anal verge has been considered.

Significant lesions

Significant lesions were found in 54 young patients (30.5%) and 67 patients (29.8%) in the older group ($P > 0.5$). The potential diagnostic yields of different approaches (based on the location of the lesions) for the diagnosis of significant lesions are compared in Table 2.

Findings in young patients who underwent flexible sigmoidoscopy

There were 94 young patients (41 male, 53 female; aged 27.6 ± 5.8 years), who met the eligibility criteria, but did not agree to undergo colonoscopy. Evaluation of these patients revealed hemorrhoids in 46 (48.9%), anal fissures in 20 (21.3%), IBD in 7 (7.4%), solitary rectal ulcer syndrome in 6 (6.4%), and diverticulosis in 1 (1.1%). There were no cases of carcinoma, polyps or angiodysplasia. Normal results were found in 21 patients (22.3%).

DISCUSSION

Our study showed that significant lesions in the proximal colon are infrequent in patients with minimal BRBPR. Colonoscopy is recommended for the evaluation of rectal bleeding in patients who are at increased risk for colorectal neoplasms ('red flags')[3], but there are no specific recommendations for the appropriate evaluation of the majority of patients who lack these risk factors. The decision about the extent of the evaluation of these patients should be based on the prevalence of clinically significant lesions, potential need for a repeat procedure, costs and availability of the facility. Some experts recommend that young patients do not require further evaluation, if the presentation and history do not suggest an increased risk of cancer and a potential source of bleeding (such as hemorrhoids or an anal fissure) is identified in the clinical evaluation[3]. Several studies have concluded that flexible sigmoidoscopy is initially appropriate[5,8-10], while others have recommended colonoscopy in this age group[7]. Contrasting opinions are also expressed in the guidelines prepared by the American Society for Gastrointestinal Endoscopy (ASGE) and the European Panel for Appropriateness of Gastrointestinal Endoscopy (EPAGE): While the former specify that middle-aged or older individuals must always undergo a total colonoscopy, even in the presence of an anal lesion that could justify the hematochezia[11], the latter consider total colonoscopy inappropriate when the source of bleeding has been ascertained by ano- or sigmoidoscopy[12].

IBD was found in 16.4% of our patients. Other studies have reported lower rates of IBD in their patients[5,7,9-10]. Detection of ulcerative colitis is not a problem, because the rectum is almost always involved. Our 10 patients with Crohn's disease also had distal colonic involvement (less than 30 cm from the anal verge). Thus, our results show that IBD can be readily diagnosed in patients with minimal BRBPR with any of the available procedures.

Colorectal cancer has been reported as low as 0%-4% and adenomatous polyps in 9.9%-30% in patients with minimal BRBPR from Western countries[5,7-10]. Some of the differences in these results may be explained by the differences in their study populations. In a recent study from Iran, Sotoudehmanesh *et al*[13] found no cancer and

4 adenomatous polyps (3%) in 134 average-risk patients with minimal bright red bleeding from midline anal fissures. We found colorectal carcinoma in 6.5% of our patients and adenomatous polyps in 7.5%. Our findings may be overestimated, because we excluded 94 patients from analysis who underwent only flexible sigmoidoscopy and there were no neoplastic lesions in this group. Nevertheless, minimal BRBPR should be regarded as an 'alarm symptom' for neoplastic colorectal lesions.

Patients with minimal BRBPR from colorectal cancer are likely to have left-sided lesions[3]. Almost all of neoplastic lesions in our patients were located in the distal colon. There was one patient with hemorrhoids and an adenocarcinoma in the transverse colon, but we believe that the bleeding may have been caused by the hemorrhoids and the tumor was incidentally found during colonoscopy. The distribution of polyps was similar to colorectal cancer in our patients. Thus, we conclude that average risk patients with minimal BRBPR of any age may not be at an increased risk for proximal neoplastic colonic lesions.

The choice of the appropriate diagnostic evaluation depends mainly on the age of the patient. According to our results in Table 2, young patients should at least be evaluated up to the distal 30 cm of the colon. Physical examination (including digital rectal examination), anoscopy and rectoscopy are simple and low cost maneuvers that do not require bowel preparation. In fact, anoscopy has a higher sensitivity for the detection of hemorrhoids than flexible video endoscopy[9]. However, these approaches would fail to diagnose most neoplastic lesions in our young patients, even if a potential anorectal source of bleeding was identified. Rigid sigmoidoscopy is a widely used modality as a preliminary investigation to exclude colorectal pathology and is usually done in outpatient clinics on unprepared bowel[14]. All significant lesions of our young patients were in the reach of rigid sigmoidoscopy; however, flexible sigmoidoscopy has been shown to be superior in terms of diagnostic value and patient discomfort[14]. Thus, we suggest flexible sigmoidoscopy for young patients with minimal BRBPR regardless of identified anorectal pathologies and rigid sigmoidoscopy may be an appropriate alternative in settings, where flexible sigmoidoscopy is not accessible.

Colorectal cancer screening recommendations should be considered, when deciding about the evaluation of middle-aged or older individuals with minimal BRBPR. Both flexible sigmoidoscopy and colonoscopy have been recommended for this purpose and the decision about which option to select should be made between the patient and physician[15]. Although, clinically significant lesions of 97% of our older patients were in the reach of flexible sigmoidoscopy; colonoscopy is also an appropriate option for patients over 50 years willing to undergo screening for colorectal cancer simultaneously. Therefore, patients should be informed that minimal BRBPR does not place them at an increased risk for proximal neoplastic colonic lesions and the costs and availability of the facility should also be considered. Another important factor is the need for a repeat procedure. About 30% of patients who undergo initial flexible sigmoidoscopy will eventually require colonoscopy.

Our findings should be interpreted in the context of the limitations of our study. First, not all patients with minimal BRBPR are referred to gastroenterologists for evaluation, and this is particularly true for younger patients[10]. Second, any recommendation about the appropriate extent of evaluation of patients with minimal BRBPR should be made from randomized clinical trials with follow-up data.

We suggest flexible sigmoidoscopy for the evaluation of average risk patients for colorectal cancer with minimal BRBPR. Rigid sigmoidoscopy may be used as an alternative in patients less than 40 years of age in settings where the former is not available. The choice of colonoscopy over flexible sigmoidoscopy in patients aged over 50 years should be individualized.

ACKNOWLEDGMENTS

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COMMENTS

Background

Minimal bright red bleeding per rectum (BRBPR) is a clinical problem frequently found in adults of all ages. The etiology of bleeding is highly variable and depends upon the nature of the population studied.

Research frontiers

It is a source of controversy as to whether minimal BRBPR necessitates total colonoscopy as a first-line procedure or a 60 cm flexible sigmoidoscopy. We performed this study to investigate the prevalence of clinically significant lesions in patients with minimal BRBPR.

Innovations and breakthroughs

Hemorrhoids, anal fissures and ulcerative colitis were the most common lesions. Almost all patients with clinically significant lesions had at least one lesion in the distal colon, which can be examined by flexible sigmoidoscopy.

Applications

We suggest flexible sigmoidoscopy for the evaluation of patients with minimal BRBPR who are at average risk for colorectal cancer. Rigid sigmoidoscopy may be used as an alternative in patients less than 40 years of age in settings where the former is not available. The choice of colonoscopy over flexible sigmoidoscopy in patients aged over 50 years should be individualized.

Terminology

Patients less than 40 years of age are referred to as 'young' patients. The part of colon situated between the rectum and the splenic flexure was defined as distal colon. Neoplastic polyps, colorectal carcinoma, and inflammatory bowel disease were defined as "significant lesions".

Peer review

They assess the prevalence of clinically significant lesions in patients with minimal bright red bleeding per rectum. It is a very interested topic for the readers of WJG, it is written in correct English, with valuable results and conclusions.

REFERENCES

1 **Dent OF**, Goulston KJ, Zubrzycki J, Chapuis PH. Bowel symptoms in an apparently well population. *Dis Colon Rectum* 1986; **29**: 243-247

2 **Talley NJ**, Jones M. Self-reported rectal bleeding in a United States community: prevalence, risk factors, and health care seeking. *Am J Gastroenterol* 1998; **93**: 2179-2183

3 **Penner RM**, Majumdar SR. Approach to minimal bright red bleeding per rectum. In: Rose BD (ed). Waltham: UpToDate,

2007
4 **Helfand M**, Marton KI, Zimmer-Gembeck MJ, Sox HC Jr. History of visible rectal bleeding in a primary care population. Initial assessment and 10-year follow-up. *JAMA* 1997; **277**: 44-48
5 **Carlo P**, Paolo RF, Carmelo B, Salvatore I, Giuseppe A, Giacomo B, Antonio R. Colonoscopic evaluation of hematochezia in low and average risk patients for colorectal cancer: A prospective study. *World J Gastroenterol* 2006; **12**: 7304-7308
6 **Goulston KJ**, Cook I, Dent OF. How important is rectal bleeding in the diagnosis of bowel cancer and polyps? *Lancet* 1986; **2**: 261-265
7 **Wong RF**, Khosla R, Moore JH, Kuwada SK. Consider colonoscopy for young patients with hematochezia. *J Fam Pract* 2004; **53**: 879-884
8 **Church JM**. Analysis of the colonoscopic findings in patients with rectal bleeding according to the pattern of their presenting symptoms. *Dis Colon Rectum* 1991; **34**: 391-395
9 **Korkis AM**, McDougall CJ. Rectal bleeding in patients less than 50 years of age. *Dig Dis Sci* 1995; **40**: 1520-1523
10 **Spinzi G**, Fante MD, Masci E, Buffoli F, Colombo E, Fiori G, Ravelli P, Ceretti E, Minoli G. Lack of colonic neoplastic lesions in patients under 50 yr of age with hematochezia: a multicenter prospective study. *Am J Gastroenterol* 2007; **102**: 2011-2015
11 **The role of endoscopy in the patient with lower gastrointestinal bleeding**. American Society for Gastrointestinal Endoscopy. *Gastrointest Endosc* 1998; **48**: 685-688
12 **Gonvers JJ**, De Bosset V, Froehlich F, Dubois RW, Burnand B, Vader JP. 8. Appropriateness of colonoscopy: hematochezia. *Endoscopy* 1999; **31**: 631-636
13 **Sotoudehmanesh R**, Ainechi S, Asgari AA, Kolahdoozan S. Endoscopic lesions in low-to average-risk patients with minimal bright red bleeding from midline anal fissures. How much should we go in? *Tech Coloproctol* 2007; **11**: 340-342
14 **Rao VS**, Ahmad N, Al-Mukhtar A, Stojkovic S, Moore PJ, Ahmad SM. Comparison of rigid vs flexible sigmoidoscopy in detection of significant anorectal lesions. *Colorectal Dis* 2005; **7**: 61-64
15 **Winawer S**, Fletcher R, Rex D, Bond J, Burt R, Ferrucci J, Ganiats T, Levin T, Woolf S, Johnson D, Kirk L, Litin S, Simmang C. Colorectal cancer screening and surveillance: clinical guidelines and rationale-Update based on new evidence. *Gastroenterology* 2003; **124**: 544-560

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RAPID COMMUNICATION

Abnormal liver function and central obesity associate with work-related fatigue among the Taiwanese workers

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Abstract

AIM: To examine the associations between objective health indicators and high need for recovery (NFR) after work, one of the subjective presentations of work related-fatigue, among apparently healthy workers in modern workplaces.

METHODS: From October to December, 2007, an annual health examination was performed for the workers from an electronics manufacturing factory in Taiwan. Health records of 1216 workers with a relatively homogeneous socioeconomic status were used for analysis. The health checkups included personal and NFR scale questionnaires, physical examinations, blood tests for biochemistry and hematology. The workers within the top tertile NFR score were defined as high-NFR workers.

RESULTS: After adjusted for potential confounders, the workers with elevated alanine aminotransferase (ALT) and central obesity had a significantly higher NFR after work, with increased risks of 1.4-fold [95% confidence interval (CI) = 1.01-2.0] and 1.8-fold (95% CI = 1.2-2.7), respectively. Shiftworkers had a 2.0-fold (95% CI = 1.5-2.6) increased risk for high-NFR. The associations between high-NFR and lipid profiles, blood sugar, hematology indexes or blood pressure were insignificant after controlling for confounders.

CONCLUSION: For apparently healthy workers, high NFR after work is not simply a subjective experience. Objective health measures, such as elevated ALT and increased waist circumference, should be carefully evaluated for the apparently healthy workers having a higher NFR after work.

Key words: Liver enzyme; Need for recovery after work; Obesity; Work-related fatigue

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INTRODUCTION

Work-related fatigue is a common complaint encountered by the occupational physician in the industrialized societies[1-3]. Most past investigations of work-related fatigue focused on age[4], subjective discomforts, social-economical factors[3,5] or on work styles[6]. However, the associations between work-related fatigue and general objective health measures, such as liver function tests, metabolic syndrome components (waist circumference, blood pressure, sugar and lipids profile) or hematology test results are yet to be declared among the modern workplaces in Taiwan. Since most factories in Taiwan provide routine health check-ups for their workers, we had the opportunity to examine the association between work-related fatigue and the objective health measures.

The need for recovery (NFR) scale questionnaire is a validation tool used for the evaluation of work-related fatigue[4,7,8]. In the present study, we surveyed the potential risk factors for work-related fatigue by utilizing NFR scale questionnaire and health examination records. The records were collected from the workers of an electronics manufacturing factory. All the participants had similar salary and educational levels. Our focus was put on the association between the NFR after

work and the objective health measures. Data analyses were controlled for the confounders of gender, age, working years, smoking status, shiftwork, liver function, hematology tests and metabolic syndrome components.

MATERIALS AND METHODS

Participants

Most of the blue collar workers in this electronics manufacturing factory were residents of north Taiwan, aged from 23 to 56 years. These workers took periodic health checkups according to the Labor Health Protection Regulation of the Labor Safety and Health Act. The exclusion criteria of our analysis were workers' records with a past medical history of malignancy, uncompleted questionnaire or any illness on the day of health checkups. This periodic health examination was performed at the healthcare unit in the workplace between October and December, 2007. All the health examinees were suggested to avoid drastic physical exercises, such as long-distance (marathon or endurance) running, heavy weight lifting training, within 3 d before the health checkups. The records of 1216 eligible apparently healthy workers were collected for the final analysis.

Methods

The 11-item NFR scale questionnaire containing yes/no questions representing short-term effects of a day of work[8] was translated into Chinese and then conducted by three trained nurses at the start of health examinations. The NFR scale score was calculated by adding the individual's scores on the 11 recoded items, and transformed into a scale ranging 0-100[9]. A higher scale score indicates a higher degree of NFR after work (high-NFR). For calculating relative risks, the scale scores were divided into tertiles. Workers within the top tertile NFR scale score were defined as high-NFR workers or as suffering from work-related fatigue. A questionnaire about personal history, including current smoking (recent one year and more than one pack consumed a day: Yes *vs* no), shiftwork involving night duty (yes *vs* no) was completed by the examinees.

The physical examination records used for analysis included measurements of waist circumference, weight, height and blood pressure. The definition of central obesity was waist circumference > 90 cm for males and > 80 cm for females, based on the Taiwanese criteria[10]. Elevated blood pressure was defined as systolic blood pressure (SBP) ≥ 130 mmHg or diastolic blood pressure (DBP) ≥ 85 mmHg[11]. The biochemistry blood tests for analysis included: Aspartate aminotransferase (AST), alanine aminotransferase (ALT), fasting plasma glucose, levels of triglyceride, and high-density lipoprotein (HDL) cholesterol. Elevated ALT was defined as > 40 U/mL, according to the standard reference limits used at the Tao-Yuan General Hospital and other studies[12,13]. The definitions of hyperglycemia, hypo-HDL cholesterolemia and hypertriglyceridemia were fasting sugar ≥ 100 mg/dL, HDL < 40 mg/dL for males or HDL < 50 mg/dL for females and triglyceride ≥ 150 mg/dL based on the modified ATPIII criteria[11]. Significant anemia was defined as hemoglobin (Hb) < 11 for females or < 12 for males[14].

Student's *t*-test was used to analyze the continuous variables. Cochran-Armitage trend test was used for analyzing the categorical variables among NFR score tertiles. Multivariate logistic regression was utilized to examine the association between high-NFR and potential risk factors. SAS version 8.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

Table 1 Baseline data and distribution of characteristics

Characteristic/Potential risk factor	Total (n = 1216)
NFR score	27.8 (21.6)
Age (yr)	34.6 (7.1)
Working year (yr)	9.1 (6.5)
Blood tests	
ALT (U/dL)	31.5 (31.8)
AST (U/dL)	23.4 (16.1)
HDL-cholesterol (mg/dL)	56.2 (12.4)
Triglyceride (mg/dL)	122.3 (81.8)
Fasting blood sugar (mg/dL)	91.6 (22.1)
Hemoglobin (mg/dL)	14.7 (1.6)
White blood count (× 10^3/mL)	6.3 (1.7)
Anthropometric measures	
Waist (cm)	81.0 (11.1)
Body mass index (kg/m^2)	24.3 (3.8)
Systolic blood pressure (mmHg)	124.9 (15.8)
Diastolic blood pressure (mmHg)	80.5 (11.5)
Male gender *n* (%)	852 (70)
Shiftwork *n* (%)	400 (33)
Smoking[1] *n* (%)	126 (11)
Blood tests	
Abnormal ALT *n* (%)	249 (21)
Hypo-HDL[2] *n* (%)	134 (11)
Hypertriglyceridemia[2] *n* (%)	309 (25)
Elevated fasting blood sugar[2] *n* (%)	167 (14)
Anemia[3] *n* (%)	29 (2)
Anthropometric measures	
Central obesity[2] *n* (%)	274 (23)
Elevated blood pressure[2] *n* (%)	543 (45)

Abnormal ALT: > 40 mg/dL. [1]Recent one year and more than one pack a day: Yes *vs* no; [2]Taiwanese metabolic syndrome criteria; [3]Significant anemia: Hb < 11 for females or < 12 mg/dL for males.

RESULTS

The records of a total of 1216 workers (852 males, 364 females) were used for the final analysis. Thirty-three percent of participants in this study were shiftworkers. The overall characteristics and abnormal prevalence rates are summarized in Table 1. The mean age, working years and NFR score for this population were 34.6 years, 9.1 years and 27.8, respectively. The prevalence of elevated ALT, hypo-HDL cholesterolemia, elevated triglyceride, elevated fasting sugar, significant anemia, central obesity, elevated blood pressure and current smoking were 21%, 19%, 25%, 14%, 2%, 23%, 45% and 11%, respectively.

The objective health measures were compared according to the NFR scale score tertiles (Table 2). From the bottom to the top tertile NFR scale scores (means, 6.8, 22.4 and 52.6), ALT (29.2, 31.2 and 34.0 U/dL) and HDL cholesterol (57.6, 56.0 and 55.1 mg/dL) were significantly

Table 2 Distribution of potential risk factors according to the NFR after work score

Potential risk factor	NFR Tertiles			P^1
	1st tertile (n = 409)	2nd tertile (n = 379)	3rd tertile (n = 428)	
NFR score	6.8 (3.9)	22.4 (4.5)	52.6 (15.4)	-
AGE (yr)	34.6 (7.2)	34.3 (7.1)	34.8 (6.9)	0.571
Working year (yr)	8.9 (6.9)	8.7 (6.3)	9.5 (6.4)	0.251
Blood tests				
ALT (U/dL)	29.2 (22.4)	31.2 (27.3)	34.0 (41.6)	0.035
AST (U/dL)	22.4 (8.9)	23.3 (14.2)	24.4 (21.9)	0.092
HDL-cholesterol (mg/dL)	57.6 (12.9)	56 (12.4)	55.1 (11.8)	0.004
Triglyceride (mg/dL)	120.6 (76.9)	121.7 (82.7)	124.5 (85.5)	0.489
Fasting blood sugar (mg/dL)	92.3 (28.1)	90.8 (14.1)	91.7 (21.3)	0.725
Hemoglobin (mg/dL)	14.7 (1.6)	14.6 (1.6)	14.7 (1.5)	0.898
White blood count (× 10^3/mL)	6.3 (1.9)	6.4 (1.6)	6.3 (1.6)	0.566
Anthropometric measures				
Waist (cm)	80.7 (10.8)	80.9 (11.2)	81.4 (11.6)	0.394
Body mass index (kg/m^2)	24.2 (3.7)	24.2 (4)	24.4 (3.8)	0.413
Systolic blood pressure (mmHg)	124.8 (15.3)	125.6 (16.3)	124.3 (15.9)	0.678
Diastolic blood pressure (mmHg)	80.1 (11.6)	80.9 (11.5)	80.4 (11.4)	0.725
Male gender n (%)	300 (73)	258 (68)	294 (69)	0.072
Shiftwork n (%)	87 (22)	125 (33)	188 (44)	< 0.001
Smoking[2] n (%)	32 (8)	38 (10)	56 (13)	0.007
Blood tests				
Abnormal ALT n (%)	70 (17)	78 (21)	101 (24)	0.010
Hypo-HDL[3] n (%)	44 (11)	39 (10)	51 (12)	0.294
Hypertriglyceridemia[3] n (%)	103 (25)	91 (24)	115 (27)	0.285
Elevated fasting blood sugar[3] n (%)	58 (14)	51 (14)	58 (14)	0.397
Anemia[4] n (%)	11 (3)	10 (3)	8 (2)	0.217
Anthropometric measures				
Central obesity[3] n (%)	84 (21)	76 (20)	114 (27)	0.017
Elevated blood pressure[3] n (%)	169 (41)	186 (49)	188 (44)	0.232

Abnormal ALT: > 40 mg/dL. [1]P of student's t-test between the top- *vs* bottom-NFR tertile for the continuous variables; Cochran-Armitage Trend Test for categorical variables among tertiles; [2]Recent one year and more than one pack a day: Yes *vs* no; [3]Taiwanese metabolic syndrome criteria; [4]Significant anemia: Hb < 11 for females or < 12 mg/dL for males.

unfavorable in the top-tertile, in contrast to the subjects within the bottom-tertile of NFR scale score. The means of triglyceride (120.6, 121.7 and 124.5 mg/dL), AST (22.4, 23.4 and 24.2 U/dL), BMI (24.2, 24.2 and 24.4 kg/m^2) and waist circumference (80.7, 80.9 and 81.4 cm) were unfavorable in the top-tertile, but did not reach a statistical significance. The prevalence rates of elevated ALT (17%, 20% and 27%; P = 0.010), central obesity (21%, 20% and 26%; P = 0.017) and the percentages of shiftwork (22%, 33% and 44%; P < 0.001), smoking (8%, 10% and 13%; P = 0.007) rose along with the increased NFR scale scores. The abnormal rates of significant anemia and the other metabolic syndrome components were insignificantly different among workers stratified by tertiles of the NFR scale score.

As demonstrated in Figure 1, after controlling for confounders, shiftworkers had a 2.0-fold [95% confidence interval (CI) = 1.5-2.6], centrally obese employees had a 1.8-fold (95% CI = 1.2-2.7) and the workers with elevated ALT had a 1.4-fold (95% CI = 1.01-2.0) increased risk of high NFR. Smokers had a 1.4-fold increased risk of high-NFR, though we could not confirm its statistical significance (95% CI = 0.9-2.1). Anemic status and metabolic syndrome components, except for central obesity, could not be demonstrated to have a statistically significant association with high-NFR from the present analysis of our early-middle-aged workers.

DISCUSSION

To our knowledge, this is the first observation from the electronics manufacturing industry to report that the apparently health workers with elevated serum ALT, an objective measure for liver function[13], require a significantly higher NFR after work. Liver function impairment has been suggested to be a developer of oxidative stress[12,15-17], and its levels are raised among patients with chronic fatigue syndrome and associated with clinical symptoms[18-20]. In the general Taiwanese, elevated serum ALT is prevalent and predominantly caused by asymptomatic chronic hepatovirus infection, steatohepatitis[21] or by hepatic toxic chemical exposure in some workplaces[22]. The present findings might motivate further studies on the relationships between each etiological entity of elevated ALT and work-related fatigue. Since liver problems are prevalent in Taiwan and elevated ALT is closely connected to high-NFR in our workers, careful liver function evaluations should be performed for those apparently healthy workers with work-related fatigue in the modern workplaces.

Central obesity was found to be an independent risk factor for a significantly higher NFR in our multivariate risk analysis. A similar phenomenon has been observed in many studies of fatigue and self-reported fatigue is associated with a higher waist circumference among Western adults[23,24]. Physical fatigue of healthy adults is

Risk factor	OR[1]	95% CI	*P*
Shiftwork	2.0	1.5-2.6	< 0.001
Central obesity[2]	1.8	1.2-2.7	0.003
Abnormal ALT[3]	1.4	1.01-2.0	0.044
Smoking[4]	1.4	0.9-2.0	0.103
Hypo-HDL[2]	1.1	0.7-1.6	0.756
Hypertriglyceridemia[2]	1.1	0.8-1.5	0.616
Elevated fasting blood sugar[2]	0.9	0.6-1.3	0.478
Elevated blood pressure[2]	0.9	0.6-1.1	0.269
Anemia[5]	0.4	0.1-1.1	0.076

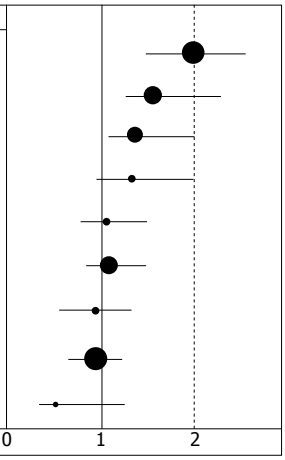

Figure 1 Odds ratio of risk factors for high NFR after work among workers, analyzed using logistic regression. Size of dark circle represents approximate overall prevalence for each risk factor. [1]Odds ratio adjusted for age, gender, working years, metabolic syndrome components, liver function test, anemia and smoking status; [2]Taiwanese metabolic syndrome criteria; [3]Alanine aminotransferase > 40 mg/dL; [4]Recent one year and more than one pack a day: Yes *vs* no; [5]Significant anemia: Hb < 11 for females or < 12 mg/dL for males.

associated with obesity independent of psychological factors[25], and visceral obesity related sleep apnea can result in general fatigue[26]. Whether waist circumference reduction helps our workers to moderate the work-related fatigue is worthy of subsequent exploration.

The high-NFR workers in our analysis had a significant larger percentage of doing shiftwork. As one of the independent risk factors for high-NFR, shiftwork carries the highest odds ratio for high-NFR when compared with the other risk factors. In our analysis, no significant association was observed between shiftwork and obesity or elevated ALT. Since previous studies of work style have highlighted the adverse effects of shiftwork on workers' physical and mental health, shiftworkers must adapt their life pattern to shiftwork styles, which can result in an increased fatigue level, or even illnesses[27-29]. Since shiftwork style is common in the electronics manufacturing industry and it was reported that fatigued workers are at a high risk of being injured in occupational accidents[2], the shiftworkers' health and safety should be emphasized in our modern workplaces. Our analysis did not address the linkage of high-NFR and shiftwork patterns, such as clockwise *vs* anticlockwise patterns, interval of shifts, or working hours in a shift. Further detailed investigations should be necessary.

Smoking, or even exposure to second-hand smoke, has been identified as a risk factor for fatigue in adults[30,31]. The present result also indicates that the percentage of current heavy smoking among the high-NFR workers was significantly greater than that in the low-NFR workers. However, the prevalence rate of smoking was lower in our study than in other studies[32], and the analysis for smoking merely demonstrated an insignificantly increased risk for high-NFR. A partial reason may be that we emphasized more than one pack of cigarettes consumed per day in our self-report questionnaires, so that we might have failed to take account of other smokers consuming less than one pack per day and underrated the risk of work-related fatigue in smokers. Further studies about work-related fatigue associated with smoking might take into consideration all the subjects' exposure to smoke.

One of the limitations of our study is that we did not survey the job types, work titles or leisure-time physical activity included in the study, thus we could not answer the question that workers differ in terms of physical activity that may influence both risks of central obesity and fatigue. The majority of participants in this study were early-middle-aged (mean age, 34.6 years). However, there are many different characteristics between younger and older workers in the NFR after work[4]. The present analysis results for a younger population may underestimate the impact of aging-related risk factors including metabolic syndrome on work-related fatigue[5,33] and our findings from a mostly male worker population may lack power for women, thus, some applications of our conclusion to the general working population should be cautious.

In conclusion, for the apparently healthy workers, high NFR after work is not simply a subjective experience. Objective measures such as elevated ALT and increased waist circumference are significantly associated with high NFR after work for the apparently healthy workers. We suggest that careful evaluations should focus on abnormal liver function and central obesity for those apparently healthy workers with higher NFR after work in the modern workplaces. Further studies should address if the prevalence of risk factors, particularly central obesity and elevated ALT, would reduce the risk of work-related fatigue.

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COMMENTS

Background

Work-related fatigue is a common complaint encountered by the occupational physician in industrialized societies. The associations between work-related fatigue and general objective health measures such as liver function tests, metabolic syndrome components (waist circumference, blood pressure, sugar and lipids profile) or hematology test results are yet to be declared among the modern workplaces.

Research frontiers

The need for recovery (NFR) scale questionnaire is a validation tool used for the evaluation of work-related fatigue. In the present study, the authors surveyed the potential risk factors for work-related fatigue by utilizing NFR

scale questionnaire and health examination records. Their focus was put on the association between the NFR after work and the objective health measures.

Innovations and breakthroughs

The workers with elevated alanine aminotransferase (ALT) and central obesity had a significantly higher NFR after work, with increased risks of 1.4-fold [95% confidence interval (CI) = 1.01-2.0] and 1.8-fold (95% CI = 1.2-2.7), respectively.

Applications

The authors suggest that careful evaluations should focus on abnormal liver function and central obesity for those apparently healthy workers with a higher NFR in the modern workplaces. Reducing the prevalence of risk factors, particularly central obesity and elevated ALT, might reduce the risk of work-related fatigue.

Terminology

The 11-item NFR scale questionnaire containing yes/no questions, representing short-term effects of a day of work, is used for the evaluation of work-related fatigue.

Peer review

This article supports previous reports in the literature relating liver function to fatigue in the workplace. The significant results about work-related fatigue of this study for ALT and obesity are after adjusting for potential confounders.

REFERENCES

1 **Sluiter JK**, de Croon EM, Meijman TF, Frings-Dresen MH. Need for recovery from work related fatigue and its role in the development and prediction of subjective health complaints. *Occup Environ Med* 2003; **60** Suppl 1: i62-i70

2 **Swaen GM**, Van Amelsvoort LG, Bultmann U, Kant IJ. Fatigue as a risk factor for being injured in an occupational accident: results from the Maastricht Cohort Study. *Occup Environ Med* 2003; **60** Suppl 1: i88-i92

3 **van Amelsvoort LG**, Kant IJ, Bultmann U, Swaen GM. Need for recovery after work and the subsequent risk of cardiovascular disease in a working population. *Occup Environ Med* 2003; **60** Suppl 1: i83-i87

4 **Kiss P**, De Meester M, Braeckman L. Differences between younger and older workers in the need for recovery after work. *Int Arch Occup Environ Health* 2008; **81**: 311-320

5 **Prescott E**, Godtfredsen N, Osler M, Schnohr P, Barefoot J. Social gradient in the metabolic syndrome not explained by psychosocial and behavioural factors: evidence from the Copenhagen City Heart Study. *Eur J Cardiovasc Prev Rehabil* 2007; **14**: 405-412

6 **Winwood PC**, Winefield AH, Lushington K. Work-related fatigue and recovery: the contribution of age, domestic responsibilities and shiftwork. *J Adv Nurs* 2006; **56**: 438-449

7 **de Croon EM**, Sluiter JK, Frings-Dresen MH. Need for recovery after work predicts sickness absence: a 2-year prospective cohort study in truck drivers. *J Psychosom Res* 2003; **55**: 331-339

8 **van Veldhoven M**, Broersen S. Measurement quality and validity of the "need for recovery scale". *Occup Environ Med* 2003; **60** Suppl 1: i3-i9

9 **de Croon EM**, Sluiter JK, Frings-Dresen MH. Psychometric properties of the Need for Recovery after work scale: test-retest reliability and sensitivity to detect change. *Occup Environ Med* 2006; **63**: 202-206

10 **Chu NF**. Prevalence of obesity in Taiwan. *Obes Rev* 2005; **6**: 271-274

11 **Tan CE**, Ma S, Wai D, Chew SK, Tai ES. Can we apply the National Cholesterol Education Program Adult Treatment Panel definition of the metabolic syndrome to Asians? *Diabetes Care* 2004; **27**: 1182-1186

12 **Schindhelm RK**, Dekker JM, Nijpels G, Bouter LM, Stehouwer CD, Heine RJ, Diamant M. Alanine aminotransferase predicts coronary heart disease events: a 10-year follow-up of the Hoorn Study. *Atherosclerosis* 2007; **191**: 391-396

13 **Schindhelm RK**, Diamant M, Dekker JM, Tushuizen ME, Teerlink T, Heine RJ. Alanine aminotransferase as a marker of non-alcoholic fatty liver disease in relation to type 2 diabetes mellitus and cardiovascular disease. *Diabetes Metab Res Rev* 2006; **22**: 437-443

14 **Cawood TJ**, Buckley U, Murray A, Corbett M, Dillon D, Goodwin B, Sreenan S. Prevalence of anaemia in patients with diabetes mellitus. *Ir J Med Sci* 2006; **175**: 25-27

15 **Koenig W**. Heart disease and the inflammatory response. *BMJ* 2000; **321**: 187-188

16 **Videla LA**, Rodrigo R, Orellana M, Fernandez V, Tapia G, Quinones L, Varela N, Contreras J, Lazarte R, Csendes A, Rojas J, Maluenda F, Burdiles P, Diaz JC, Smok G, Thielemann L, Poniachik J. Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clin Sci* (Lond) 2004; **106**: 261-268

17 **Ioannou GN**, Weiss NS, Boyko EJ, Mozaffarian D, Lee SP. Elevated serum alanine aminotransferase activity and calculated risk of coronary heart disease in the United States. *Hepatology* 2006; **43**: 1145-1151

18 **Jones EA**. Fatigue complicating chronic liver disease. *Metab Brain Dis* 2004; **19**: 421-429

19 **Swain MG**. Fatigue in liver disease: pathophysiology and clinical management. *Can J Gastroenterol* 2006; **20**: 181-188

20 **Kennedy G**, Spence VA, McLaren M, Hill A, Underwood C, Belch JJ. Oxidative stress levels are raised in chronic fatigue syndrome and are associated with clinical symptoms. *Free Radic Biol Med* 2005; **39**: 584-589

21 **Lin YC**, Hsiao ST, Chen JD. Sonographic fatty liver and hepatitis B virus carrier status: synergistic effect on liver damage in Taiwanese adults. *World J Gastroenterol* 2007; **13**: 1805-1810

22 **Luo JC**, Kuo HW, Cheng TJ, Chang MJ. Abnormal liver function associated with occupational exposure to dimethylformamide and hepatitis B virus. *J Occup Environ Med* 2001; **43**: 474-482

23 **Resnick HE**, Carter EA, Aloia M, Phillips B. Cross-sectional relationship of reported fatigue to obesity, diet, and physical activity: results from the third national health and nutrition examination survey. *J Clin Sleep Med* 2006; **2**: 163-169

24 **Vgontzas AN**, Bixler EO, Chrousos GP. Obesity-related sleepiness and fatigue: the role of the stress system and cytokines. *Ann N Y Acad Sci* 2006; **1083**: 329-344

25 **Lim W**, Hong S, Nelesen R, Dimsdale JE. The association of obesity, cytokine levels, and depressive symptoms with diverse measures of fatigue in healthy subjects. *Arch Intern Med* 2005; **165**: 910-915

26 **Vgontzas AN**, Papanicolaou DA, Bixler EO, Hopper K, Lotsikas A, Lin HM, Kales A, Chrousos GP. Sleep apnea and daytime sleepiness and fatigue: relation to visceral obesity, insulin resistance, and hypercytokinemia. *J Clin Endocrinol Metab* 2000; **85**: 1151-1158

27 **Srithongchai S**, Intaranont K. A study of impact of shift work on fatigue level of workers in a sanitary-ware factory using a fuzzy set model. *J Hum Ergol* (Tokyo) 1996; **25**: 93-99

28 **Smith L**, Mason C. Reducing night shift exposure: a pilot study of rota, night shift and age effects on sleepiness and fatigue. *J Hum Ergol* (Tokyo) 2001; **30**: 83-87

29 **Shen J**, Botly LC, Chung SA, Gibbs AL, Sabanadzovic S, Shapiro CM. Fatigue and shift work. *J Sleep Res* 2006; **15**: 1-5

30 **Corwin EJ**, Klein LC, Rickelman K. Predictors of fatigue in healthy young adults: moderating effects of cigarette smoking and gender. *Biol Res Nurs* 2002; **3**: 222-233

31 **Hicks RA**, Fernandez C, Hicks GJ. Fatigue and exposure to cigarette smoke. *Psychol Rep* 2003; **92**: 1040-1042

32 **Lin YC**, Chen JD. Association between sonographic fatty liver and ischemic electrocardiogram among non-obese Taiwanese male adults. *Zhonghua Yixue Chaocheng Zazhi* 2006; **14**: 58-66

33 **Rodriguez A**, Muller DC, Metter EJ, Maggio M, Harman SM, Blackman MR, Andres R. Aging, androgens, and the metabolic syndrome in a longitudinal study of aging. *J Clin Endocrinol Metab* 2007; **92**: 3568-3572

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RAPID COMMUNICATION

Comparison of four models for end-stage liver disease in evaluating the prognosis of cirrhosis

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Abstract

AIM: To investigate the prognostic value of the model for end-stage liver disease (MELD) and three new MELD-based models combination with serum sodium in decompensated cirrhosis patients-the MELD with the incorporation of serum sodium (MELD-Na), the integrated MELD (iMELD), and the MELD to sodium (MESO) index.

METHODS: A total of 166 patients with decompensated cirrhosis were enrolled into the study. MELD, MELD-Na, iMELD and MESO scores were calculated for each patient following the original formula on the first day of admission. All patients were followed up at least 1 year. The predictive prognosis related with the four models was determined by the area under the receiver operating characteristic curve (AUC) of the four parameters. Kaplan-Meier survival curves were made using the cut-offs identified by means of receiver operating characteristic (ROC).

RESULTS: Out of 166 patients, 38 patients with significantly higher MELD-Na (28.84 ± 2.43 *vs* 14.72 ± 0.60), iMELD (49.04 ± 1.72 *vs* 35.52 ± 0.67), MESO scores (1.59 ± 0.82 *vs* 0.99 ± 0.42) compared to the survivors died within 3 mo ($P < 0.001$). Of 166 patients, 75 with markedly higher MELD-Na (23.01 ± 1.51 *vs* 13.78 ± 0.69), iMELD (44.06 ± 1.19 *vs* 34.12 ± 0.69), MESO scores (1.37 ± 0.70 *vs* 0.93 ± 0.40) than the survivors died within 1 year ($P < 0.001$). At 3 mo of enrollment, the iMELD had the highest AUC (0.841), and was followed by the MELD-Na (0.766), MESO (0.723), all larger than MELD (0.773); At 1 year, the iMELD still had the highest AUC (0.783), the difference between the iMELD and MELD was statistically significant ($P < 0.05$). Survival curves showed that the three new models were all clearly discriminated the patients who survived or died in short-term as well as intermediate-term ($P < 0.001$).

CONCLUSION: Three new models, changed with serum sodium (MELD-Na, iMELD, MESO) can exactly predict the prognosis of patients with decompensated cirrhosis for short and intermediate period, and may enhance the prognostic accuracy of MELD. The iMELD is better prognostic model for outcome prediction in patients with decompensated cirrhosis.

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Key words: Cirrhosis; Model for end-stage liver disease; Serum sodium; Prognosis; Survival time

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INTRODUCTION

The model for end-stage liver disease (MELD) was developed as a prognostic model of short-term mortality in patients with cirrhosis treated with transjugular intrahepatic portosystemic shunt (TIPS)[1]. The scoring system has been widely applied in recent years and shown to predict mortality across a broad spectrum of liver diseases in most studies. But, there is not any parameter correlated with complications of cirrhosis in this formula. Its ability of prognosis is decreased. Some studies have indicated that serum sodium is the independent predictor of mortality in patients with cirrhosis[2,3]. And the incorporation of Na into the MELD may enhance its prognostic accuracy[4,5]. Then some scholars had successively introduced three new mathematical equations based on both MELD and Na,

known as the MELD with the incorporation of serum sodium (MELD-Na)[6], the integrated MELD (iMELD) score[7] and the MELD to sodium (MESO) index[8]. In this study, we compared the value of MELD and three new MELD-based models in combination with serum sodium in to evaluate the short-term and intermediate-term prognosis of decompensated cirrhosis patients through retrospective analysis of 166 decompensated cirrhosis cases.

MATERIALS AND METHODS

Patients

From October, 2005 to May, 2007, 166 patients with decompensated cirrhosis who had been in Department of Gastroenterology of Shanghai East Hospital affiliated to Tongji University were evaluated, and their medical profiles were retrospectively analyzed in this study. The clinical diagnosis was all based on the program of 2000 for the prevention and treatment of virus hepatitis established in Xi'an Congresses[9]. We excluded patients with past or current hepatocellular carcinoma, serious diseases in other systems, admission to hospital repeatedly and incomplete case records. This study included 105 (63.7%) males and 61 (36.3%) females, with mean age 62.3 ± 12.9 (range 29-87) years.

Clinical data

Baseline laboratory results of all the patients obtained at admission (i.e. serum bilirubin, serum creatinine, serum sodium, INR) were retrieved from the medical records. All patients were followed up for 1 year. The outcome was assessed as the 3-, 6- and 12-mo mortality.

Calculation of the MELD, MELD-Na, iMELD and MESO index

All prognostic models were calculated based on laboratory results obtained on the first day of admission. The MELD equation was used to calculate the severity score: $9.6 \times \log_e$ [creatinine (mg/dL)] + $3.8 \times \log_e$ [bilirubin (mg/dL)] + $11.2 \times \log_e$ (INR) + 6.43[10]. The MELD-Na equation was based on the MELD and Na: MELD + $1.59 \times (135 - \text{Na})$[6], with maximum and minimum Na values of 135 and 120 mmol/L, respectively. The iMELD equation was based on the MELD score, age (years), and Na (mmol/L): MELD + $(0.3 \times \text{age})$ - (0.7 + Na) + 100[7]. The MESO index was defined as [MELD/Na (mmol/L)] × 10[8].

Statistical analysis

All statistical analyses were conducted with the SPSS for Windows version 13 release. Categorical variables were compared by Pearson Chi-squared test and continuous variables were compared by Student's *t*-test. To assess the ability of the four MELD-based models in predicting the risk of mortality at 3, 6 and 12 mo, our analysis was performed by the measurement of the *c*-statistic equivalent to the area under the receiver operating characteristic curve (AUC). The cumulative transplant-free survival at different cut-offs were performed by Kaplan-Meier analysis and compared by log rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical features between the survival group and the death group

Thirty-eight (22.9%) patients died at 3 mo, and 75 (45.2%) patients died at 1 year. At 3 mo of enrollment, 38 patients with significantly higher MELD-Na (28.84 ± 2.43 *vs* 14.72 ± 0.60), iMELD (49.04 ± 1.72 *vs* 35.52 ± 0.67), MESO scores (1.59 ± 0.82 *vs* 0.99 ± 0.42) compared to the survivors died ($P < 0.001$). At 1 year of enrollment, 75 patients with markedly higher MELD-Na (23.01 ± 1.51 *vs* 13.78 ± 0.69), iMELD (44.06 ± 1.19 *vs* 34.12 ± 0.69), MESO scores (1.37 ± 0.70 *vs* 0.93 ± 0.40) than the survivors died within 1 year ($P < 0.001$). The differences of age and serum sodium, two parameters incorporated into three new models, between the survival group and the death group, were also statistically significant at 3 mo and 1 year, especially serum sodium ($P < 0.001$) (Table 1).

Comparison of the AUC and predictive accuracy between four MELD-based prognostic models

At 3 mo of enrollment, the iMELD had the highest AUC (0.841), followed by the MELD-Na (0.766), MESO (0.723) and MELD (0.712) (Figure 1A). At 6 mo and 1 year, the iMELD still had the highest AUC (0.806 and 0.783, respectively), followed by the MELD-Na (0.738 and 0.714, respectively), MESO (0.715 and 0.694, respectively) and MELD (0.708 and 0.689, respectively) (Figure 1B and C). The iMELD had a significantly higher AUC in comparison with MELD at 3 mo, 6 mo and 1 year ($P < 0.05$).

Kaplan-Meier fractional survival curves of MELD-Na, iMELD and MESO

The most discriminative cut-offs from the ROC with the *c*-statistic and 1 year mortality for MELD-Na, iMELD and MESO were 20, 40 and 1.6, respectively. According to these cut-offs, survival curves are given in Figure 2. The cut-offs of three new models were indicated to discern between the patients who would be survived and dead in 3 mo and 1 year ($P < 0.001$).

DISCUSSION

MELD was initially created to predict survival following elective placement of TIPS[1]. The MELD scoring system has been widely applied in recent years and shown to predict mortality across a broad spectrum of liver diseases in most studies[11-14]. MELD has been demonstrated to have a better ability in short-term or intermediate-term outcome prediction in comparison with the Child-Turcotte-Pugh (CTP) system[15-17]. Nonetheless, MELD still has potential limitations[18-21]. Hepatic encephalopathy, esophageal varices bleeding and spontaneous bacterial peritonitis are common

Table 1 The clinical features of 166 patients with cirrhosis at 3 mo and 1 year follow-up[1]

Clinical features	3-mo follow-up		1-yr follow-up	
	Survival group	Death group	Survival group	Death group
Age (yr)	61.2 ± 12.7	66.1 ± 13.0[a]	60.3 ± 13.0	64.8 ± 12.4[a]
Bilirubin (μmol/L)	44.2 ± 40.1	106.2 ± 117.9[a]	43.1 ± 43.7	77.0 ± 91.0[a]
Creatinine (μmol/L)	85.5 ± 34.0	115.3 ± 67.7[a]	82.8 ± 32.2	103.8 ± 55.8[a]
INR	1.64 ± 0.51	2.21 ± 1.38[a]	1.58 ± 0.49	2.00 ± 1.07[a]
Serum sodium (mmol/L)	137.3 ± 4.9	130.4 ± 6.8[b]	137.7 ± 5.2	133.2 ± 6.3[b]
MELD	13.2 ± 5.6	20.5 ± 10.5[b]	8.63 ± 2.13	10.5 ± 2.52[b]
MELD-Na	14.7 ± 6.8	28.8 ± 15.0[b]	14.2 ± 5.31	20.5 ± 9.42[b]
iMELD	35.5 ± 7.6	49.0 ± 10.6[b]	34.1 ± 7.5	44.1 ± 10.3[b]
MESO	0.99 ± 0.42	1.59 ± 0.82[b]	0.93 ± 0.40	1.37 ± 0.70[b]

[1]Data expressed as mean ± SD. MELD: Model for end-stage liver disease. [a]$P < 0.05$, [b]$P < 0.01$ *vs* the survival group.

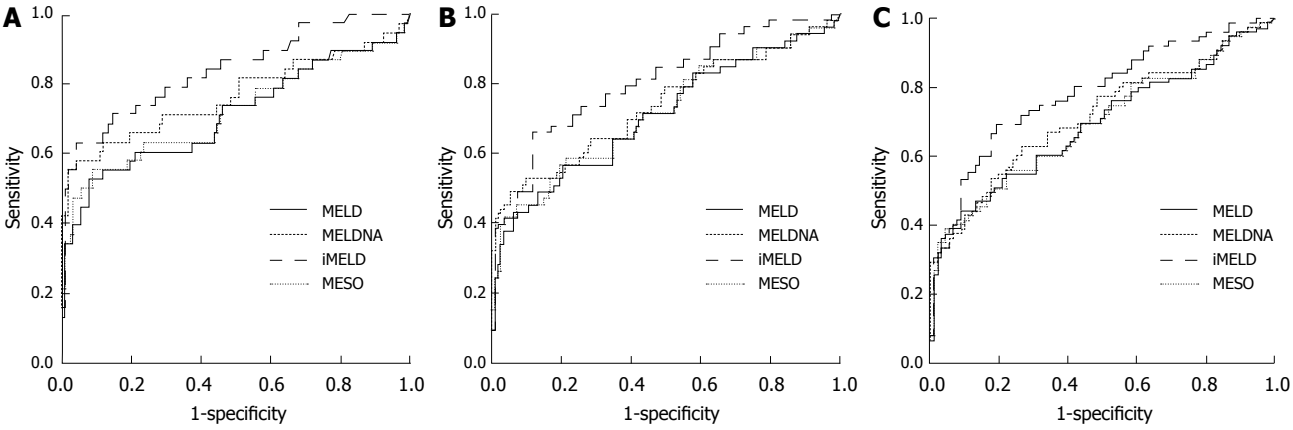

Figure 1 The area under the receiver operating characteristic curve (AUC) of MELD, MELD-Na, iMELD and MESO. A: At 3 mo of enrollment; B: At 6 mo of enrollment; C: At 1 year of enrollment. The comparison between iMELD and MELD at 3 mo, 6 mo and 1 year showed significant differences ($P < 0.05$).

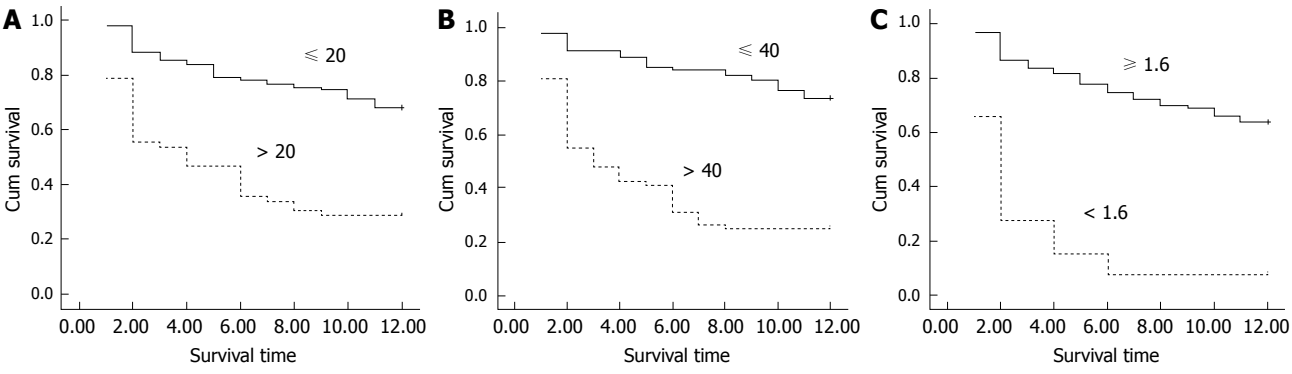

Figure 2 Kaplan-Meier fractional survival curves using the cut-offs identified by means of ROC for 1 year and compared by log rank test. All comparisons showed significant differences (all $P < 0.01$). A: MELD-Na; B: iMELD; C: MESO.

complications with cirrhosis, which had been considered one of the allocation policies of liver providing. The patients with these complications all had relatively ideal long-term survival rates. But, there is no parameter correlated with these complications in MELD. Portal hypertension is responsible for above-mentioned complications[22,23]. Hyponatremia is a common event in liver cirrhosis. It develops primarily as a result of free water retention, which is positively correlated with the severity of portal hypertension[24]. Consequently, the serum sodium (SNa) level may inversely reflect the severity of portal hypertension. Those with low MELD scores who have persistent ascites and low SNa are at a disadvantage. This group of patients has a higher mortality than that predicted by the MELD score alone[15]. Many studies have proposed serum sodium can be used to exactly evaluate the prognosis and mortality of patients with cirrhosis, which is objective, quantitative, and reproducible. The incorporation of Na into the MELD may enhance prognostic accuracy[4,5,25].

In 2006, Biggins *et al*[6] first established "MELD-Na". Under the new system, a patient with serum Na of

130 mEq/L and a MELD score of 14 will have a "MELD-Na" score of 22 and will be allocated an organ, whereas another patient with serum Na of 135 mEq/L and a MELD score of 20 ("MELD-Na" score of 20) will be given a lower priority. Obviously, the latter patient with a higher MELD score is being given a higher priority under the new system. Thus, this analysis suggests that a significant number of patients with low serum Na will benefit by receiving a priority score corresponding to their mortality risk. Studies in Korea and Hongkong both confirmed that MELD-Na performed better than MELD in predicting 3-mo and 1-year mortality[26,27]. Later, Huo *et al*[8] developed MESO as MELD to SNa ratio. The AUC was 0.860 for SNa, 0.795 for the MESO index and 0.789 for MELD at 3 mo of enrollment. Among patients with Child-Pugh class A or B, the MESO index had a significantly higher AUC compared with MELD ($P < 0.001$). In survival analysis, MESO index > 1.6 independently predicted a higher mortality rate (relative risk: 3.32; $P < 0001$) using the Cox model. The latest study[7] incorporated both serum sodium and age into the new formula: iMELD. The iMELD was better than original MELD in evaluating the mortality of cirrhosis patients 1 year after TIPS: AUC increased by 13.4% and the likelihood ratio statistic from 23.5 to 48.2; it was demonstrated for patients with cirrhosis on the waiting list for liver transplantation by increasing auROC (+8%) and likelihood ratio statistic (from 41.4 to 82.0).

Our study compared MELD with three new MELD-based models containing Na. It is discovered that the AUCs of MELD-Na, iMELD, MESO were all larger than MELD in evaluating the short-term and intermediate-term prognosis of decompensated cirrhosis patients. Among the four models, iMELD had the biggest AUC at different periods and showed significant differences with MELD. The iMELD was demonstrated to be better prognostic model for outcome prediction in patients with cirrhosis, which is similar to that reported by Huo *et al*[28]. Interestingly, in addition to the MELD and Na, the iMELD also takes into account the factor of age. Age was associated to the risk of mortality as a continuous variable, with older patients having worse survival. The association of aging with mortality in cirrhosis has been shown in the past[29,30]. Most recently, a systematic review of 118 prognostic studies in patients with cirrhosis showed that age is the most important independent prognostic factor of survival[31]. It has been suggested that aging may reflect a longer duration of cirrhosis and a more severe liver disease.

One of the important aspects of the MELD and its derived models is that they are continuous variables and account for the spectrum of disease severity. However, using the most discriminative cut-off from the ROC for different models may provide additional information in certain clinical settings. From the survival curves, it was indicated that the cut-offs of three new models may discern between the patients who would be survived and dead in 3 mo and 1 year. In our study, although the AUCs of MELD-Na and MESO were larger than MELD at 3 mo, 6 mo and 1 year, the comparisons showed no significant differences. The research should be improved more thoroughly and objectively using larger series of patients.

In conclusion, three new models combination with serum sodium (MELD-Na, iMELD, MESO) can all exactly predict the prognosis of patients with decompensated cirrhosis for short and intermediate period, and may enhance the prognostic accuracy of MELD. The iMELD is better prognostic model for outcome prediction in patients with decompensated cirrhosis.

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COMMENTS

Background

The model for end-stage liver disease (MELD) has been widely applied in recent years and shown to predict mortality across a broad spectrum of liver diseases. But MELD still has potential limitations and its ability of prognosis is decreased. In order to further improve the formula, many researches have been performed recent years.

Research frontiers

Some studies have indicated that serum sodium is the independent predictor of mortality in patients with cirrhosis. And the incorporation of Na into the MELD may enhance its prognostic accuracy. Some scholars had successively introduced three new mathematical equations based on both MELD and Na.

Innovations and breakthroughs

Limited data are available for a direct comparison of the performance of the MELD-Na and MELD, and the predictive ability of the other models has not yet been confirmed. In this study, authors compare the short- and intermediate-term prognostic ability of the 4 models-MELD, MELD-Na, iMELD, and MESO index - in a single institute to determine if Na-containing MELD systems have a better predictive accuracy in patients with cirrhosis.

Applications

The result of our study showed the prognostic value of the four models for end-stage liver disease. It will provide us the ideal formula which can exactly evaluate the prognosis of cirrhosis in clinic.

Terminology

The area under a receiver operating characteristic (ROC) curve (AUC) is a commonly used index for summarizing the ability of a continuous diagnostic test to discriminate between healthy and diseased subjects.

Peer review

This is a good paper with some practical value. The authors investigated the prognostic value of MELD and three new MELD-based models combination with serum sodium in decompensated cirrhosis patients.

REFERENCES

1 **Kamath PS**, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; **33**: 464-470

2 **Wang YW**, Huo TI, Yang YY, Hou MC, Lee PC, Lin HC, Lee FY, Chi CW, Lee SD. Correlation and comparison of the model for end-stage liver disease, portal pressure, and serum sodium for outcome prediction in patients with liver cirrhosis. *J Clin Gastroenterol* 2007; **41**: 706-712

3 **Selcuk H**, Uruc I, Temel MA, Ocal S, Huddam B, Korkmaz M, Unal H, Kanbay M, Savas N, Gur G, Yilmaz U, Haberal M. Factors prognostic of survival in patients awaiting liver

transplantation for end-stage liver disease. *Dig Dis Sci* 2007; **52**: 3217-3223

4 **Biggins SW**, Rodriguez HJ, Bacchetti P, Bass NM, Roberts JP, Terrault NA. Serum sodium predicts mortality in patients listed for liver transplantation. *Hepatology* 2005; **41**: 32-39

5 **Ruf AE**, Kremers WK, Chavez LL, Descalzi VI, Podesta LG, Villamil FG. Addition of serum sodium into the MELD score predicts waiting list mortality better than MELD alone. *Liver Transpl* 2005; **11**: 336-343

6 **Biggins SW**, Kim WR, Terrault NA, Saab S, Balan V, Schiano T, Benson J, Therneau T, Kremers W, Wiesner R, Kamath P, Klintmalm G. Evidence-based incorporation of serum sodium concentration into MELD. *Gastroenterology* 2006; **130**: 1652-1660

7 **Luca A**, Angermayr B, Bertolini G, Koenig F, Vizzini G, Ploner M, Peck-Radosavljevic M, Gridelli B, Bosch J. An integrated MELD model including serum sodium and age improves the prediction of early mortality in patients with cirrhosis. *Liver Transpl* 2007; **13**: 1174-1180

8 **Huo TI**, Wang YW, Yang YY, Lin HC, Lee PC, Hou MC, Lee FY, Lee SD. Model for end-stage liver disease score to serum sodium ratio index as a prognostic predictor and its correlation with portal pressure in patients with liver cirrhosis. *Liver Int* 2007; **27**: 498-506

9 **The societies of communicable diseases and parasitic diseases of Chinese Medical Association**. The program for the prevention and treatment of virus hepatitis. *Zhonghua Ganzangbing Zazhi* 2000; **8**: 324-329

10 **Wiesner R**, Edwards E, Freeman R, Harper A, Kim R, Kamath P, Kremers W, Lake J, Howard T, Merion RM, Wolfe RA, Krom R. Model for end-stage liver disease (MELD) and allocation of donor livers. *Gastroenterology* 2003; **124**: 91-96

11 **Dunn W**, Jamil LH, Brown LS, Wiesner RH, Kim WR, Menon KV, Malinchoc M, Kamath PS, Shah V. MELD accurately predicts mortality in patients with alcoholic hepatitis. *Hepatology* 2005; **41**: 353-358

12 **Ahmad J**, Downey KK, Akoad M, Cacciarelli TV. Impact of the MELD score on waiting time and disease severity in liver transplantation in United States veterans. *Liver Transpl* 2007; **13**: 1564-1569

13 **Huo TI**, Wu JC, Lin HC, Lee FY, Hou MC, Lee PC, Chang FY, Lee SD. Evaluation of the increase in model for end-stage liver disease (DeltaMELD) score over time as a prognostic predictor in patients with advanced cirrhosis: risk factor analysis and comparison with initial MELD and Child-Turcotte-Pugh score. *J Hepatol* 2005; **42**: 826-832

14 **Yu JW**, Wang GQ, Li SC. Prediction of the prognosis in patients with acute-on-chronic hepatitis using the MELD scoring system. *J Gastroenterol Hepatol* 2006; **21**: 1519-1524

15 **Srikureja W**, Kyulo NL, Runyon BA, Hu KQ. MELD score is a better prognostic model than Child-Turcotte-Pugh score or Discriminant Function score in patients with alcoholic hepatitis. *J Hepatol* 2005; **42**: 700-706

16 **Durand F**, Valla D. Assessment of the prognosis of cirrhosis: Child-Pugh versus MELD. *J Hepatol* 2005; **42** Suppl: S100-S107

17 **Liu F**, Xiong WJ, Liu YB. The value of delta model of end stage liver disease in predicting the prognosis of patients with decompensated liver cirrhosis. *Zhonghua Xiaohua Zazhi* 2007; **27**: 371-373

18 **Mishra P**, Desai N, Alexander J, Singh DP, Sawant P. Applicability of MELD as a short-term prognostic indicator in patients with chronic liver disease: an Indian experience. *J Gastroenterol Hepatol* 2007; **22**: 1232-1235

19 **Neuberger J**. Allocation of donor livers--is MELD enough? *Liver Transpl* 2004; **10**: 908-910

20 **Freeman RB**. MELD: the holy grail of organ allocation? *J Hepatol* 2005; **42**: 16-20

21 **Cholongitas E**, Senzolo M, Triantos C, Samonakis D, Patch D, Burroughs AK. MELD is not enough--enough of MELD? *J Hepatol* 2005; **42**: 475-477; author reply 478-479

22 **Bosch J**, Garcia-Pagan JC. Complications of cirrhosis. I. Portal hypertension. *J Hepatol* 2000; **32**: 141-156

23 **Ripoll C**, Banares R, Rincon D, Catalina MV, Lo Iacono O, Salcedo M, Clemente G, Nunez O, Matilla A, Molinero LM. Influence of hepatic venous pressure gradient on the prediction of survival of patients with cirrhosis in the MELD Era. *Hepatology* 2005; **42**: 793-801

24 **Freeman RB**, Wiesner RH, Edwards E, Harper A, Merion R, Wolfe R. Results of the first year of the new liver allocation plan. *Liver Transpl* 2004; **10**: 7-15

25 **Heuman DM**, Abou-Assi SG, Habib A, Williams LM, Stravitz RT, Sanyal AJ, Fisher RA, Mihas AA. Persistent ascites and low serum sodium identify patients with cirrhosis and low MELD scores who are at high risk for early death. *Hepatology* 2004; **40**: 802-810

26 **Kim SY**, Yim HJ, Lee J, Lee BJ, Kim DI, Jung SW, Han WS, Lee JS, Koo JS, Seo YS, Yeon JE, Lee HS, Lee SW, Um SH, Byun KS, Choi JH, Ryu HS. [Comparison of CTP, MELD, and MELD-Na scores for predicting short term mortality in patients with liver cirrhosis] *Korean J Gastroenterol* 2007; **50**: 92-100

27 **Wong VW**, Chim AM, Wong GL, Sung JJ, Chan HL. Performance of the new MELD-Na score in predicting 3-month and 1-year mortality in Chinese patients with chronic hepatitis B. *Liver Transpl* 2007; **13**: 1228-1235

28 **Huo TI**, Lin HC, Huo SC, Lee PC, Wu JC, Lee FY, Hou MC, Lee SD. Comparison of four model for end-stage liver disease-based prognostic systems for cirrhosis. *Liver Transpl* 2008; **14**: 837-844

29 **Gines P**, Quintero E, Arroyo V, Teres J, Bruguera M, Rimola A, Caballeria J, Rodes J, Rozman C. Compensated cirrhosis: natural history and prognostic factors. *Hepatology* 1987; **7**: 122-128

30 **de Jongh FE**, Janssen HL, de Man RA, Hop WC, Schalm SW, van Blankenstein M. Survival and prognostic indicators in hepatitis B surface antigen-positive cirrhosis of the liver. *Gastroenterology* 1992; **103**: 1630-1635

31 **D'Amico G**, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. *J Hepatol* 2006; **44**: 217-231

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RAPID COMMUNICATION

Study on protecting effects of Baicalin and Octreotide on hepatic injury in rats with severe acute pancreatitis

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Abstract

AIM: To investigate the protective effects and mechanisms of Baicalin and Octreotide on hepatic injury in rats with severe acute pancreatitis (SAP).

METHODS: The SAP rat models were prepared and randomly assigned to the model control group, Baicalin treated group, and Octreotide treated group while other healthy rats were assigned to the sham-operated group. Rat mortality, levels of ALT, AST, liver and pancreas pathological changes in all groups were observed at 3, 6 and 12 h after operation. Tissue microarray (TMA) sections of hepatic tissue were prepared to observe expression levels of Bax, Bcl-2 protein and Caspase-3, and changes of apoptotic indexes.

RESULTS: Rat survival at 12 h, expression levels of Bax, Caspase-3 protein and apoptotic indexes of liver were all significantly higher in treated groups than in model control group. While the liver and pancreas pathological scores, contents of ALT, AST, and expression levels of Bcl-2 protein were all lower in treated groups than in the model control group.

CONCLUSION: Both Baicalin and Octreotide can protect rats with SAP by decreasing the contents of ALT, AST and expression levels of Bcl-2 protein, and improving the expression levels of Bax protein, Caspase-3 protein, and inducing apoptosis.

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Key words: Baicalin; Octreotide; Severe acute pancreatitis; Hepatic injury; Tissue microarray; Apoptosis

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INTRODUCTION

Severe acute pancreatitis (SAP) can cause systemic inflammatory response syndromes (SIRS) such as effusion of blood vessel, shock and multiple organ functional disturbances or even multiple organ dysfunction syndrome (MODS)[1-3]. SAP with extremely hazardous onset process and quite high mortality clinically remains unclear till now[4-7]. The main cause of early death is multiple organ failure, which most frequently affects liver, *etc.* Studies prove the incidence of hepatic injury and its severity degree are positively

correlated with the severity of pancreatitis and hepatic injury prolongs the course of pancreatitis[8,9].

Presently, the main medications for SAP in clinical practices are Somatostatin and its analogue Octreotide. Their mechanism of action is maily to inhibit pancreatin secretion, decreasing the generation of endotoxin, inhibiting the release of inflammatory mediators, and inhibiting platelet aggregation and other steps[10-12]. However, their high price, short half life and inconvenient administration have made it difficult to popularize their clinical application in economically poor and remote areas, resulting in the necessity of finding other cheap and effective alternatives that should be able to protect multiple organs[13-16]. There is a great prospect for developing and utilizing traditional Chinese medicine to treat SAP[17,18]. Its main advantages include cheap price, extensive pharmacological actions and fewer side effects. In this study, Baicalin, the main effective ingredient of baikal skullcap root has been chosen to treat rats with SAP. Antibacterial and anti-inflammatory Baicalin can inhibit platelet aggregation, eliminate oxygen free radicals and reduce the generation of endotoxin. Baicalein, the metabolite of Baicalin in body, is also potent at inhibiting pancreatin. Baicalin can block multiple phases during SAP onset. Baicalin also has many effects similar to those of Somatostatin and its analogues. Therefore, Baicalin can be used more extensively[13-16].

In this experiment, tissue microarray (TMA) was adopted to study the protective effects of Baicalin on the liver of rats with SAP. The therapeutic effects of Baicalin and Octreotide were compared to prove the therapeutic effects of Baicalin on SAP.

MATERIALS AND METHODS

Material

Experimental animals: Clean grade healthy male Sprague-Dawley (SD) rat in 250-300 g of body weight were purchased from the Experimental Animal Center of Medical School, Zhejiang University (China).

Experimental medicine and reagents: Sodium taurocholate and sodium pentobarbital purchased from USA Sigma Company, Octreotide purchased from Swiss pharmaceutical company Novartis, 5% Baicalin injection (China national invention patent number ZL200310122673.6) prepared by the first author with 305 mmol/L osmotic pressure. Bax and Bcl-2 antibody purchased from Santa Cruz Company. The main reagent Takara *in situ* Apoptosis detection Kit purchased from TaKaRa Biotechnology Co., Ltd, PK (protease K) purchased from Sigma Company, DAB (biphenyldiamine) purchased from China Huamei Company. The above determinations were all operated according to the instructions of the kits.

Experimental methods

Preparation methods of animal models: Prepared 135 SAP rat models *via* retrograde injection of 3.5% sodium taurocholate to the pancreatic duct through epidural catheter and duodenal papilla.

Rats grouping: The 135 SAP rat models were randomly assigned to the model control group, Baicalin treated group and Octreotide treated group, 45 rats in each group while other 45 rats were assigned to the sham-operated group. In sham-operated group, only exploratory laparotomy was performed, namely after entering abdominal cavity, checking pancreas and duodenum and then closing abdomen. After that, the above-mentioned groups were randomly divided into 3 h group, 6 h group and 12 h group, 15 rats in each group[13-16].

Dosage and methods

Baicalin treated group: The animal experiments of 5% Baicalin injection have been completed including the acute toxicity test and SAP rat treated by small, middle and large dose. The large dose can achieve the best therapeutic effect (dose is 10 mg/h per 100 g) and the dosage referred to the result of the previous preliminary experiment. Ten min after successful modeling, Baicalin treated group was first injected 5% Baicalin injection 10 mg/100 g *via* external jugular vein passage followed by continuous intravenous administration (10 mg/h per 100 g) by microinfusion pump[13-16].

Octreotide treated group: The octreotide treated group was first injected Octreotide 0.2 μg/100g *via* external jugular vein passage followed by continuous intravenous transfusion by a microinfusion pump at a transfusion speed of 0.2 μg/h per 100 g. All above dosages have been proved as effective dosages in the previous preliminary experiment.

Sham-operated group and model control group: Both groups were injected saline of equivalent volume at the corresponding time points after operation.

Observing indexes and methods

Observations were made at 2, 6 and 12 h after the operation. Mortalities of rats in all groups followed by batch execution of rats with observation of gross liver pathological changes.

Hepatic tissue samples were collected and fixed in accordance with relevant requirements and the pathological score changes of liver and pancreas under HE staining observed.

Changes of ALT (GPT) and AST (GOT) in serum *via* blood sampling from heart were determined.

Bax, Bcl-2 and Caspase-3 protein expression: TMA was applied to prepare the hepatic TMA sections (2 mm in diameter) and the SP method for immunohistochemical staining. After Bax, Bcl-2 and Caspase-3 protein expression of lung tissue was observed under light microscope, the comprehensive judgment was carried out basing on the percentage of positive cells: (-) in case positive cell count < 10%; (+) in case positive cell count 10%-20%; (++) in case positive

cell count 20%-50%; (+++) in case positive cell count > 50%.

TUNEL staining technique was performed to observe the changes of hepatic apoptotic cells and the apoptotic indexes were calculated. Apoptotic index = apoptotic cell count/total cell count × 100%.

Statistical analysis

Values were presented as mean and standard deviation for nomal distribution variables or median and quartile range for highly skewed variables. The significance of differences among the four groups was tested using the Kruskal-Wallis test for highly skewed data and analysis of variance (ANOVA) for normal distribution data. Mutiple comparisons were subjected to Bonfferoni correction test. The Chi-square test was used to evalute equality of frequencies for discrete variables. Correlations were tested using the Spearman rank correlation coefficients. $P < 0.05$ was considered statistical significant and all statistical analyses were conducted using SPSS version 11.5 for windows.

RESULTS

Survival rate

The mortalities of the model control group were respectively 0% (0/15), 13.33% (2/15) and 33.33% (5/15) at 3, 6 and 12 h, all the mortalities of Baicalin treated group and Octreotide treated group were 0% at different time points. The entire sham-operated group survived at the different time points. The survival of model control group was 66.67% (10/15) at 12 h while the survivals of both Baicalin treated group and Octreotide treated group were 100% at 12 h, indicating marked difference ($P < 0.05$)[13-16].

Pathological changes of liver

Model control group: (1) Gross changes. 3 h group: Mild swelling of liver, local grey plaques in liver of individual rat, obscure boundary; at 6 h and 12 h pale and muddy liver color or hemostasis change, part with scattered grey plaques or necrosis manifestation in irregular shapes, especially obvious at edge of liver; (2) Changes under light microscope. 3 h group: Swelling and apomorphosis of liver cells, inflammatory cell infiltration in portal area, dilation and hyperemia of sinus hepaticus, scattered focal or punctate necrosis in hepatic lobules; 6 h group: Obvious swelling of liver cells, collapse of liver cell cord within hepatic lobules caused by integrality damage due to relatively large area of focal necrosis of liver cells, local sinus hepaticus narrowing or vanishing, increased range and area of liver cell necrosis, visible focal or large lamellar necrosis, mainly hemorrhage necrosis and some coagulation necrosis, inflammatory cell infiltration within necrosis focus, obvious congestion in partial sinus hepaticus (Figure 1A); 12 h group: Obviously damaged hepatic lobule structure, further increased range and area of cell necrosis, residual metamorphic liver cells only at periphery of partial hepatic lobules; relatively large area of inflammatory cell infiltration within lobules or portal area, obvious congestion in sinus hepaticus (Figure 1B).

Baicalin and Octreotide treated group: (1) Gross changes. The gross liver pathological changes of the Baicalin and Octreotide treated group were milder than those of the model control group; (2) Changes under light microscope. Baicalin treated group at all time section: mild swelling of liver cells, mild dilation and hyperemia change of sinus hepaticus, scattered inflammatory cell infiltration in portal area; 6 h and 12 h group: Punctate necrosis and/or mild focal necrosis of liver cells, no obvious lamellar necrosis, inflammatory cell infiltration in portal area. The gross pathological changes in all groups were milder than those of the model control group; there was no marked difference between the Baicalin and Octreotide treated group, but Baicalin treated group had milder pathological manifestations (Figure 1C and D).

Sham-operated group: (1) Gross changes. No obvious swelling of liver, normal color; (2) Changes under light microscope. Complete structure of hepatic lobules, occasional inflammatory cell infiltration in portal area. Most liver cells have normal morphous, some local swelling of liver cells, cholestasis and stenosis of sinus hepaticus (Figure 1E).

Comparison of liver pathological scores in all groups: The pathohistological severity score standard made by first author[19]. The scores of the model control group, Baicalin treated group and Octreotide treated group were significantly exceeded the sham-operated group at the different time points ($P < 0.001$). The scores of the Baicalin treated group and Octreotide treated group were significantly lower than that of the model control group at 12 h ($P < 0.05$). There was no marked difference between the Baicalin treated group and Octreotide treated group at the different time points ($P > 0.05$) (Table 1).

Pathological severity score of pancreas: A modified Schmidt's severity score standard of pancreas made by us was referred[19]. The pancreatic severity score in model control group, Baicalin treated group and Octreotide treated group significantly exceeded than that in sham-operated group ($P_{3, 6, 12\,h} < 0.001$). The pancreatic severity score of Baicalin treated group was significantly lower than that in model control group at 12 h ($P_{12\,h} < 0.01$), and Octreotide treated group was significantly lower than those in model control group at 6 h and 12 h ($P_{6, 12\,h} < 0.01$). There was no marked difference in pancreatic severity score between Baicalin treated group and Octreotide treated group at 3, 6 and 12 h ($P_{3, 6, 12\,h} > 0.05$) (Table 1).

Comparison of serum ALT (GPT) contents in all groups: The model control group and treated groups were significantly higher than the sham-operated group

Table 1 Comparison of different pathological indexes [$M\ (Q_R)$]

Indexes	Sham-operated group			Model control group			Baicalin treated group			Octreotide treated group		
	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h
Pathological score of liver	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.0[1] (1.0)	3.0[1] (1.0)	3.0[1] (0.25)	1.0[1] (2.0)	2.0[1] (2.0)	2.0[1,c] (2.0)	2.0[1] (2.0)	2.0[1] (2.0)	2.0[1,c] (1.0)
Pathological score of pancreas	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)	8.0[1] (2.0)	9.0[1] (3.0)	10.5[1] (1.5)	7.0[1] (1.5)	7.0[1] (3.0)	9.0[1,d] (4.0)	7.0[1] (2.0)	6.0[1,d] (2.0)	8.0[1,d] (2.0)
Bax	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (1.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.5)	0.0[a,c] (1.0)	0.0 (0.0)	0.0 (1.0)	0.0[a] (1.0)	0.0[b] (1.0)
Bcl-2	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0[a] (1.0)	3.0[a] (2.5)	0.0[a] (2.0)	0.0[a,e] (1.0)	0.0[c] (0.0)	0.0[c] (0.0)	0.0[c] (0.0)	0.0[c] (0.0)	0.0[c] (0.0)
Caspase-3	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0[a] (1.0)	0.0[a] (1.0)	0.0[a] (1.0)	0.0[b] (1.0)	0.0[b] (1.0)	1.0[bc] (1.0)	1.0[a] (1.0)	0.0[a] (1.0)	0.0[a] (1.0)
Apoptosis indexes	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.02[a,d] (0.10)	0.00 (0.00)	0.00 (0.04)	0.02[a,c] (0.04)	0.00[a,c] (0.06)

[1]$P < 0.001$, *vs* sham-operated group. [a]$P < 0.05$, [b]$P < 0.01$, *vs* sham-operated group; [c]$P < 0.05$, [d]$P < 0.01$, *vs* model control group; [e]$P < 0.05$, *vs* octreotide treated group.

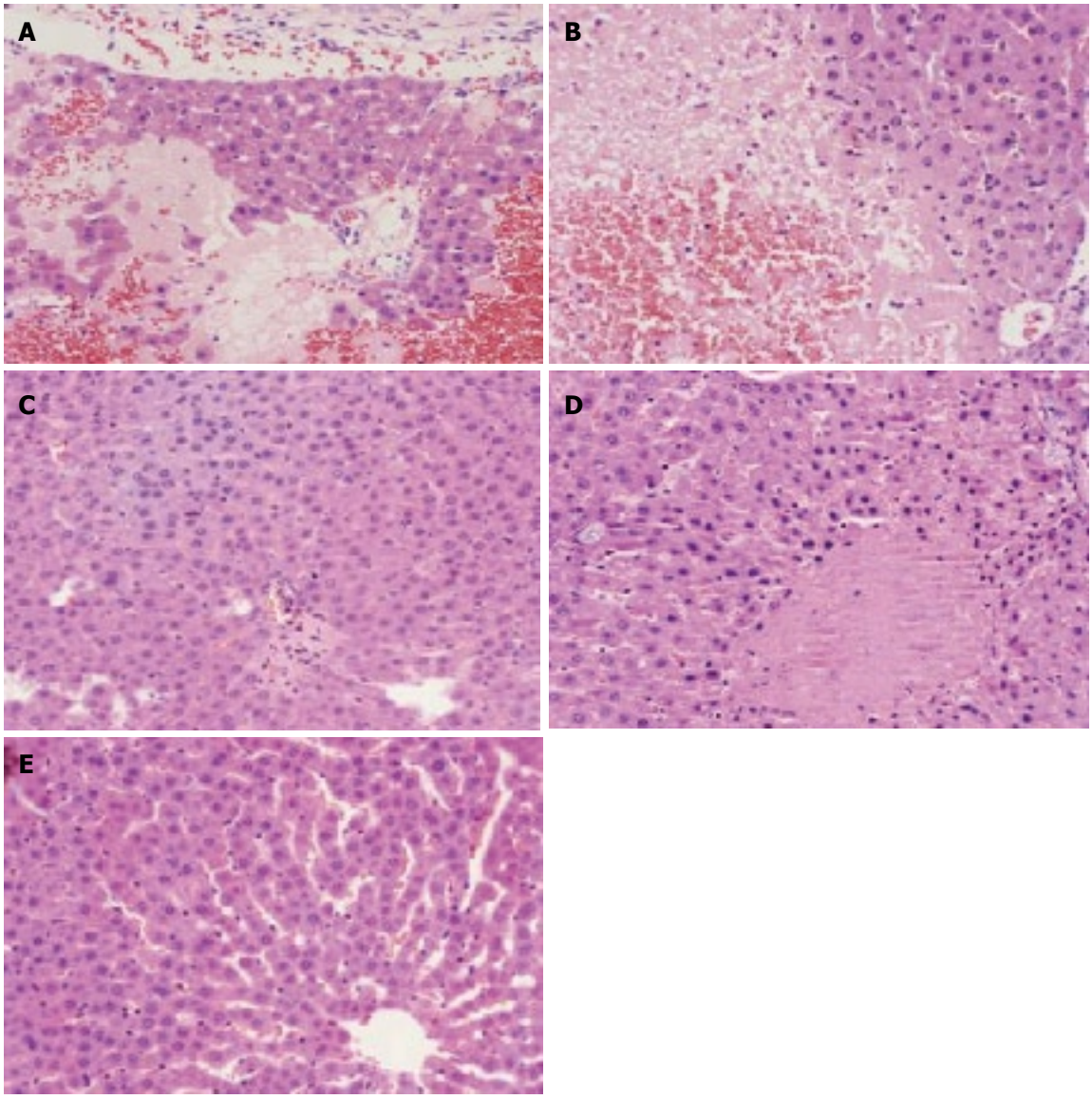

Figure 1 Pathological changes of liver under light microscope. A: Lamellar hemorrhagic necrosis, model control group (6 h); B: Massive hemorrhagic necrosis, model control group (12 h); C: Spotty necrosis of liver cell, Baicalin treated group (12 h); D: Piecemeal necrosis, Octreotide treated group (12 h); E: Normal liver, sham-operated group (12 h). (HE, × 200).

at all time points ($P < 0.001$). The Baicalin treated group was significantly lower than the model control group at all

Table 2 Comparison of ALT, AST contents in blood [$M\ (Q_R)$]

Indexes	Sham-operated group			Model control group			Baicalin treated group			Octreotide treated group		
	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h
ALT	28 (12)	29 (10)	35 (9)	332[1] (140)	623[1] (193)	641[1] (163)	102[1,2,d] (99)	150[1,2] (69)	178[1,2,c] (111)	208[1,2] (128)	158[1,2] (57)	226[1,2] (50)
AST	130 (37)	142 (26)	158 (29)	528[1] (270)	975[1] (242)	987[1] (230)	221[1,2,3] (205)	306[1,2,c] (225)	472[1,2] (114)	423[1,a] (229)	420[1,2] (163)	513[1,2] (258)

[1]$P < 0.001$, *vs* sham-operated group; [2]$P < 0.001$, *vs* model control group; [3]$P < 0.001$, *vs* octreotide treated group. [a]$P < 0.05$, *vs* model control group; [c]$P < 0.05$, [d]$P < 0.01$, *vs* octreotide treated group.

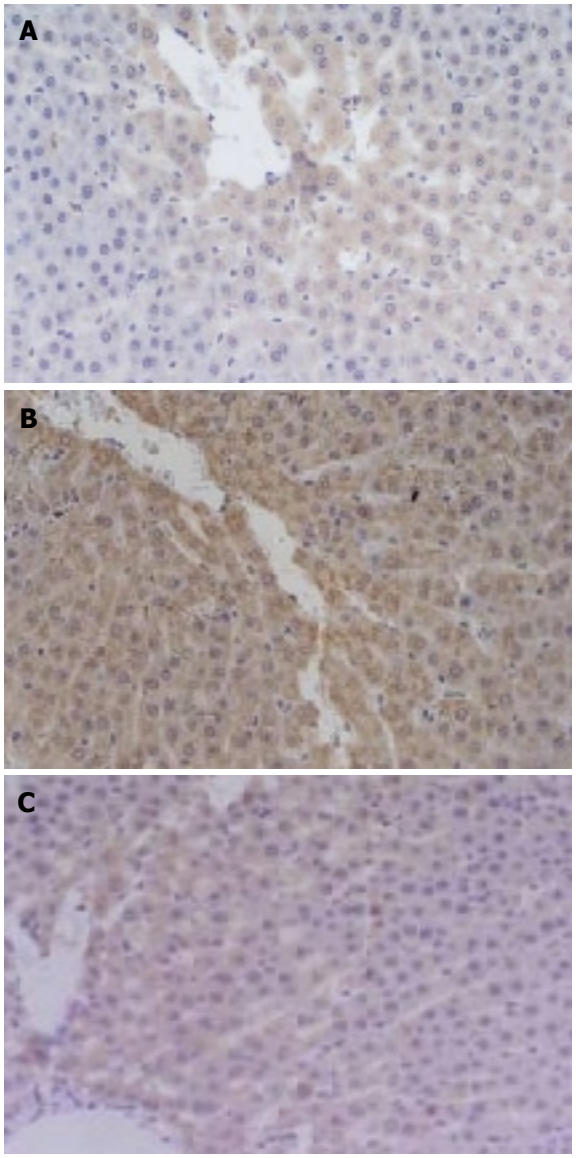

Figure 2 TMA of liver was prepared and immunohistochemical staining. A: Bax protein expression level was "++", model control group (3 h); B: Bcl-2 protein expression level was "+++", model control group (6 h); C: Caspase 3 protein expression level was "++", Octreotide treated group (6 h). (× 200).

time points ($P < 0.001$), the Octreotide treated group were significantly lower than the model control group ($P < 0.001$). The Baicalin treated group was significantly lower than the Octreotide treated group at 3 h ($P < 0.01$), no marked difference between the Baicalin treated group and Octreotide treated group at 6 h ($P > 0.05$), the Baicalin treated group was significantly lower than the Octreotide treated group at 12 h ($P < 0.05$) (Table 2).

Comparison of serum AST (GOT) contents in all groups: The model control group and treated groups were significantly higher than the sham-operated group at all time points ($P < 0.001$). The Baicalin treated group was significantly lower than the model control group ($P < 0.001$), the Octreotide treated group significantly lower than the model control group ($P < 0.05$), and the Baicalin treated group significantly lower than the Octreotide treated group ($P = 0.001$) at 3 h. The Baicalin treated group was significantly lower than the model control group ($P < 0.001$), the Octreotide treated group significantly lower than the model control group ($P < 0.001$) at 6 h and 12 h. The Baicalin treated group was significantly lower than the Octreotide treated group at 6 h ($P < 0.05$), no marked difference between the Baicalin treated group and Octreotide treated group at 12 h ($P > 0.05$) (Table 2).

Comparison of the expression level of Bax protein in liver: The positive Bax protein staining position was in cytoplasm of liver cell. There was no marked difference in all groups at 3 h ($P > 0.05$). The Octreotide treated group significantly exceeded the sham-operated group at 12 h ($P < 0.01$). Both the Baicalin treated group and Octreotide treated group significantly exceeded the sham-operated group ($P < 0.05$) and the Baicalin treated group significantly exceeded the model control group ($P < 0.05$) at 6 h (Table 1 and Figure 2A).

Comparison of the expression level of Bcl-2 protein in liver: The positive Bcl-2 protein staining position was in cytoplasm of liver cell. There was no marked difference between the Octreotide treated group and sham-operated group, the model control group significantly exceeded the sham-operated group ($P < 0.05$), the Octreotide treated group significantly lower than the model control group ($P < 0.05$) at different time points. The Baicalin treated group significantly exceeded the sham-operated group at 3 h ($P < 0.05$), the Baicalin treated group significantly lower than the model control group at 6 h ($P < 0.05$), the Octreotide treated group significantly lower than the Baicalin treated group at 3 h ($P < 0.05$) (Table 1 and Figure 2B).

Comparison of the expression level of Caspase-3 protein in liver: The positive staining signal of Caspase-3 protein was localized in the cytoplasm of hepatic cells. At all time points after operation, the staining intensity of Caspase-3 protein in liver in the model control group, the Baicalin treated group and

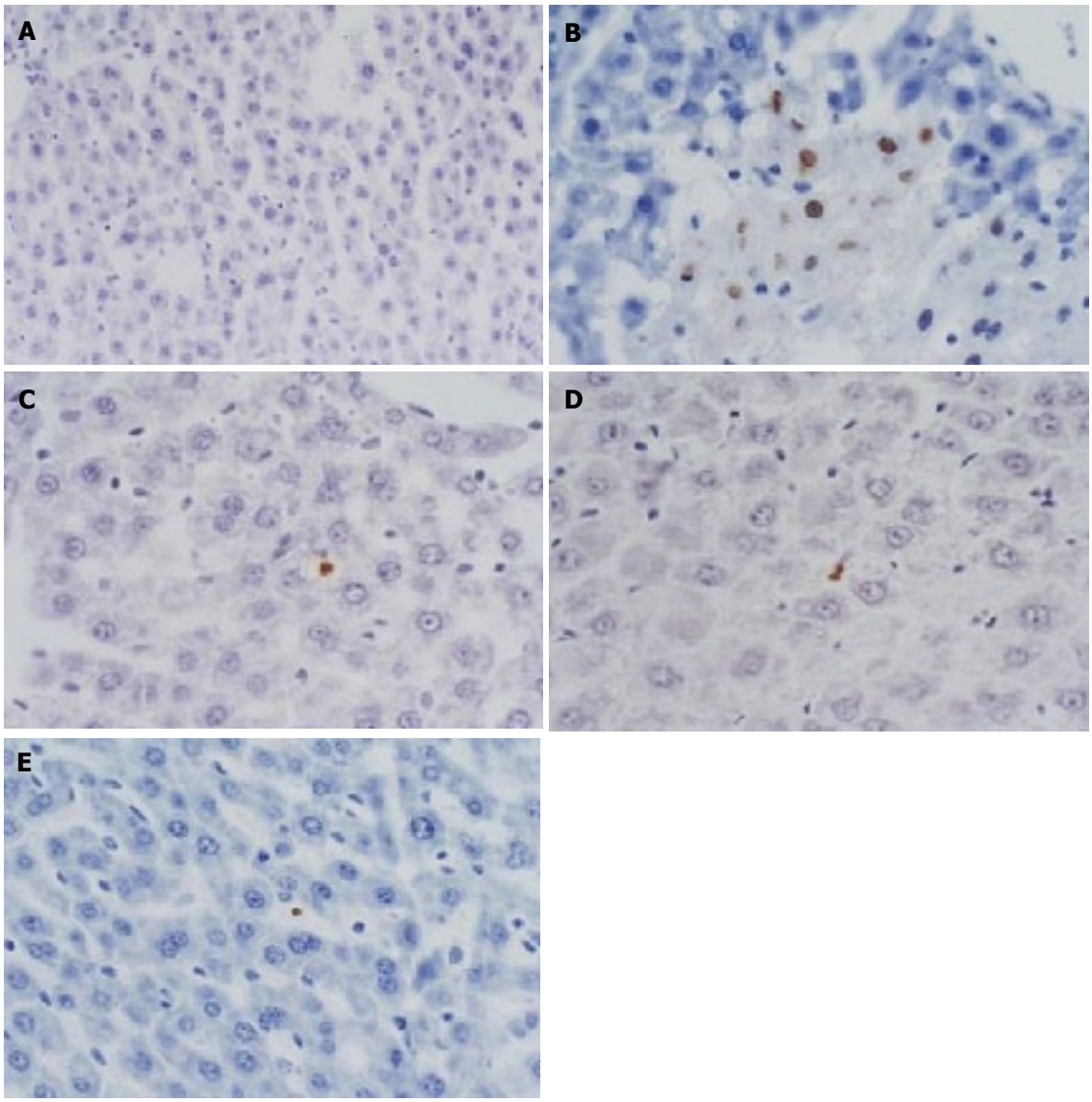

Figure 3 TMA of liver was prepared and conduced TUNEL staining, observing the changes of apoptotic indexes. A: model control group (3 h), there was no apoptotic cell; B: Baicalin treated group (3 h), several hepatic apoptotic cells appeared; C: Baicalin treated group (6 h), several apoptotic hepatic Kupffer cells; D: Baicalin treated group (12 h), several apoptotic hepatic Kupffer's cells; E: Octreotide treated group (3 h), several apoptotic hepatic Kupffer's cells. (TUNEL, × 400).

the Octreotide treated group were significantly higher than that in the sham-operated group ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively); at 12 h after operation, the staining intensity in the Baicalin treated group was obviously higher than that in the model control group ($P < 0.05$); at all time points after operation, no marked difference was noted between the Baicalin treated group and the Octreotide treated group ($P > 0.05$) (Table 1 and Figure 2C).

Comparison of apoptosis indexes in liver: Apoptosis occurred to liver Kupffer cells and liver cells. There was no marked difference in all groups at 3 h ($P > 0.05$); the Baicalin treated group significantly exceeded the sham-operated group ($P < 0.05$) and model control group ($P < 0.01$) at 6 h; Octreotide treated group significantly exceeded the sham-operated group and model control group at 6 h and 12 h ($P < 0.05$) (Table 1 and Figure 3).

Comparison of correlations among all indexes

Correlations between hepatic apoptosis indexes and Bax, Bcl-2: The 3 h apoptosis indexes of the Baicalin treated group was positively correlated with Bax ($P < 0.001$); the 12 h apoptosis indexes of the Baicalin treated group was positively correlated with Bax ($P < 0.05$); the 3 h apoptosis indexes of the Octreotide treated group was positively correlated with Bax ($P < 0.01$); There was no correlation between apoptosis indexes in all groups and Bcl-2 ($P > 0.05$).

Correlations between pathological score of hepatic injury and ALT, AST: The 6 h pathological score of the model control group was positively correlated with ALT ($P < 0.01$). The 3 h pathological score of the Baicalin treated group was positively correlated with AST ($P < 0.01$); the 12 h pathological score of the Baicalin treated group was positively correlated

with ALT ($P < 0.05$). The 3 h pathological score of the Octreotide treated group was positively correlated with ALT ($P < 0.05$); the 6 h pathological score of the Octreotide treated group was positively correlated with ALT ($P < 0.001$) and meanwhile AST ($P < 0.05$); the 12 h pathological score of the Octreotide treated group was positively correlated with ALT ($P < 0.01$).

DISCUSSION

As the morbidity and mortality of SAP increases, improving the clinical therapeutic effects on SAP and finding cheap alternates with precise therapeutic effects and fewer side effects have been the hot spots of clinical research[20-23]. Our study results show the main mechanism of MODS onset during AP is the abnormal activation of mononuclear-macrophage system which generates excessive cytokines[24]. Kupffer cells of liver, which is the largest fixed macrophage population in body, account for 80%-90% of the total mononuclear macrophage system. They must affect the progression of SAP state. Therefore, it is necessary to find cheap alternates with precise therapeutic effects and fewer side effects to protect rats with SAP from hepatic injury.

As one of the common clinical SAP medications at present, Octreotide can effectively reducing SAP complications and improve survival[12,25]. However, it is also expensive and inconvenient. It is still not sure whether Octreotide can protect liver, leading to the need of further study. However, Baicalin is cheap and convenient. Baicalin also can protect liver. At present, our hospital is still the only hospital that has submitted a study report on SAP treated by Baicalin injection[13-16]. We have been granted a Chinese invention patent number for our Baicalin injection prescription, which has paved the way for further developing and utilizing the new drug.

This study showed the pancreatic score of Baicalin treated group was significantly lower than that in model control group at 12 h, which mentioned that Baicalin can relieve the severity of pancreas in SAP procedure. The protective effect of Baicalin may be related to inhibit secretion of pancreatin and decrease the contents of endotoxin and TNF-α. From the prospect of hepatic pathological score, spotty necrosis and/or mild focal necrosis, visible inflammatory cell infiltration in portal area and no lamellar necrosis were observed in Baicalin treated group microscopically. The gross pathological changes were milder in Baicalin treated group than in model control group. And there was no difference between Baicalin treated group and Octreotide treated group in gross changes[15].

ALT is the abbreviation for alanine aminotransferase. It is found mainly in liver cells. AST is the abbreviation for aspartate aminotransferase, which is found mainly in cardiac muscle, followed by the liver. Both are non-specific intracellular functional enzymes and are normally found in the serum at very low levels. When hepatic cells are damaged, their membrane permeability increases. As a consequence, cytoplasmic ALT and AST are released into the blood, resulting in an increase in the activities and contents of serum ALT and AST. Therefore, serum ALT and AST levels are sensitive parameters for the evaluation of hepatic cell damage. The correlation analysis has demonstrated a positive correlation between 6 h and 12 h pathological scores and ALT ($P < 0.01$ and $P < 0.05$), and a positive correlation between 3 h score and AST ($P < 0.01$) in Baicalin treated group, which means Baicalin can alleviate the hepatic injury of rats with SAP, and protect rats from SAP hepatic injury. We believe these effects of Baicalin are related to its many capacities. For instance, Baicalin can lower AST and AST content in serum, and down regulate the expression level of hepatic Bcl-2 protein. According to our results from a series of experiments, we believe that the protective effect of Baicalin and Octreotide on the liver may be mediated by reducing the contents of inflammatory mediators such as NO, MDA, TNF-α, IL-6 and PLA_2 *etc*[14-16].

Bax and Bcl-2 are two important components of apoptosis regulating system. When Bax forms dimers, it will induce apoptosis. As Bcl-2 expression increases, the apoptosis promoting effect of Bax dimers are inhibited. In this experiment, it was found that the expression levels of both Bax protein and Bcl-2 protein rose during SAP. It is possible that both apoptosis induction and inhibition have been enhanced so equally that no marked difference occurred between model control group and sham-operated group in apoptotic index. In addition, according to the experimental results, the expression level of Bax protein at 6 h was higher in Baicalin treated group than in model control group ($P < 0.05$). The expression levels of Bcl-2 protein at 6 h and 12 h were lower in Baicalin treated group than in model control group ($P < 0.05$). These findings indicate that Baicalin can induce Bax and inhibit the expression of Bcl-2 protein to let apoptosis inducing factor prevail in treated group. Therefore, Baicalin can protect liver and alleviate pathological changes. The expression levels of Bcl-2 protein were lower in Octreotide treated group than in model control group at different time points. The apoptotic indexes at 6 h and 12 h were higher in Octreotide treated group than in model control group ($P < 0.05$). These findings indicate that Octreotide can induce apoptosis in hepatic cells. From the prospect of correlation analysis, the apoptotic indexes at 3 h and 12 h were positively correlated with Bax, and not correlated with Bcl-2 in treated group, which means that *Bax* gene participates in the regulation of apoptosis of hepatic cell while *Bcl-2* gene may prevent the apoptosis of hepatic cell and promote its hyperplasia during hepatic injury of rats with SAP.

Caspase-3 is an interleukin-converting enzyme that belongs to a member of caspase family (cysteine protease)[26,27]. Some studies have demonstrated that cell apoptosis is negatively correlated with the severity of SAP[28,29]. Activated Caspase-3 is the main executor of cell apoptosis[30,31] and works as the final common signal molecule of various apoptotic mechanisms[32]. During apoptosis signal transduction, caspases function

as downstream signal molecules. The binding of caspases to cytochrome c can induce the activation of Caspase-3 which enables cell apoptosis completed. Some characteristic markers for cell apoptosis, such as chromosome condensation and DNA fragmentation, are directly related to Caspase-3[33,34]. This study showed that, at 12 h after operation, the expression level of Caspase-3 protein in liver in the Baicalin treated group was significantly higher than that in the model control group, suggesting that Baicalin is able to effectively upregulate the expression of Caspase-3 protein and promote apoptosis. Although some studies have indicated that cell apoptosis is positively correlated with hepatic injury[35-37], the results of the present study showed that low proportion of hepatic apoptosis at the early stage was favorable to the body. Considering that apoptotic cells do not release inflammatory mediators and, therefore, reduce their contents and weaken the body's inflammatory reaction[14], we believe that cell apoptosis which occurred at early stage of SAP has some protective effect on the liver.

The TMA we adopted has surpassed the traditional tissue pathological section technique that is inefficient only using a single sample[38]. There is a great potential for TMA in oncopathological research since it can achieve high-throughput, reliable results and so on. This field is also the focus of current study[39-41].

In conclusion, Baicalin and Octreotide can enhance the apoptosis promoting effect of Bax dimmers by lowering the expression level of Bcl-2 protein, leading to the apoptosis of hepatic Kupffer cells and hepatic cells, thus alleviating the pathological changes of liver. Baicalin and Octreotide also can lower the content of ALT, AST in serum to improve the survival of rats with SAP. Baicalin is more advantageous than Octreotide in many aspects. TMA is time and energy saving, highly efficient and representative for the pathological examination of pancreatitis. We believe that as a cheap new drug with precise therapeutic effects and fewer side effects, Baicalin can play certain role in future SAP treatment.

COMMENTS

Background

Severe acute pancreatitis (SAP), which is a systemic disease, seriously life-threatening with acute onset, severe state and multiple complications can cause multiple organ injuries, especially to liver. Octreotide mainly treats SAP by inhibiting trypsin secretion, lowering endotoxin generation, inhibiting the release of inflammatory mediators and platelet aggregation, *etc*. As the main effective ingredient of Scutellaria Baicalensis Georgi, the pharmacologic effects of Baicalin (monomer) can antagonize multiple phases during SAP onset, Baicalin is a possible medication for treating SAP. This article has observed the effects of Baicalin and Octreotide on SAP with hepatic injury at different time points, and compared their therapeutic effects and mechanisms, providing the theoretical basis for application of Baicalin to treat SAP with hepatic injury.

Research frontiers

Tissue microarray (TMA) has become a new member of the chip family recently. This technique has been extensively applied to the technological fields. It surpasses the concept of traditional histopathologic slide which can afford the relevant biological function study at the three levels including gene, genetic transcription and related expression product. Presently, there are some reports on Baicalin treatment of SAP made by the authors. This experiment is one of series studies about Baicalin treatment to SAP. The authors applied TMA technique to prepare the sections of liver samples, and conducted pathological examinations of different indexes.

Innovations and breakthroughs

Firstly, the authors applied TMA technique to study the pathological changes in SAP complicated with hepatic injury; Secondly, they found Baicalin injectin has good effects to treat SAP rats, and has a similar function with Octreotide.

Applications

The application of hepatic TMA in the study of SAP is economical and efficient, and it is worthy to be further popularized. This article will make us realize the value of applying Baicalin in SAP treatment.

Terminology

Caspase-3 is an interleukin-converting enzyme that belongs to a member of caspase family (cysteine protease). Activated Caspase-3 is the main executor of cell apoptosis and works as the final common signal molecule of various apoptotic mechanisms.

Peer review

This article showed that the Baicalin and Octreotide can protect rats with SAP by improving the expression levels of Bax protein, Caspase-3 protein, *etc*. This is a well-written paper.

REFERENCES

1 **Zhang Q**, Ni Q, Cai D, Zhang Y, Zhang N, Hou L. Mechanisms of multiple organ damages in acute necrotizing pancreatitis. *Chin Med J* (Engl) 2001; **114**: 738-742

2 **Zhang XP**, Ye Q, Jiang XG, Ma ML, Zhu FB, Zhang RP, Cheng QH. Preparation method of an ideal model of multiple organ injury of rat with severe acute pancreatitis. *World J Gastroenterol* 2007; **13**: 4566-4573

3 **Rau BM**, Bothe A, Kron M, Beger HG. Role of early multisystem organ failure as major risk factor for pancreatic infections and death in severe acute pancreatitis. *Clin Gastroenterol Hepatol* 2006; **4**: 1053-1061

4 **Garcea G**, Jackson B, Pattenden CJ, Sutton CD, Neal CP, Dennison AR, Berry DP. Early warning scores predict outcome in acute pancreatitis. *J Gastrointest Surg* 2006; **10**: 1008-1015

5 **Granger J**, Remick D. Acute pancreatitis: models, markers, and mediators. *Shock* 2005; **24** Suppl 1: 45-51

6 **Lytras D**, Manes K, Triantopoulou C, Paraskeva C, Delis S, Avgerinos C, Dervenis C. Persistent early organ failure: defining the high-risk group of patients with severe acute pancreatitis? *Pancreas* 2008; **36**: 249-254

7 **Yousaf M**, McCallion K, Diamond T. Management of severe acute pancreatitis. *Br J Surg* 2003; **90**: 407-420

8 **Zhang HY**, Xia Q. [Clinical study on severe acute pancreatitis complicated by hepatic insufficiency] *Zhongxiyi Jiehe Xuebao* 2006; **4**: 17-19

9 **Wang G**, Sun B, Gao Y, Meng QH, Jiang HC. The effect of emodin-assisted early enteral nutrition on severe acute pancreatitis and secondary hepatic injury. *Mediators Inflamm* 2007; **2007**: 29638

10 **Shor NA**, Levina VP, Ioffe IV, Andreeva IV, Chumak IuF, Zhadanov VI, Zelenyi II. [Application of octreotide in patients with acute pancreatitis] *Klin Khir* 2004: 15-17

11 **Suzuki M**, Shimizu T, Kudo T, Shoji H, Ohtsuka Y, Yamashiro Y. Octreotide prevents L-asparaginase-induced pancreatic injury in rats. *Exp Hematol* 2008; **36**: 172-180

12 **Paran H**, Mayo A, Paran D, Neufeld D, Shwartz I, Zissin R, Singer P, Kaplan O, Skornik Y, Freund U. Octreotide treatment in patients with severe acute pancreatitis. *Dig Dis Sci* 2000; **45**: 2247-2251

13 **Zhang XP**, Zhang L, He JX, Zhang RP, Cheng QH, Zhou YF, Lu B. Experimental study of therapeutic efficacy of Baicalin in rats with severe acute pancreatitis. *World J Gastroenterol* 2007; **13**: 717-724

14 **Zhang XP**, Tian H, Lai YH, Chen L, Zhang L, Cheng QH, Yan W, Li Y, Li QY, He Q, Wang F. Protective effects and

mechanisms of Baicalin and octreotide on renal injury of rats with severe acute pancreatitis. *World J Gastroenterol* 2007; **13**: 5079-5089
15 **Zhang XP**, Zhang L, Yang P, Zhang RP, Cheng QH. Protective effects of baicalin and octreotide on multiple organ injury in severe acute pancreatitis. *Dig Dis Sci* 2008; **53**: 581-591
16 **Zhang XP**, Tian H, Chen HQ, Chen L, Wang ZW, Wang KY, Yan W, Li Y, Li QY, He Q, Wang F. The protecting effects and mechanisms of Baicalin and Octreotide on heart injury in rats with SAP. *Mediators Inflamm* 2007; **2007**: 19469
17 **Gu XD**, Zhang Q. Clinical progress in the treatment of severe acute pancreatitis with integrative Chinese and Western medicine. *Chin J Integr Med* 2007; **13**: 235-240
18 **Li YY**, Sibaev A, Zhou MZ, Zhu GY, Yuce B, Storr M. The Chinese herbal preparation Qing Yi Tang (QYT) improves intestinal myoelectrical activity and increases intestinal transit during acute pancreatitis in rodents. *Phytother Res* 2007; **21**: 324-331
19 **Zhang XP**, Zhang L, Wang Y, Cheng QH, Wang JM, Cai W, Shen HP, Cai J. Study of the protective effects of dexamethasone on multiple organ injury in rats with severe acute pancreatitis. *JOP* 2007; **8**: 400-412
20 **Lese M**, Tamasan A, Stoicescu B, Branduse M, Puia I, Mare C, Lazar C. [Surgical treatment in severe acute pancreatitis. Last 15 years of experience in Emergency County Hospital of Baia Mare] *Chirurgia* (Bucur) 2005; **100**: 445-450
21 **Cappell MS**. Acute pancreatitis: etiology, clinical presentation, diagnosis, and therapy. *Med Clin North Am* 2008; **92**: 889-923, ix-x
22 **Lankisch PG**, Lerch MM. Pharmacological prevention and treatment of acute pancreatitis: where are we now? *Dig Dis* 2006; **24**: 148-159
23 **Hochman D**, Louie B, Bailey R. Determination of patient quality of life following severe acute pancreatitis. *Can J Surg* 2006; **49**: 101-106
24 **Norman J**. The role of cytokines in the pathogenesis of acute pancreatitis. *Am J Surg* 1998; **175**: 76-83
25 **Czakó L**, Hegyi P, Takács T, Góg C, Farkas A, Mándy Y, Varga IS, Tiszlavicz L, Lonovics J. Effects of octreotide on acute necrotizing pancreatitis in rabbits. *World J Gastroenterol* 2004; **10**: 2082-2086
26 **Yang J**, Fier A, Carter Y, Liu G, Epling-Burnette PK, Bai F, Loughran TP Jr, Mastorides S, Norman JG, Murr MM. Liver injury during acute pancreatitis: the role of pancreatitis-associated ascitic fluid (PAAF), p38-MAPK, and caspase-3 in inducing hepatocyte apoptosis. *J Gastrointest Surg* 2003; **7**: 200-207; discussion 208
27 **Meyerholz DK**, Samuel I. Morphologic characterization of early ligation-induced acute pancreatitis in rats. *Am J Surg* 2007; **194**: 652-658
28 **Bhatia M**. Apoptosis of pancreatic acinar cells in acute pancreatitis: is it good or bad? *J Cell Mol Med* 2004; **8**: 402-409
29 **Xu XF**, Lou WH, Wang DS, Jin da Y, Ni XL, Wu ZH. Influence of glutamine on pancreatic blood flow and apoptosis of pancreatic acinar in rats with severe acute pancreatitis. *Chin J Dig Dis* 2006; **7**: 121-126
30 **Li P**, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; **91**: 479-489
31 **Yasuda T**, Takeyama Y, Ueda T, Shinzeki M, Kishi S, Sawa H, Nakajima T, Kuroda Y. Protective effect of caspase inhibitor on intestinal integrity in experimental severe acute pancreatitis. *J Surg Res* 2007; **138**: 300-307
32 **Ashkenazi A**, Dixit VM. Death receptors: signaling and modulation. *Science* 1998; **281**: 1305-1308
33 **Porter AG**, Jänicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999; **6**: 99-104
34 **Peng Y**, Sigua CA, Gallagher SF, Murr MM. Protein kinase C-zeta is critical in pancreatitis-induced apoptosis of Kupffer cells. *J Gastrointest Surg* 2007; **11**: 1253-1261
35 **Zhang XP**, Wang L, Zhang J. Study progress on mechanism of severe acute pancreatitis complicated with hepatic injury. *J Zhejiang Univ Sci B* 2007; **8**: 228-236
36 **Takeyama Y**. Significance of apoptotic cell death in systemic complications with severe acute pancreatitis. *J Gastroenterol* 2005; **40**: 1-10
37 **Takeyama Y**, Hori Y, Takase K, Ueda T, Yamamoto M, Kuroda Y. Apoptotic cell death of hepatocytes in rat experimental severe acute pancreatitis. *Surgery* 2000; **127**: 55-64
38 **Kononen J**, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; **4**: 844-847
39 **Waddell SJ**, Butcher PD. Microarray analysis of whole genome expression of intracellular Mycobacterium tuberculosis. *Curr Mol Med* 2007; **7**: 287-296
40 **Das K**, Mohd Omar MF, Ong CW, Bin Abdul Rashid S, Peh BK, Putti TC, Tan PH, Chia KS, Teh M, Shah N, Soong R, Salto-Tellez M. TRARESA: a tissue microarray-based hospital system for biomarker validation and discovery. *Pathology* 2008; **40**: 441-449
41 **Yu G**, Wang J, Chen Y, Wang X, Pan J, Li Q, Xie K. Tissue microarray analysis reveals strong clinical evidence for a close association between loss of annexin A1 expression and nodal metastasis in gastric cancer. *Clin Exp Metastasis* 2008; **25**: 695-702

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RAPID COMMUNICATION

Risk factors for operative morbidity and mortality in gastric cancer patients undergoing total gastrectomy

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Abstract

AIM: To study the risk factors for morbidity and mortality following total gastrectomy.

METHODS: We retrospectively reviewed the records of 125 consecutive patients who underwent total gastrectomy for gastric cancer at the Second Affiliated Hospital of Zhejiang University School of Medicine between January 2003 and March 2008.

RESULTS: The overall morbidity rate was 20.8% (27 patients) and the mortality rate was 3.2% (4 patients). Morbidity rates were higher in patients aged over 60 [odds ratio (OR) 4.23 (95% confidence interval (CI) 1.09 to 12.05)], with preoperative comorbidity [with *vs* without, OR 1.25 (95% CI 1.13 to 8.12)], when the combined resection was performed [combined resection *vs* total gastrectomy only, OR 2.67 (95% CI 1.58 to 5.06)].

CONCLUSION: Age, preoperative comorbidity and combined resection were independently associated with the rate of morbidity after total gastrectomy for gastric cancer.

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Key words: Gastric cancer; Total gastrectomy; Morbidity; Mortality; Risk factor

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INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death worldwide[1]. It ranks first among all causes of death from cancer in China, with an annual mortality rate of approximately 25.2 per 100 000 people[2].

Despite the best efforts of clinicians, gastric cancer is usually diagnosed at a fairly advanced stage in most countries[3]. Complete surgical resection is the only potentially curative treatment for gastric cancer[4,5].

In most Western countries, there has been a rapid rise in the incidence of tumors at or close to the gastrooesophageal junction over the past 20 to 30 years[3]. Based on tumor location and growth pattern, a total gastrectomy is the procedure of choice for patients with middle and proximal third gastric cancer[6].

Preoperative preparation, anesthesiology, operative techniques, and postoperative care have improved considerably in recent years. However, total gastrectomy has been reported to have higher morbidity and mortality rates than subtotal gastrectomy[7–9].

The aim of this study is to document the frequency and nature of operative morbidity and mortality after total gastrectomy, and to identify factors that are predictive of complications and death.

MATERIALS AND METHODS

We retrospectively reviewed the records of 125 consecutive patients who underwent total gastrectomy for gastric cancer at the Second Affiliated Hospital of Zhejiang University School of Medicine between January, 2003 and March, 2008. The median age of the patients was 60 (range 29-78). All patients had histologically confirmed gastric cancer, without previous or coexisting cancer. None had received preoperative chemotherapy or radiation therapy. Abdominal terminolateral esophagojejunal anastomosis

was performed with staples in every patient. Criteria for exclusion from this study were surgery for gastric stump carcinoma and other nonresective palliative operations for gastric cancer including bypass procedures. Roux-en-Y oesophagojejunostomy with or without a Paulino pouch was performed in all patients after radical or palliative total gastrectomy. A D2 or D2+ lymph node dissection was performed in patients with radical total gastrectomy. All patients received prophylactic antibiotics starting half an hour before the laparotomy and continuing for over 72 h.

From these data, risk factors were analyzed with postoperative morbidity and mortality. Analyzed risk factors included sex, age, America Society of Anesthesiologists (ASA) grade, tumor site, tumor size, pTNM stage, intraoperative transfusion volume, intraoperative loss of blood, preoperative comorbidity, operative time, combined organ resection, and procedure. Classification of pTNM stage followed the 5th edition of the International Union Against Cancer (UICC) criteria. The operative time was defined as the time between initiation of skin incision and the completion of wound closure. Postoperative hospital stay was defined as the number of days in hospital from operation to discharge. Combined organ resection was referred to as splenectomy, pancreaticosplenectomy, transverse colectomy, cholecystectomy, and hepatic segment resection.

Operative complications analyzed in this study included both immediate postoperative minor complications and major complications that occurred during the same hospitalization. Late complications, such as gallstone formation, anemia, dumping syndrome, and weight loss, were not included in the scope of this study. Bleeding was defined by the need for postoperative transfusion. A fluid collection was defined by the presence of septic fluid in the abdominal cavity causing fever higher than 38℃ and verified by computer tomography (CT) or B-type ultrasound. Wound infection included the presence of septic fluid or pus at the incision leading to delayed suture removal or the need for wound re-suture. Pleural effusion was defined by the presence of fluid in the thoracic cavity requiring drainage. Pulmonary embolus was verified by pulmonary angiography. Pulmonary infection was verified by chest X-ray. The diagnosis of fungus infection and urinary tract infection was determined by sample microbiology culture.

Operative mortality in this study included all hospital deaths within 30 d.

Statistical analysis

The χ^2 test, Fisher's exact test and a binary logistic regression model were used for statistical analysis. $P < 0.05$ (two sided) was regarded as significant. SPSS® version 11.5 (SPSS, Chicago, IL, USA) was used for data analysis.

RESULTS

The postoperative morbidity of the 125 patients studied is listed in Table 1. The overall morbidity rate was 20.8% (27 patients), and the mortality rate was 3.2% (4 patients) and mean postoperative stay was 18.34 d. Univariate analysis showed that age, sex, extension of resection (combined or not), perioperative transfusion, and preoperative comorbidity were all significantly associated with operative morbidity (Table 2). Multiple logistic regression analysis identified older age [odds ratio (OR) 4.23 (95% confidence interval (CI) 1.09 to 12.05)], preoperative comorbidity [with *vs* without, OR 1.25 (95% CI 1.13 to 8.12)] and combined resection [combined resection *vs* total gastrectomy only, [OR 2.67 (95% CI 1.58 to 5.06)] as independent predictors of a higher operative morbidity rate.

Table 1 Postoperative morbidity

Morbidity	No. of patients
Pulmonary infection	7
Wound infection	4
Abdominal abscess	3
Intra-abdominal bleeding	3
Upper digestive tract bleeding	3
Duodenal stump leakage	3
Jejunum stump leakage	1
Chylous leaks	2
Pulmonary embolus	2
Arrhythmia	4
Pleural effusion	4
Ascite	3
Urinary tract infection	2
Fungus infection	3
Total	44

Four patients (3.2%) with preoperative comorbidity died in hospital. All four had undergone palliative or radical total gastrectomy without combined organ resection. The first of these patients died of a pulmonary infection with preoperative chronic bronchitis. The second patient, who died of sudden cardiac arrest, had preoperative coronary heart disease for 5 years. The third patient died of multiple organ dysfunction syndrome (MODS) with preoperative complete right bundle-branch heart block. The fourth patient, who died of heart failure, had preoperative pleural effusion.

DISCUSSION

Gastric cancer is the second leading cause of cancer-related death worldwide[1]. It ranks first among all causes of death from cancer in China, with an annual mortality rate of approximately 25.2 per 100 000[2]. In most Western countries, there has been a rapid rise in the incidence of tumors at or close to the gastrooesophageal junction over the past 20 to 30 years[3]. Based on tumor location and growth pattern, a total gastrectomy is the procedure of choice in patients with middle and proximal third gastric cancer[6].

Although morbidity and mortality rates for gastrectomy for gastric cancer were different in past studies, many recent studies show that they have now decreased significantly to less than 23% and 6%, respectively[8,10-14]. Total gastrectomy has been reported

Table 2 Factors related to operative morbidity *n* (%)

	No. of patients	No. with complications	P^1	P^2
Age (yr)			0.005	< 0.01
< 60	62	7 (11.29)		
⩾ 60	63	20 (31.75)		
Sex			0.038	
Male	86	23 (26.74)		
Female	39	4 (10.26)		
ASA			0.154	
1	37	5 (16.51)		
2-3	88	22 (25)		
Tumor size (cm)			0.821	
⩽3	19	5 (26.32)		
⩽ 6	45	10 (22.22)		
> 6	61	12 (19.67)		
Tumor site			0.887	
Upper	46	9 (19.56)		
Middle	24	4 (16.67)		
Low	17	4 (23.53)		
Two thirds or more	38	10 (26.32)		
pTNM stage			0.135	
Ⅰ-Ⅱ	27	3 (11.11)		
Ⅲ-Ⅳ	98	22 (22.45)		
Transfusion (U)			0.036	
0	71	8 (11.27)		
⩽ 3	34	8 (23.53)		
⩾ 4	20	7 (35)		
Operative hemorrhage (mL)			0.947	
⩽ 400	45	10 (22.22)		
⩽ 800	54	12 (22.22)		
> 800	26	5 (19.23)		
Operative time (h)			0.214	
⩽ 4	75	19 (25.33)		
> 4	50	8 (16)		
Combined resection			0.009	0.012
Yes	47	16 (34.04)		
No	78	31 (39.74)		
Procedure			0.585	
Palliative	28	5 (17.86)		
Radical	97	22 (22.68)		
Comorbidity			0.003	0.011
With	56	19 (33.93)		
Without	69	8 (11.59)		

Values in parentheses are percentages. [1]χ^2 test; [2]Multiple regression analysis.

to have higher morbidity and mortality rates than subtotal gastrectomy[7-9]. In our study, the morbidity and mortality rates for total gastrectomy was 20.8% and 3.2%, respectively. The most common complication was pulmonary infection, in accordance with other reports[9,12].

In keeping with other studies showing that postoperative morbidity and mortality rates are associated with age, sex and combined resection[15], univariate analysis in our study demonstrated that age, sex, and combined resection contributed to postoperative complications. Furthermore, besides combined resection, age was an independent contributor to postoperative complications, because elderly patients may harbor occult heart disease, and have reduced respiratory and liver function reserves.

Opelz *et al*[16] reported that homologous blood transfusion was associated with immunosuppression in renal allograft transplantation. Clinical evidence of a relationship between perioperative transfusion and postoperative septic complications has been reported in some studies of gastric cancer[17-20]. Although the exact mechanism of immunosuppression is still to be elucidated, reduced tumor necrosis factor-α levels, interleukin 10 (IL-10) induction, impairment of natural killer cells, increases in certain other cytokines, complement activation, decreased macrophage function, decreased CD4/CD8 ratio, and decreased IL-2 secretion are all involved[21-23]. Our univariate analysis also showed that perioperative transfusion was associated with postoperative morbidity.

Our study found that preoperative comorbidity was an independent predictor of postoperative morbidity, and that all of the 4 patients who died in hospital were preoperatively suffering from respiratory or cardiac disease. This relationship was also reported in other studies[9,24,25]. Thus, we believe that prior treatment of preoperative comorbid conditions is critical to the postoperative recovery of patients with gastric cancer.

There are some common drawbacks to undertaking any retrospective study, including sample insufficiency and operations performed by different surgeons, which apply to this study. This study also demonstrates that relatively low morbidity and mortality rates for total gastrectomy can be achieved in a large-volume hospital by experienced surgeons with careful perioperative treatment.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer-related death worldwide. In most Western countries, there has been a rapid rise in the incidence of tumors at or close to the gastrooesophageal junction. A total gastrectomy is the procedure of choice for patients with middle and proximal third gastric cancer. However, total gastrectomy has been reported to have higher morbidity and mortality rates than subtotal gastrectomy. It is significant to identify factors that are predictive of complications and death.

Research frontiers

Although morbidity and mortality rates for gastrectomy for gastric cancer were different in past studies, many recent reports show that they have now decreased significantly to less than 23% and 6%, respectively. Furthermore, some reports showed that several risk factors were associated with the complications and death.

Innovations and breakthroughs

Age, preoperative comorbidity and combined resection were independently associated with the rate of morbidity after total gastrectomy for gastric cancer. Perioperative transfusion was also associated with postoperative morbidity.

Applications

This study demonstrates that relatively low morbidity and mortality rates for total gastrectomy can be achieved in a large-volume hospital by experienced surgeons and with careful perioperative treatment.

Terminology

Immunosuppression is defined as deliberate prevention or diminution of the host's immune response. It may be nonspecific as in the administration of immunosuppressive agents (drugs or radiation) or by lymphocyte depletion or may be specific as in desensitization or the simultaneous administration of antigen and immunosuppressive drugs.

Peer review

To document the frequency and nature of operative morbidity and mortality after total gastrectomy, the authors retrospectively reviewed the records of 125

consecutive patients who underwent total gastrectomy for gastric cancer. As a clinical experience, the results may provide some helpful information for the readers.

REFERENCES

1 **Boring CC**, Squires TS, Tong T, Montgomery S. Cancer statistics, 1994. *CA Cancer J Clin* 1994; **44**: 7-26
2 **Sun XD**, Mu R, Zhou YS, Dai XD, Zhang SW, Huangfu XM, Sun J, Li LD, Lu FZ, Qiao YL. [Analysis of mortality rate of stomach cancer and its trend in twenty years in China] *Zhonghua Zhongliu Zazhi* 2004; **26**: 4-9
3 **McCulloch P**. The role of surgery in patients with advanced gastric cancer. *Best Pract Res Clin Gastroenterol* 2006; **20**: 767-787
4 **Meyer HJ**, Jahne J. Lymph node dissection for gastric cancer. *Semin Surg Oncol* 1999; **17**: 117-124
5 **Roukos DH**. Current status and future perspectives in gastric cancer management. *Cancer Treat Rev* 2000; **26**: 243-255
6 **Verreet PR**. [Stage-adapted radical principles in gastric carcinoma] *Praxis* (Bern 1994) 1998; **87**: 447-450
7 **Bozzetti F**, Marubini E, Bonfanti G, Miceli R, Piano C, Crose N, Gennari L. Total versus subtotal gastrectomy: surgical morbidity and mortality rates in a multicenter Italian randomized trial. The Italian Gastrointestinal Tumor Study Group. *Ann Surg* 1997; **226**: 613-620
8 **Degiuli M**, Sasako M, Ponti A, Soldati T, Danese F, Calvo F. Morbidity and mortality after D2 gastrectomy for gastric cancer: results of the Italian Gastric Cancer Study Group prospective multicenter surgical study. *J Clin Oncol* 1998; **16**: 1490-1493
9 **McCulloch P**, Ward J, Tekkis PP. Mortality and morbidity in gastro-oesophageal cancer surgery: initial results of ASCOT multicentre prospective cohort study. *BMJ* 2003; **327**: 1192-1197
10 **Sano T**, Katai H, Sasako M, Maruyama K. One thousand consecutive gastrectomies without operative mortality. *Br J Surg* 2002; **89**: 123
11 **Zhang XF**, Huang CM, Lu HS, Wu XY, Wang C, Guang GX, Zhang JZ, Zheng CH. Surgical treatment and prognosis of gastric cancer in 2,613 patients. *World J Gastroenterol* 2004; **10**: 3405-3408
12 **Bittner R**, Butters M, Ulrich M, Uppenbrink S, Beger HG. Total gastrectomy. Updated operative mortality and long-term survival with particular reference to patients older than 70 years of age. *Ann Surg* 1996; **224**: 37-42
13 **Wu CW**, Hsieh MC, Lo SS, Wang LS, Hsu WH, Lui WY, Huang MH, P'eng FK. Morbidity and mortality after radical gastrectomy for patients with carcinoma of the stomach. *J Am Coll Surg* 1995; **181**: 26-32
14 **Jähne J**, Piso P, Meyer HJ. 1114 total gastrectomies in the surgical treatment of primary gastric adenocarcinoma--a 30-year single institution experience. *Hepatogastroenterology* 2001; **48**: 1222-1226
15 **Park DJ**, Lee HJ, Kim HH, Yang HK, Lee KU, Choe KJ. Predictors of operative morbidity and mortality in gastric cancer surgery. *Br J Surg* 2005; **92**: 1099-1102
16 **Opelz G**, Sengar DP, Mickey MR, Terasaki PI. Effect of blood transfusions on subsequent kidney transplants. *Transplant Proc* 1973; **5**: 253-259
17 **Bellantone R**, Sitges-Serra A, Bossola M, Doglietto GB, Malerba M, Franch G, Pacelli F, Crucitti F. Transfusion timing and postoperative septic complications after gastric cancer surgery: a retrospective study of 179 consecutive patients. *Arch Surg* 1998; **133**: 988-992
18 **Grossmann EM**, Longo WE, Virgo KS, Johnson FE, Oprian CA, Henderson W, Daley J, Khuri SF. Morbidity and mortality of gastrectomy for cancer in Department of Veterans Affairs Medical Centers. *Surgery* 2002; **131**: 484-490
19 **Rovera F**, Dionigi G, Boni L, Imperatori A, Tabacchi A, Carcano G, Diurni M, Dionigi R. Postoperative infections after oesophageal resections: the role of blood transfusions. *World J Surg Oncol* 2006; **4**: 80
20 **Vamvakas EC**, Carven JH. Allogeneic blood transfusion, hospital charges, and length of hospitalization: a study of 487 consecutive patients undergoing colorectal cancer resection. *Arch Pathol Lab Med* 1998; **122**: 145-151
21 **Biedler AE**, Schneider SO, Seyfert U, Rensing H, Grenner S, Girndt M, Bauer I, Bauer M. Impact of alloantigens and storage-associated factors on stimulated cytokine response in an in vitro model of blood transfusion. *Anesthesiology* 2002; **97**: 1102-1109
22 **Kirkley SA**. Proposed mechanisms of transfusion-induced immunomodulation. *Clin Diagn Lab Immunol* 1999; **6**: 652-657
23 **Hyllner M**, Tylman M, Bengtson JP, Rydberg L, Bengtsson A. Complement activation in prestorage leucocyte-filtered plasma. *Transfus Med* 2004; **14**: 45-52
24 **Roviello F**, Marrelli D, De Stefano A, Messano A, Pinto E, Carli A. Complications after surgery for gastric cancer in patients aged 80 years and over. *Jpn J Clin Oncol* 1998; **28**: 116-122
25 **Gretschel S**, Estevez-Schwarz L, Hünerbein M, Schneider U, Schlag PM. Gastric cancer surgery in elderly patients. *World J Surg* 2006; **30**: 1468-1474

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RAPID COMMUNICATION

Low level of galacto-oligosaccharide in infant formula stimulates growth of intestinal *Bifidobacteria* and *Lactobacilli*

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Abstract

AIM: To investigate the effect of a new infant formula supplemented with a low level (0.24 g/100 mL) of galacto-oligosaccharide (GOS) on intestinal micro-flora (*Bifidobacteria*, *Lactobacilli* and *E. coli*) and fermentation characteristics in term infants, compared with human milk and a standard infant formula without GOS.

METHODS: Term infants (n = 371) were approached in this study in three hospitals of China. All infants started breast-feeding. Those who changed to formula-feeding within 4 wk after birth were randomly assigned to one of the two formula groups. Growth and stool characteristics, and side effects that occurred in recruited infants were recorded in a 3-mo follow-up period. Fecal samples were collected from a subpopulation of recruited infants for analysis of intestinal bacteria (culture technique), acetic acid (gas chromatography) and pH (indicator strip).

RESULTS: After 3 mo, the intestinal *Bifidobacteria*, *Lactobacilli*, acetic acid and stool frequency were significantly increased, and fecal pH was decreased in infants fed with the GOS-formula or human milk, compared with those fed with the formula without GOS. No significant differences were observed between the GOS formula and human milk groups. Supplementation with GOS did not influence the incidence of crying, regurgitation and vomiting.

CONCLUSION: A low level of GOS (0.24 g/100 mL) in infant formula can improve stool frequency, decrease fecal pH, and stimulate intestinal *Bifidobacteria* and *Lactobacilli* as in those fed with human milk.

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Key words: Human milk; Prebiotic; Probiotic; Safety; Chinese

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INTRODUCTION

Breastfeeding is the primary choice for newborns. However, for some reason, many infants are formula fed. Breast milk is superior over artificial formula in many aspects, including its effect on the development of intestinal microflora. Breast-fed infants have a higher level of intestinal *Bifidobacteria* and *Lactobacilli*[1,2], both of which are known to be potentially beneficial to the health of their hosts[3,4]. It is likely that oligosaccharides in human milk are more beneficial to intestinal flora[5-7].

The amount of oligosaccharides in mature human milk is in range of 12-15 g/L. Most of oligosaccharides are fermented in the large intestine[8,9]. Galacto-oligosaccharide (GOS) contained in oligosaccharides, is a short-chain galactose with a terminal glucose molecule[10], which is produced from lactose and commercially available. Studies have shown that GOS can selectively stimulate development of *Bifidobacteria*[11]. However, only a few studies are available on oligosaccharides in infant

formula[12,13], and there is no study using only GOS in infant formula.

Recently, Moro *et al*[13] showed that the number of *Bifidobacteria* and *Lactobacilli* increases significantly compared with a control formula with maltodextrin instead of the oligosaccharides in term infants fed with formula at the dose of 0.4 g and 0.8 g of oligosaccharide per 100 mL (90% GOS and 10% low-molecular weight fructo-oligosaccharides). There was no difference between the two supplemented groups. These values have been adopted by the Scientific Committee on Food (SCF: April, 2003) of the European Commission and probably will be implemented in European regulations. In preterm infants, the same mixture of oligosaccharide (1.0 g/100 mL) could stimulate growth of *Bifidobacteria* and result in stool characteristics as seen in human milk-fed infants[12]. Recently, however, a rat study[14] showed that the intestinal cell wall is irritated and the risk of bacterial translocation increases (following orally *Salmonella enteritidis* infection) when fructooligosaccharides are provided at a high (3% and 6% of dry matter), but not an unrealistic amount (maximum level adopted by the SCF is about 6% of dry matter). Therefore, positive effects of oligosaccharides on intestinal bacteria may not always justify the levels tested.

Our hypothesis is that a prebiotic effect of the human milk-like GOS is already present at a much lower level than that currently adopted by the SCF. This study was to investigate the effect of 0.24 g GOS per 100 mL on intestinal microflora colonization and fermentation in formula-fed term infants compared with breast-fed and control formula-fed counterparts, and to detect the lowest and safe effective level of GOS.

MATERIALS AND METHODS

Subjects

Three hundred and seventy-one healthy term infants, appropriate for gestational age, were approached in the study by Nanjing Children's Hospital of Nanjing Medical University (n = 181), Nanjing Maternal Hospital of Nanjing Medical University (n = 90) and Affiliated Hospital of South East University (n = 100). Finally, 164 infants were recruited for the 3-mo follow-up (Table 1). Those excluded from the study were due to refusal of their parents and failure in taking the fresh fecal samples. The Ethics Committees of the three hospitals approved the study and all the parents gave their informed consent before enrolment in the study.

Diets

When not breast-fed, the infants were randomly assigned to test formula group and control formula group (Table 1). The test formula (Frisolac Advanced, Friesland Nutrition, Netherlands) group was supplemented with GOS (0.24 g/100 mL), while the control formula (Frisolac H, Friesland Nutrition, Netherlands) group was not supplemented with GOS. The other infants were either breastfed or received a combination of breast milk and test formula.

Growth and stool characteristics of, and side effects in the infants were recorded during the 3-mo follow-up. The body weight of all infants was measured using a scale with an accuracy of ± 5 g. The crown-heel length and head circumference were measured using a special board for newborn infants with an accuracy of ± 1 mm. Stool consistency (score 1-4: 1 = watery, 2 = loose/mushy, 3 = soft formed, 4 = hard formed) and frequency were recorded based on an interview with the mother. Crying (score 1-3: 1 = practically not crying, 2 = crying in connection with feeding, 3 = crying independently from the meals), regurgitation (score 1-3: 1 = no regurgitation, 2 = 1-2 regurgitations, 3 = > 2 regurgitations per day), and vomiting (score 1-3: 1 = no vomiting, 2 = 1 episode of vomiting, 3 = > 1 episode of vomiting per day) were also recorded based on an interview with the mother.

Fecal samples were collected from a subpopulation (Tables 2 and 3) for analysis of intestinal bacteria (n = 82), short chain fatty acids (SCFAs) (n = 96) and pH (n = 112). For analysis of *Bifidobacteria* and *Lactobacilli*[1,12,13], one gram of fresh feces was homogenized and diluted in 10 mL of a pre-reduced brain and heart infusion broth in an anaerobic glove box within 1 h of collection. Ten μL of each dilution was spread on the surfaces of Rogosa SL agar (Difco, Detroit, USA) dishes and incubated anaerobically at 37℃. Colony forming units (cfu) of *Lactobacilli* were marked after 2 d, whereas cfu of *Bifidobacteria* were counted after 4 d. For detection of *E. coli*, 1 g of fresh feces was homogenized and diluted in 10 mL of a pre-reduced brain and heart infusion broth in a clean airflow bench. Ten μL of each dilution was spread on the surfaces of MacConkey agar (Difco, Detroit, USA) dishes and incubated aerobically at 37℃ for 1 d. Cfu were expressed as per gram of feces.

Concentration of SCFA acetic acid was determined by gas chromatography as previously described[15]. Briefly, weighed fecal samples were diluted at approximately 1:10 in a 0.1 mol/L sodium phosphate broth (pH 6.5), and suspensions were used to determine the concentration of acetate using a Hewlett-Packard 5890A Series Ⅱ gas chromatograph (Agilent, Wilmington, DE) and a glass column (180 cm × 4 mm i.d.) packed with 10% SP-1200/1% H_3PO_4 on 80/100 mesh Chromosorb WAW (Supelco, Bellefonte, PA). Nitrogen was the carrier gas with a flow rate of 75 mL/min. Temperature of the oven, detector and injector was 125℃, 175℃ and 180℃, respectively. SCFA concentrations in the blank tube were used to correct non-substrate SCFA production. pH of the fresh stool sample was measured with a piece of multicolor indicator paper with an accuracy of 0.2 U (Spezialindicatorpapier Merck Eurolab GmbH, Darmstadt, Germany).

Statistical analysis

All data were given as mean ± SD. An overall group effect on a measured variable was evaluated by ANOVA with F value. When significant, this was followed by t test for single factor group comparisons. $P < 0.05$ was considered statistically significant. SPSS 12 (SPSS Institute Inc., Chicago, USA) was used in analysis of data.

Table 1 Clinical data of the infants enrolled in the study

	GOS formula	GOS formula & human milk	Human milk	Control formula	*F*/*P* value
Babies recruited in 3 mo follow-up	*n* = 37	*n* = 58	*n* = 24	*n* = 45	
Male/Female	20/17	30/28	13/11	24/21	
Gestational age (wk)	38.7 ± 0.6	39.1 ± 0.8	39.4 ± 0.7	38.8 ± 0.8	1.40/0.24
Weight at birth (kg)	3.30 ± 0.42	3.20 ± 0.43	3.34 ± 0.43	3.35 ± 0.45	1.28/0.28
Length at birth (cm)	49.50 ± 0.96	49.47 ± 1.15	49.61 ± 1.23	49.57 ± 1.03	0.13/0.94
Head circumference at birth (cm)	34.15 ± 0.55	33.98 ± 0.72	34.07 ± 0.81	34.25 ± 0.77	1.35/0.26
Feeding volume (mL/kg-BW/d)	162 ± 27			157 ± 34	
Weight gain during study period (g/d)	41.26 ± 5.22	43.35 ± 4.87	40.97± 5.06	40.59 ± 3.95	1.54/0.21
Length gain during study period (cm/wk)	0.95 ± 0.11	1.01 ± 0.11	0.93 ± 0.10	0.96 ± 0.11	1.94/0.13

Data are expressed as mean ± SD. GOS formula: Galactooligosaccharides in an amount of 0.24 g/dL. Control formula does not contain added GOS.

Table 2 Levels of intestinal bacteria at the end of a 3-mo feeding period as measured in fresh feces

	GOS formula (*n* = 20)	GOS formula & human milk (*n* = 29)	Human milk (*n* = 15)	Control formula (*n* = 18)	*F*/*P* value
Bifidobacteria	9.01 ± 1.18	8.97 ± 0.85	9.25 ± 0.93	8.16 ± 0.99	4.08/0.01
Lactobacilli	5.91 ± 1.61	5.99 ± 2.12	5.45 ± 2.16	4.27 ± 2.02	3.17/0.03
E. coli	6.35 ± 1.59	5.90 ± 1.84	5.74 ± 1.68	5.68 ± 2.11	0.52/0.67

Data are presented as mean ± SD Log_{10} cfu/g wet faeces. Control formula does not contain added GOS.

Table 3 Fecal concentration of acetic acid and pH values at the end of a 3-mo feeding period

	GOS formula	GOS formula & human milk	Human milk	Control formula	*F*/*P* value
Acetic acid (*n*)	25.93 ± 6.84 (21)	25.09 ± 5.49 (34)	23.76 ± 5.65 (17)	19.42 ± 5.35 (24)	6.03/< 0.01
pH (*n*)	5.22 ± 0.25 (25)	5.27 ± 0.25 (41)	5.32 ± 0.24 (19)	5.56 ± 0.51 (27)	5.57/< 0.01

Data of acetic acid are presented as mean ± SD mmol/g wet faeces. Control formula does not contain added GOS.

Table 4 Scores of stool characteristics and intensity of digestive symptoms in the infants enrolled in the study (mean ± SD)

	GOS formula	GOS formula & human milk	Human milk	[1]Control formula	*F*/*P* value
Babies recruited in 3 mo follow-up	*n* = 37	*n* = 58	*n* = 24	*n* = 45	
Stool consistency	2.46 ± 0.62	2.55 ± 0.66	2.37 ± 0.83	3.11 ± 0.34	3.27/0.02
Crying	1.06 ± 0.03	1.04 ± 0.02	1.08 ± 0.05	1.05 ± 0.03	1.29/0.27
Regurgitation	1.34 ± 0.55	1.28 ± 0.63	1.41 ± 0.58	1.35 ± 0.67	1.18/0.34
Vomiting	1.22 ± 0.43	1.18 ± 0.34	1.14 ± 0.46	1.25 ± 0.38	1.24/0.30

[1]Control formula does not contain added GOS.

RESULTS

At the end of a 3-mo feeding period, the number of intestinal *Bifidobacteria* and *Lactobacilli* was significantly increased both in GOS-supplemented formula-fed infants and in breast-fed infants, compared with those fed with the control formula. No difference was seen between the GOS formula-feeding and breast feeding groups. The number of cfu in *E. coli* did not differ between the 3 groups (Table 2).

Intestinal acetic acid production and stool frequency were significantly increased in infants fed with GOS formula or with breast milk, compared with those fed with standard formula. Fecal pH was significantly higher in those fed with the control formula. No difference in stool frequency, fecal pH and intestinal acetic acid production was found between GOS formula-fed and breast-fed infants, (Tables 3 and 4).

The GOS formula did not influence the incidence of side effects (crying, regurgitation, vomiting) (Table 4). Weight gain and body height increase were similar among the groups (Table 1).

DISCUSSION

This study showed that a 3-mo feeding period of a relatively low amount of GOS (0.24 g/dL) in infant formula stimulated the growth of *Bifidobacteria* and *Lactobacilli* as seen in breast-fed infants but not the growth of potentially harmful *E. coli*. Stool frequency, fecal pH and amount of produced acetic acid were also comparable in the breast-fed infants, indicating hat a low GOS formula has the same prebiotic effect as a high GOS formula, but a minimized risk of intestinal irritation.

Breast-fed infants have an intestinal microflora dominated by *Bifidobacteria* and *Lactobacilli*[1,2] and are

quite different from those fed with a standard infant formula[16]. Both *Bifidobacteria* and *Lactobacilli* are beneficial to infants. As a result, manufacturers of formula try to mimic the gastrointestinal flora in breast-fed infants by adding probiotics and/or prebiotics such as GOS. Although the addition of probiotics is able to manipulate the infants' microflora towards the breast-fed infants' microflora[17,18], this concept may be regarded as unphysiological since breast milk itself does not contain bacteria. Prebiotics are, therefore, the first choice.

Prebiotics serve as food for *Bifidobacteria* and *Lactobacilli* and increase their number by their competitive edge over the pathogenic bacteria. During the fermentation of prebiotics, organic acids (lactic acid and SCFA) are produced that can inhibit the growth (increase colonization resistance) of acid-sensitive pathogens like salmonella[19]. SCFA, also the preferred source of energy for colonic epithelial cells, may stimulate colonocyte proliferation, and are suggested to enhance small intestinal glucose uptake[20]. On the other hand, the same organic acids may induce injury to the intestinal mucosa and impair its barrier function, as indicated by increased cytotoxicity of fecal water and fecal mucin excretion[14]. The fermentation rate might play an important role in this detrimental effect. Since slow fermentation as seen in case of resistant starch does not increase fecal mucin and luminal cytotoxicity, providing a lower amount of fast fermentable prebiotics to the intestine may also prohibit irritation[21].

Ten Bruggencate *et al*[14], in a rat study, showed that 6% of FOS on dry matter increases the number of *Bifidobacteria*, but 3% of FOS significantly (100-fold) increases the number of Enterobacteria, indicating that the selectivity of fibers can be questioned. The increased levels of Enterobacteria in combination with an impaired barrier function may increase the risk of bacterial translocation[14]. Moro *et al*[13] reported that the number of *Bifidobacteria* and *Lactobacilli* increases significantly in full term infants following oligosaccharide supplementation of 0.4 g/dL and 0.8 g/dL (3% and 6% on dry matter respectively). Although the authors reported no significant increase in the number of infants with positive culture of Enterobacteria, this statement does not say anything about the levels of these gram negative bacteria in those with a positive culture. Boehm *et al*[22] showed that 1.0 g of a mixture of GOS and FOS per 100 mL preterm formula has a bifidogenic effect but no significant effect on the number of *Lactobacilli* and Enterobacteria.

In the present study, the effect of only 0.24 g/dL of GOS on intestinal microflora and fermentation was observed in term infants. The results show that even such a low amount of GOS could stimulates the growth of *Bifidobacteria* and *Lactobacilli* as in breast-fed counterparts, decrease fecal pH, and increase the production of intestinal SCFA. The frequency of stools was shorter and the stools became softer, as seen in breast milk-fed infants. These changes in stool characteristics could not be explained by the increased osmolarity (about 1 mOsmol/L) of the formula because of the addition of GOS, and are, therefore, probably related to the changes in bacterial flora and fermentation. Studies in adults showed that a greater amount of dietary oligosaccharides may lead to adverse effects, flatulence in particular[23]. In the present study, no adverse side effects were reported.

This study certainly has its limitations. The number of infants involved in bacterial and SCFA analysis as very small mainly due to the refusal of parents and the failure in taking fresh fecal samples. However, despite such limitations, the difference between GOS-fortified and non-fortified groups was significant. Furthermore, files of infants are not complete because of the poor communication and traffic facility for the follow-up. Therefore, this study was a pilot study with promising results that need to be confirmed in a larger and more appropriate study.

In summary, supplementation of GOS stimulates the growth of *Bifidobacteria* and *Lactobacilli*. Both bacteria are beneficial to infants. However, an increase in Enterobacteria cannot be excluded, although it may be dose-dependent. A small amount of GOS can stimulate the growth of *Bifidobacteria* and *Lactobacilli*, but not the growth of Enterobacteria in breast-fed infants.

COMMENTS

Background

Breast milk is superior over artificial formula in terms of newborn nutrition. Breast-fed infants have a higher level of intestinal *Bifidobacteria* and *Lactobacilli*, both of which are potentially beneficial to the health of their hosts. Oligosaccharides in human milk are more beneficial to intestinal flora. The amount of oligosaccharides in mature human milk ranges 12-15 g/L. Galacto-oligosaccharide (GOS) is a short chain galactose with a terminal glucose molecule. Studies have shown that GOS can selectively stimulate the development of *Bifidobacteria*. However, a large amount GOS (3%-6% of dry matter) supplementation to the artificial formula showed irritation of the intestinal cell wall and increased risk of bacterial translocation. This study investigated the effects of artificial formula supplemented with 0.24 g GOS per 100 mL (1.8% of dry matter) on intestinal microflora colonization and fermentation in infants, and detected the lowest and safe effective level of GOS.

Research frontiers

Moro *et al*[13] showed that in term infants fed with formula at the doses of 0.4 and 0.8 g of oligosaccharides per 100 mL (90% GOS and 10% low-molecular weight fructo-oligosaccharides), the number of *Bifidobacteria* and *Lactobacilli* increased significantly compared with a control formula with maltodextrin instead of oligosaccharides. These values have been adopted by the Scientific Committee on Food of the European Commission and probably will be implemented in European regulations. In preterm infants, the same mixture of oligosaccharides (1.0 g/100 mL) stimulated the growth of *Bifidobacteria* and resulted in stool characteristics as seen in human milk-fed infants. However, a recent rat study showed irritation of the intestinal cell wall and increased risk of bacterial translocation (following orally *Salmonella enteritidis* infection) when a large amount of fructooligosaccharide (3% and 6% of dry matter), but not an unrealistic amount (maximum level adopted by the SCF is about 6% of dry matter) was provided. Therefore, positive effects of oligosaccharides on intestinal bacteria may not always justify the levels tested.

Innovations and breakthroughs

This study showed that a 3 mo feeding period of a relatively small amount of GOS (0.24 g/dL) in infant formula could stimulate the growth of *Bifidobacteria* and *Lactobacilli*, but not the growth of potentially harmful *E. coli* in breast-fed infants. Stool frequency, fecal pH and the amount of produced acetic acid were also comparable, indicating that low GOS formula may have the same prebiotic effect as high GOS formula, but a minimized risk of intestinal irritation.

Applications

The present study was designed to investigate the effect of only 0.24 g/dL

of GOS on intestinal microflora and fermentation in term infants. The data show that even such a small amount of GOS could stimulate the growth of *Bifidobacteria* and *Lactobacilli*, decrease fecal pH, and increase the production of intestinal SCFA. Stools came more frequently and became softer in breast milk-fed infants, indicating that a mall amount of GOS (0.24 g/dL) supplementation can stimulate the growth of *Bifidobacteria* and *Lactobacilli*, but not the growth of *Enterobacteria* in breast-fed infants. It is, therefore, safe and effective when used in artificial infant formula.

Terminology

Prebiotics are "selectively fermented ingredients that allow specific changes both in composition and/or activity of gastrointestinal microflora that confers benefits to host well being and health". Probiotics are defined viable microorganisms, a sufficient amount of which can reach the intestine in an active state and thus exerting positive health effects.Synergistic combinations of pro- and prebiotics are called synbiotics. Today, only bifidogenic, non-digestible oligosaccharides (particularly inulin, its hydrolysis product oligofructose, and galactooligosaccharides), fulfill all the criteria for prebiotic classification.

Peer review

This study determined the effect of a lower-than-normal dose of a prebiotics on the gastrointestinal tract of infants. Its results show that a mall amount of GOS (0.24 g/dL) in artificial formula could stimulate the growth of intestinal *Bifidobacteria* and *Lactobacilli* but not *E. coli* in term infants, indicating that it is safe and effective when used in artificial infant formula.

REFERENCES

1 **Fanaro S**, Vigi V, Chierici R, Boehm G. Fecal flora measurements of breastfed infants using an integrated transport and culturing system. *Acta Paediatr* 2003; **92**: 634-635

2 **Harmsen HJ**, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, Welling GW. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* 2000; **30**: 61-67

3 **Gronlund MM**, Arvilommi H, Kero P, Lehtonen OP, Isolauri E. Importance of intestinal colonisation in the maturation of humoral immunity in early infancy: a prospective follow up study of healthy infants aged 0-6 months. *Arch Dis Child Fetal Neonatal Ed* 2000; **83**: F186-F192

4 **Saavedra J**. Probiotics and infectious diarrhea. *Am J Gastroenterol* 2000; **95**: S16-S18

5 **Newburg DS**. Oligosaccharides in human milk and bacterial colonization. *J Pediatr Gastroenterol Nutr* 2000; **30** Suppl 2: S8-S17

6 **Picciano MF**. Nutrient composition of human milk. *Pediatr Clin North Am* 2001; **48**: 53-67

7 **Miller JB**, McVeagh P. Human milk oligosaccharides: 130 reasons to breast-feed. *Br J Nutr* 1999; **82**: 333-335

8 **Collins MD**, Gibson GR. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr* 1999; **69**: 1052S-1057S

9 **Engfer MB**, Stahl B, Finke B, Sawatzki G, Daniel H. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am J Clin Nutr* 2000; **71**: 1589-1596

10 **Kobata A**, Yamashita K, Tachibana Y. Oligosaccharides from human milk. *Methods Enzymol* 1978; **50**: 216-220

11 **Fanaro S**, Boehm G, Garssen J, Knol J, Mosca F, Stahl B, Vigi V. Galacto-oligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: a review. *Acta Paediatr Suppl* 2005; **94**: 22-26

12 **Boehm G**, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B, Marini A. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch Dis Child Fetal Neonatal Ed* 2002; **86**: F178-F181

13 **Moro G**, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B, Boehm G. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *J Pediatr Gastroenterol Nutr* 2002; **34**: 291-295

14 **Ten Bruggencate SJ**, Bovee-Oudenhoven IM, Lettink-Wissink ML, Van der Meer R. Dietary fructo-oligosaccharides dose-dependently increase translocation of salmonella in rats. *J Nutr* 2003; **133**: 2313-2318

15 **Bouhnik Y**, Flourie B, D'Agay-Abensour L, Pochart P, Gramet G, Durand M, Rambaud JC. Administration of transgalacto-oligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. *J Nutr* 1997; **127**: 444-448

16 **Rubaltelli FF**, Biadaioli R, Pecile P, Nicoletti P. Intestinal flora in breast- and bottle-fed infants. *J Perinat Med* 1998; **26**: 186-191

17 **Langhendries JP**, Detry J, Van Hees J, Lamboray JM, Darimont J, Mozin MJ, Secretin MC, Senterre J. Effect of a fermented infant formula containing viable bifidobacteria on the fecal flora composition and pH of healthy full-term infants. *J Pediatr Gastroenterol Nutr* 1995; **21**: 177-181

18 **Parracho H**, McCartney AL, Gibson GR. Probiotics and prebiotics in infant nutrition. *Proc Nutr Soc* 2007; **66**: 405-411

19 **Bovee-Oudenhoven IM**, Termont DS, Heidt PJ, Van der Meer R. Increasing the intestinal resistance of rats to the invasive pathogen Salmonella enteritidis: additive effects of dietary lactulose and calcium. *Gut* 1997; **40**: 497-504

20 **Chen CC**, Walker WA. Probiotics and prebiotics: role in clinical disease states. *Adv Pediatr* 2005; **52**: 77-113

21 **Bovee-Oudenhoven IM**, ten Bruggencate SJ, Lettink-Wissink ML, van der Meer R. Dietary fructo-oligosaccharides and lactulose inhibit intestinal colonisation but stimulate translocation of salmonella in rats. *Gut* 2003; **52**: 1572-1578

22 **Boehm G**, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B, Marini A. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch Dis Child Fetal Neonatal Ed* 2002; **86**: F178-F181

23 **Bouhnik Y**, Vahedi K, Achour L, Attar A, Salfati J, Pochart P, Marteau P, Flourie B, Bornet F, Rambaud JC. Short-chain fructo-oligosaccharide administration dose-dependently increases fecal bifidobacteria in healthy humans. *J Nutr* 1999; **129**: 113-116

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CASE REPORT

Isolated intestinal neurofibromatous proliferations in the absence of associated systemic syndromes

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Abstract

Gastrointestinal tract involvement by neurofibromatous lesions is rare and occurs most frequently as one of the systemic manifestations of generalized neurofibromatosis type 1 (NF1). In this setting, the lesions may manifest as focal scattered neurofibromas or as an extensive diffuse neural hyperplasia designated ganglioneuromatosis. Occasionally, such lesions may be the initial sign of NF1 in patients without any other clinical manifestations of the disease. Rarely, cases of isolated neurofibromatosis of the large bowel with no prior or subsequent evidence of generalized neurofibromatosis have been documented. We present the case of a 52 year-old female with abdominal pain and alternating bowel habits. Colonoscopic evaluation revealed multiple small polyps in the cecum and the presence of nodular mucosa in the colon and rectum. Pathologic evaluation of the biopsies from the cecum, descending colon, sigmoid colon, and rectum revealed tangled fascicles of spindle cells expanding the lamina propia leading to separation of the intestinal crypts. Immunohistochemical stains helped confirm the diagnosis of diffuse intestinal neurofibromatosis. A thorough clinical evaluation failed to reveal any stigmata of generalized neurofibromatosis. This case represents a rare presentation of isolated intestinal neurofibromatosis in a patient without classic systemic manifestations of generalized neurofibromatosis and highlights the need in such cases for close clinical follow-up to exclude neurofibromatosis type Ⅰ or multiple endocrine neoplasia type Ⅱ.

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INTRODUCTION

Neurofibromatous lesions of the lower gastrointestinal tract may manifest in multiple forms and have accordingly been given various descriptive designations including intestinal neurofibromatosis, ganglioneuromatosis, diffuse plexiform neurofibromatosis, neuronal intestinal dysplasia, and diffuse colonic ganglioneuromatous polyposis. These lesions may be seen in up to 25% of cases of neurofibromatosis type 1 (NF1) and multiple endocrine neoplasia type 2b (MEN 2b), and their occurrence in the absence of other clinical features of NF1 and MEN 2b is extremely rare[1]. Neurofibromatous lesions of the gastrointestinal tract, unassociated with systemic features of NF1 or MEN 2b, have also been documented in distributions isolated to the esophagus and stomach[2]. No consensus has yet been reached in the medical community as to whether these isolated lesions represent different phenotypic manifestations of the neurocutaneous and multiple endocrine neoplasia syndromes or whether they represent separate and distinct entities.

CASE REPORT

A 51-year-old female presented to our institution with a three-year history of worsening gastroesophageal reflux. She stated that she had progressively developed dysphagia to solid foods and had begun to regurgitate solid food particles, but the episodes of vomiting were unassociated with nausea, epigastric pain, or abdominal pain. The patient denied weight loss and reported no

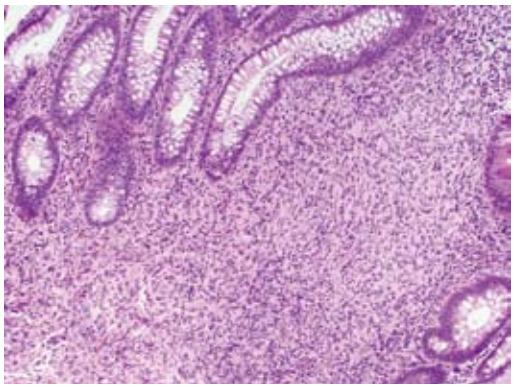

Figure 1 Proliferation of spindle cells admixed with scattered chronic inflammatory cells expanding the lamina propria and leading to separation of the intestinal crypts (HE, x 200).

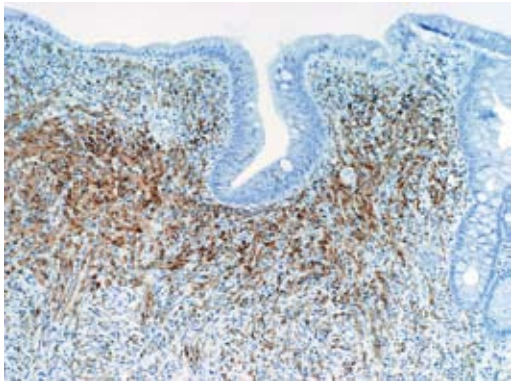

Figure 2 Immunohistochemical stain for S-100 protein (x 200).

improvement of her symptoms with smaller meals. For the last year, the patient also reported the development of frequent non-bloody diarrhea with associated abdominal cramping and bloating. There was no history of coffee-ground emesis, hematemesis, melena, or hematochezia. The patient's additional past medical history included Sjogren's syndrome, fibromyalgia, and cervical radiculitis. The patient had previously undergone colonoscopy with biopsy at a separate institution. At that time, multiple small polyps of the cecum and a 5-8 cm segment of mucosal nodularity of the descending colon were identified. Biopsies of the cecum and colon were diagnosed histologically as hyperplastic changes and as an inflammatory fibroid polyp. At our institution, the patient was placed on a lactose-free diet due to suspected lactose intolerance, but over the course of three months, she experienced an 8 lb. (3.63 kg) weight loss and continuing gastrointestinal distress. A subsequent colonoscopy again showed multiple cecal polyps and nodular large bowel mucosa. Biopsies were obtained from the cecum, descending colon, sigmoid colon, and rectum. Histologic analysis of the biopsies showed a proliferation of tangled fascicles of spindle cells admixed with scattered chronic inflammatory cells expanding the lamina propria and leading to separation of the intestinal crypts (Figure 1). Immunohistochemical stains for S-100 protein supported the diagnosis of neurofibroma for each of the biopsies (Figure 2). The biopsy specimens from the patients' prior colonoscopy were obtained from the outside institution, and immunohistochemical analysis confirmed a retrospective diagnosis of neurofibromas. A thorough physical examination failed to reveal any clinical signs of neurofibromatosis or multiple endocrine neoplasia. Following her diagnosis of isolated intestinal neurofibromatosis, the patient was lost to clinical follow-up in our healthcare system.

DISCUSSION

Isolated intestinal neurofibromatous proliferations (IINP) are benign neural lesions of the lower gastrointestinal tract which may be the initial manifestation of generalized systemic NF1 or MEN 2b. In these settings, IINP may manifest as single or multiple well-defined stromal neoplasms or as a diffuse neuronal hyperplasia, commonly termed ganglioneuromatosis. The clinical, radiographic, and histologic findings of such lesions are not specific to NF1 or MEN 2b, and identical features have been reported in association with juvenile and adenomatous colonic polyposis[3,4] and as an isolated finding in patients with no additional clinical evidence of neurocutaneous, intestinal polyposis, or multiple endocrine neoplasia syndromes[5,6].

The clinical presentation of neurofibromatous lesions of the lower gastrointestinal tract are myriad and are dependent upon the focal or diffuse nature of the lesions, their location, their effect on gastrointestinal motility, and their possible impingement on adjacent structures. Affected patients may present with altered bowel habits including constipation or diarrhea[7,8], abdominal pain[9], intestinal obstruction[10], or with palpable abdominal masses[6]. In the setting of NF1, associated clinical findings including the classic dermal neurofibromas, café-au-lait macules, and Lisch nodules may be seen. In the setting of MEN 2b, the characteristic facies including thickened lips from mucosal neuromas, marfanoid habitus, and even features of medullary carcinoma of the thyroid may be present. In IINP, the intestinal symptoms manifest without demonstrable clinical evidence of associated systemic syndromes.

Radiographically, lower gastrointestinal neurofibromatous lesions may manifest as diffuse, confluent thickening of portions of the intestine or as single or multiple discrete lesions of the intestinal wall or mesentery. The radiographic differential diagnosis of single or multiple nodular neurofibromatous lesions is wide and includes many epithelial and stromal neoplasms as well as nodular lymphomas. The diffuse forms of IINP may mimic the radiographic appearance of Crohn's disease on barium studies and computed tomography scans. In a report by Charagundla *et al*[7], a patient with signs and symptoms of colitis was found to have segmental thickening of an extended portion of the distal ileum radiographically. Clinically, Crohn's disease was suspected, but subsequent small bowel resection showed diffuse IINP. At least two other reports have documented similar findings[11,12].

The endoscopic appearance of IINP depends on

the focal or diffuse nature of the lesions. As the lesions arise deep to the epithelium, they manifest as single or multiple subepithelial masses of variable size and distribution. In some cases, numerous small lesions carpet a portion of the lower gastrointestinal tract, while in others, a larger lesion may predominate and significantly stenose the lumen of the affected area. Endoscopic biopsies are the mainstay of diagnosis, but when used in an attempt to sample deep-seated lesions, the biopsies may yield only unaffected overlying bowel mucosa or minimally diagnostic superficial lesional tissue. Ulceration, due to erosion of the epithelium over the surface of lesions, has been described, particularly in larger solitary lesions, but occasional cases have been described in which ulceration was sufficiently widespread and significant to raise the endoscopic differential diagnosis of idiopathic inflammatory bowel disease. In the current case, the endoscopic appearance was that of multiple small cecal polyps and nodular colonic mucosa, and endoscopic sampling yielded only the most superficial portion of the neurofibromatous proliferation. No associated ulceration was identified.

Grossly, the intestinal neurofibromatous proliferations tend to be firm, solid, and white to tan throughout. Hemorrhage and necrosis are exceptional, but superficial ulceration has been reported in lesions of varying size. The lesions may manifest as confluent neurofibromatous proliferations involving contiguous sections of bowel or as focal polypoid lesions with a sporadic distribution. The varied gross manifestations of IINP generally have a similar histologic appearance, consisting of a proliferation of neural elements including nerve fibers and supporting cells which may be diffusely intermingled or arranged in fascicles of bland spindle-shaped cells. The cells exhibit elongated nuclei with inconspicuous nucleoli and moderate amounts of eosinophilic cytoplasm. Ganglion cells may be present in variable numbers. Despite their sometimes striking cellularity and occasional cellular pleomorphism, mitotic activity in these lesions is minimal. Immunohistochemically, the cells show a variable degree of expression for S100 protein and synaptophysin.

IINP most frequently involve the colon, terminal ileum, and appendix[13], but similar neurofibromatous proliferations isolated to the upper gastrointestinal tract have been described. Siderits *et al*[2] reported a case of sporadic ganglioneuromatosis of the gastroesophageal junction in a patient with gastroesophageal reflux, and solitary neurofibromas of the esophagus have also been documented[14]. These lesions show endoscopic and biopsy features identical to their counterparts in the lower gastrointestinal tract.

The treatment of IINP is primarily surgical and is dependent on the location and size of the lesions. Solitary, well-circumscribed lesions may be only incidental findings at the time of screening endoscopy and require no further therapy, but larger solitary lesions may come to clinical attention due to intestinal obstruction or impingement on adjacent structures and require resection. For lesions that are more diffusely distributed, treatment strategies are dictated by the symptomatology. Intractable abdominal pain, constipation, and diarrhea may require palliative resection of the involved segment of lower gastrointestinal tract. Accurate histopathologic categorization of biopsy and resection specimens is required to guide appropriate surgical therapy.

REFERENCES

1 **Hochberg FH**, Dasilva AB, Galdabini J, Richardson EP Jr. Gastrointestinal involvement in von Recklinghausen's neurofibromatosis. *Neurology* 1974; **24**: 1144-1151
2 **Siderits R**, Hanna I, Baig Z, Godyn JJ. Sporadic ganglioneuromatosis of esophagogastric junction in a patient with gastro-esophageal reflux disorder and intestinal metaplasia. *World J Gastroenterol* 2006; **12**: 7874-7877
3 **Mendelsohn G**, Diamond MP. Familial ganglioneuromatous polyposis of the large bowel. Report of a family with associated juvenile polyposis. *Am J Surg Pathol* 1984; **8**: 515-520
4 **Weidner N**, Flanders DJ, Mitros FA. Mucosal ganglioneuromatosis associated with multiple colonic polyps. *Am J Surg Pathol* 1984; **8**: 779-786
5 **Bononi M**, De Cesare A, Stella MC, Fiori E, Galati G, Atella F, Angelini M, Cimitan A, Lemos A, Cangemi V. Isolated intestinal neurofibromatosis of colon. Single case report and review of the literature. *Dig Liver Dis* 2000; **32**: 737-742
6 **Hirata K**, Kitahara K, Momosaka Y, Kouho H, Nagata N, Hashimoto H, Itoh H. Diffuse ganglioneuromatosis with plexiform neurofibromas limited to the gastrointestinal tract involving a large segment of small intestine. *J Gastroenterol* 1996; **31**: 263-267
7 **Charagundla SR**, Levine MS, Torigian DA, Campbell MS, Furth EE, Rombeau J. Diffuse intestinal ganglioneuromatosis mimicking Crohn's disease. *AJR Am J Roentgenol* 2004; **182**: 1166-1168
8 **Kim HR**, Kim YJ. Neurofibromatosis of the colon and rectum combined with other manifestations of von Recklinghausen's disease: report of a case. *Dis Colon Rectum* 1998; **41**: 1187-1192
9 **Boldorini R**, Tosoni A, Leutner M, Ribaldone R, Surico N, Comello E, Min KW. Multiple small intestinal stromal tumours in a patient with previously unrecognised neurofibromatosis type 1: immunohistochemical and ultrastructural evaluation. *Pathology* 2001; **33**: 390-395
10 **Urschel JD**, Berendt RC, Anselmo JE. Surgical treatment of colonic ganglioneuromatosis in neurofibromatosis. *Can J Surg* 1991; **34**: 271-276
11 **Urbano U**, Farina P. [Intestinal ganglioneuromatosis in a case of terminal ileitis] *Pathologica* 1967; **59**: 547-550
12 **Tobler A**, Maurer R, Klaiber C. [Stenosing ganglioneuromatosis of the small intestine with ileus and ileal rupture] *Schweiz Med Wochenschr* 1981; **111**: 684-688
13 **Shekitka KM**, Sobin LH. Ganglioneuromas of the gastrointestinal tract. Relation to Von Recklinghausen disease and other multiple tumor syndromes. *Am J Surg Pathol* 1994; **18**: 250-257
14 **Lee R**, Williamson WA. Neurofibroma of the esophagus. *Ann Thorac Surg* 1997; **64**: 1173-1174

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CASE REPORT

Albumin liver dialysis as pregnancy-saving procedure in cholestatic liver disease and intractable pruritus

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Abstract

Progressive familial intrahepatic cholestasis type 3 (PFIC3) is a rare cholestatic liver disease. Such liver disease can get worse by female hormone disorder. Albumin dialysis or Molecular Adsorbent Recirculating System (MARS) has been reported to reverse severe cholestasis-linked pruritus. Here, we report the first use of MARS during a spontaneous pregnancy and its successful outcome in a patient with PFIC3 and intractable pruritus. Albumin dialysis could be considered as a pregnancy-saving procedure in pregnant women with severe cholestasis and refractory pruritus.

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Key words: Albumin dialysis; Intractable pruritus; Pregnancy; Cholestatic liver disease

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INTRODUCTION

Progressive familial intrahepatic cholestasis type 3 (PFIC3) is a rare autosomal recessive cholestatic liver disease due to mutations in the adenosine triphosphate-binding-cassette, subfamily B, member 4 gene (*ABCB4*) involved in biliary phospholipid excretion. A few years ago, we reported a case of a young female with PFIC3 revealed by oestro-progestative contraceptive pill-related cholestasis[1]. Here, we report, in the same patient, her first spontaneous pregnancy that resulted in a successful outcome after the use of Molecular Adsorbent Recirculating System (MARS) for intractable pruritus.

CASE REPORT

A 23-year-old woman, gravida 1, was referred to our liver unit for pruritus and recent diagnosis of pregnancy in November, 2005. This woman was diagnosed with PFIC3 five years ago and it was revealed by contraceptive pill consumption[1]. After stopping the contraceptive pill and the starting of a treatment with ursodeoxycholic acid (UDCA, 20 mg/kg per day), the patient remained asymptomatic with a normal physical examination, normal liver tests, no endoscopic signs of portal hypertension and no abdominal ultrasonographic abnormalities.

In November, 2005, the patient was at the 7th week of gestation. Because of potential teratogenic risks, UDCA was stopped until the end of the first trimester. As a consequence, itching recurred and moderate liver tests abnormalities were observed. Despite administration of emollients, hydroxyzine, zolpidem tartrate, cetirizine and cholestyramine, pruritus remained very intense leading to sleep disturbance. At the 11th week of gestation, UDCA (20 mg/kg per day) was started again. Within 15 d, no clinical effectiveness was

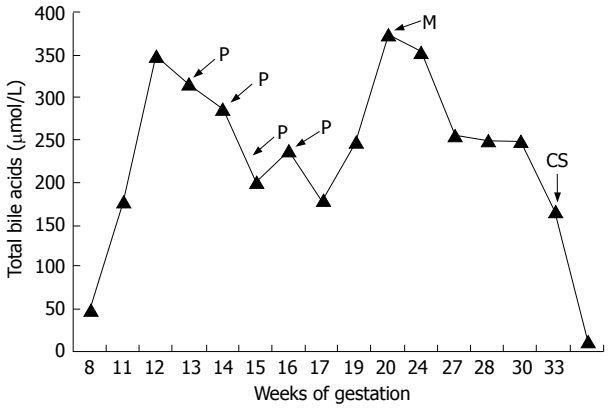

Figure 1 Course of total Bile Acids during the pregnancy. P: Plasmapheresis; M: MARS: Molecular Adsorbent Recirculating System; CS: Caesarean section.

observed: Pruritus became unbearable with insomnia and depressed mood. In addition, total serum bile acids rose to 316 μmol/L. At the 13th week of gestation, a first plasmapheresis was performed, and then continued weekly for 1 mo. The patient reported improvement in pruritus immediately after each procedure and total bile acids slowly decreased, but rose again over 300 μmol/L (Figure 1). Despite the four plasma exchanges (with 2000 mL using 5% albumin as replacement fluid) without any maternal or fetal complications, the pruritus worsened again. Procedure of extracorporeal albumin dialysis using the MARS was decided after giving explanations to the patient regarding the fetal risks. The fetus was careful monitored every week using ultrasonography, Doppler velocimetry and cardiotocography. Two consecutive sessions of MARS were performed at the 20th week of gestation. The procedure was well tolerated without maternal or fetal hemodynamic changes. The pruritus improved without recurrence until the delivery despite high serum total bile acids levels (Figure 1). During pregnancy, several ultrasound scans showed normal fetus growth without morphologic abnormalities and arterial Doppler showed normal uterine arteries and umbilical flow velocity. The patient was hospitalized for preterm labour at 33 wk of gestation and received nifedipine tocolysis, antibiotic prophylaxis and antenatal corticosteroids. Because fetal bowel dilatation observed by ultrasonography and maternal persistent cholestasis with high bile acids, a caesarean section was performed at 34 wk of gestation and the mother gave birth of a healthy, Apgar 10/10 (at 1 and 5 min), boy weighting 1960 g (20th percentile for gestational age), with a height of 42 cm (15th percentile) who was admitted in the neonatology unit. The newborn was discharged on the 15th day, his development was normal and 6 mo later he was still healthy. No intestinal pathology was found and his liver tests were normal. The histopathology analysis of the placenta showed multiple microscopic hypoxic lesions. The postoperative course of the mother was uncomplicated. UDCA was reintroduced just after the delivery and the mother bile acids normalized in few days in postpartum (Figure 1).

DISCUSSION

Regarding the medical literature, the course of pregnancy in PFIC3 has never been reported. In pregnant patients with chronic liver disease, such as primary biliary cirrhosis, primary sclerosing cholangitis or viral hepatitis, the high systemic and hepatic sex-hormones load levels may induce cholestasis and pruritus[2]. Our patient, who was affected by a cholestatic liver disease clearly aggravated by female hormones rapidly complained of intractable pruritus during the first half of pregnancy. Most likely, the withdrawn of UDCA at the 7th week of gestation triggered the onset of cholestasis with pruritus. Although UDCA has been widely and successfully used in the second and third trimesters of pregnancy in women with intrahepatic cholestasis of pregnancy (ICP)[3,4], we stopped its administration early during the first trimester of pregnancy of our patient for the lack of teratogenic effects of the drug is not well established in early pregnancy.

Refractory pruritus is a serious manifestation of cholestasis and its physiopathology remains unknown. Interventions using extra corporeal removal of pruritogens (bile acids, histamine) have been proposed including hemodialysis, charcoal hemoperfusion, or plasmapheresis[5]. Among these methods, only plasmapheresis provided relief, but with transient efficiency[6]. Plasmapheresis, an extracorporeal exchange procedure used to remove large-molecular-weight substances from the plasma, is generally safe although rare complications have been reported (bleeding, infection, coagulation abnormalities and electrolyte disturbances)[7]. Only one case of plasmapheresis has been reported in a woman with ICP-related pruritus[7]. Although four sessions of plasmapheresis were performed in our patient, the pruritus recurred and the serum bile acids levels remained high, largely above 40 μmol/L, a threshold value that has been shown to be associated with an increased risk preterm delivery, fetal distress and fetal loss[8]. Therefore, considering the failure of plasmapheresis to control the dangerous consequences of the cholestasis of our patient during her pregnancy, we decided to use MARS as a pregnancy-saving procedure.

MARS was developed as an extracorporeal hemofiltration system using an albumin-enriched dialysate to remove albumin-bound substances in patients with liver failure. However, MARS was also reported as a valuable and safe procedure for intractable pruritus either after orthotopic liver transplantation or in patients with primary biliary cirrhosis[9-11]. To date, 25 cases of successful use of the MARS in patients with refractory pruritus have been reported[9-13]. The mechanisms by which MARS ameliorates pruritus remain unclear. In patients with symptomatic cholestasis, removal of bile acids from serum should lead to a decline of their concentration in the skin causing amelioration of pruritus. However, several studies did not observe changes of serum bile acids concentration. A further possible explanation for efficacy of MARS

in cholestasis-related pruritus is the clearance of other lipophilic, albumin-bound substances from the patient's plasma that could induce pruritus, either as peripheral pruritogens or by binding to central serotonin or opioid receptors. To our knowledge, the use of MARS as a pregnancy-saving therapy in cholestasis-induced pruritus during pregnancy was not previously reported. In our patient, MARS led to a spectacularly rapid and protracted improvement in pruritus and allowed significant prolongation of pregnancy.

In conclusion, the successful use of MARS in our patient suggests that the procedure could be considered as a pregnancy-saving procedure in pregnant women with severe cholestasis and intractable pruritus.

REFERENCES

1 **Ganne-Carrié N**, Baussan C, Grando V, Gaudelus J, Cresteil D, Jacquemin E. Progressive familial intrahepatic cholestasis type 3 revealed by oral contraceptive pills. *J Hepatol* 2003; **38**: 693-694

2 **Janczewska I**, Olsson R, Hultcrantz R, Broome U. Pregnancy in patients with primary sclerosing cholangitis. *Liver* 1996; **16**: 326-330

3 **Palma J**, Reyes H, Ribalta J, Iglesias J, Gonzalez MC, Hernandez I, Alvarez C, Molina C, Danitz AM. Effects of ursodeoxycholic acid in patients with intrahepatic cholestasis of pregnancy. *Hepatology* 1992; **15**: 1043-1047

4 **Palma J**, Reyes H, Ribalta J, Hernandez I, Sandoval L, Almuna R, Liepins J, Lira F, Sedano M, Silva O, Toha D, Silva JJ. Ursodeoxycholic acid in the treatment of cholestasis of pregnancy: a randomized, double-blind study controlled with placebo. *J Hepatol* 1997; **27**: 1022-1028

5 **Jones EA**, Bergasa NV. The pathogenesis and treatment of pruritus and fatigue in patients with PBC. *Eur J Gastroenterol Hepatol* 1999; **11**: 623-631

6 **Cohen LB**, Ambinder EP, Wolke AM, Field SP, Schaffner F. Role of plasmapheresis in primary biliary cirrhosis. *Gut* 1985; **26**: 291-294

7 **Warren JE**, Blaylock RC, Silver RM. Plasmapheresis for the treatment of intrahepatic cholestasis of pregnancy refractory to medical treatment. *Am J Obstet Gynecol* 2005; **192**: 2088-2089

8 **Glantz A**, Marschall HU, Mattsson LA. Intrahepatic cholestasis of pregnancy: Relationships between bile acid levels and fetal complication rates. *Hepatology* 2004; **40**: 467-474

9 **Bellmann R**, Graziadei IW, Feistritzer C, Schwaighofer H, Stellaard F, Sturm E, Wiedermann CJ, Joannidis M. Treatment of refractory cholestatic pruritus after liver transplantation with albumin dialysis. *Liver Transpl* 2004; **10**: 107-114

10 **Pares A**, Cisneros L, Salmeron JM, Caballeria L, Mas A, Torras A, Rodes J. Extracorporeal albumin dialysis: a procedure for prolonged relief of intractable pruritus in patients with primary biliary cirrhosis. *Am J Gastroenterol* 2004; **99**: 1105-1110

11 **Macia M**, Aviles J, Navarro J, Morales S, Garcia J. Efficacy of molecular adsorbent recirculating system for the treatment of intractable pruritus in cholestasis. *Am J Med* 2003; **114**: 62-64

12 **Huster D**, Schubert C, Achenbach H, Caca K, Mossner J, Berr F. Successful clinical application of extracorporal albumin dialysis in a patient with benign recurrent intrahepatic cholestasis (BRIC). *Z Gastroenterol* 2001; **39** Suppl 2: 13-14

13 **Mullhaupt B**, Kullak-Ublick GA, Ambuhl PM, Stocker R, Renner EL. Successful use of the Molecular Adsorbent Recirculating System (MARS) in a patient with primary biliary cirrhosis (PBC) and treatment refractory pruritus. *Hepatol Res* 2003; **25**: 442-446

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CASE REPORT

Clear cell adenocarcinoma of the colon is a unique morphological variant of intestinal carcinoma: Case report with molecular analysis

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Abstract

Here we report a new case of clear cell adenocarcinoma (CCA) of the colon in a 54-year-old Caucasian man. Despite of the previous reported cases, the lesion was located in the right colon and was not associated with the conventional adenoma. We performed immunohistochemical and molecular analyses in order to explore whether the CCA had the molecular features generally associated with conventional colorectal carcinoma. The immunohistochemical and molecular analyses showed that the different morphology of CCA does not reflect a distinct biological entity but only an unusual morphological variant of intestinal carcinoma.

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Key words: Clear cell carcinoma; Colon carcinoma; Beta-catenin; *KRAS*; Molecular analysis

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INTRODUCTION

Clear cell adenocarcinomas (CCAs) are very rare in the colon[1-3]. They generally affect elderly men, are preferentially located in the left colon and almost all form part of a larger conventional adenoma[4,5].

It is not known whether they are biologically different from morphologically conventional colorectal adenocarcinomas[6-9].

Here, an intriguing variant of CCA, unusual in term of location and morphology is described. Moreover, we have evaluated some pathways reported as deregulated in the conventional colorectal adenocarcinoma[10]. To this, we performed an extensive immunohistochemical analysis and for the first time a molecular analysis, including the genomic sequencing of *KRAS* gene and the main deregulated genes belonging to the Wnt pathway such as APC and β-catenin.

CASE REPORT

In September, 2000, a 54-year-old Caucasian man with a family history of gastrointestinal cancer and a personal 7-year history of multiple colon polyps, came to our Institution for a routine annual control colonoscopy. On this occasion 14 flat lesions with a diameter of 5-15 mm were found extending throughout the large intestine, some of which were endoscopically removed and histologically analysed, including one located in the left colon that was histologically a tubulo-villous adenoma with extensive clear cell aspects. The patient underwent subsequent colonoscopies with polyp resections in 2001, 2002, 2003, 2004 and 2005. All of these lesions were tubular adenomas. At the time of the last control colonoscopy in September 2005, a flat 0.9 cm lesion of the hepatic flexure was endoscopically revealed and biopsied, and was found to be a high-grade dysplastic adenoma with extensive clear cell features. The same

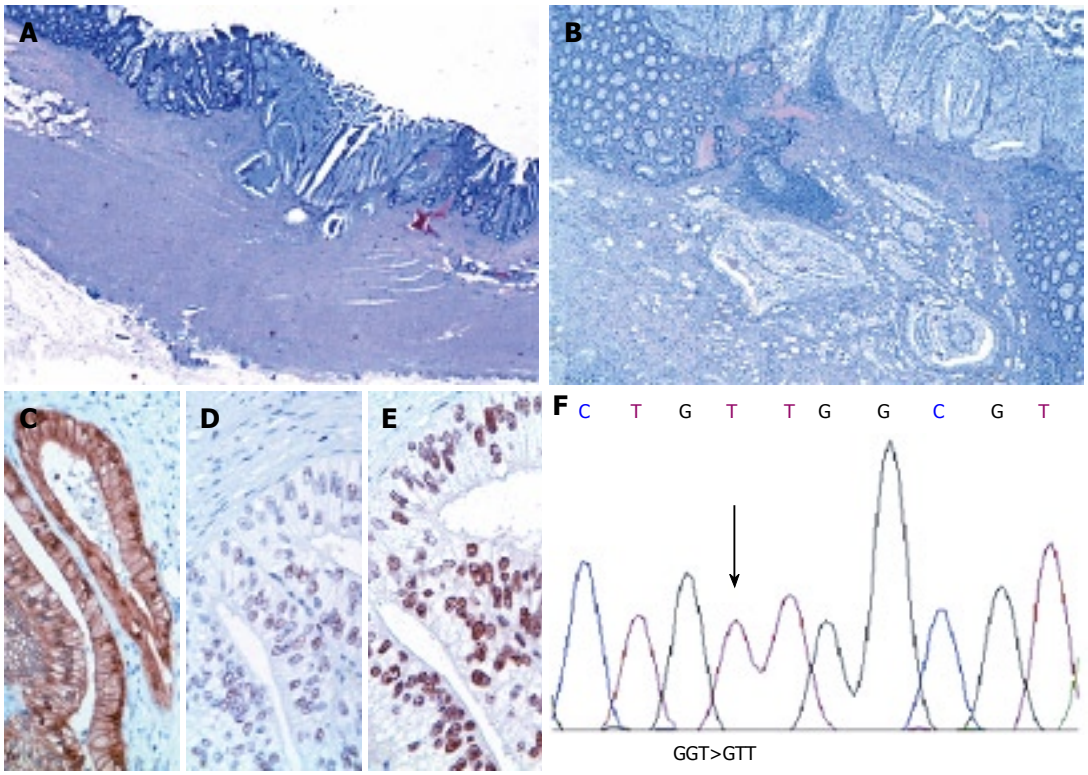

Figure 1 HE and KRAS sequencing. A: The 0.9 cm diameter lesion at the hepatic flexure was a CCA with surface erosion, focal invasion of the muscularis propria, and an abrupt transition from the normal adjacent mucosa (HE, × 4); B: Clear cells with a solid growth on the surface and budding with single-cell growth at the periphery; no conventional adenoma was seen near to the CCA (HE, × 2); C: Strong β-catenin nuclear positivity (× 20); D: Nuclear hMLH1 positivity (× 40); E: Nuclear hMSH2 positivity (× 40); F: *KRAS* sequencing revealed the point mutation GGT>GTT that leads to the activating aminoacid substitution Gly12Val.

lesion was re-biopsied in 2006 and 2007 with the same result. No conventional adenoma was identified. After undergoing total colectomy in June, 2007, the patient now feels well.

The gross specimen was a total colectomy of 115 cm with the flat, previously biopsied 0.9 cm lesion at the hepatic flexure and other pedunculated lesions in the rest of the colon. Microscopic examination showed that the pedunculated lesions were multiple tubular adenomas, and the 0.9 cm lesion of the hepatic flexure was a CCA invading the muscularis propria (Figure 1A). The CCA had a solid growth at the surface and a tendency to grow as single cells at the periphery (cellular budding); there was neither intratumoral inflammatory infiltration nor vascular invasion and no residual classic adenoma at the periphery (Figure 1B). Nodes were negative.

Five-micrometer thick, formalin-fixed (10% buffered formalin), paraffin-embedded tissue sections were immunohistochemically studied for CK20 (mouse KS20.8, BiOptica; 1:100; 6 min 95℃ heated in 0.01 mol/L citrate buffer, pH 6.0), CEA polyclonal antibody (rabbit poly, DAKO; 1:4000; 0.1% trypsin 15 min), CK7 (clone K72.7, NeoMarkers; 1:400; 0.1% trypsin 15 min), alpha-feto protein (rabbit poly, DAKO; 1:2000; 0.1% trypsin 15 min), CD 10 (mouse 56C6, Neo Markers, 1:20, 6 min 95℃ heated in 0.1 mol/L citrate buffer, pH6.0), vimentin (clone V9, DAKO; 1:400; 6 min 95℃ heated in 0.1 mol/L citrate buffer, pH 6.0), β-catenin (clone 14, Transduction; 1:4000; 6 min 95℃ heated in 0.1 mol/L citrate buffer, pH 6.0), hMLH1 (G168-15, Santa Cruz; 1:10; 2 min 120℃ heated in 0.1 mol/L citrate buffer, pH 6.0), hMSH2 (NA27-100 μg, Oncogene; 1:40; 2 min 120℃ heated in 0.1 mol/L citrate buffer pH 6.0) and p53 (clone DO7, Novocastra; 1:400; 6 min 95℃ heated in 0.1 mol/L citrate buffer, pH 6.0). The positive controls were a sporadic aggressive fibromatosis sample with a known mutation in the *CTNNB1* gene[11] for *β-catenin*; two colorectal cancer samples from hereditary non-polyposis colorectal cancer patients carrying *MLH1* or *MSH2* germline mutations for hMLH1 and hMSH2; a serous ovarian carcinoma with a known *TP53* mutation for p53. The samples were strongly positive for CK20 and CEA polyclonal antibody, and negative for CK7, alpha-feto protein, CD10 and vimentin, thus supporting the intestinal origin of the CCA[12]. They also showed p53 focal nuclear positivity, strong β-catenin nuclear positivity in the glands (Figure 1C) and nuclear positivity for hMLH1 and hMSH2 (Figure 1D to E). The proliferation index was high (> 90%).

After genomic DNA extraction (QIAmp DNA mini Kit, Qiagen, Chatsworth, CA, USA), exon 1 of the *KRAS* gene was amplified by means of polymerase chain reaction (PCR) in order to seek potential mutations on the two foremost codons[12-13], which have been reported to be mutated in morphologically conventional colorectal carcinoma. Given the strong β-catenin nuclear positivity revealed by immunohistochemistry, exon 3 of the *β-catenin* gene was sequenced. The PCR amplifications were carried out using a standard

protocol, and previously described primers and conditions[11,13]. In addition, specific primers were designed by means of Primer3 software to amplify codons 1368-1679 located in exon 15 of the *APC* gene and encompassing the mutation cluster region (MCR), where more than 60% of *APC* mutations have been detected in conventional colorectal cancer[14]. All of the PCR products were directly sequenced using an ABI Prism 377 (Applied Biosystems, Foster City, CA, USA) and evaluated by means of Sequence Navigator software (Applied Biosystems, Foster City, CA, USA). *KRAS* sequencing revealed the previously described point mutation GGT>GTT at codon 12, which leads to the activating aminoacid substitution Gly12Val (Figure 1F), whereas no mutations were found in the MCR of the *APC* gene or in the *β-catenin* gene.

DISCUSSION

Colon CCAs are rare, usually affect the left colon of elderly men, and are treated by means of polypectomy (in most cases) or segmental resection. Almost all CCAs form part of a larger conventional adenoma, thus supporting the hypothesis of a linear carcinogenetic sequence from conventional adenoma, to clear cell-type adenomas and CCAs.

However, our patient was a middle-aged man, the lesion was located in the right colon (hepatic flexure) and was not accompanied by a conventional adenoma, although many other tubular adenomas affected the whole colon, and the patient underwent total colectomy. Histologically, the CCA showed superficial solid growth and diffuse cellular budding at the periphery, but no vascular invasion. Immunohistochemistry ruled out other possible origin from extracolonic primary tumors, including renal clear cell carcinoma and carcinoma arising from the mullerian system.

We subsequently undertook immunohistochemical and molecular analyses in order to explore whether the CCA had the molecular features generally associated with conventional colorectal carcinoma. The positive nuclear immunostaining for hMLH1 and hMSH2 was consistent with microsatellite stability; the focal p53 nuclear immunoreactivity strongly suggested the absence of disabled p53 protein; and the β-catenin nuclear immunostaining was in line with activation of the Wnt pathway reported in conventional colorectal carcinoma[15]. However, neither *APC* nor *β-catenin* mutations were found, a result that we are inclined to attribute to the incomplete sequencing of *APC* exon 15 due to the very small amount of tumour tissue available.

However, molecular analysis of the *KRAS* gene by means of genomic DNA automatic sequencing revealed the activating KRAS mutation Gly12Val. This suggests that the analyzed CCA shares the KRAS genotype characterizing most (approximately 40%) conventional colorectal carcinomas.

The different morphology of CCA, therefore, does not seem to reflect a distinct biological entity, but an unusual morphological variant with similar molecular profile of conventional colorectal carcinoma.

REFERENCES

1 **Hamilton SR**, Aaltonen LA (eds): World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of the Digestive System. Lyon: IARC Press, 2000: 110
2 **Hellstrom HR**, Fisher ER. Physaliferous variant of carcinoma of colon. *Cancer* 1964; **17**: 259-263
3 **Soga K**, Konishi H, Tatsumi N, Konishi C, Nakano K, Wakabayashi N, Mitsufuji S, Kataoka K, Okanoue T, Mukaisho K, Hattori T. Clear cell adenocarcinoma of the colon: a case report and review of literature. *World J Gastroenterol* 2008; **14**: 1137-1140
4 **Suzuki H**, Ohta S, Tokuchi S, Moriya J, Fujioka Y, Nagashima K. Adenoma with clear cell change of the large intestine. *J Surg Oncol* 1998; **67**: 182-185
5 **Domoto H**, Terahata S, Senoh A, Sato K, Aida S, Tamai S. Clear cell change in colorectal adenomas: its incidence and histological characteristics. *Histopathology* 1999; **34**: 250-256
6 **Hao LS**, Zhu X, Zhao LH, Qian K, Zhou Y, Bu J, Wu XT. Clear cell adenocarcinoma of colorectum: a case report and review of the literature. *Acta Gastroenterol Belg* 2007; **70**: 235-238
7 **Ko YT**, Baik SH, Kim SH, Min BS, Kim NK, Cho CH, Lee SK, Kim HG. Clear cell adenocarcinoma of the sigmoid colon. *Int J Colorectal Dis* 2007; **22**: 1543-1544
8 **Braumann C**, Schwabe M, Ordemann J, Jacobi CA. The clear cell adenocarcinoma of the colon: case report and review of the literature. *Int J Colorectal Dis* 2004; **19**: 264-267
9 **Rubio CA**. Clear cell adenocarcinoma of the colon. *J Clin Pathol* 1995; **48**: 1142-1144
10 **Frattini M**, Balestra D, Suardi S, Oggionni M, Alberici P, Radice P, Costa A, Daidone MG, Leo E, Pilotti S, Bertario L, Pierotti MA. Different genetic features associated with colon and rectal carcinogenesis. *Clin Cancer Res* 2004; **10**: 4015-4021
11 **Signoroni S**, Frattini M, Negri T, Pastore E, Tamborini E, Casieri P, Orsenigo M, Da Riva L, Radice P, Sala P, Gronchi A, Bertario L, Pierotti MA, Pilotti S. Cyclooxygenase-2 and platelet-derived growth factor receptors as potential targets in treating aggressive fibromatosis. *Clin Cancer Res* 2007; **13**: 5034-5040
12 **D'Amato A**, Gentili V, Santella S, Pronio A, Montesani C. [Synchronous neoplasms of the colon and kidney: analysis of 2 case reports] *Chir Ital* 2000; **52**: 83-86
13 **Frattini M**, Ferrario C, Bressan P, Balestra D, De Cecco L, Mondellini P, Bongarzone I, Collini P, Gariboldi M, Pilotti S, Pierotti MA, Greco A. Alternative mutations of BRAF, RET and NTRK1 are associated with similar but distinct gene expression patterns in papillary thyroid cancer. *Oncogene* 2004; **23**: 7436-7440
14 **Fearnhead NS**, Britton MP, Bodmer WF. The ABC of APC. *Hum Mol Genet* 2001; **10**: 721-733
15 **Segditsas S**, Tomlinson I. Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* 2006; **25**: 7531-7537

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CASE REPORT

Colonic perforation in Behçet's syndrome

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Abstract

A 17-year-old gentleman was admitted to our hospital for headache, the differential diagnosis of which included Behçet's syndrome (BS). He developed an acute abdomen and was found to have air under the diaphragm on erect chest X-ray. Subsequent laparotomy revealed multiple perforations throughout the colon. This report describes an unusual complication of Behcets syndrome occurring at the time of presentation and a review of the current literature of reported cases.

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Key words: Colonic perforation; Behçet's syndrome

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INTRODUCTION

Behçet's syndrome (BS) is a multisystemic vasculitic disorder which can affect a number of different systems. It was first described by the Turkish dermatologist, Halushi Behçet in 1937, who described a triad of oral ulceration, genital ulceration and ocular inflammation[1]. BS is now recognized as a chronic, inflammatory, multisystemic disorder, which relapses in nature. It is a vasculitic process which can affect large and small vessels of both the venous and arterial systems[2].

Manifestations of BS are widespread and varied, the most common of which are cutaneous including oral and genital ulceration, erythema nodosum, pyoderma gangrenosum and many more. Up to half of patients will have musculoskeletal complications which include arthralgia and arthritis[3]. The diagnosis of BS is made using major and minor criteria devised by the International Study Group Criteria for Behçets disease in 1990[4]. The disease mainly affects young males and has a higher prevalence along the historic "silk route", i.e. from the eastern rim of Asia to the Eastern Mediterranean[5].

Gastrointestinal symptoms, such as nausea, vomiting and abdominal pain, can sometimes occur, but the presence of intestinal ulceration is rare (< 1%)[6]. Our report describes a case of extensive colonic perforation due to intestinal involvement.

CASE REPORT

A 17-year-old man was admitted to the Accident and Emergency Department due to a six-week history of occipital headache associated with vomiting and poor balance. He also reported decreased appetite and weight loss of approximately 28 lbs over a two-month period. He described transient tender skin nodules, which were present on the anterior tibial area, eight weeks prior to presentation. On direct questioning, he did reveal that he had oral ulceration previously, but this resolved spontaneously. Background medical history was unremarkable, as was his family history.

On examination in the Emergency Department, he appeared unwell. However, all his vital signs were within normal limits. Positive findings on examination included bilateral papilloedema and a seventh lower motor neuron lesion. There was bilateral scarring anteriorly

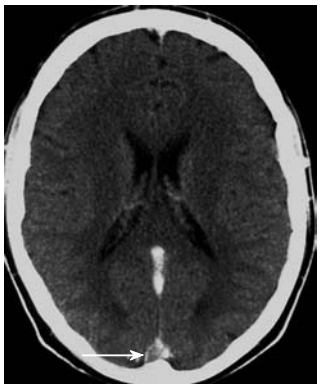

Figure 1 MRI post contrast with "Empty Delta" sign indicating a cerebral venous thrombosis.

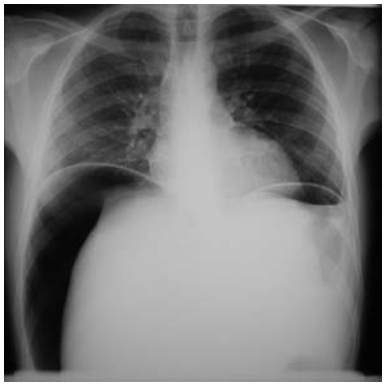

Figure 2 Erect chest X-ray demonstrating free air under the right hemidiaphragm.

on his skins. There was no evidence of oral or genital ulceration and abdominal examination at this time was normal. Hematological investigations were essentially normal except for a raised C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) at 119 mg/L and 84 mm/h, respectively. Further investigations performed included a lumbar puncture, Ziehl-Neelsen stain, autoantibody and infectious mononucleosis screen, all of which were non-contributory. He proceeded to have a CT brain and magnetic resonance studies, which demonstrated evidence of cerebral venous thrombosis. The "Empty Delta" sign on the post contrast MRI image is a characteristic finding in cerebral venous thrombosis (Figure 1).

A working diagnosis of vasculitis was formulated at this stage and the patient was placed on anticoagulation and steroids with proton-pump inhibitor cover. His symptoms gradually improved, but on day eight, he developed acute abdominal pain. On examination, his abdomen was distended, diffusely tender with absent bowel sounds. An erect chest X-ray demonstrated air under the right hemidiaphragm (Figure 2).

Marked fecal peritonitis was observed at laparotomy. There were five separate colonic perforations in the cecum, ascending, transverse and sigmoid colon. Surrounding these perforations were multiple areas of gangrenous colon with punched-out ulcerations extending throughout the colon to the level of the proximal sigmoid colon (Figure 3). A sub-total colectomy and terminal ileum resection with an end ileostomy were performed.

The histology specimen demonstrated a normal terminal ileum, but extensive ulceration in the proximal

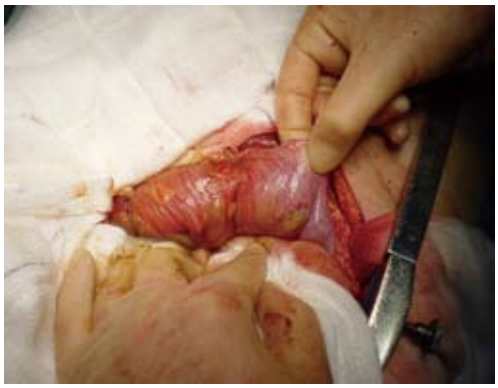

Figure 3 Punched-out ulcer at laparotomy.

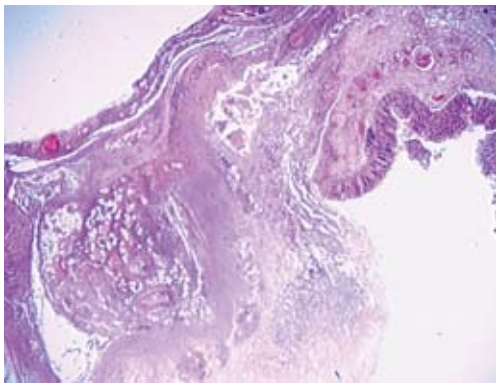

Figure 4 Microscopic specimen demonstrating loss of normal mucosa.

colon. The colon had numerous punched-out areas of ulceration, many of which were deeply penetrating with perforation. The intervening mucosa showed areas of normal mucosa. The distal segment of bowel, descending and sigmoid colon showed less ulceration than the proximal segment. However, there were nine distinct areas of necrosis, the largest of which was 2 cm in diameter. There was active inflammation with vasculitis, consistent with changes described in BS (Figure 4).

The patient made a good post-operative recovery, but developed a pelvic collection four weeks after the procedure. This was treated with a transrectal drain and intravenous antibiotic therapy. The patient was discharged home after a 51-d hospital stay on warfarin, azathioprine and a decreasing dose of corticosteroids. He remained well two months post discharge on immunosuppressant therapy.

DISCUSSION

BS involves the gastrointestinal tract in 10%-50% of patients, manifesting as diarrhoea, nausea, anorexia and abdominal pain. However, ulcerative changes are found in less than 1% of patients with BS[7]. Two types of ulceration can occur: localized and diffuse. Localized lesions tend to occur in the ileocaecal region, and are deep, often penetrating the serosal surface. In contrast, diffuse lesions are seen more commonly in the colon and may occur as multiple discrete, punched-out ulcers.

The main sites of involvement are the terminal ileum and cecum. Sometimes, BS can present with a cecal mass, anemia and weight loss, and thus mimick a cecal carcinoma[8]. In a recent literature review by Turan *et al*[9], the distribution of diffuse ulcers in twenty patients with BS was reviewed. Of these 20 patients, 9 had ulceration involving the entire colon, and only two cases (including their case) involved the sigmoid colon. Kyle *et al*[10] described the other case in 1991. Our case is the third reported case of sigmoid perforation in BS. In both previous cases, the diagnosis of BS was established. However, our case illustrates this rare complication at the time of presentation.

In a review by Kasahara *et al*[6], the most common site of involvement of the small intestine was the terminal ileum. Our case is also unusual in that the terminal ileum was unaffected.

BS often represents a diagnostic challenge, as an incomplete disease can be confused with other conditions which have similar clinical manifestations, for example inflammatory bowel disease (IBD). Not only do they share gastrointestinal manifestations, but the extra-intestinal features of both BS and IBD are very similar. Gastrointestinal disease in BS is associated with an overall poor prognosis, as approx 5%-10% will require surgery[3]. This poses a number of problems for patients with BS. Recurrent ulceration of the stoma is a relatively common complication, as is recurrence of disease adjacent to or at the surgical anastomosis. Most of these recurrent ulcers appear within two years of the resection. There are very few reports on the long term prognosis of intestinal BS. Naganuma *et al*[11] looked at 2-year follow-up in 20 patients with BS in Japan, and found that the postoperative recurrence rate is as high as 87.5%.

The pathergy phenomenon seen in BS, that is a cutaneous hyperreactivity following minor trauma[12], means that patients with BS are prone to problems at the incision site[13]. Fortunately, our patient has not experienced any such complications.

As BS is a relapsing condition, careful follow-up is necessary to detect any late complications or reactivation of disease. This report describes a rare complication of BS at the time of presentation, and highlights the importance of considering BS in the differential diagnosis of a complex patient presenting with such gastrointestinal manifestations.

REFERENCES

1 **Behçet H**. Uber rezivirde, aphtose, durch virus verursachte. Geshwure am Mund, am Auge, und den Genitalien. *Dermatol Wochenschr* 1937; 1153-1157

2 **Koç Y**, Güllü I, Akpek G, Akpolat T, Kansu E, Kiraz S, Batman F, Kansu T, Balkanci F, Akkaya S. Vascular involvement in Behçet's disease. *J Rheumatol* 1992; **19**: 402-410

3 **Bradbury AW**, Milne AA, Murie JA. Surgical aspects of Behçet's disease. *Br J Surg* 1994; **81**: 1712-1721

4 **International study group for Behcet's disease**. Criteria for diagnosis of Behcet's disease. *Lancet* 1990; **335**: 1078-1080

5 **O'Duffy JD**. Behçet's syndrome. *N Engl J Med* 1990; **322**: 326-328

6 **Kasahara Y**, Tanaka S, Nishino M, Umemura H, Shiraha S, Kuyama T. Intestinal involvement in Behçet's disease: review of 136 surgical cases in the Japanese literature. *Dis Colon Rectum* 1981; **24**: 103-106

7 **Baba S**, Maruta M, Ando K, Teramoto T, Endo I. Intestinal Behçet's disease: report of five cases. *Dis Colon Rectum* 1976; **19**: 428-440

8 **Ha HK**, Lee HJ, Yang SK, Ki WW, Yoon KH, Shin YM, Jung HY, Yu E, Lee SI, Kim KW, Auh YH. Intestinal Behçet syndrome: CT features of patients with and patients without complications. *Radiology* 1998; **209**: 449-454

9 **Turan M**, Sen M, Koyuncu A, Aydin C, Arici S. Sigmoid colon perforation as an unusual complication of Behçet's syndrome: report of a case. *Surg Today* 2003; **33**: 383-386

10 **Kyle SM**, Yeong ML, Isbister WH, Clark SP. Bechet's colitis: a differential diagnosis in inflammations of the large intestine. *Aust N Z J Surg* 1991; **61**: 547-550

11 **Naganuma M**, Iwao Y, Inoue N, Hisamatsu T, Imaeda H, Ishii H, Kanai T, Watanabe M, Hibi T. Analysis of clinical course and long-term prognosis of surgical and nonsurgical patients with intestinal Behçet's disease. *Am J Gastroenterol* 2000; **95**: 2848-2851

12 **Gilhar A**, Winterstein G, Turani H, Landau J, Etzioni A. Skin hyperreactivity response (pathergy) in Behçet's disease. *J Am Acad Dermatol* 1989; **21**: 547-552

13 **Bozkurt M**, Torin G, Aksakal B, Ataoglu O. Behçet's disease and surgical intervention. *Int J Dermatol* 1992; **31**: 571-573

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CASE REPORT

Laparoscopic approach to retrorectal cyst

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Author contributions: Gunkova P assisted the operation and wrote the paper; Martinek L operated on the patients; Dostalik J head of the Surgical Clinic; Gunka I, Vavra P and Mazur M examined the patients.

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Abstract

Retrorectal cysts are rare benign lesions in the presacral space which are frequently diagnosed in middle-aged females. We report here our experience with two symptomatic female patients who were diagnosed as having a retrorectal cyst and managed using a laparoscopic approach. The two patients were misdiagnosed as having an ovarian cystic lesion after abdominal ultrasonography. Computer tomography (CT) scan was mandatory to establish the diagnosis. The trocar port site was the same in both patients. An additional left oophorectomy was done for a coexisting ovarian cystic lesion in one patient in the same setting. There was no postoperative morbidity or mortality and the two patients were discharged on the 5th and 6th post operative days, respectively. Our cases show that laparoscopic management of retrorectal cysts is a safe approach. It reduces surgical trauma and offers an excellent tool for perfect visualization of the deep structures in the presacral space.

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Key words: Presacral space; Retrorectal cyst; Laparoscopy

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INTRODUCTION

The retrorectal space (Figure 1) is defined as the space bounded by the sacrum posteriorly, the rectum anteriorly, the peritoneal reflection superiorly, the levator ani and coccygeus muscles inferiorly. Its lateral margins are formed by the ureters and iliac vessels.

Cystic lesions arising in this space are known as retrorectal or presacral cysts and their incidence rate is one in approximately 40 000 patients[1]. Although they can be found in all age groups including infants, they are frequently diagnosed in women (81%), in their middle-age[2]. They are benign in most of the cases, but malignant degeneration is rare. If they turn malignant, they are commonly diagnosed histopathologically as adenocarcinoma or carcinoid[3]. In cases of malignancy, prognosis depends upon acheiving total surgical excision with clear margins as well as their histopathological criteria. The prognosis of adenocarcinoma is unfavourable.

Retrorectal cysts can be uni or multilocular. The content in retrorectal cysts varies from clear fluid to dense mucus. Calcifications can occur in the wall of the cyst and septa can be found inside. The lining consists of more than one type of epithelium. It is often surrounded by scattered bundles of smooth muscle but has no myenteric plexus or serosa.

CASE REPORT

Case 1

The first case was a 43-year-old woman. She underwent a diagnostic laparoscopy procedure after a suspecious cyst was found in the left ovary at sonography. The laparoscopic diagnosis was negative. The patient suffered from permanent abdominal pain and was further investigated using a computer tomography (CT) scan which showed a 9.5 cm × 4 cm × 6 cm well circumscribed cystic lesion in the presacral space, to the left of the rectum. Rectoscopy did not demonstrate any extraluminal pressure and coloscopy was also negative. The patient submitted to a laparoscopic operation for cyst excision. A 10 cm × 8 cm × 6 cm cyst with white mucous content was extracted laparoscopically (Figure 2).

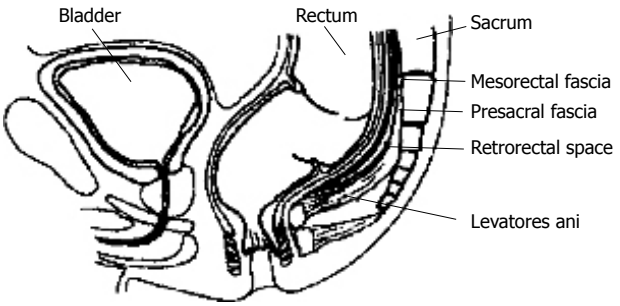

Figure 1 Retrorectal space.

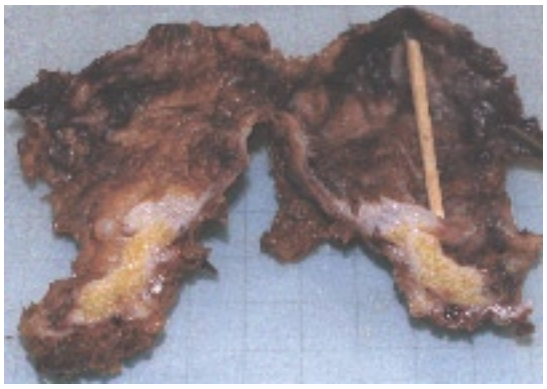

Figure 2 Specimen of retrorectal cyst.

Histopathological examination of the cyst showed dysontogenetic origin with tuboendometrial metaplasia. Postoperative recovery of the patient was smooth with no complications. She was discharged on the 6th postoperative day.

The second case was a 28-year-old woman complaining of anal pain. She underwent repeated gynecological laparoscopic operations for cysts in both ovaries. A CT scan showed cystic lesions in the left ovary and another 9.3 cm × 5.8 cm × 6.4 cm cystic mass in the presacral space (Figure 3). A 10 cm × 5.5 cm × 5 cm cyst was extracted laparoscopically. Left oophorectomy was performed in the same setting. Histopathological examination of the cyst confirmed its benign origin which is lined by simple columnar ciliated epithelium. The postoperative recovery was smooth and the patient was discharged on the 5th postoperative day.

The same laparoscopic approach was used in both cases. After peritoneal insufflation, the pressure was maintained at 11 mmHg. A 10 mm umbilical trocar, a 12 mm trocar in the right hypogastrium and a 5 mm trocar in the right mesogastrium were used for camera and the surgeon working hands, respectively. A 10 mm trocar in the left hypogastrium and a 5 mm trocar in the left mesogastrium were used for the assistance. The retrorectal space was opened using a harmonic scalpel at the sacral promontory level, the mesorectum was dissected free and the cystic lesion was identified. The cyst was resected using a harmonic scalpel after its complete dissection and separation from the surrounding structures. The pelvic floor was reconstructed with suture and a drain was placed in the Douglas pouch. The total operation time was 75 min for the first case and 90 min for the second case.

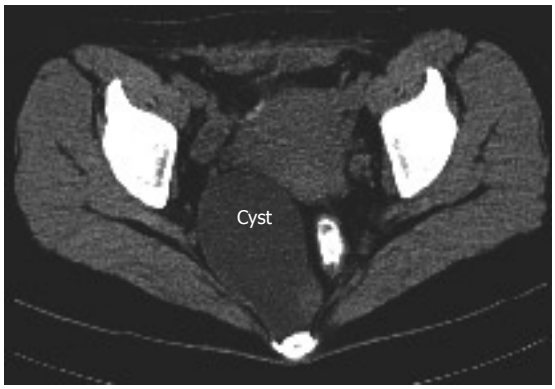

Figure 3 CT scan of cystic mass in the presacral space.

DISCUSSION

Retrorectal cystic hamartoma (hindgut cyst) is a rare developmental lesion arising from the vestiges of the embryonic hind gut[4]. Other developmental cysts (epidermoid, dermoid with skin appendages and rectal cystic duplication with well-defined muscle layers, myenteric plexus and serosa) can occur in retrorectal space[5]. Anal gland cysts can develop near the anal sphincter. All these cysts are similar to retrorectal cystic hamartoma and their exact diagnosis depends on the histopathological examination including immunohistochemical profile.

Retrorectal cysts are asymptomatic in 50% of the cases and the lesion is an incidental finding. A cyst becomes symptomatic due to its mass effects on surrounding organs (rectal fullness, painful defecation, dysuria). Cysts with secondary infection have typical symptoms of abscess and fistula. Perianal changes and a draining sinus in the sacrococcygeal area can be found. The presence of retrorectal cyst can bring about the risk of complicated labor.

Diagnosis of cysts by ultrasound is difficult, and cysts can be easily misdiagnosed as an ovarian lesion, especially if the ovaries are not visualized precisely. Since diagnostic laparoscopy can be false-negative because of retroperitoneal localization of the cyst, it is necessary to think of a retrorectal cyst when ultrasound shows a cyst and laparoscopic findings are negative.

Computed tomography (CT) and MR imaging are necessary to confirm the diagnosis of cysts[6]. CT scans usually show the cyst as a well-circumscribed hypodense retrorectal lesion without invasion of its adjacent structures. Infection of a cyst may cause wall thickening. Parietal calcifications can be found, but they are more typical in dermoid cysts, teratoma, neuroblastoma, chordoma and mucinous adenocarcinoma. The cyst signal on MR imaging depends on its content. Malignant changes are suspected in a case of focal parietal thickening or with invasion of the adjacent organs[7].

Endorectal ultrasound can also be useful[8]. Rectoscopy or sigmoidoscopy can verify external

compression on the rectum. Digital rectal examination is simple, but essential for cyst diagnosis because 75% of retrorectal masses are palpable.

Routine preoperative biopsy (transrectal or transcutaneous) is not recommended because it carries a significant hazard of spillage of possible malignant cells into the peritoneal cavity and can lead to infection of cyst[2,8]. CT-guided extrarectal or presacral approach is indicated in case of inoperable or locally advanced lesion or in patients at a high surgical risk[2,8]. Histopathological examination is necessary to determine adjuvant therapy.

The differential diagnosis of a retrorectal cyst includes malignant and inflammatory lesions which can occur in presacral space. Chordoma is the most common primary tumor, followed by leiomyosarcoma, neuroblastoma, ganglioneuroblastoma, chondrosarcomas, hemangiopericytoma, teratocarcinoma, squamous-cell carcinoma, neuroendocrine carcinoma[9], carcinoid[10] and adenocarcinoma. Adnexal tumor or endometriosis is difficult to diagnose[11]. Malignant lesions are solid with signs of local invasion and bone destruction. Sacrococcygeal teratoma with cystic and solid components and calcification is usually discovered in neonates. Anterior sacral meningocele is associated with sacral defect. Primary retrorectal adenocarcinoma arising from cystic lesions (hind gut cysts) is rare[12]. Lipoma, myelolipoma and hemangioma are rare in presacral space too. For completeness, it is necessary to mention bone tumors including osteosarcoma, chondrosarcoma, giant cell tumor, bone cysts and bone metastases. Infected hindgut cyst may be misdiagnosed as a pilonidal cyst, an anorectal fistula or a recurrent rectal abscess.

Surgery is the first line in management. Simple cyst marsupialisation with drainage is insufficient and leads to recurrence and possible infection. Surgical approaches used for excision are as follows: anterior (abdominal), posterior (intersphincteric, parasacrococcygeal, transsphincteric, transsacral, transsacrococcygeal[13], transanorectal, transvaginal) or combinated. The approach depends on location, size of the lesion and its relationship with adjacent structures[14]. Digital rectal examination is helpful for the choice of approach. If the superior border of tumor can be palpated, the posterior approach can be performed successfully. The posterior (most often transsacral or parasacral) approach is indicated for low-lying tumors (below the sacral promontory) and the abdominal approach is recommended for lesions above the sacral promontory. Laparoscopic transabdominal approach presents a minimally invasive approach with reduced surgical trauma and an excellent means for visualization of the presacral space and its contents[15,16].

In conclusion, laparoscopic excision of retrorectal systs is a safe and efficient option. Laparoscopic approach minimizes the surgical trauma and offers perfect visualization of the deep structures in the presacral space.

REFERENCES

1 **Jao SW**, Beart RW Jr, Spencer RJ, Reiman HM, Ilstrup DM. Retrorectal tumors. Mayo Clinic experience, 1960-1979. *Dis Colon Rectum* 1985; **28**: 644-652

2 **Singer MA**, Cintron JR, Martz JE, Schoetz DJ, Abcarian H. Retrorectal cyst: a rare tumor frequently misdiagnosed. *J Am Coll Surg* 2003; **196**: 880-886

3 **Prasad AR**, Amin MB, Randolph TL, Lee CS, Ma CK. Retrorectal cystic hamartoma: report of 5 cases with malignancy arising in 2. *Arch Pathol Lab Med* 2000; **124**: 725-729

4 **Hjermstad BM**, Helwig EB. Tailgut cysts. Report of 53 cases. *Am J Clin Pathol* 1988; **89**: 139-147

5 **Leborgne J**, Guiberteau B, Lehur PA, Le Goff M, Le Neel JC, Nomballais MF. [Retro-rectal cystic tumors of developmental origin in adults. Apropos of 2 cases] *Chirurgie* 1989; **115**: 565-571

6 **Liessi G**, Cesari S, Pavanello M, Butini R. Tailgut cysts: CT and MR findings. *Abdom Imaging* 1995; **20**: 256-258

7 **Lim KE**, Hsu WC, Wang CR. Tailgut cyst with malignancy: MR imaging findings. *AJR Am J Roentgenol* 1998; **170**: 1488-1490

8 **Wolpert A**, Beer-Gabel M, Lifschitz O, Zbar AP. The management of presacral masses in the adult. *Tech Coloproctol* 2002; **6**: 43-49

9 **Mourra N**, Caplin S, Parc R, Flejou JF. Presacral neuroendocrine carcinoma developed in a tailgut cyst: report of a case. *Dis Colon Rectum* 2003; **46**: 411-413

10 **Edelstein PS**, Wong WD, La Valleur J, Rothenberger DA. Carcinoid tumor: an extremely unusual presacral lesion. Report of a case. *Dis Colon Rectum* 1996; **39**: 938-942

11 **Rana S**, Stanhope RC, Gaffey T, Morrey BF, Dumesic DA. Retroperitoneal endometriosis causing unilateral hip pain. *Obstet Gynecol* 2001; **98**: 970-972

12 **Puccio F**, Solazzo M, Marciano P, Fadani R, Regina P, Benzi F. Primary retrorectal adenocarcinoma: report of a case. *Tech Coloproctol* 2003; **7**: 55-57

13 **Canessa CE**. Dorsal transsacrococcygeal rectal approach. *Dis Colon Rectum* 2005; **48**: 1663-1665

14 **Lev-Chelouche D**, Gutman M, Goldman G, Even-Sapir E, Meller I, Issakov J, Klausner JM, Rabau M. Presacral tumors: a practical classification and treatment of a unique and heterogeneous group of diseases. *Surgery* 2003; **133**: 473-478

15 **Bax NM**, van der Zee DC. The laparoscopic approach to sacrococcygeal teratomas. *Surg Endosc* 2004; **18**: 128-130

16 **Konstantinidis K**, Theodoropoulos GE, Sambalis G, Georgiou M, Vorias M, Anastassakou K, Mpontozoglou N. Laparoscopic resection of presacral schwannomas. *Surg Laparosc Endosc Percutan Tech* 2005; **15**: 302-304

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CASE REPORT

Adult T-cell leukemia/lymphoma presenting multiple lymphomatous polyposis

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Abstract

Multiple lymphomatous polyposis (MLP) is an unusual form of non-Hodgkin's lymphoma characterized by polyps throughout the gastrointestinal tract. It has been reported that most MLP are observed in cases with mantle cell lymphoma of B-cell type. We herein present a case of a 66-year-old man with adult T-cell leukemia/lymphoma (ATLL). Colonoscopy revealed MLP throughout the colon and histopathological findings of ATLL cell infiltration. The patient died despite combination of chemotherapy. The literature of manifestations of colonic involvement of ATLL is reviewed and the importance of endoscopic evaluation to differentiate ATLL intestinal lesions from opportunistic infectious enterocolitis is discussed.

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Key words: Adult T-cell leukemia/lymphoma; Multiple lymphomatous polyposis; Human T-cell lymphotropic virus type 1; *Strongyloides stercoralis*; Colonoscopy

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Hokama A, Tomoyose T, Yamamoto Y, Watanabe T, Hirata T, Kinjo F, Kato S, Ohshima K, Uezato H, Takasu N, Fujita J. Adult T-cell leukemia/lymphoma presenting multiple lymphomatous polyposis. *World J Gastroenterol* 2008; 14(42): 6584-6588 Available from: URL: http://www.wjgnet.com/1007-9327/14/6584.asp DOI: http://dx.doi.org/10.3748/wjg.14.6584

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) is a malignancy associated with retrovirus, human T-cell lymphotropic virus type 1 (HTLV-1)[1-3]. Although it is well-known that ATLL cells infiltrate into systemic organs including gastrointestinal (GI) tract[4], colonic involvement has not been fully documented[5]. We herein report a case of ATLL presenting MLP and provide a literature review on this rare entity.

CASE REPORT

A 48-year-old man presented with fever and watery diarrhea of a history for three weeks. He had been diagnosed as having smouldering ATLL with erythematopapular cutaneous lesions, in which monoclonal integration of proviral DNA of HTLV-1 into the host genome was confirmed by the Southern blot analysis. He had been managed conservatively without leukemic change or visceral invasion for

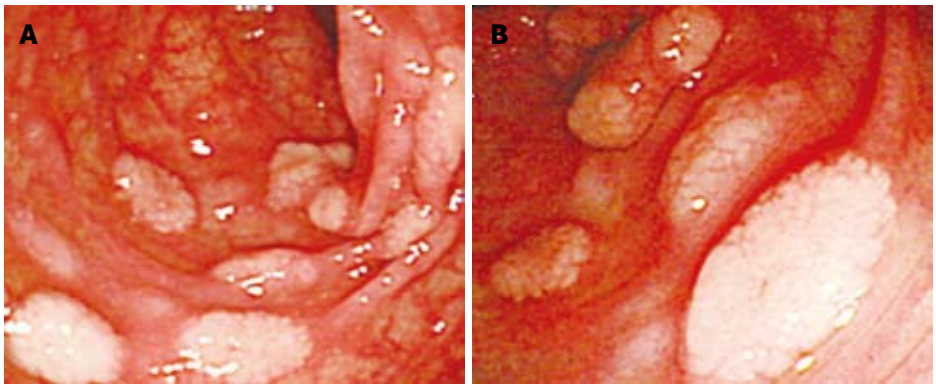

Figure 1 Colonoscopic images of the transverse colon. A: Multiple small polypoid lesions were presented; B: Closer observation revealed tiny submucosal tumors as well as whitish polypoid lesions.

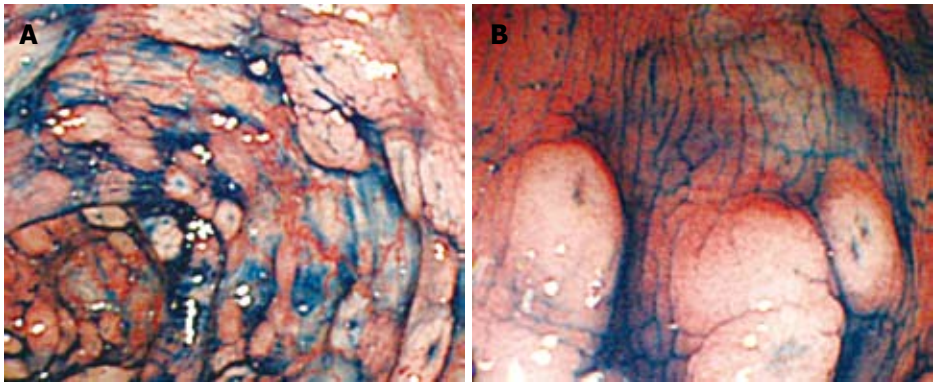

Figure 2 Colonoscopic images with indigo carmine dye. A: Multiple lymphomatous polyposis was clearly depicted; B: Closer observation revealed polypoid and aphthoid lesions had tiny central depression.

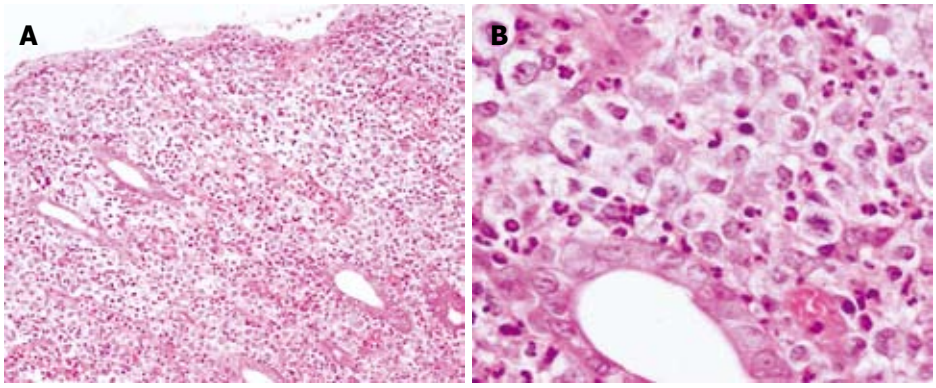

Figure 3 Pedunculated colonic mucosal tissue. Biopsy specimens of the polypoid lesion showing diffuse proliferation of atypical lymphoid cells in the mucosal layer (A, HE, × 100). The lymphoma cells display pleomorphic nuclei and pale cytoplasm. (B, HE, × 400).

20 years. Physical examination revealed mandibular lymphadenopathy and multiple erythema and papules on the skin of face, abdomen and back. Abdominal examination showed hyper bowel sounds, but no hepatosplenomegaly. The white blood cell count was 7600/mm^3 (normal: 3500-8000/mm^3), with a normal differential. Serum lactate dehydrogenase and calcium were normal. The soluble interleukin 2 receptor was 23070 U/mL (normal: 220-530 U/mL) and anti-HTLV-1 antibody was positive. Stool cultures and parasites including *Strongyloides stercoralis* were negative. Colonoscopy disclosed multiple whitish polyps throughout the colon (Figure 1). Indigo carmine dye spraying showed a central depression on the polyps (Figure 2). Pedunculated colonic mucosal tissue was replaced by diffuse proliferation of large lymphoid cells with pleomorphic nuclei and pale cytoplasm (Figure 3). Immunohistochemically, these cells were positive for CD3, CD25, and CD30, but not for CD20, suggesting anaplastic large cell variant of ATLL (Figure 4). All of these findings indicated the diagnosis of anaplastic variant of ATLL with colonic involvement presenting MLP. Subsequent ATLL infiltration to the heart, lungs and central nervous system occurred rapidly. An esophagogastroduodenoscopy was not performed. Despite combination chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine and prednisolone, the patient died 3 mo later.

DISCUSSION

HTLV-1 infection is endemic in southern Japan, Caribbean, West Africa, South America, and the Middle East. Worldwide 10-20 million people are infected approximately[1,2]. Although the majority of HTLV-1 carriers remain asymptomatic, the virus is associated with severe diseases that can be subdivided into three categories: neoplastic diseases (ATLL and

Table 1 Clinical features and colonic findings of adult T-cell leukemia/lymphoma reported in the English literature

Author	Age/gender	Gastroduodenal findings	Colonic findings	Treatment	Outcome
Utsunomiya[5]	55/M	Gastric small nodules	Edematous mucosa	ND	ND
	55/M	Gastric redness and petechiae	Diffuse petechiae	ND	ND
	67/M	Gastric ulcer and erosions	Granular appearance	ND	ND
	49/M	Gastroduodenal erosions	MLP	ND	ND
Tokunaga[8]	60/M	ND	Ulcerated tumor	ND	15 mo, died
Itsuno[9]	43/M	Gastric ulcerated tumors	MLP	CPA, DXR, VCR	8 mo, died
Gakiya[10]	62/M	Duodenal MLP	MLP	CPA, PSL	1 mo, alive
Isomoto[11]	70/M	Duodenal polypoid tumor	MLP	CPA, DXR, VDS, VP-16	4 mo, alive
Isomoto[12]	47/M	Gastric protruding masses	Granular, friable, and hyperemic mucosa	CPA, DXR, VCR, PSL	12 mo, died
Isomoto[13]	58/F	Normal	MLP	CPA, DXR, VDS	7 mo, died
Asada[14]	78/F	ND	Reddish, flat or slightly elevated lesion	CPA, THP, VCR, VP-16	died
Hidaka[15]	68/M	ND	Ulcerated tumor	surgery, CPA, DXR, VDS, PSL	10 mo, alive
Onishi[16]	41/F	ND	Redness and erosion	ND	ND
Present case	68/M	NE	MLP	CPA, DXR, VCR, PSL	3 mo, died

MLP: Multiple lymphomatous polyposis; ND: Not described; NE: Not examined; CPA: Cyclophosphamide; DXR: Doxorubicin; VCR: Vincristine; VDS: Vindesine; PSL: Prednisolone; THP: Pirarubicin.

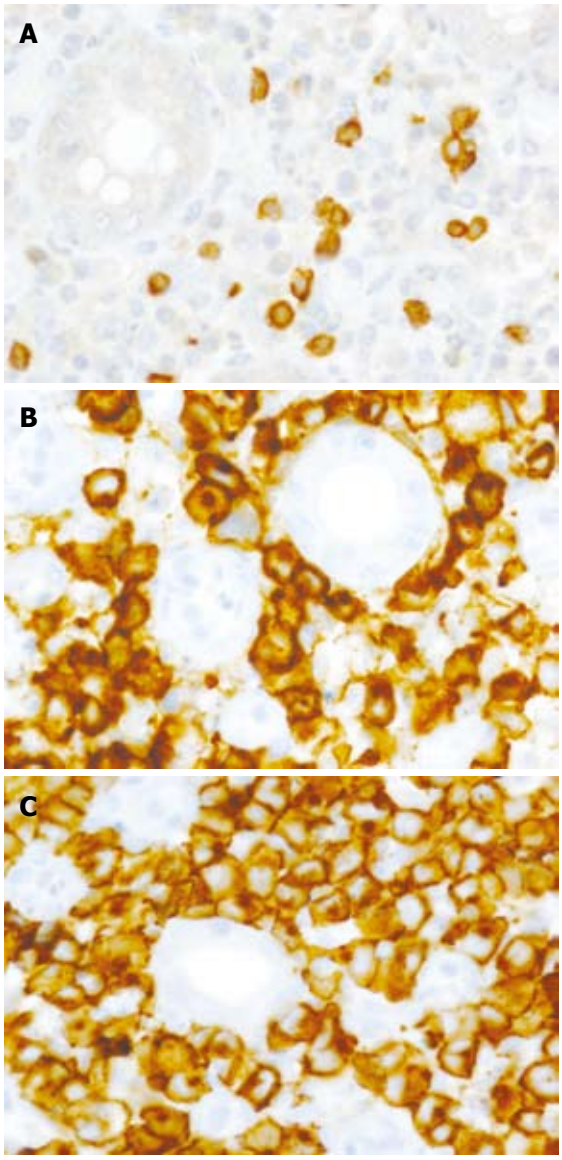

Figure 4 Immunohistochemical studies. The lymphoma cells were positive for CD3 (A, × 400), CD25 (B, × 400) and CD30 (C, × 400), consistent with ATLL, anaplastic large cell variant.

cutaneous T-cell lymphoma), inflammatory syndromes (HTLV-1 myelopathy, uveitis, arthropathy, polymyositis and thyroiditis), and opportunistic infections (*S. stercoralis* hyperinfection, scabies, tuberculosis and leprosy)[1,2].

ATLL is a systemic multiple lymphoma with a strong tendency to infiltrate various organs or to manifest as leukemic change. Based on the disease manifestations, ATLL is classified into four subtypes: smouldering type, chronic type, lymphoma type, and acute type[6]. Disease progression to the acute or lymphoma from in patients with smouldering and chronic ATLL sometimes occurs, which has a poor prognosis with median survival time of less than 1 year. The case we present was smouldering ATLL with progression to the lymphoma form. A study of 47 autopsied patients with ATLL revealed that tumor cells infiltrate various organs including the spleen (85.1%), bone marrow (72.3%), lungs (72.3%), and GI tract (70.2%). In the GI tract, the stomach is most frequently involved (40.4%), followed by the colon (38.3%) and the small intestine (34.0%)[4]. Precise mechanisms for ATLL cell infiltration in GI tract have not been clarified; however, Chen *et al*[7] described that ATLL cells from patients with GI tract involvement showed considerably higher expression of an adhesion molecule integrin β7, suggesting a critical role of this molecule in adhesion and subsequent infiltration of a certain type of ATLL cells into intestinal mucosa. Regarding colonic lesions, Utsunomiya *et al*[5] first described radiographic and endoscopic manifestations of colonic involvement of ATLL in the English literature. They classified the colonic lesions into three types: Edema with erosion, granular appearance, and multiple polypoid lesions with central depression. Table 1 shows colonic involvement of ATLL reported in the English literature. In addition to the prior categories, tumor type[8,15] and colitis type[12] have been reported. A search of the MEDLINE database retrieved only 5 reported cases of ATLL with MLP[5,9-11,13]. Although the molecular mechanisms which may influence phenotypes

of colonic lesions have not been clarified, colonic MLP may be correlated with coexistence of upper gastrointestinal MLP or polypoid lesions (Table 1). In addition, patterns and degree of ATLL cell infiltration have been suspected to be reflected to the morphological differences[17]. MLP was coined by Cornes, in 1961, to describe polypoid involvement of long segments of the gastrointestinal tract by lymphoma[17]. Although most of MLP were observed in cases with mantle cell lymphoma (MCL) of B-cell type[18-20], recent studies have shown that follicular lymphoma (FL) and mucosa-associated lymphoid tissue (MALT) lymphoma cases also showed MLP with emphasis of the importance of differentiating MCL, FL and MALT presenting MLP because of distinct prognosis among them[21]. Based on prior reports, definite differences of endoscopic morphology have not been observed between MLP of various types of lymphoma. ATLL may have the worst prognosis of MLP; therefore, pathological diagnosis will be much more important.

Patients with ATLL are immunocompromised and develop opportunistic infections that complicate the disease course[2]. Among these infections, there is an increasing body of evidence regarding a strong association between HTLV-1 and *S. stercoralis* co-infection[22]. Endemic region of HTLV-1 is overlapped with that of *S. stercoralis* worldwide and co-infestation by *S. stercoralis* can be severe and fatal[23]. Therefore, great attention should be paid to immunocompromised ATLL patients with refractory diarrhea and malabsorption to differentiate ATLL colonic lesions from opportunistic infectious enterocolitis caused by *S. stercoralis*, *Entamoeba histolytica*, *Isospora belli*, cytomegalovirus, or *Mycobacterium tuberculosis*.

The prognosis of ATLL is still poor with a median survival of less than one year for the acute and lymphoma forms despite advanced therapy including new multiagent chemotherapy, allogenic hematopoietic stem cell transplantation, and antiretroviral therapy[1,2]. Especially, gastrointestinal involvement of ATLL was reported to be one of the poor prognostic factors in acute type ATLL[11,24]. Therefore, endoscopic evaluation may be important for estimating prognosis of ATLL patients.

In conclusion, although this condition is rare, ATLL should be included in the differential diagnosis of MLP. We also emphasize the importance of endoscopic evaluation to differentiate neoplastic intestinal lesions from infectious enterocolitis for abdominal symptoms in patients with leukemia/lymphoma.

REFERENCES

1 **Matutes E**. Adult T-cell leukaemia/lymphoma. *J Clin Pathol* 2007; **60**: 1373-1377

2 **Verdonck K**, Gonzalez E, Van Dooren S, Vandamme AM, Vanham G, Gotuzzo E. Human T-lymphotropic virus 1: recent knowledge about an ancient infection. *Lancet Infect Dis* 2007; **7**: 266-281

3 **Ohshima K**. Pathological features of diseases associated with human T-cell leukemia virus type I. *Cancer Sci* 2007; **98**: 772-778

4 **Suzumiya J**, Marutsuka K, Nabeshima K, Nawa Y, Koono M, Tamura K, Kimura N, Hisano S, Tachibana N, Inoue S. Autopsy findings in 47 cases of adult T-cell leukemia/lymphoma in Miyazaki prefecture, Japan. *Leuk Lymphoma* 1993; **11**: 281-286

5 **Utsunomiya A**, Hanada S, Terada A, Kodama M, Uematsu T, Tsukasa S, Hashimoto S, Tokunaga M. Adult T-cell leukemia with leukemia cell infiltration into the gastrointestinal tract. *Cancer* 1988; **61**: 824-828

6 **Shimoyama M**. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-87). *Br J Haematol* 1991; **79**: 428-437

7 **Chen H**, Hori T, Maeda M, Uchiyama T. Identification of an adhesion molecule expressed on adult T cell leukemia cells derived from a patient with gastrointestinal involvement: implication for a possible role of integrin beta 7 in leukemic cell infiltration into intestinal mucosa. *J Clin Immunol* 1999; **19**: 186-193

8 **Tokunaga O**, Watanabe T, Shimamoto Y, Tokudome S. Primary T-cell lymphoma of the gastrointestinal tract associated with human T-cell lymphotropic virus type I. An analysis using in situ hybridization and polymerase chain reaction. *Cancer* 1993; **71**: 708-716

9 **Itsuno M**, Makiyama K, Muta K, Furukawa K, Hara K, Tabata S, Soda H, Ikeda S, Takashima H, Fukuda Y. Adult T-cell leukemia with multiple lymphomatous polyposis of the gastrointestinal tract. *Endoscopy* 1995; **27**: 700-703

10 **Gakiya I**, Kugai Y, Hayashi S, Nimura S, Zaha O, Kouchi A, Oshiro J, Sakugawa H, Kitukawa K, Kinjou F, Saitou A, Araki K. Varioliform mucosal polypoid lesions in intestinal tract in a patient with adult T-cell leukemia. *J Gastroenterol* 1997; **32**: 553-557

11 **Isomoto H**, Ohnita K, Mizuta Y, Maeda T, Onizuka Y, Miyazaki M, Omagari K, Takeshima F, Murase K, Haraguchi M, Murata I, Kohno S. Clinical and endoscopic features of adult T-cell leukemia/lymphoma with duodenal involvement. *J Clin Gastroenterol* 2001; **33**: 241-246

12 **Isomoto H**, Furusu H, Onizuka Y, Kawaguchi Y, Mizuta Y, Maeda T, Kohno S. Colonic involvement by adult T-cell leukemia/lymphoma mimicking ulcerative colitis. *Gastrointest Endosc* 2003; **58**: 805-808

13 **Isomoto H**, Maeda T, Ohnita K, Nakayama T, Kohno S. Colonic lymphomatous polyposis. *Gastrointest Endosc* 2004; **59**: 261

14 **Asada Y**, Isomoto H, Shikuwa S, Ito M, Momita S, Matsumura N, Ohba K, Ohnita K, Nakamura T, Mizuta Y, Ishibashi H, Kohno S. Adult T-cell leukemia with colorectal involvement. *Gastrointest Endosc* 2004; **60**: 983-984

15 **Hidaka H**, Hotokezaka M, Iwamura T, Moriguchi S, Marutsuka K, Toyama T, Chijiiwa K. Primary, solitary, adult T-cell leukemia/lymphoma of the descending colon. *J Gastroenterol* 2004; **39**: 788-792

16 **Onishi T**, Tamura S, Onishi S. A case of adult T-cell leukemia with colon involvement. *Gastrointest Endosc* 2007; **65**: 712-713

17 **Cornes JS**. Multiple lymphomatous polyposis of the gastrointestinal tract. *Cancer* 1961; **14**: 249-257

18 **Isaacson PG**, MacLennan KA, Subbuswamy SG. Multiple lymphomatous polyposis of the gastrointestinal tract. *Histopathology* 1984; **8**: 641-656

19 **Lavergne A**, Brouland JP, Launay E, Nemeth J, Ruskone-Fourmestraux A, Galian A. Multiple lymphomatous polyposis of the gastrointestinal tract. An extensive histopathologic and immunohistochemical study of 12 cases. *Cancer* 1994; **74**: 3042-3050

20 **Hokama A**, Kishimoto K, Tomiyama R, Hirata T, Kinjo F, Fujita J, Masuda M. An unusual cause of polyposis. *Gut* 2006; **55**: 1574, 1591

21 **Kodama T**, Ohshima K, Nomura K, Taniwaki M, Nakamura

N, Nakamura S, Kohno S, Yamamoto J, Karube K, Yamasita Y, Shirakusa T, Kikuchi M. Lymphomatous polyposis of the gastrointestinal tract, including mantle cell lymphoma, follicular lymphoma and mucosa-associated lymphoid tissue lymphoma. *Histopathology* 2005; **47**: 467-478

22 **Hirata T**, Uchima N, Kishimoto K, Zaha O, Kinjo N, Hokama A, Sakugawa H, Kinjo F, Fujita J. Impairment of host immune response against strongyloides stercoralis by human T cell lymphotropic virus type 1 infection. *Am J Trop Med Hyg* 2006; **74**: 246-249

23 **Kishimoto K**, Hokama A, Hirata T, Ihama Y, Nakamoto M, Kinjo N, Kinjo F, Fujita J. Endoscopic and histopathological study on the duodenum of Strongyloides stercoralis hyperinfection. *World J Gastroenterol* 2008; **14**: 1768-1773

24 **Sakata H**, Fujimoto K, Iwakiri R, Mizuguchi M, Koyama T, Sakai T, Inoue E, Tokunaga O, Shimamoto Y. Gastric lesions in 76 patients with adult T-cell leukemia/lymphoma. Endoscopic evaluation. *Cancer* 1996; **78**: 396-402

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CASE REPORT

Endoscopic diverticulotomy with an isolated-tip needle-knife papillotome (Iso-Tome) and a fitted overtube for the treatment of a Killian-Jamieson diverticulum

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Abstract

A Killian-Jamieson diverticulum (KJD) is an unfamiliar and rare cervical esophageal diverticulum. This diverticulum originates on the anterolateral wall of the proximal cervical esophagus through a muscular gap (the Killian-Jamieson space) below the cricopharyngeal muscle and lateral to the longitudinal muscle of the esophagus. To date, only surgical treatment has been recommended for a symptomatic KJD due to its close proximity to the recurrent laryngeal nerve and the concern of possible nerve injury. Recently, traditional open surgery for a symptomatic KJD is being challenged by the development of new endoscopic techniques and devices. We present here a case of a symptomatic KJD that was successfully treated with the flexible endoscopic diverticulotomy using two new devices. An isolated-tip needle-knife papillotome (Iso-Tome) was used for the dissection of the tissue bridge of the diverticulum. And a flexible overtube with a modified distal end (a fitted overtube) was used for adequate visualization of the tissue bridge of the diverticulum and protection of the surrounding tissue during dissection of the tissue bridge. Our successful experience suggests that the flexible endoscopic diverticulotomy with the use of appropriate endoscopic devices can be a safe and effective method for the treatment of a symptomatic KJD.

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Key words: Esophagus; Diverticulum; Killian-Jamieson diverticulum; Endoscopy; Diverticulotomy

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INTRODUCTION

A Killian-Jamieson diverticulum (KJD) is an unfamiliar and rare disease entity as compared with Zenker's diverticulum (ZD), which is the most commonly encountered diverticulum of the cervical esophagus[1-4]. These two types of cervical esophageal diverticula can be diagnosed and can be differentiated by radiological studies or by the use of endoscopy. A KJD originates on the anterolateral wall of the proximal cervical esophagus through a muscular gap (the Killian-Jamieson space) below the cricopharyngeal muscle and lateral to the longitudinal muscle of the esophagus[1-4]. A ZD develops at the anatomically weak posterior zone (the Killian's triangle) just above the cricopharyngeal muscle[1-4]. Although a KJD is anatomically distinct from a ZD, the symptoms attributable to a KJD are similar to those of a ZD[3]. To date, only surgical management has been recommended in symptomatic patients with a KJD[2]. Very recently, the first report of the flexible endoscopic treatment of a KJD was published[4].

We present here a rare case of a symptomatic KJD that was successfully treated with the flexible endoscopic diverticulotomy using an isolated-tip needle-knife papillotome (Iso-Tome) and a fitted overtube.

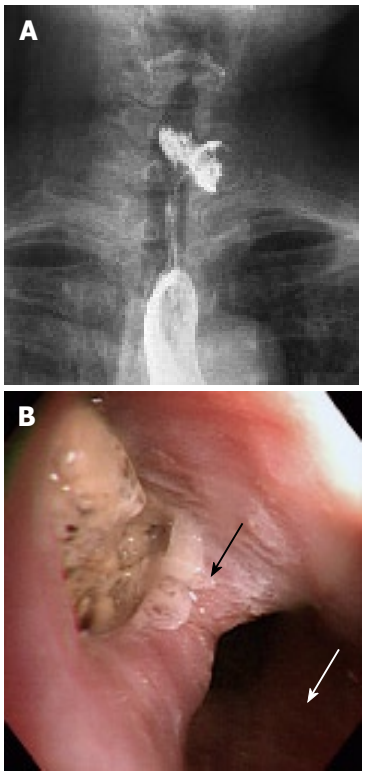

Figure 1 Barium swallow and endoscopic images. A: An antero-posterior view of a barium swallow image, showing a diverticular sac filled with contrast and food debris in the left lateral side of the cervical esophagus; B: An endoscopic view of the KJD that originates on the anterolateral wall of the cervical esophagus, which was filled with impacted food debris (black arrow: the tissue bridge; white arrow: The esophageal lumen).

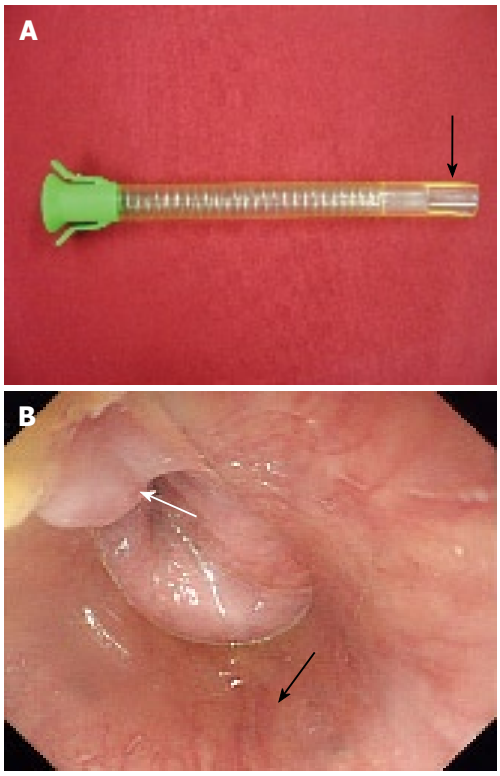

Figure 2 A fitted overtube. A: A photograph of a fitted overtube. The distal end of a standard overtube is snipped off with a scissor in a rectangular form according to the size of the diverticular opening (arrow); B: An endoscopic view following insertion of a fitted overtube (white arrow: Tissue bridge; Black arrow: Overtube). The tissue bridge of the diverticulum is exposed in the fixed operational field. The surrounding esophageal wall is completely protected and stabilized.

CASE REPORT

A 55-year-old woman was referred from a local clinic for a 6-mo history of pharyngeal discomfort and an abnormal contrast esophagogram. An anteroposterior projection of a barium swallow image revealed a 1.6 cm left-sided KJD with a wide neck (Figure 1A). The diverticulum was filled with contrast and food debris, as demonstrated by filling defects in the contrast pool. Contrast retention was persistent regardless of the barium swallow. An initial esophagoscopy showed a diverticulum protruding into the anterolateral wall of the cervical esophagus (Figure 1B). Despite overnight fasting, the diverticular wall was not clearly identified as food debris, was fully impacted inside the diverticular sac. The opening of the esophageal lumen was identified at the posterolateral side of the diverticular opening. Based on the esophagogram and the endoscopic finding, we could diagnose a true KJD. There was no coexisting ZD.

An endoscopic diverticulotomy, with the use of a forward-viewing flexible endoscope (GIF-Q260; Olympus Optical, Tokyo, Japan), was performed on an inpatient basis under conscious sedation with the administration of midazolam and propofol. Informed written consent was obtained before the procedure. In order to obtain good visualization of the tissue bridge of the KJD and to maintain a clear operational field, we used an overtube designed for endoscopic variceal ligation (Flexible Overtube; Sumitomo Bakelite Inc, Tokyo, Japan). Before the procedure, the distal end of an overtube was snipped off with a scissor in a rectangular form (Figure 2A). This 'fitted' overtube was advanced into the esophageal lumen under an endoscopic view. The position of the modified distal end of the overtube was adjusted so that it was possible to locate the tissue bridge of the KJD within the hollow rectangle (Figure 2B).

The diverticular sac was not clearly visualized during the procedure due to the presence of impacted food debris. We used an isolated-tip needle-knife papillotome (Iso-Tome, MTW Endoskopie, Wesel, Germany) and Endo-cut (VIO 300D, ERBE, Tübingen, Germany) for dissection of the tissue bridge (Figure 3A). The direction of the cut was from the diverticular lumen towards the esophageal lumen. Dissection of the tissue bridge was performed at its midpoint by direct contact of the needle of the Iso-Tome with the tissue bridge of the diverticulum in a slightly vertical manner (Figure 3B). About three-fourths of the height of the tissue bridge was separated after the dissection. Minor blood oozing developed at the incised wound, but the bleeding was successfully controlled with the use of argon plasma coagulation (APC). The total time of the procedure from insertion of the endoscope to complete hemostasis with APC was 11 min. The patient was discharged two days after the procedure without any procedure-related complications.

A follow up esophagoscopy was performed at three months after the procedure. The esophagoscopy revealed a wide communication between the diverticular sac and the esophageal lumen without a significant tissue bridge (Figure 4). There was no residual food debris inside the diverticular sac and the patient denied having any of the previous symptoms.

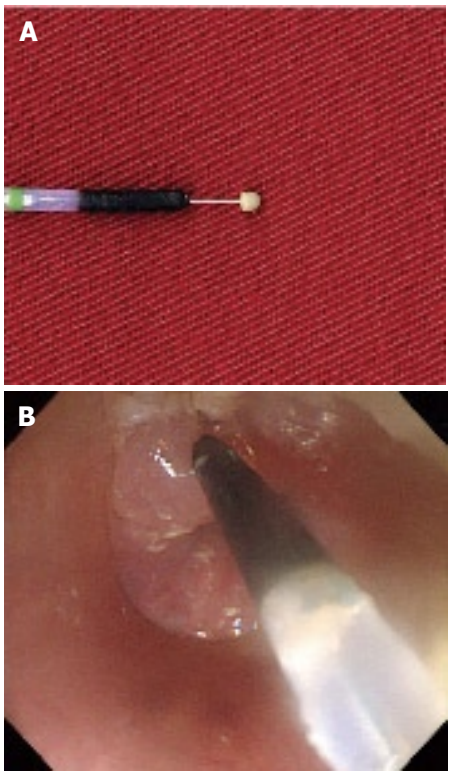

Figure 3 Isolated-tip needle-knife papillotome. A: A photograph of an isolated-tip needle-knife papillotome (Iso-tome). Note the semi-oval shaped, isolated-tip composed of epoxide adhesive. B: An endoscopic view during the diverticulotomy. The Iso-Tome is inserted into the diverticular sac before dissection.

DISCUSSION

A KJD is an uncommon cervical esophageal diverticulum located on the proximal lateral side of the cervical esophagus (also called as proximal lateral cervical diverticulum)[1-4]. Because of the unfamiliarity and rarity of this entity, a KJD is often unrecognized and misdiagnosed as a ZD on an endoscopic finding. Accurate differentiation between a ZD and KJD relies on radiological studies, especially the use of a contrast esophagogram. Unfortunately, we had no initial lateral view of the esophagogram, but we finally confirmed the presence of a KJD after a serial esophagogram and interpretation of the endoscopic findings at our institution. Although the pathogenesis of a KJD is unclear, only surgical treatment has been recommended for a symptomatic KJD due to its close proximity to the recurrent laryngeal nerve and the concern of possible nerve injury[3]. Recently, the use of flexible endoscopic diverticulotomy for the treatment of a symptomatic KJD was introduced by Tang *et al*[4]. The technique is primarily based upon a needle-knife incision of the esophageal circular muscle under the assistance of an auxiliary transparent hood as reported by the investigators. The investigators recommended that endoscopic management of a symptomatic KJD was designed to widen the opening of the diverticulum and allow subsequent fluent drainage of food material. The basic principle is not different from that for a ZD, to dissect the septum between the diverticular sac and the esophagus and then allow an overflow of food material from the diverticular sac into the esophageal lumen[5]. The aim of performing the diverticulotomy in the present case was the creation of a communication between the diverticular sac and the esophageal lumen for fluent drainage of food debris without retention inside the diverticulum, thereby resolving the patient symptoms.

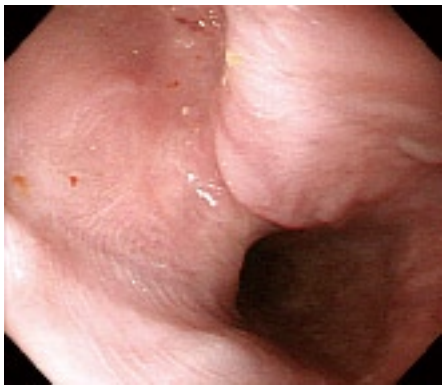

Figure 4 An endoscopic view at three months after the diverticulotomy. A wide communication between the diverticular sac and the esophageal lumen without a significant tissue bridge or retained food debris is shown.

Endoscopic treatment of the KJD was successful with a good symptomatic resolution in this case. We want to introduce two new devices for a safe diverticulotomy. First, we used an isolated-tip needle-knife papillotome (Iso-Tome) for the dissection of the tissue bridge of the diverticulum instead of a classical needle-knife. The most feared complication of needle-knife based endotherapy of the esophageal diverticula is mediastinitis[6]. The Iso-Tome was originally introduced in our institution for a safe and effective pre-cut papillotomy during an ERCP[7]. The Iso-Tome has a semi-oval shaped isolated-tip made of epoxide adhesive, which prevents electrical leakage from the tip of the incising needle, thereby preventing an unintended deep cut and perforation. This definite merit has been reported for the endoscopic treatment of an intraluminal duodenal diverticulum[8]. The semi-oval shape of the tip is also advantageous for placing the Iso-Tome into a narrow space, such as a diverticular sac filled with impacted food debris. A diverticular sac is not often clearly visualized on endoscopy due to impacted food debris inside the diverticulum. If the depth of the diverticulum is not measured endoscopically, a blind dissection with a needle-knife incision may be associated with overextension beyond the inferior border of the diverticulum and subsequent mediastinitis. In our practice, the Iso-Tome was inserted through the biopsy channel of the endoscope. The tip of the Iso-Tome was intentionally pushed into the gap between the food material and the inner wall of the diverticulum, and was then positioned at the bottom of the diverticulum (Figure 3B). The lower bottom of the diverticulum could be assumed by placement of the Iso-Tome inside the diverticulum before dissection. Another advantage of the use of the Iso-Tome is stable control of the direction of dissection. The dissection with the use of the Iso-Tome is made from the diverticular lumen

towards the esophageal lumen. Unintended slippage of the knife on the inner wall of the diverticulum can be prevented by hooking the inner diverticular wall by the shoulder of the semi-oval tip of the Iso-Tome. These advantages suggest that the use of the Iso-Tome is a safe and effective alternative to the use of the classical needle-knife in the treatment of cervical esophageal diverticula.

As a second device, we used a fitted overtube for adequate visualization of the tissue bridge of the diverticulum and protection of the surrounding tissue during dissection of the tissue bridge. Several accessory tools (a nasogastric tube, transparent hood and diverticulosope) have been introduced for flexible endoscopic treatment of cervical esophageal diverticula to overcome technical problems, especially for a ZD[9-12]. All of these accessory tools are commercially available and can be useful for flexible endoscopic treatment of a ZD. In our case, the use of a flexible overtube with a modified distal end allowed clear isolation and better exposure of the tissue bridge within the fixed operational field (Figure 2B). The surrounding esophageal wall, except for the operational field, could be prevented from exposure to unintended electrical damage. Effective stabilization of esophageal motility allowed free movement of the tip of the endoscope inside the overtube. Direct compression of the more proximal esophageal wall by the overtube could prevent aspiration of undigested food debris during the procedure. Modification of the distal end of the overtube was easily adjusted according to the form and size of the diverticular opening before the procedure.

In conclusion, the flexible endoscopic diverticulotomy can be a safe and effective method for the treatment of a symptomatic KJD. We expect that the two new devices can assist a safer and easier flexible endoscopic treatment for any kind of cervical esophageal diverticula, including both a KJD and a ZD.

REFERENCES

1 **Ekberg O**, Nylander G. Lateral diverticula from the pharyngo-esophageal junction area. *Radiology* 1983; **146**: 117-122

2 **Rodgers PJ**, Armstrong WB, Dana E. Killian-Jamieson diverticulum: a case report and a review of the literature. *Ann Otol Rhinol Laryngol* 2000; **109**: 1087-1091

3 **Rubesin SE**, Levine MS. Killian-Jamieson diverticula: radiographic findings in 16 patients. *AJR Am J Roentgenol* 2001; **177**: 85-89

4 **Tang SJ**, Tang L, Chen E, Myers LL. Flexible endoscopic Killian-Jamieson diverticulotomy and literature review (with video). *Gastrointest Endosc* 2008; **68**: 790-793

5 **Mulder CJ**, Costamagna G, Sakai P. Zenker's diverticulum: treatment using a flexible endoscope. *Endoscopy* 2001; **33**: 991-997

6 **Vogelsang A**, Preiss C, Neuhaus H, Schumacher B. Endotherapy of Zenker's diverticulum using the needle-knife technique: long-term follow-up. *Endoscopy* 2007; **39**: 131-136

7 **Park SH**, Kim HJ, Park DH, Kim JH, Lee JH, Lee SH, Chung IK, Kim HS, Kim SJ. Pre-cut papillotomy with a new papillotome. *Gastrointest Endosc* 2005; **62**: 588-591

8 **Lee SH**, Park SH, Lee JH, Park DH, Chung IK, Kim SJ, Kim HC. Endoscopic diverticulotomy with an isolated-tip papillotome (Iso-Tome) in a patient with intraluminal duodenal diverticulum. *Gastrointest Endosc* 2005; **62**: 817-819

9 **Hashiba K**, de Paula AL, da Silva JG, Cappellanes CA, Moribe D, Castillo CF, Brasil HA. Endoscopic treatment of Zenker's diverticulum. *Gastrointest Endosc* 1999; **49**: 93-97

10 **Sakai P**, Ishioka S, Maluf-Filho F, Chaves D, Moura EG. Endoscopic treatment of Zenker's diverticulum with an oblique-end hood attached to the endoscope. *Gastrointest Endosc* 2001; **54**: 760-763

11 **DOHLMAN G**, MATTSSON O. The endoscopic operation for hypopharyngeal diverticula: a roentgencinematographic study. *AMA Arch Otolaryngol* 1960; **71**: 744-752

12 **Evrard S**, Le Moine O, Hassid S, Devière J. Zenker's diverticulum: a new endoscopic treatment with a soft diverticuloscope. *Gastrointest Endosc* 2003; **58**: 116-120

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CASE REPORT

Giant sporadic fundic gland polyp: Endoscopic and endosonographic features and management

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Abstract

Fundic gland polyps are the most common gastric polyps. They are usually small in size, sporadic and asymptomatic. We present a case of giant fundic gland polyp. Our case is particular because of the clinical presentation, the endoscopic and endosonographic documented findings, and the treatment options followed.

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Key words: Fundic gland polyp; Anemia; Endoscopic ultrasonography

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INTRODUCTION

Fundic gland polyps (FGPs) account for about 47% of all gastric polyps[1]. They are found in up to 1.9% of the general population, 5% of patients presenting for upper gastrointestinal endoscopy, 84% of patients with familial adenomatous polyposis (FAP), and in 93% of those with attenuated-FAP (AFAP)[1,2]. FGPs are usually asymptomatic, though some patients may complain of dyspepsia. Most FGPs are sporadic and number less than 10 in a given patient. Typically their size is between 1 and 5 mm; however, larger polyps are occasionally observed[3]. FGPs tend to occur mostly in women during the 5th or 6th decade of life[4], but they have been described as early as 8 years of age in patients with FAP[1].

We present a case of giant sporadic FGP that was associated with positive fecal occult blood test (FOBT). We describe its endoscopic, endosonographic and histologic features and discuss the decision for surveillance.

CASE REPORT

An asymptomatic 63-year-old male was referred for evaluation of borderline microcytic anemia and positive FOBT. Past medical history was positive for long-standing systemic lupus erythematosus (SLE) for which he was maintained on prednisone 5 mg daily, hydroxychloroquine, oral calcium, Vitamin D, and alendronate. Physical exam was unremarkable. Laboratory tests revealed: Hemoglobin, 12.9 g/dL; hematocrit, 37.5%; mean corpuscular volume (MCV), 79 fl; serum iron, 34 μg/dL (37-160); total iron binding capacity (TIBC), 309 μg/dL (270-450); and ferritin 36.7 ng/mL (20-280). Colonoscopy was performed and was normal. Upper endoscopy showed a sessile, broad-based, smooth-surfaced polyp, similar in color to the surrounding mucosa and extending around 8 cm (with a width of 2 cm) from the cardia along the lesser curvature with two small satellite polyps (Figure 1). Multiple biopsies from the polyp were compatible with FGP (Figure 2). Rapid urease test for *Helicobacter pylori* (*H pylori*) was negative. Endoscopic ultrasound (EUS), performed at a frequency of 12 MHz using radial sector transducer (GF-UM-240, Olympus Tokyo, Japan), revealed a wide-based, large, predominantly hypoechoic polyp that involved the mucosa without reaching the submucosa or muscularis propria (Figure 3). Small intestinal series (SIS)

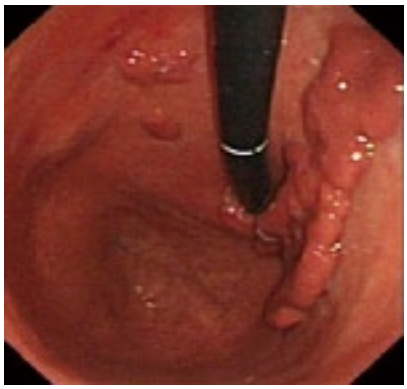

Figure 1 Endoscopic appearance of the giant FGP and the two small satellite polyps.

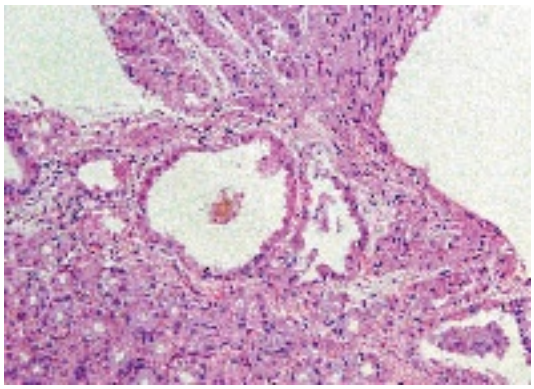

Figure 2 Fundic gland polyp biopsy showing numerous fundic glands, several of which are cystically dilated (HE, ×100).

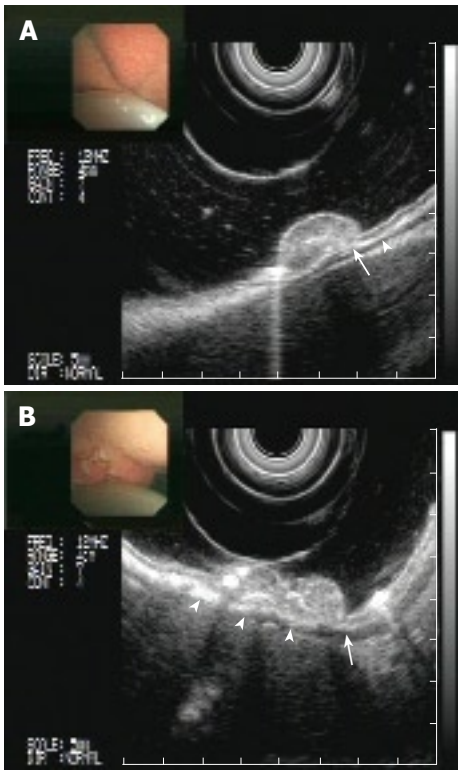

Figure 3 EUS images of the gastric polyp. A: The polyp involves the mucosa without reaching the submucosa (arrow) or muscularis propria (arrowhead); B: Mixed echogenicity of the polyp (EUS view at a different level).

exam was normal. The treatment was conservative and the patient was started on iron supplementation.

The patient was re-evaluated at 3 and 8 mo. He remained asymptomatic. Repeat stool occult blood was positive on 3 occasions. Laboratory studies showed normal hemogram and iron studies. A repeat esophagogastrod uodenoscopy (EGD) and EUS confirmed the previous findings. Biopsies from the polyp were unchanged, while biopsies of the surrounding mucosa revealed mild chronic active gastritis and rare *H pylori*-like organisms for which the patient received eradication therapy.

DISCUSSION

FGPs are the most common gastric polyps. The size of FGPs varies between 1 and 5 mm. Unlike adenomatous polyps where a size more than 2 cm has been suggested as critical in the determination of malignant potential, the size of sporadic FGPs is not considered a risk factor for malignancy[5]. Six cases of giant FGPs of different sizes have been reported in the literature[6-11]. The histologic diagnosis of sporadic FGP was reported in only one of the cases[8], making our case the second case of giant FGP.

The diagnosis of FGPs is based on pathology. The presence of cystically dilated fundic glands that are lined by attenuated, but otherwise normal-appearing chief and parietal cells is characteristic. The overlying foveolae are usually shortened. Inflammatory changes in the surrounding mucosa are usually absent[3]. In our patient, the surrounding mucosa was involved by an *H pylori*-positive mild chronic active gastritis. This association between sporadic FGPs and *H pylori* gastritis has been rarely reported. It seems like patients with sporadic FGPs are largely protected from *H pylori* colonization and that these patients rarely acquire the infection after the regression of the polyps[12].

Sporadic FGPs have distinct endoscopic features[13]. They are confined to the acid secreting oxyntic mucosa of the gastric fundus and gastric body. They are small, sessile, smooth-surfaced, hemispherical or dome-shaped, and similar in color to the surrounding mucosa. The role of EUS in the evaluation of gastric mucosal and submucosal lesions is well established. However, its use in the evaluation of giant gastric polyps has been described in four cases only[7-9,14]. It may be helpful in defining the depth and the extension of the lesion through the layers of the gastric wall, ruling out a possible malignancy.

The natural history of FGPs is unclear and there are no guidelines for surveillance and treatment. The number and size of FGPs in the FAP and sporadic forms can slowly increase, decrease or remain the same[3]. Since forceps biopsy sampling may fail to provide adequate tissue for histological diagnosis, it is recommended to remove all gastric polyps larger than 5 mm in diameter[15]. Whether this recommendation applies to FGPs is still unclear. Endoscopic mucosal resection (EMR) seems to be a safe and fast procedure for the diagnosis and treatment of gastric polypoid lesions[16]. However, a very high bleeding rate of 32% may complicate EMR for polyps greater than 3 cm in

size[17]. Moreover, surgical resection (partial gastrectomy) of large polyps may be associated with serious acute and chronic complications[18].

In our patient, the polyp is most likely the cause of positive stool occult blood. The question whether to remove such a giant polyp by EMR or surgical resection or to observe is debatable. We opted for surveillance of the polyp for the following reasons: The patient is asymptomatic and his hemogram normalized on iron therapy; FGPs may regress, progress or remain unchanged in size, FGPs carry no malignant potential, and finally endoscopic or surgical resection of such a polyp carries a high risk of bleeding and complications respectively. We felt that keeping this large polyp imposes a negligible risk to the patient.

REFERENCES

1 **Oberhuber G**, Stolte M. Gastric polyps: an update of their pathology and biological significance. *Virchows Arch* 2000; **437**: 581-590

2 **Kinoshita Y**, Tojo M, Yano T, Kitajima N, Itoh T, Nishiyama K, Inatome T, Fukuzaki H, Watanabe M, Chiba T. Incidence of fundic gland polyps in patients without familial adenomatous polyposis. *Gastrointest Endosc* 1993; **39**: 161-163

3 **Burt RW**. Gastric fundic gland polyps. *Gastroenterology* 2003; **125**: 1462-1469

4 **Marcial MA**, Villafaña M, Hernandez-Denton J, Colon-Pagan JR. Fundic gland polyps: prevalence and clinicopathologic features. *Am J Gastroenterol* 1993; **88**: 1711-1713

5 **Ginsberg GG**, Al-Kawas FH, Fleischer DE, Reilly HF, Benjamin SB. Gastric polyps: relationship of size and histology to cancer risk. *Am J Gastroenterol* 1996; **91**: 714-717

6 **Sekine S**, Shimoda T, Nimura S, Nakanishi Y, Akasu T, Katai H, Gotoda T, Shibata T, Sakamoto M, Hirohashi S. High-grade dysplasia associated with fundic gland polyposis in a familial adenomatous polyposis patient, with special reference to APC mutation profiles. *Mod Pathol* 2004; **17**: 1421-1426

7 **McGarrity TJ**, Ruggiero FM, Chey WY, Bajaj R, Kelly JE, Kauffman GL Jr. Giant fundic polyp complicating attenuated familial adenomatous polyposis. *Am J Gastroenterol* 2000; **95**: 1824-1828

8 **Winkler A**, Hinterleitner TA, Langner C. Giant fundic gland polyp mimicking a gastric malignancy. *Endoscopy* 2007; **39** Suppl 1: E34

9 **Nagata S**, Tanaka S, Ito M, Yoshihara M, Haruma K, Chayama K. Cardiac glands hyperplastic polyp of the stomach. *J Gastroenterol Hepatol* 2005; **20**: 1461-1463

10 **Sebastian S**, Addley J, Crotty P, Buckley M. Giant gastric polyp. *Gastrointest Endosc* 2004; **59**: 398-399

11 **Geller AJ**, Achem SR, Kolts BE. Giant inflammatory fibroid polyp mimicking gastric cancer. *J Clin Gastroenterol* 1992; **15**: 352-354

12 **Declich P**, Tavani E, Bellone S, Porcellati M, Pastori L, Omazzi B, Gozzini C, Bortoli A, Prada A. Sporadic fundic gland polyps: what happened before? *Gut* 2004; **53**: 1721

13 **Weston BR**, Helper DJ, Rex DK. Positive predictive value of endoscopic features deemed typical of gastric fundic gland polyps. *J Clin Gastroenterol* 2003; **36**: 399-402

14 **Müller-Höcker J**, Rellecke P. Chief cell proliferation of the gastric mucosa mimicking early gastric cancer: an unusual variant of fundic gland polyp. *Virchows Arch* 2003; **442**: 496-500

15 **Muehldorfer SM**, Stolte M, Martus P, Hahn EG, Ell C. Diagnostic accuracy of forceps biopsy versus polypectomy for gastric polyps: a prospective multicentre study. *Gut* 2002; **50**: 465-470

16 **Gencosmanoglu R**, Sen-Oran E, Kurtkaya-Yapicier O, Avsar E, Sav A, Tozun N. Gastric polypoid lesions: analysis of 150 endoscopic polypectomy specimens from 91 patients. *World J Gastroenterol* 2003; **9**: 2236-2239

17 **Ahmad NA**, Kochman ML, Long WB, Furth EE, Ginsberg GG. Efficacy, safety, and clinical outcomes of endoscopic mucosal resection: a study of 101 cases. *Gastrointest Endosc* 2002; **55**: 390-396

18 **Woodfield CA**, Levine MS. The postoperative stomach. *Eur J Radiol* 2005; **53**: 341-352

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
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E-mail: ngm2008@mci-group.com
www.ngm2008.com

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Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

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www.falkfoundation.de

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N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

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Interventional GI Endoscopy Techniques
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E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

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Issue with no volume

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Books

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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

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Contents

Contents

World Journal of Gastroenterology
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Contents

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EDITORIAL

Interaction of major genes predisposing to hepatocellular carcinoma with genes encoding signal transduction pathways influences tumor phenotype and prognosis

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Abstract

Studies on rodents and humans demonstrate an inherited predisposition to hepatocellular carcinoma (HCC). Analysis of the molecular alterations involved in the acquisition of a phenotype resistant or susceptible to hepatocarcinogenesis showed a deregulation of G1 and S phases in HCC of genetically susceptible F344 rats and a G1-S block in lesions of resistant Brown norway (BN) rats. Unrestrained extracellular signal-regulated kinase (ERK) activity linked to proteasomal degradation of dual-specificity phosphatase 1 (DUSP1), a specific ERK inhibitor, by the CKS1-SKP2 ubiquitin ligase complex occurs in more aggressive HCC of F344 rats and humans. This mechanism is less active in HCC of BN rats and human HCC with better prognosis. Up-regulation of iNos cross-talk with IKK/NF-κB and RAS/ERK pathways occurs in rodent liver lesions at higher levels in the most aggressive models represented by HCC of F344 rats and c-Myc-TGF-α transgenic mice. iNOS, IKK/NF-κB, and RAS/ERK upregulation is highest in human HCC with a poorer prognosis and positively correlates with tumor proliferation, genomic instability and microvascularization, and negatively with apoptosis. Thus, cell cycle regulation and the activity of signal transduction pathways seem to be modulated by HCC modifier genes, and differences in their efficiency influence the susceptibility to hepatocarcinogenesis and probably the prognosis of human HCC.

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Key words: Hepatocarcinogenesis; Genetic predisposition; Polygenic disease; Redifferentiation; Signal transduction pathways; Cell cycle; Cell proliferation; Apoptosis; Proteasomal degradation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent human cancers, with 1 million of newly diagnosed cases each year. The highest frequencies are found in sub-Saharan Africa and far eastern Asia, where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are endemic, and in regions where food contaminated with Aflatoxin B1 is consumed[1-3]. Other risk factors associated with the development of HCC include: long term use of oral contraceptive (female), high dose of androgen steroids, type 2 diabetes, genetic disorders such as hemochromatosis, hereditary tyrosinemia, glycogen storage disease (types 1 and 2), α1-antitrypsin deficiency, Wilson's disease; porphyria cutanea tarda, galactosemia, orotic aciduria, congenital cholestatic syndrome, and environmental agents (Thorotrast, Aflatoxins, Cycasin, Pyrrolizidine alkaloids, Vinyl chloride, tobacco smoke, N-nitrosylated compounds). HCC incidence appears to be rising, even in countries with relatively low incidence[4]. HCC is prevalently male associated with M:F ratios ranging from 1.3 to 12.9 according to the geographic area[1]. It is a rapidly fatal disease, with a life expectancy of about 6 mo from the time of diagnosis. Partial liver resection or liver transplantation are potentially curative, but only a minority of the cases is amenable to these treatments.

The frequency of HCC, similarly to that of other

tumor types, shows great differences within a human population as a response to environmental risk agents[5]. This suggests that additional environmental and/or genetic factors may be involved in the pathogenesis of the disease. Genetic polymorphisms of Cytochromes P450 2E1 and 2D6, Aldehyde dehydrogenase, Arylamine N-acetyltransferase 2, Epoxide hydrolase and L-*MYC*, and mutation of the Glutathione S-transferase gene have been associated with increased risk of HCC[6-10]. The genetic susceptibility has been shown to be one of the factors involved in familial aggregations of HCCs, even in HBV endemic areas where perinatal transmission of HBV is mainly responsible of familial HBV clustering[11]. The absence of an obviously inherited predisposition to the majority of liver cancers and of familial aggregations of HCC, independent of environmental agents such as HBV and HCV infections indicates that high-penetrance mutations are rare for this tumor, and suggests instead the involvement of low-penetrance genetic variants. Indeed, studies of families at risk seem to confirm the implication of a polygenic control of cancer incidence[12,13], and are in keeping with a polygenic model of autosomal recessive inheritance with a major gene involved in the genetic predisposition of HCC onset at an earlier age[14].

The development of cancers in mammals depends on accumulation within somatic cells of a number of genetic alterations including activation of proto-oncogenes and inactivation of oncosuppressor genes. Genetic instability plays an important role in the accumulation of these alterations[1]. Although various interindividual and interspecies differences have been documented, several morphological, biochemical and biological commonalties have been found in human and rodent preneoplastic and neoplastic liver lesions[3,15-17]. This suggests that the basic mechanisms of HCC development, in different species, are closely similar. Furthermore, studies with experimental models of complex diseases, different from cancer, indicate that the genetic variants controlling susceptibility map to orthologous regions of the mouse and human genomes, and various diseases are caused by polymorphism of equivalent mouse and human genes[18]. On the basis of these observations and considerations; in recent years, mouse and rat hepatocarcinogenesis models have been used to map susceptibility genes, define the genetic model responsible for an increased risk of HCC, and examine the effector mechanisms of tumor susceptibility genes.

RESISTANT PHENOTYPE

Liver carcinogenesis is a multistage process[1,15-17,19]. The clonal expansion of carcinogen-initiated cells (promotion) leads to the development of foci of altered hepatocytes (FAHs) that, in the rat, can be identified by immunohistochemical staining of glutathione S-transferase 7-7 (GST 7-7). Most liver preneoplastic lesions re-differentiate or no further evolve to cancer, whereas a subset of lesions acquires the capacity of autonomous growth and progress to neoplastic nodules (dysplastic nodules, adenomas) and HCCs[19,20]. Genomic instability, up-regulation of oncogenes and downregulation of oncosuppressor genes, and in late stages, alteration of cell adhesion mechanisms drive the process evolution[15,21].

A striking behavior of rodents' genetic resistance to hepatocarcinogenesis is the fact that resistance genes apparently do not affect the initiation of the process, but only the capacity of initiated cells to grow autonomously[22-25]. In recent years the Brown norway (BN)[26] and Copenhagen (Cop)[27,28] rat strains have been shown to be strongly resistant to hepatocarcinogenesis. Both strains, crossed with F344 rats, susceptible to hepatocarcinogenesis, dominantly transmit their resistance to (BN × F344) F1 (BFF1) and (Cop × F344) F1 (CFF1) rats. Treatment of rats with DENA/AAF/partial hepatectomy, according to the "resistant hepatocyte" model of hepatocarci nogenesis[19], results in the development in the liver of resistant strains of an elevated number of fast-growing early preneoplastic lesions, positive for GST 7-7 expression. After exhaustion of the promoting stimulus, GST 7-7 positive lesions undergo a progressive decrease in growth capacity and intensive phenotypic reversion (remodeling)[29], thus showing inability to grow autonomously. In carcinogen-treated Cop rat strain, the block of progression of preneoplastic lesions exclusively depends on re-differentiation, whereas DNA synthesis in these lesions proceeds at the same rate as in the lesions of susceptible strains[27,28,30]. The analysis of BN and Cop rats excluded apoptosis in the preneoplastic lesions as a mechanism of resistance to hepatocarcinogenesis and attributed a preeminent role to redifferentiation[26-28]. However, apoptosis occurs in HCC from the resistant BN[31] and DHB[32] rats. These observations indicate that in the presence of dominant resistance alleles the subset of autonomously growing preneoplastic lesions, selected during promoting treatments, is very small or absent.

MAPPING OF THE LOCI CONTROLLING THE SUSCEPTIBILITY TO HEPATOCARCINOGENESIS

Linkage analysis experiments, made in various models of rodent hepatocarcinogenesis, led to the identification of different hepatocarcinogenesis susceptibility (*Hcs*) and resistance (*Hcr*) loci[25]. Mouse *Hcs1*, *Hcs2*, and *Hcs3* loci were identified on chromosomes 7, 8, and 12, respectively, in urethane-treated F2 male mice generated by crossing the susceptible C3H/HeJ strain with the resistant A/J strain[33]. Interspecific testcrosses between the phylogenetically distant C3H/HeJ and *Mus spretus* mice, followed by the cross of the resulting F1 with the resistant C57BL/6J (B6) strain, to increase interstrain polymorphism[23], led to the identification of 3 additional *Hcs* loci (numbered from 4 to 6), mapping to chromosomes 2, 5, and 19, respectively. More recently a seventh *Hcs* locus (*Hcs7*) was mapped to

distal chromosome 1 by analysis of the backcrosses and intercrosses between the susceptible C3H/HeJ or CBA/J strains and the resistant B6 strain[35]. Congenic B6.C3H (*D1Mit5-D1Mit17*) and B6.BR (*D1Mit5-D1Mit17*) were generated in which a approximate 70 cm segment (between *D1Mit5* and *D1Mit17*) from C3H or C57BR/cdj (Br) susceptible strains, was introgressed onto a B6 background. These recombinant congenic strains (RCSs) develop more liver tumors than B6 mice, indicating that distal chromosome 1 carries potent modifier gene(s) sufficient to confer susceptibility to liver cancer.

Two loci involved in the susceptibility to HCC have been identified in crosses between BR and B6 mice[35]. BR females are extremely sensitive to HCC induction, since they are genetically insensitive to the inhibition of hepatocarcinogenesis exerted by ovarian hormones. This property is dominantly transmitted to B6BRF1 and BRB6F1 mice. BR alleles at two loci, on chromosomes 17 and 1, identified in backcrosses and F2 progeny, are associated with increased susceptibility in both sexes. They were denominated *Hcf1* and *Hcf2* (hepatocarcinogenesis in females) loci. *Hcf1* and at a lower extent *Hcf2* accounted for the higher sensitivity of BR mice to hepatocarcinogenesis.

In addition to susceptibility loci, two resistance loci, with negative phenotypic effects have been discovered in mouse genome. *Hcr1* and *Hcr2* loci map on chromosomes 4 and 10, respectively[36]. Further work[37] has shown that a resistant F1 mouse may be generated by crossing the resistant BXD-15 recombinant inbred mouse, presumably carrying *Hcr* genes contributed by the parental strain DBA/2J, to susceptible recombinant BXD-11 mice, which should carry DBA/2J *Hcs* genes. This strongly suggests that *Hcr* genes may modify the activity of several sensitivity loci.

The genome of the BALB/c mouse strain provides alleles that semi dominantly inhibit hepatocellular tumor development in F1 crosses with the highly hepatocarcinogenesis-susceptible C3H/He strain[39]. Recent genome-wide linkage analysis in a F2 population produced by intercrossing the BALB/c to the C3H/He mouse strain revealed a hepatocarcinogen resistance 3 (*Hpcr3*) locus with a major role in the resistance to urethane-induced hepatocarcinogenesis[40]. This locus, mapping to central Chromosome 15, accounts for 40% of the phenotypic variance. A gene expression profile of normal adult mouse liver showed a significant association with susceptibility of BALB/c, C3H/He, and F1 mice to hepatocarcinogenesis, and identified the genes expressed in the *Hpcr3* locus region. This analysis implicated the E2F1 pathway in the modulation of the phenotype susceptibility to hepatocarcinogenesis.

The first locus regulating the susceptibility of rats to chemical hepatocarcinogenesis, denominated *rcc* locus, has been identified in the telomeric end of chromosome 20 of MHC-recombinant rat strains, congenic for the MHC genes and its linked region *grc* (growth reproduction complex)[41,42]. The rcc^+ locus has many properties in common with tumor-suppressor genes: it is recessive, its deletion causes phenotypic susceptibility to various carcinogens, and it inhibits tumor development in many organs and tissues, including liver, skin, kidney and mesenchyma[41].

Numerous other loci have been identified by linkage analysis of male backcrosses and intercrosses of resistant BN and/or Cop rats to susceptible F344 rats. Seven susceptibility loci have been identified: rat *Hcs1* and *Hcs2* loci on chromosomes 7 and 1 respectively, in BN × BFF1 backcross progeny[43], *Hcs3* and *Hcs4* loci in BFF2 rats[44], and *Hcs3*, *Hcs5*, *Hcs6* and *Hcs7* in CFF2 intercrosses[45]. *Hcr* loci numbered 1 to 3 have been mapped to chromosomes 10, 4, and 8, respectively, in BN × BFF1 backcrosses[43]. Four additional *Hcr* loci, numbered from 9 to 12, (Rat genome database, www.rgd.mcw.edu/; previously numbered from 4 to 7) were identified on chromosomes 4, 6, and 8 of BFF2 rats[44]. *Hcr13* and *Hcr14* (RGD; previously numbered 8 and 9) were mapped to chromosomes 4 and 18 of CFF2 rats[45].

The results of genomic scanning of crosses of BN and Cop rats with F344 rats are consistent with some observations on a resistant mutant of Donryu rats strain, the DRH rats[46,47], indicating the presence of two clusters of genes on chromosomes 1 and 4 of (DRH × F344) F2 rats, designated collectively as *Drh1* and *Drh2*, and considered to be resistance genes. These genes are transmitted dominantly from DHR rats to the F2 progeny. The *Drh1* locus affects the development of FAH induced by 3'-Me-DAB[46,47], whereas *Drh2* seems to control the progression of FAH to carcinoma. On the basis of the chromosomal localization, *Dhr2* seems to correspond to *Hcr2* on chromosome 4, while *Drh1* corresponds to *Hcs3* and *Hcs5*.

The phenotypic effect of *Hcs3* locus in BFF2 rats, consisting in a marked increase in the volume of neoplastic nodules, accounts for 49% of the total phenotypic traits[44]. In CFF2 rats, *Hcs3* and *Hcs5* loci apparently account for only 14.6% and 8.4% of the phenotypic trait, respectively, consisting in about a 100% rise in number of non-remodeling nodules[45]. These nodules represent less than 20% of the total lesions in these rats. Thus, a diluting effect of the large number of remodeling lesions may be responsible for the apparently low penetrance of the *C* allele at the *Hcs3* locus. In DRH rats, the presence of *F* alleles at the *Drh1* locus has a dominant positive effect on the number of FAH (about 100% increases)[46]. These phenotypic effects of *Hcs3*/*Drh1* have been recently confirmed in a congenic DRH.F344-*Drh1* strain in which an about 43 cm segment of Drh1 from F344 rats has been introgressed onto a DRH background[32]. Above observations are consistent with a major role of *Hcs3* in the predisposition to liver cancer. It should be noted that the analysis of phenotypic effects of susceptibility/resistance loci in rats showed the presence of susceptibility loci in resistant BN rats. Given that BFF1 rats, heterozygous at all loci, are resistant to hepatocarcinogenesis[26], the behavior of backcross rats suggests the existence of inhibitory mechanisms of susceptibility genes in these animals.

Interestingly, the *Hpcr3* locus in the middle region of mouse chromosome 15 is synthetic to the rat *Hcs1*

locus which has been linked with dominant resistance to hepatocarcinogenesis in a backcross between the genetically resistant BN and the susceptible F344 rat strains[43]. Thus, it is possible that the mouse *Hpcr3* and the rat *Hcs1* locus modify hepatocarcinogenesis susceptibility by functional alleles of the same gene in both species. Moreover, both the mouse *Hpcr3* and the rat *Hcs1* regions are homologous to the human chromosome 8q region which undergoes frequent structural alterations in HCCs, including copy number gains that have been associated with tumor growth[48-50]. Consistent with these findings, the gain of chromosome 15 occurs in primary HCCs of B6C3F1 mice[51]. These findings suggest that *Hpcr3* represents a major modifier locus for hepatocarcinogenesis.

The progressive disappearance of molecular markers of preneoplastic lesions, followed by the disappearance of histological evidence of these lesions (phenotypic reversion, remodeling), during rat liver carcinogenesis, has been interpreted, at least in some instances, as re-differentiation[19,20,26-28]. The regulation of marker expression may involve the activity of various genes. We have identified in BFF2 rats' two loci, denominated liver neoplastic nodule remodeling, *Lnnr1* and *Lnnr2* whose phenotypic effect was the reduction in the percentage of remodeling lesions[52]. Due to an intensive remodeling of preneoplastic lesions in the Cop strain, more detailed information was obtained by genomic scanning of CFF2 rats, in which four *Lnnr* loci were discovered: *Lnnr3* downregulated the number of remodeling neoplastic nodule, whereas *Lnnr4* and *Lnnr5* had the opposite effect. An additional locus, on chromosome 6, *Lnnr6*, had a negative phenotypic effect on the volume of remodeling nodules[45]. These observations are consistent with a model of hepatocarcinogenesis in which only a relatively small subset of early preneoplastic lesions is genetically programmed to evolve to HCC, whereas the remainder undergoes phenotypic reversion. The importance of this phenomenon is underlined by the observation of hepatocarcinogenesis prevention by compounds inducing remodeling[20], as well as by its implication in some cases of spontaneous regression of liver nodules and carcinomas in humans[53].

The recent construction of the F344.BN-*Hcs4* RCS, by introgressing a 4.41 cm portion of Hcs4 from BN strain in an isogenic F344 background, allowed the important identification of a high penetrance gene(s), activated by estrogens and inhibited/unaffected by testosterone, conferring resistance to females towards liver cancer[54]. The volume and positivity for Proliferating Cell Nuclear Antigen (PCNA) were much higher in chemically induced preneoplastic lesions of F344 than BN rats, of both sexes. These parameters were lower in females than males. It was found that lesion volume and PCNA values of male RCS were similar to those of F344 rats, but corresponded in females to those of BN females. Carcinomatous nodules and HCC developed, at 32 and 60 wk, respectively, in male F344 and congenics and, rarely, in F344 females. Gonadectomy of congenic males, followed by β-estradiol administration, caused decrease in *Ar* (Androgen receptor) gene expression, increase in *Er-α* (Estrogen receptor-α) expression, and development of preneoplastic lesions comparable to those from BN females. Administration of testosterone to gonadectomized females leads to *Ar* increase and development of preneoplastic lesions as in F344 males. This indicates a role of homozygous *B* alleles at *Hcs4* in determination of phenotypic patterns of female RCS.

Research on rodent models of liver cancer have clearly shown a model-based on the polygenic inheritance of low penetrance genes with a few predominant susceptibility/resistance loci. Furthermore, the study of epistatic interactions between microsatellite loci, inducing phenotypic effects not predictable on the basis of the sum of their separate effect, resulted in the identification of several novel tumor modifier loci in rats, indicating that gene-gene interactions have a major role in hepatocarci nogenesis[25]. We can envisage the existence of different subsets of low-penetrance genes at play in different subsets of population.

Overall, these findings prove the existence of a great complexity of the inherited predisposition to liver cancer. A complex combination and interplay of susceptibility or resistance alleles determines the individual risk. Some individuals may inherit a predominance of susceptibility alleles and/or a major allele and may be highly cancer prone. However, since human individuals are generally casually assorted, independently of genetic factors, a situation of high or low genetic risk should be rare, and most humans should be at average risk. A corollary of this situation is that the effect of polygenic inheritance can be masked by a predominant presence of environmental high risk factors. Nevertheless, taking into account the effect of susceptibility/resistance genes on the proliferative activity and re-differentiation of initiated cells, it may be expected that the genetic substrate largely influences the prognosis.

PUTATIVE TARGETS OF SUSCEPTIBILITY/RESISTANCE GENES

Cell cycle deregulation

The deregulation of signal transduction pathways, cell cycle control, and genes involved in cell death signals characterizes initiated cells and influences their evolution to malignancy[1-3,15]. Several genes implicated in these activities, in mouse and rats, can be targeted by the susceptibility/resistance genes. This mechanism could be responsible for the acquisition of a phenotype susceptible or resistant to HCC development.

According to recent evidence HCCs developing in c-*Myc* transgenic mice undergo sustained regression, associated with re-differentiation of tumor cells, following inactivation of c-*Myc* transgene expression[55]. The re-differentiation is not terminal, and tumor growth starts again after restoration of c-*Myc* expression. These results, linking re-differentiation of tumor cells to the expression of c-*Myc*, a gene located at *Lnnr1*[52], are in keeping with the observation that

inhibition of c-*Myc* expression by antisense strategy contributes to the deregulation of E2f1 expression, and blocks *in vitro* growth of human and rat HCC cells[56]. In addition, *in vivo* studies have shown c-*Myc* hypomethylation in preneoplastic and neoplastic rat liver lesions[57]. The decrease in c-*Myc* expression, induced by prolonged administration of the methyl donor S-adenosylmethionine to rats, is associated with growth restraint and re-differentiation of liver lesions[20,57,58]. No differences in c-*Myc* expression occur in the liver of normal F344 and BN rats[56,58,59]. However, the expression of this gene is much higher in preneoplastic and neoplastic lesions of F344 rats than in the lesions of BN rats[56,58,59]. c-*Myc* is frequently amplified in preneoplastic and neoplastic lesions of the susceptible strain, but not in the lesions of the resistant BN and Wistar strains[60]. Taken together, these observations suggest the existence of some connections between c-*Myc* and the susceptibility genes that regulate re-differentiation.

Since c-*Myc* regulates the pRb-E2F pathway, we evaluated cell cycle gene expression in neoplastic nodules and HCCs, induced by initiation/selection protocols, 40 and 70 wk after diethylnitrosamine treatment, in susceptible F344 rats, and resistant Wistar and BN rats[59]. No interstrain differences in gene expression were observed in normal livers. Overexpression of c-*Myc*, *Cyclins D1*, *E*, and *A*, and *E2f1* genes, at mRNA and protein levels, rise in Cyclin D1-Cyclin-dependent kinase 4 (CDK4), Cyclin E-CDK2 and E2f1-DP1 complexes, and pRb hyperphosphorylation occurred in nodules and HCCs of F344 rats. In nodules and/or HCCs of Wistar and BN rats, low or no increases in c-*Myc*, *Cyclins D1*, *E*, and *A*, and *E2f1* expression, and Cyclin-CDKs complexes formation were associated with pRb hypophosphorylation. These results are consistent with a deregulation of the G1 and S phases in liver lesions of susceptible rats, and a block of G1-S transition in lesions of resistant strains, which explains their low progression capacity.

Autonomously growing preneoplastic liver nodules develop in susceptible rat strains, initiated by diethylnitrosamine and subjected to the initiation/selection treatments of the "resistant hepatocyte" protocol, after the cessation (about 6 wk after initiation) of the promotion stimulus represented by partial hepatectomy[19,20,61]. In contrast, only very few preneoplastic lesions of resistant BN rats grow autonomously after the end of the promoting stage; the majority of lesions remodel or no further evolve to neoplasia[61]. To evaluate the molecular mechanisms underlying the appearance of the resistant phenotype in BN rats[61], the behavior of the *p16*^{INK4A} and some genes regulating cell cycle inhibition by p16^{INK4A} have been analyzed. Preneoplastic liver (7 wk after initiation), neoplastic nodules (32 wk) and HCC (57 wk) showed high *p16*^{INK4A} expression at mRNA and protein levels, in both F344 and BN rat strains. This was associated with increase in the expression of Heat shock protein 90 (Hsp90) and Cell division cycle 37 (Cdc37) protein,

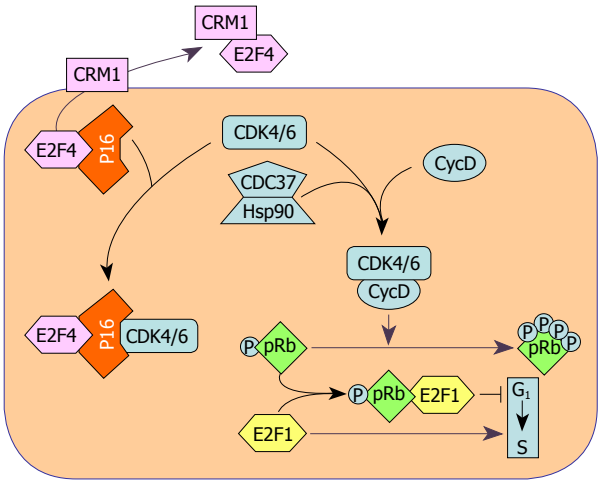

Figure 1 Cell cycle protection from inhibition by P16^{INK4A} through the CDC37-HSP90 complex and CRM1 transporter protein. P16^{INK4A} forms complexes with CDK4 and CDK6 which, as a consequence, cannot by activated by Cyclin D1 and cannot phosphorylate pRb. The chaperons CDC37 and HSP90 form complexes with CDKs protecting them from inactivation by P16^{INK4A}. CRM1 forms a complex with E2F4, a P16^{INK4A} effector, transporting it outside of the nucleus, thus inactivating P16^{INK4A}.

and Cdc37-Cdk4 complex. The HSP90-CDC37 complex protects several kinases, including Cdk4 and Cdk6 from the formation of inhibitory complexes with p16^{INK4A}[62-64] (Figure 1). Consequently, the increase in Cdc37-Cdk4 complex resulted in a decrease in p16^{INK4A}-Cdk4 complex in the lesions of F344 rats, whereas lower/no changes occurred in BN rats[61].

The transcription factor E2f4, is a p16^{INK4A} effector, acting as a growth repressor[65], equally expressed in the lesions of both F344 and BN rats. Required for chromosome region maintenance 1 protein (Crm1) is a receptor for various proteins containing a specific nuclear export sequence, including E2f4[66]. Crm1 and the cytoplasmic E2f4-Crm1 complex are highest in preneoplastic and neoplastic lesions of F344 rats. This indicates more elevated nuclear E2f4 efflux in the susceptible rats leading to a decrease in the interaction of p16^{INK4A} with G1 kinases (Figure 1). Furthermore, lower P16^{INK4A} level and highest upregulation of the HSP90/CDC37, and E2F4/CRM1 systems occur in human HCCs with a poorer prognosis (HCCP), based on survival rate, compared to HCCs with a better prognosis (HCCB)[61]. Accordingly, the P16^{INK4A}-CDK4 complex is higher in HCCB than in HCCP, whereas the complexes of CDK4 with HSP90 and CDC37 are higher in HCCP than in HCCB. Consistent with a protective role of CDC37 against growth inhibition is the observation that a decrease in its expression, induced by specific siRNAs, leads to inhibition of DNA synthesis in HepG2 cells, without modifying P16^{INK4A} expression[61]. This suggests that *CDC37* could be a target for HCC chemoprevention and therapy. These findings underline the role of the Hsp90/Cdc37 and E2f4/Crm1 systems in the acquisition of a susceptible or resistant phenotype in rats and suggest that the protection by CDC37 and CRM1 against cell cycle inhibition by P16^{INK4A} may influence the prognosis

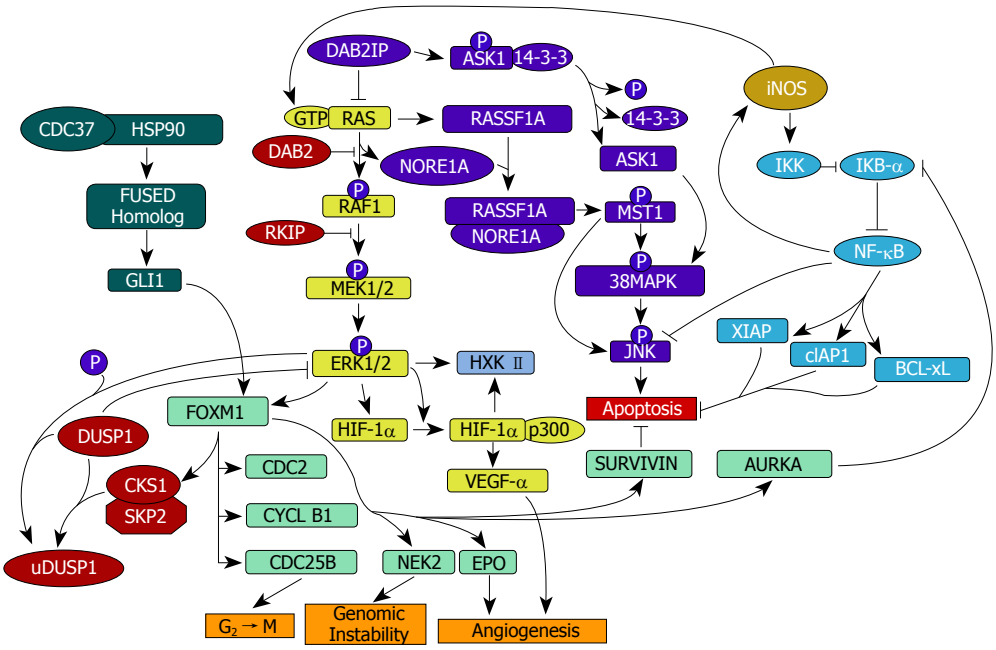

Figure 2 Schematic representations of the activated RAS-MAPK, RASSF1A/NORE1A, and Dab2IP/ASK1 pathways, iNOS signalling, and FOXM1-related pathways involved in the dysregulation of cell growth and apoptosis in HCC. Active Ha-RAS (GTP-RAS) triggers the MAPK pathway leading to activation of ERK1/2. Active ERK can down-regulate DUSP1 by phosphorylation at ser296 allowing the formation of the SKP2/CSK1/DUSP1 complex, which facilitates DUSP1 ubiquitination and proteasomal degradation. In addition, ERK may further contribute to DUSP1 proteolysis *via* induction of its target FOXM1, leading to transcriptional activation of *SKP2* and *CKS1*. These mechanisms result in decreased inhibition of ERK by DUSP1 (blunt arrow). Moreover, FOXM1 favors the growth of neoplastic cells by targeting genes involved in $G_2 \rightarrow M$ transition, genomic instability, angiogenesis, NF-κB activation, and anti-apoptosis. iNOS activates IKK that allows proteasomal degradation of the NF-κB inhibitor, IkB-α. This results in NF-κB activation. iNOS also activates Ha-RAS, thus triggering the MAPK pathway leading to activation of ERK1/2. NF-κB activation by ERK may occur through AURORA-A (AURKA) which inhibits IkB-α. NF-κB activates various antiapoptotic genes (*XIAP*, *cIAP1*, *BCL-xL*) and inhibits the proapoptotic gene *JNK*. The inhibition of RAS activation by DAB2IP leads to the activation of ASK1, whereas active RAS favors the formation of the RASSF1-NORE1A complex. Both the ASK1 and RASSF1-NORE1A complexes trigger pro-apoptotic pathways. ASK1: Apoptosis signal-regulating kinase 1; BCL-Xl: BCL2-related protein, long isoform; CDC37: Cell division cycle 37; CKS1: CDC28 protein kinase 1b; cIAP: Iinhibitor-of-apoptosis protein 1; DAB2: Disabled homolog 2; DAB2IP: DAB2-interacting protein; DUSP1: Dual-specificity phosphatase 1; EPO: Erythropoietin; ERK: Extracellular signal-regulated kinase; GLI1: Glioblastoma associated oncogene 1; HIF-1α: Hypoxia-inducible factor 1 α; HSP90: Heat shock protein 90; HXK Ⅱ: Hexokinase Ⅱ; IKK: Inhibitor of kB kinase; iNOS: Inducible nitric oxide synthase; MST1: Mammalian sterile twenty kinase 1; NEK2: NIMA-related kinase 2; NF-κB: Nuclear factor-κB; NORE1A: Novel RAS effector 1A; RASSF1A: RAS association domain family 1A; RKIP: RAF kinase inhibitory protein; SKP2: S-phase kinase-associated protein 2; VEGF-α: Vascular endothelial growth factor α; XIAP: Inhibitor of apoptosis, X-linked. Pointed and blunt arrows indicate activation and inhibition, respectively.

of human HCC.

Overall, these observations indicate that susceptible F344 rats develop various adaptive mechanisms for protection against stress-responsive tumor suppressors, such as $p16^{INK4A}$, that confer to their liver cells the ability to proliferate under stressful conditions, such as hypoxia, oxidative stress, DNA damage, abnormal conditions of growth and differentiation, inappropriate extracellular matrix, and improper cell-to-cell contracts. In the resistant BN strain, a shut off of these mechanisms, associated with cell cycle deregulation and growth inhibition, occurs in coincidence with the exhaustion of promoting stimuli.

Mitogen activated protein kinase pathway

The best characterized *RAS* effector promoting cell cycle progression is the mitogen activated protein kinase (MAPK) pathway[67] (Figure 2). Active RAS (RAS-GTP) drives the RAF1-MAPK kinase kinase 1/2 (MEK1/2)-extracellular signal-regulated kinase 1/2 (ERK1/2) cascade which mediates proliferative and survival signals and, through the binding to RAS association domain family 1A (RASSF1A) and the related protein Novel RAS effector 1A (NORE1A), may induce apoptosis[68,69]. RASSF1A and NORE1A are members of the RASSF family of *RAS* inhibitors and form homo- and hetero-dimers, which activate the mammalian sterile twenty kinase 1 (MST1 kinase), an upstream effector of the p38MAPK and JNK pathways[67-72]. Following activation, phosphorylated MST1 induces apoptosis *via* caspase-dependent and -independent mechanisms[60-72]. Furthermore, RASSF1A may inhibit the ERK pathway by its association with the plasma membrane calcium pump (PMCA) 4b protein[73]. Finally, p38MAPK may be also activated by Disabled homolog 2 (DAB2)-interacting protein (DAB2IP), a RAS-GTP inhibitor, *via* activation of Apoptosis signal-regulating kinase 1 (ASK1), which phosphorylates p38MAPK[74].

Studies on the role of the inhibitors of RAS/ERK pathway in the acquisition of a phenotype resistant or susceptible to hepatocarcinogenesis, showed moderate activation of Ras, Raf1, and Mek1/2 proteins, paralleled by strong induction of Dab2 and Raf kinase inhibitory protein (Rkip) inhibitors, in neoplastic nodules and HCC of both F344 and BN rats, induced by the "resistant hepatocyte" protocol[31]. This is compatible with the limitation of Ras-GTP-mediated activation of Raf1

due to the upregulation of the Raf1 inhibitor, Dab2[31,75] (Figure 2). Indeed, in the lesions developed in the resistant BN strain, lower Dab2 expression is associated with relatively low Ras-GTP and Raf1 expression and, consequently, low pRaf1 level[31]. Presumably, similar mechanisms may be envisaged for the inhibitory effect of Rkip1 on Mek activation[76] and determine the absence of interstrain differences in pMek1/2 expression[31]. However, the possibility that other phosphatases interfere with Raf and Mek activation, during rat liver carcinogenesis, cannot be excluded and should be the object of further research.

High levels of Dual-specificity phosphatase 1 (Dusp1), a specific Erk inhibitor (Figure 2), may be found only in neoplastic nodules and HCC of the resistant BN rat lesions, leading to modest Erk activation, whereas a progressive Dusp1 decline occurs in corresponding lesions from susceptible F344 rats and is accompanied by elevated Erk activation[31]. Interestingly, Dusp1 is slightly upregulated in preneoplastic liver of both F344 and BN strain, but its level progressively decreases in early liver nodules (12 wk after initiation), as well as in neoplastic nodules and HCC of F344 rats[31], i.e. in coincidence with the development of autonomously growing lesions in susceptible rats[25,61]. In contrast, Dusp1 further increases in the lesions of BN rats after the 12th week. This suggests that even in the presence of a limited increase in the levels of upstream activators (Raf1, Mek1/2) of Erk1/2, a failure of Dusp1 induction might sustain Erk1/2 activation and contributes to the development of autonomously growing liver lesions in F344 rats. This conclusion is further substantiated by the observed overexpression of pErk1/2 target genes, such as *Hif1-α* (hypoxia-inducible factor 1α) and *Vegf-α* (Vascular endothelial growth factor α), regulating angiogenesis, and *Hxk II*[31] (Hexokinase Ⅱ). The latter gene is a key glycolytic enzyme whose expression is correlated with *Hif1-α* mRNA, and may promote HCC development by different mechanisms, including enhanced energy production, overproduction of antiapoptotic enzymes and metabolic precursors for cell growth[77]. In BN rats, progressive increases in Dusp1 expression in preneoplastic and neoplastic lesions is associated with low expression of pErk1/2 and its target genes. This could contribute to the low propensity of BN rat lesions to progress.

The mechanisms underlying the effect of susceptibility genes on the deregulation of the inhibitors of Ras/Erk cascade in rat hepatocarcinogenesis are not yet completely known. The progressive rise in expression of the inhibitors of Ras/Erk pathway, including Dab2 and Rkip, in preneoplastic and neoplastic liver lesions of F344 rats, suggests a compensatory mechanism controlling (at least in part) Ras-GTP, pRaf1, and pMek1/2 upregulation in this rat model. Sustained Erk activation in F344 nodules and HCC promotes the phosphorylation of Dusp1 at the ser296 residue. This is followed by proteasomal degradation of Dusp1[78,79] (see below) and enhancement of Erk-driven HCC growth. The presence of *Dusp1* overexpression in late lesions of BN rats speaks in favor of a possible low proteasomal disruption of Dusp1 protein, thus resulting in a further inhibition of pErk1/2. These findings support the hypothesis that *Dusp1* is at least one of the genes involved in the acquisition of a resistant phenotype. Interestingly, *Dusp1* co-localizes with the resistance locus *Hcr1* on chromosome 10, in correspondence of the LOD score peak, a region frequently affected by loss of heterozygosis (LOH) during rat hepatocarcinogenesis[80]. Nevertheless, the existence of localization and functional plausibility does not prove *per se* that *Dusp1* is a hepatocarcinogenesis "modifier" gene[25]. *Dusp1* could be indeed controlled by modifiers of hepatocarcinogenesis. In accordance with the latter statement, the absence of functional polymorphisms at the *Dusp1* in F344 and BN rat liver tissues as detected by DNA sequencing (Feo *et al*, unpublished data) speaks against a role of *Dusp1* as a tumor modifier gene. Further work is necessary to clarify this point.

A gradual increase of Rassf1A/Nore1A/Mst1-driven apoptosis has been detected in HCC of both F344 and BN strains, with highest levels in BN HCC, whereas loss of Dab2IP (Figure 2) occurs only in F344 rat HCC[31]. These changes are associated with significantly higher apoptosis in BN than F344 HCC. Taken together, these results indicate a control of the Ras/Erk pathway, as well as of the pro-apoptotic Rassf1A/Nore1A and Dab2IP/Ask1 pathways by HCC susceptibility genes. Dusp1 possesses a prominent role in the acquisition of the phenotype resistant to HCC by BN rats, whereas late activation of RassF1A/Nore1A and Dab2IP/Ask1 axes is implicated in the highest apoptosis characteristic of BN HCC[31].

Sustained ERK activity is associated with various types of human tumors, including lung, breast, colon, pancreas, and kidney[81-83]. This frequently depends on upregulation of the RAS/MEK cascade. However, constitutive ERK overexpression may also occur independently of the RAS/MEK signaling[84,85]. Recent studies[86] indicate that prolonged activation of ERK promotes phosphorylation at the Ser296 residue of DUSP1. Phosphorylation of this specific residue renders the DUSP1 protein susceptible to ubiquitination and proteasomal degradation by the Skp1/Cul1/F-box protein SKP2 (SCFSKP2)[79]. On the other hand, constitutive ERK expression may induce SKP2/CSK1 ubiquitin ligase which can phosphorylate DUSP1 and determine its ubiquitination (Figure 2). This mechanism, which contributes to maintain elevated ERK expression, could be at least partially attributed to induction by ERK of the *FOXM1* (Forkhead box M1B)[86,87] (Figure 2) which, in turn, upregulates the SKP2/CSK1 ligase[88]. It should be noted, however, that transient activation of ERK induces the catalytic activation of DUSP1, followed by inactivation of ERK[87,88]. This body of evidence indicates that DUSP1 feedback inhibits its activation by ERK and that DUSP1 might be a crucial regulator of ERK activity in the cell. DUSP1 inactivation is frequent in prostate and urothelial tumors[89,90], and recent observations indicate that immunohistochemical

positivity for DUSP1 in human HCC is associated with longer patients' survival[91].

The interactions of DUSP1 with CKS1-SKP2 ubiquitin ligase have been recently evaluated in human HCC subtypes with different survival times, in the attempt to correlate the effects of DUSP1 molecular interactions with tumor growth and patients' survival, and explore DUSP1 prognostic role[92]. It was found that the levels of DUSP1 are significantly higher in HCCB when compared with both normal and non-tumorous surrounding livers, whereas DUSP1 protein expression sharply declines in HCCP[92]. In the latter subtype, DUSP1 inactivation is due to either ERK/CKS1/SKP2-dependent ubiquitination or promoter hypermethylation associated with loss of heterozygosity at the DUSP1 locus. Notably, expression levels of DUSP1 inversely correlate with those of activated ERK as well as with HCC proliferation index and microvessel density, and directly correlate with HCC apoptosis and patients' survival rate. Functional studies revealed that DUSP1 reactivation leads to suppression of ERK, CKS1 and SKP2 activities, inhibition of proliferation and induction of apoptosis in human hepatoma cell lines. Taken together, these data indicate that ERK achieves unrestrained activity during HCC progression by triggering ubiquitin-mediated proteolysis of its specific inhibitor DUSP1. Thus, DUSP1 may represent a valuable prognostic marker and ERK, CKS1 or SKP2 potential therapeutic targets for human HCC.

FOXM1: A pleiotropic regulator of hepatocarcinogenesis

FOXM1 transcription factor is a major downstream effector of ERK whose overexpression occurs in various experimental and human tumors[93,94]. FOXM1 promotes cell proliferation through its ability to influence various cell cycle phases. Indeed, FOXM1 triggers the activation of SKP2/CKS1 ubiquitin ligase, which targets $P21^{WAF1}$, $P27^{KIP1}$ and $P57^{KIP2}$ proteins for degradation during the G1-S transition[93-98] (Figure 2). Furthermore, FOXM1 induces transcription of genes promoting cell cycle progression (*AURKA*, *CDC2*, *CYCLIN B1*, *NEK2*, and *CDC25B*), genomic instability generators (*NEK2*, *CDC25B*), suppressors of cell cycle inhibitors (*SKP2*, *CKS1*), and apoptosis inhibitors (*SURVIVIN*)[93-97]. In the mouse liver, Foxm1 depletion results in block of proliferation and resistance to hep atocarcinogenesis[98-102]. Recently, the role of *FOXM1* during hepatocarcinogenesis has been studied in both, the susceptible/resistant comparative rat model and the human HCC (Feo *et al*, unpublished results). Activation of Foxm1 and its targets (*AurkA*, *Cdc2*, *Cyclin* B1, *Nek2*) occurs earlier and is most pronounced in liver lesions from F344 than BN rats, leading to highest Cdc2-Cyclin B1 complexes (implying highest G2-M transition) in F344 rats. In humans, FOXM1 is ubiquitously and progressively induced from surrounding non-tumorous liver to HCC, reaching highest levels in tumors with poorer prognosis and its expression levels directly correlate with proliferation index, genomic instability rate, and microvessel density, and inversely correlate with apoptosis. Interestingly, the strong correlation between FOXM1 levels and both genomic instability rate and adverse outcome in HCC agrees with the existence of a molecular signature, including *FOXM1* overexpression, which is significantly associated with degree of genomic instability and accurately predicts patients survival in multiple tumors[87,103,104].

Some reports showed *FOXM1* upregulation following either ERK or Glioblastoma associated oncogene 1 (*GLI1*) induction[105-107] (Figure 2). FOXM1 is a direct transcriptional target of *GLI1*[105,106]. GLI family proteins, including GLI1, 2, and 3, are the terminal effectors of the Hedgehog signaling[107,108]. The interaction of Sonic hedgehog (SHH) with its plasmamembrane receptor PTCH1, releases PTCH-induced inhibition of the membrane protein Smoothened (SMO). This results in GLI proteins activation and nuclear translocation, where they activate target gene transcription[109]. According to recent observations GLI2 is overexpressed in some HCC cell lines, and its inhibition by antisense oligonucleotides inhibits cell proliferation[110]. GLI1 overexpression occurs in a lower number of HCC cell lines and its inhibition causes lower decrease in growth rate[110].

High levels of phosphorylated ERK1/2 (pERK1/2) and Gli1 proteins occur in neoplastic nodules and HCC induced by the resistant hepatocyte protocol in F344 rats and, at a lower extent, in BN rats (Feo *et al*, unpublished results). Furthermore, pERK1/2 and GLI1 expression are higher in human HCC than normal and non-neoplastic surrounding livers, and most pronounced in HCCP. Silencing of either *ERK2* or *GLI1 via* siRNA in human HCC cell lines, leads to strong decreases in FOXM1 levels, whereas forced *ERK2* or *GLI1* overexpression results in a remarkable elevated rise in FOXM1 level[107]. These findings suggest a reciprocal activation of *ERK2* and *GLI1*, but the mechanisms underlying this phenomenon and its role in the activation of Hedgehog signaling are unclear and require further investigation.

Interestingly, a recent report implies the combined overexpression of HSP90 and CDC37 in sustaining elevated Fused Homolog expression[111]. Accordingly, a previous report from our laboratory showed a strong induction of HSP90 and CDC37 in F344 rat liver lesions and human HCCP[61]. Thus, it might be hypothesized a role of HSP90 and CDC37 combined activity in the highest activation of GLI1 observed in F344 neoplastic lesions and human HCCP.

The observation that FOXM1 induces SKP2 and CKS1 expression, involved in DUSP1 degradation, underlines the role of FOXM1 in the active proliferation of HCC cells, though its implication in a positive feedback loop reinforcing ERK cascade, by its ability to inhibit DUSP1[78] (Figure 2).

Inducible nitric oxide synthase signaling

Inducible nitric oxide synthase (iNOS) produces sustained nitric oxide (NO) concentrations in response to pro-inflammatory agents. NO is a major mediator of

chronic inflammation and may modulate tumorigenesis by regulating cell proliferation, survival, and migration, angiogenesis, drug resistance, and DNA repair[112,113]. In particular, iNOS might promote unrestrained cell growth *via* its ability to inactivate the retinoblastoma (pRb) pathway[114]. Some observations envisage a cross talk between iNOS and inhibitor of kB kinase (IKK)/ nuclear factor-κB (NF-κB) and RAS/ERK pathways. The Ikk and NF-κB activities are strongly reduced in *iNos* knockout mice[115]. NO activates Ha-RAS/ERK pathway in T lymphocytes[116]. Phosphorylated ERK activates iNos in melanoma[117] and *NF-κB* in HeLa cells[118].

iNOS, NF-κB, RAS, and ERK are upregulated in preneoplastic rat liver lesions[119], dysplastic and neoplastic liver from c-Myc/TGF-α transgenic mice[120], and human HCCs[121,122], and elevated NO plasma levels are present in patients with cirrhosis and HCC[123]. A recent analysis of iNos function and interactions with NF-κB and Ha-RAS/ERK signalling was performed during hepatocarcinogenesis in F344 and BN rats, possessing different genetic predisposition to HCC, and TGF-α and c-Myc-TGF-α transgenic mice, characterized by different susceptibility to HCC[124]. iNos upregulation was found always at higher levels in the most aggressive preneoplastic and neoplastic liver lesions of F344 rats and c-Myc-TGF-α transgenic mice. Moreover, the determination of iNOS expression in human HCC shows highest values in HCC with poorer prognosis[124]. The suppression of iNOS signaling by Aminoguanidine[125] in c-Myc/Tgf-α mice and human HCC cell lines results in decreased HCC growth and NF-κB and RAS/ERK expression, and increased apoptosis[124]. In contrast, NO production by Glyco-S-nitroso-N-acetyl penicillamine 2 (Glyco-Snap-2) inhibits apoptosis of *in vitro* growing human HCC cells. Conversely, the block of NF-κB signalling by Sulfasalazine[126] or siRNA, or ERK signaling by the MEK inhibitor UO126[127] causes iNOS downregulation in HCC cell lines. In transgenic mice and human HCC cell lines, iNOS anti-apoptotic effect seems to be mediated by the NF-κB cascade. The latter induces various antiapoptotic proteins, such as BCL2-related protein, long isoform (BCL-xL), Inhibitor of apoptosis, X-linked (XIAP), and Inhibitor-of-apoptosis protein 1 (cIAP1), and inhibits the proapoptotic pJNK[124]. Accordingly, iNOS suppression by Aminoguanidine triggers downregulation of NF-κB and antiapoptotic proteins, and upregulation of pJNK, in c-Myc/TGF-α and HCC cell lines. However, these findings cannot exclude the contribution of other mechanisms to the antiapoptotic action of iNOS.

Above results assign a role to iNOS upregulation in the control of the proliferative phenotype of preneoplastic and neoplastic liver cells through the activation of the IKK/NF-κB axis (Figure 2). They also imply a cross-talk between iNOS and Ha-RAS/ERK. The mechanism of NF-κB regulation by pERK1/2 is not fully understood. Recent observations show that pERK1/2 activates *AURORA-A* (AURKA), which in turn may activate NF-κB through Inhibitor of kB (IKB-α) inhibition[128]. The possibility that pERK1/2 contributes to NF-κB upregulation *via* direct activation of iNOS may also be taken into account, but requires experimental support.

The observation that the expression of iNOS and its downstream targets is highest in HCCs prone to progression both in rodents and humans, and that iNOS levels are directly correlated with genomic instability, proliferation rate and microvessel density of HCC, and inversely correlated with apoptosis and patients' survival[124], suggests that iNOS upregulation and changes in iNOS/NF-κB and iNOS/Ha-RAS/ERK cross-talks are prognostic markers for HCC. These results agree with the observation of a significant association of iNOS and Metalloproteinase-9 expression with HCC recurrence[121], and iNOS overexpression with poor prognosis for gastric cancer[129], adenoid cystic carcinoma of salivary glands[130], fibrous histiocytoma[131], colorectal cancer[132]. These iNOS effects seem to be mediated by its angiogenic properties and intensified by Cycloxygenase 2 (COX2) upregulation[133]. However, no correlation of iNOS overexpression with prognosis has been reported for pancreatic and ovarian tumors[134,135]. Furthermore, iNos ablation did not prevent hepatocarcinogenesis induced by a choline-deficient, L-aminoacid-deficient diet in mice[136], suggesting a relatively minor role of iNOS signaling. In this model of hepatocarcinogenesis, high production of lipid peroxides in hepatocyte nuclei[137] may cause DNA damage and contribute to HCC development *via* generation of genomic instability in an iNOS-independent manner.

In conclusion, iNOS overexpression contributes to growth deregulation in preneoplastic and neoplastic liver cells through a cross-talk with Ha-RAS/ERK and IKK-NF-κB axis. This does not exclude *per se* the activation of iNOS signaling by other mechanisms, such as inflammatory cytokines or the Wnt/β-catenin signaling[138]. However, the role of Wnt/β-catenin signaling in iNOS upregulation seems to be unlikely due to the observation of equal β-catenin activation (nuclear localization) in HCCs from both F344 and BN rats (M. Frau, unpublished data) expressing sharply different *iNos* mRNA levels. β-Catenin activation also occurs in a lower percentage of HCC cells from c-Myc/TGF-α than TGF-α transgenics (12% *vs* 30%)[139]. The highest iNos expression occurs in HCC from double transgenic mice.

The association of the block of iNOS signaling by a specific inhibitor such as Aminoguanidine with a consistent decrease in HCC growth and increase in apoptosis *in vitro* indicates that the key component of this pathway could represent therapeutic targets that may contribute to create networked biological therapies[140]. Thus, determination of iNOS immunoreactivity status can be proposed as a promising candidate for the identification of high risk patients who may benefit from new anticancer drugs targeting iNOS and its interplay with IKK/NF-κB and Ha-RAS/ERK signalling.

CONCLUSION

Studies on mouse and rat models of hepatocarcinogenesis have shown the existence of a great complexity of the inherited predisposition to liver cancer, and support a model based on the polygenic inheritance of low penetrance genes, with several gene-gene interactions and a main susceptibility locus (i.e. *Hcs7*, *Hpcr3* for mice, and *Hcs3*/*Hcr5* for rats)[25]. A complex combination and interplay of susceptibility or resistance alleles determines the individual risk. It has been proposed that the polymorphic variants of these "cancer modifier" genes can foster phenotypic expression of previously unexpressed alleles with consequent positive or negative influences on cell growth and differentiation[25]. Thus, the type of influence of modifiers on the carcinogenesis process may largely depend on interindividual differences in gene polymorphism, gene-gene interactions and gene-environment interactions. Epidemiologic and segregation studies strongly suggest a similar genetic model for the inherited predisposition to human HCC[11-14]. Taking into account the effect of susceptibility/resistance genes on the proliferative activity and re-differentiation of initiated cells, it may be expected that the genetic substrate largely influences the prognosis.

The mechanisms underlying the acquisition of the resistant phenotype have not been completely defined, as yet. A lower genomic instability of liver lesions developing in the resistant animals compared to the susceptible strains has been documented[80,141,142]. This may be tentatively attributed to interstrain differences in the activity of care taker and DNA repair genes, resulting in the prevention of the accumulation of DNA damage by initiated cells of the resistant rat strains. This hypothesis, however, needs experimental support. Nevertheless, accumulating evidence indicates that the predominance of susceptibility or resistance genes in individuals can largely influence the molecular control of cell proliferation and cell death. The susceptible rats develop various adaptive mechanisms for protection against stress-responsive tumor suppressors, such as $p16^{INK4A}$, that confer to their liver cells the ability to proliferate under stressful conditions. In the resistant BN strain, a shut off of these mechanisms, associated with strong cell cycle deregulation and growth inhibition, occurs in coincidence with the exhaustion of promoting stimuli.

Among the effectors promoting cell cycle progression, ERK achieves unrestrained activity during HCC progression by triggering ubiquitin-mediated proteolysis of its specific inhibitor DUSP1. This mechanism is much more active in neoplastic nodules and HCC of susceptible rats than in the lesions of the resistant rats. The observation that FOXM1 induces the expression of CKS1-SKP2 ligase, involved in DUSP1 degradation, underlines the role of FOXM1 in the active proliferation of HCC cells, though its implication in a positive feedback loop reinforcing ERK cascade, by its ability to inhibit DUSP1[77]. These data indicate that FOXM1 upregulation is associated with the acquisition of a susceptible phenotype in rats and may influence human HCC development and prognosis. A role in the control of the proliferative phenotype of preneoplastic and neoplastic liver cells has been also assigned to higher iNos upregulation, inducing higher activation of the IKK/NF-κB and Ha-RAS/ERK signaling, in neoplastic nodules and HCCs of susceptible than resistant rats[124]. In the latter rats, HCC growth is also contrasted by relatively high cell death by apoptosis, which, at least in part depends on the activation of pro-apoptotic Rassf1A/Nore1A and Dab2IP/Ask1 pathways. Thus, Dusp1 possesses a prominent role in the acquisition by BN rats of a phenotype resistant to HCC. Late activation of RassF1A/Nore1A and Dab2IP/Ask1 axes is implicated in the highest apoptosis of BN HCC.

Importantly, most of the alterations responsible for the acquisition of a resistant or susceptible phenotype by rats have also been found in human HCC with better of poorer prognosis. A link between fast growth and signaling deregulation characterizes human HCC with poor prognosis, whereas the behaviour of HCC with better prognosis is more similar to that of the lesions of resistant rats. This does not necessarily imply a genetic regulation of signaling pathways in humans like that found in rodents. Further studies are needed to clarify the influence of susceptibility genes on signaling pathways supporting tumor growth and progression in humans.

The study of signal transduction pathways in rats differently predisposed to HCC development and prone to HCC progression, allowed the identification of numerous potential prognostic markers of hepatocarcinogenesis, such as the cell cycle protective mechanisms against $p16^{INK4A}$, represented by CDC37-HSP90 complex and CRM1 protein, the ERK inhibitor DUSP1, iNOS, FOXM1. The prognostic role of these proteins was confirmed by analyzing the correlation of their expression with clinicopathological parameters of human HCC[91,124]. They therefore represent promising candidates for the identification of high risk patients who may benefit from new anticancer drugs against key components of signaling pathways.

Future work should focus on HCC prevention obtained by blocking key compounds of signal transduction network. Early blockage of signaling pathways may result in more efficient prevention, and rodent models may be useful to identify progression markers and therapeutic targets in early stages of the process, and in a large number of HCC subtypes. The study of experimental models recapitulating early preneoplastic alterations of human liver carcinogenesis may lead to the discovery of biomarkers of the risk of cirrhosis evolution to full malignancy, as well as of new key genes and signal transduction pathways involved in hepatocarcinogenesis. The existence of numerous interspecies commonalties in the biological behaviour and molecular changes of preneoplastic and neoplastic liver lesions[1-3,15,25] underlines the usefulness of this approach. The complexity of molecular changes of HCC predicts the impossibility to cure HCC

development by interfering with only one signaling pathway. To overcome this difficulty and the occurrence of resistance to therapy, networked biologic therapies have been proposed[140,143,144] in which a combination of non-cytotoxic interventions must be performed to interrupt the damage. These interventions may be directed to interfere with different cell survival pathways, enhance apoptosis, block angiogenesis and extrahepatic fibrosis, induce the lysis of tumor cells, stimulate antitumor immunity, decrease HBV and HCV replication, *etc*. Furthermore, the combination of gene therapy with conventional therapeutic approaches with cytotoxic drugs may improve the treatment and reduce the doses of toxic compounds.

REFERENCES

1 **Bruix J**, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. *Cancer Cell* 2004; **5**: 215-219
2 **Thorgeirsson SS**, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002; **31**: 339-346
3 **Farazi PA**, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer* 2006; **6**: 674-687
4 **Tanaka Y**, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, Alter HJ. Inaugural Article: A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci USA* 2002; **99**: 15584-15589
5 **Indulski JA**, Lutz W. Metabolic genotype in relation to individual susceptibility to environmental carcinogens. *Int Arch Occup Environ Health* 2000; **73**: 71-85
6 **Kato S**, Tajiri T, Matsukura N, Matsuda N, Taniai N, Mamada H, Yoshida H, Kiyam T, Naito Z. Genetic polymorphisms of aldehyde dehydrogenase 2, cytochrome p450 2E1 for liver cancer risk in HCV antibody-positive japanese patients and the variations of CYP2E1 mRNA expression levels in the liver due to its polymorphism. *Scand J Gastroenterol* 2003; **38**: 886-893
7 **Agundez JA**, Olivera M, Ladero JM, Rodriguez-Lescure A, Ledesma MC, Diaz-Rubio M, Meyer UA, Benitez J. Increased risk for hepatocellular carcinoma in NAT2-slow acetylators and CYP2D6-rapid metabolizers. *Pharmacogenetics* 1996; **6**: 501-512
8 **Hsieh LL**, Huang RC, Yu MW, Chen CJ, Liaw YF. L-myc, GST M1 genetic polymorphism and hepatocellular carcinoma risk among chronic hepatitis B carriers. *Cancer Lett* 1996; **103**: 171-176
9 **Taylor JA**, Bell DA, Nagorney D. L-myc proto-oncogene alleles and susceptibility to hepatocellular carcinoma. *Int J Cancer* 1993; **54**: 927-930
10 **Sonzogni L**, Silvestri L, De Silvestri A, Gritti C, Foti L, Zavaglia C, Bottelli R, Mondelli MU, Civardi E, Silini EM. Polymorphisms of microsomal epoxide hydrolase gene and severity of HCV-related liver disease. *Hepatology* 2002; **36**: 195-201
11 **Yu MW**, Chang HC, Liaw YF, Lin SM, Lee SD, Liu CJ, Chen PJ, Hsiao TJ, Lee PH, Chen CJ. Familial risk of hepatocellular carcinoma among chronic hepatitis B carriers and their relatives. *J Natl Cancer Inst* 2000; **92**: 1159-1164
12 **Cai RL**, Meng W, Lu HY, Lin WY, Jiang F, Shen FM. Segregation analysis of hepatocellular carcinoma in a moderately high-incidence area of East China. *World J Gastroenterol* 2003; **9**: 2428-2432
13 **Fernandez E**, La Vecchia C, D'Avanzo B, Negri E, Franceschi S. Family history and the risk of liver, gallbladder, and pancreatic cancer. *Cancer Epidemiol Biomarkers Prev* 1994; **3**: 209-212
14 **Hemminki K**, Li X. Familial risks of cancer as a guide to gene identification and mode of inheritance. *Int J Cancer* 2004; **110**: 291-294
15 **Feo F**, Pascale RM, Simile MM, De Miglio MR, Muroni MR, Calvisi D. Genetic alterations in liver carcinogenesis: implications for new preventive and therapeutic strategies. *Crit Rev Oncog* 2000; **11**: 19-62
16 **Lee JS**, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, Thorgeirsson SS. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. *Nat Genet* 2004; **36**: 1306-1311
17 **Rebouissou S**, Bioulac-Sage P, Zucman-Rossi J. Molecular pathogenesis of focal nodular hyperplasia and hepatocellular adenoma. *J Hepatol* 2008; **48**: 163-170
18 **Korstanje R**, Paigen B. From QTL to gene: the harvest begins. *Nat Genet* 2002; **31**: 235-236
19 **Farber E**, Sarma DS. Hepatocarcinogenesis: a dynamic cellular perspective. *Lab Invest* 1987; **56**: 4-22
20 **Garcea R**, Daino L, Pascale R, Simile MM, Puddu M, Frassetto S, Cozzolino P, Seddaiu MA, Gaspa L, Feo F. Inhibition of promotion and persistent nodule growth by S-adenosyl-L-methionine in rat liver carcinogenesis: role of remodeling and apoptosis. *Cancer Res* 1989; **49**: 1850-1856
21 **Zhou XD**. Recurrence and metastasis of hepatocellular carcinoma: progress and prospects. *Hepatobiliary Pancreat Dis Int* 2002; **1**: 35-41
22 **Dragani TA**, Manenti G, Della Porta G. Genetic susceptibility to murine hepatocarcinogenesis is associated with high growth rate of NDEA-initiated hepatocytes. *J Cancer Res Clin Oncol* 1987; **113**: 223-229
23 **Manenti G**, Binelli G, Gariboldi M, Canzian F, De Gregorio L, Falvella FS, Dragani TA, Pierotti MA. Multiple loci affect genetic predisposition to hepatocarcinogenesis in mice. *Genomics* 1994; **23**: 118-124
24 **Denda A**, Kitayama W, Konishi Y, Yan Y, Fukamachi Y, Miura M, Gotoh S, Ikemura K, Abe T, Higashi T, Higashi K. Genetic properties for the suppression of development of putative preneoplastic glutathione S-transferase placental form-positive foci in the liver of carcinogen-resistant DRH strain rats. *Cancer Lett* 1999; **140**: 59-67
25 **Feo F**, De Miglio MR, Simile MM, Muroni MR, Calvisi DF, Frau M, Pascale RM. Hepatocellular carcinoma as a complex polygenic disease. Interpretive analysis of recent developments on genetic predisposition. *Biochim Biophys Acta* 2006; **1765**: 126-147
26 **Pascale RM**, Simile MM, DeMiglio MR, Muroni MR, Gaspa L, Dragani TA, Feo F. The BN rat strain carries dominant hepatocarcinogen resistance loci. *Carcinogenesis* 1996; **17**: 1765-1768
27 **Wood GA**, Korkola JE, Lee VM, Sarma DS, Archer MC. Resistance of Copenhagen rats to chemical induction of glutathione S-transferase 7-7-positive liver foci. *Carcinogenesis* 1997; **18**: 1745-1750
28 **Wood GA**, Sarma DS, Archer MC. Resistance to the promotion of glutathione S-transferase 7-7-positive liver lesions in Copenhagen rats. *Carcinogenesis* 1999; **20**: 1169-1175
29 **Enomoto K**, Farber E. Kinetics of phenotypic maturation of remodeling of hyperplastic nodules during liver carcinogenesis. *Cancer Res* 1982; **42**: 2330-2335
30 **Wood GA**, Sarma DS, Archer MC. Inheritance of resistance to promotion of preneoplastic liver lesions in Copenhagen rats. *Exp Biol Med* (Maywood) 2001; **226**: 831-835
31 **Calvisi DF**, Pinna F, Pellegrino R, Sanna V, Sini M, Daino L, Simile MM, De Miglio MR, Frau M, Tomasi ML, Seddaiu MA, Muroni MR, Feo F, Pascale RM. Ras-driven proliferation and apoptosis signaling during rat liver carcinogenesis is under genetic control. *Int J Cancer* 2008; **123**: 2057-2064
32 **Liu H**, Higashi K, Hiai H. Role of resistant Drh1 locus in chemical carcinogen-induced hepatocarcinogenesis in rats:

analysis with a speed congenic strain. *Cancer Sci* 2005; **96**: 164-169

33 **Gariboldi M**, Manenti G, Canzian F, Falvella FS, Pierotti MA, Della Porta G, Binelli G, Dragani TA. Chromosome mapping of murine susceptibility loci to liver carcinogenesis. *Cancer Res* 1993; **53**: 209-211

34. **G. Manenti,** G. Binelli, M. Gariboldi, F. Canzian, L. De Gregorio, F.S. Falvella, T.A. Dragani, M.A. Pierotti, Multiple loci affect genetic predisposition to hepatocarcinogenesis in mice, *Genomics* 1994; **23:** 118-124.

35 **Bilger A**, Bennett LM, Carabeo RA, Chiaverotti TA, Dvorak C, Liss KM, Schadewald SA, Pitot HC, Drinkwater NR. A potent modifier of liver cancer risk on distal mouse chromosome 1: linkage analysis and characterization of congenic lines. *Genetics* 2004; **167**: 859-866

36 **Poole TM**, Drinkwater NR. Two genes abrogate the inhibition of murine hepatocarcinogenesis by ovarian hormones. *Proc Natl Acad Sci USA* 1996; **93**: 5848-5853

37 **Lee GH**, Bennett LM, Carabeo RA, Drinkwater NR. Identification of hepatocarcinogen-resistance genes in DBA/2 mice. *Genetics* 1995; **139**: 387-395

38 **Lee GH**, Drinkwater NR. The Hcr (hepatocarcinogen resistance) loci of DBA/2J mice partially suppress phenotypic expression of the Hcs (hepatocarcinogen sensitivity) loci of C3H/HeJ mice. *Carcinogenesis* 1995; **16**: 1993-1996

39 **Dragani TA**, Manenti G, Della Porta G. Quantitative analysis of genetic susceptibility to liver and lung carcinogenesis in mice. *Cancer Res* 1991; **51**: 6299-6303

40 **Manenti G**, Galvan A, Falvella FS, Pascale RM, Spada E, Milani S, Gonzalez Neira A, Feo F, Dragani TA. Genetic control of resistance to hepatocarcinogenesis by the mouse Hpcr3 locus. *Hepatology* 2008; **48**: 617-623

41 **Melhem MF**, Kunz HW, Gill TJ 3rd. A major histocompatibility complex-linked locus in the rat critically influences resistance to diethylnitrosamine carcinogenesis. *Proc Natl Acad Sci USA* 1993; **90**: 1967-1971

42 **Rao KN**, Shinozuka H, Kunz HW, Gill TJ 3rd. Enhanced susceptibility to a chemical carcinogen in rats carrying MHC-linked genes influencing development (GRC). *Int J Cancer* 1984; **34**: 113-120

43 **De Miglio MR**, Canzian F, Pascale RM, Simile MM, Muroni MR, Calvisi D, Romeo G, Feo F. Identification of genetic loci controlling hepatocarcinogenesis on rat chromosomes 7 and 10. *Cancer Res* 1999; **59**: 4651-4657

44 **De Miglio MR**, Pascale RM, Simile MM, Muroni MR, Calvisi DF, Virdis P, Bosinco GM, Frau M, Seddaiu MA, Ladu S, Feo F. Chromosome mapping of multiple loci affecting the genetic predisposition to rat liver carcinogenesis. *Cancer Res* 2002; **62**: 4459-4463

45 **De Miglio MR**, Pascale RM, Simile MM, Muroni MR, Virdis P, Kwong KM, Wong LK, Bosinco GM, Pulina FR, Calvisi DF, Frau M, Wood GA, Archer MC, Feo F. Polygenic control of hepatocarcinogenesis in Copenhagen x F344 rats. *Int J Cancer* 2004; **111**: 9-16

46 **Zeng ZZ**, Higashi S, Kitayama W, Denda A, Yan Y, Matsuo K, Konishi Y, Hiai H, Higashi K. Genetic resistance to chemical carcinogen-induced preneoplastic hepatic lesions in DRH strain rats. *Cancer Res* 2000; **60**: 2876-2881

47 **Yan Y**, Zeng ZZ, Higashi S, Denda A, Konishi Y, Onishi S, Ueno H, Higashi K, Hiai H. Resistance of DRH strain rats to chemical carcinogenesis of liver: genetic analysis of later progression stage. *Carcinogenesis* 2002; **23**: 189-196

48 **Fujiwara Y**, Monden M, Mori T, Nakamura Y, Emi M. Frequent multiplication of the long arm of chromosome 8 in hepatocellular carcinoma. *Cancer Res* 1993; **53**: 857-860

49 **Wong N**, Lai P, Lee SW, Fan S, Pang E, Liew CT, Sheng Z, Lau JW, Johnson PJ. Assessment of genetic changes in hepatocellular carcinoma by comparative genomic hybridization analysis: relationship to disease stage, tumor size, and cirrhosis. *Am J Pathol* 1999; **154**: 37-43

50 **Okamoto H**, Yasui K, Zhao C, Arii S, Inazawa J. PTK2 and EIF3S3 genes may be amplification targets at 8q23-q24 and are associated with large hepatocellular carcinomas. *Hepatology* 2003; **38**: 1242-1249

51 **Ogawa K**, Osanai M, Obata M, Ishizaki K, Kamiya K. Gain of chromosomes 15 and 19 is frequent in both mouse hepatocellular carcinoma cell lines and primary tumors, but loss of chromosomes 4 and 12 is detected only in the cell lines. *Carcinogenesis* 1999; **20**: 2083-2088

52 **De Miglio MR**, Simile MM, Muroni MR, Calvisi DF, Virdis P, Asara G, Frau M, Bosinco GM, Seddaiu MA, Daino L, Feo F, Pascale RM. Phenotypic reversion of rat neoplastic liver nodules is under genetic control. *Int J Cancer* 2003; **105**: 70-75

53 **Chang WY**. Complete spontaneous regression of cancer: four case reports, review of literature, and discussion of possible mechanisms involved. *Hawaii Med J* 2000; **59**: 379-387

54 **De Miglio MR**, Virdis P, Calvisi DF, Frau M, Muroni MR, Simile MM, Daino L, Careddu GM, Sanna-Passino E, Pascale RM, Feo F. Mapping a sex hormone-sensitive gene determining female resistance to liver carcinogenesis in a congenic F344.BN-Hcs4 rat. *Cancer Res* 2006; **66**: 10384-10390

55 **Shachaf CM**, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, Bachmann MH, Borowsky AD, Ruebner B, Cardiff RD, Yang Q, Bishop JM, Contag CH, Felsher DW. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature* 2004; **431**: 1112-1117

56 **Simile MM**, De Miglio MR, Muroni MR, Frau M, Asara G, Serra S, Muntoni MD, Seddaiu MA, Daino L, Feo F, Pascale RM. Down-regulation of c-myc and Cyclin D1 genes by antisense oligodeoxy nucleotides inhibits the expression of E2F1 and in vitro growth of HepG2 and Morris 5123 liver cancer cells. *Carcinogenesis* 2004; **25**: 333-341

57 **Garcea R**, Daino L, Pascale R, Simile MM, Puddu M, Ruggiu ME, Seddaiu MA, Satta G, Sequenza MJ, Feo F. Protooncogene methylation and expression in regenerating liver and preneoplastic liver nodules induced in the rat by diethylnitrosamine: effect of variations of S-adenosylmethionine:S-adenosylhomocysteine ratio. *Carcinogenesis* 1989; **10**: 1183-1192

58 **Pascale RM**, Marras V, Simile MM, Daino L, Pinna G, Bennati S, Carta M, Seddaiu MA, Massarelli G, Feo F. Chemoprevention of rat liver carcinogenesis by S-adenosyl-L-methionine: a long-term study. *Cancer Res* 1992; **52**: 4979-4986

59 **Pascale RM**, Simile MM, De Miglio MR, Muroni MR, Calvisi DF, Asara G, Casabona D, Frau M, Seddaiu MA, Feo F. Cell cycle deregulation in liver lesions of rats with and without genetic predisposition to hepatocarcinogenesis. *Hepatology* 2002; **35**: 1341-1350

60 **De Miglio MR**, Simile MM, Muroni MR, Pusceddu S, Calvisi D, Carru A, Seddaiu MA, Daino L, Deiana L, Pascale RM, Feo F. Correlation of c-myc overexpression and amplification with progression of preneoplastic liver lesions to malignancy in the poorly susceptible Wistar rat strain. *Mol Carcinog* 1999; **25**: 21-29

61 **Pascale RM**, Simile MM, Calvisi DF, Frau M, Muroni MR, Seddaiu MA, Daino L, Muntoni MD, De Miglio MR, Thorgeirsson SS, Feo F. Role of HSP90, CDC37, and CRM1 as modulators of P16(INK4A) activity in rat liver carcinogenesis and human liver cancer. *Hepatology* 2005; **42**: 1310-1319

62 **Hunter T**, Poon RY. Cdc37: a protein kinase chaperone? *Trends Cell Biol* 1997; **7**: 157-161

63 **Stepanova L**, Leng X, Parker SB, Harper JW. Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. *Genes Dev* 1996; **10**: 1491-1502

64 **Dai K**, Kobayashi R, Beach D. Physical interaction of mammalian CDC37 with CDK4. *J Biol Chem* 1996; **271**: 22030-22034

65 **Crosby ME**, Almasan A. Opposing roles of E2Fs in cell proliferation and death. *Cancer Biol Ther* 2004; **3**: 1208-1211

66 **Ohtani N**, Brennan P, Gaubatz S, Sanij E, Hertzog P, Wolvetang E, Ghysdael J, Rowe M, Hara E. Epstein-Barr virus LMP1 blocks p16INK4a-RB pathway by promoting nuclear export of E2F4/5. *J Cell Biol* 2003; **162**: 173-183

67 **Marshall CJ**. Ras effectors. *Curr Opin Cell Biol* 1996; **8**: 197-204

68 **Feig LA**, Buchsbaum RJ. Cell signaling: life or death decisions of ras proteins. *Curr Biol* 2002; **12**: R259-R261

69 **Cox AD**, Der CJ. The dark side of Ras: regulation of apoptosis. *Oncogene* 2003; **22**: 8999-9006

70 **Johnston AM**, Naselli G, Gonez LJ, Martin RM, Harrison LC, DeAizpurua HJ. SPAK, a STE20/SPS1-related kinase that activates the p38 pathway. *Oncogene* 2000; **19**: 4290-4297

71 **Ura S**, Masuyama N, Graves JD, Gotoh Y. MST1-JNK promotes apoptosis via caspase-dependent and independent pathways. *Genes Cells* 2001; **6**: 519-530

72 **Khokhlatchev A**, Rabizadeh S, Xavier R, Nedwidek M, Chen T, Zhang XF, Seed B, Avruch J. Identification of a novel Ras-regulated proapoptotic pathway. *Curr Biol* 2002; **12**: 253-265

73 **Armesilla AL**, Williams JC, Buch MH, Pickard A, Emerson M, Cartwright EJ, Oceandy D, Vos MD, Gillies S, Clark GJ, Neyses L. Novel functional interaction between the plasma membrane Ca2+ pump 4b and the proapoptotic tumor suppressor Ras-associated factor 1 (RASSF1). *J Biol Chem* 2004; **279**: 31318-31328

74 **Guicciardi ME**, Gores GJ. AIP1: a new player in TNF signaling. *J Clin Invest* 2003; **111**: 1813-1815

75 **Xu XX**, Yi T, Tang B, Lambeth JD. Disabled-2 (Dab2) is an SH3 domain-binding partner of Grb2. *Oncogene* 1998; **16**: 1561-1569

76 **Yeung K**, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, Fee F, Katsanakis KD, Rose DW, Mischak H, Sedivy JM, Kolch W. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature* 1999; **401**: 173-177

77 **Pedersen PL**, Mathupala S, Rempel A, Geschwind JF, Ko YH. Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. *Biochim Biophys Acta* 2002; **1555**: 14-20

78 **Lin YW**, Yang JL. Cooperation of ERK and SCFSkp2 for MKP-1 destruction provides a positive feedback regulation of proliferating signaling. *J Biol Chem* 2006; **281**: 915-926

79 **Wong J**, Zhang J, Si X, Gao G, Luo H. Inhibition of the extracellular signal-regulated kinase signaling pathway is correlated with proteasome inhibitor suppression of coxsackievirus replication. *Biochem Biophys Res Commun* 2007; **358**: 903-907

80 **De Miglio MR**, Muroni MR, Simile MM, Virdis P, Asara G, Frau M, Calvisi DF, Seddaiu MA, Pascale RM, Feo F. Frequent loss of heterozygosity at the Hcr1 (hepatocarcinogenesis resistance) locus on chromosome 10 in primary hepatocellular carcinomas from LFF1 rat strain. *Hepatology* 2001; **33**: 1110-1117

81 **Wang HY**, Cheng Z, Malbon CC. Overexpression of mitogen-activated protein kinase phosphatases MKP1, MKP2 in human breast cancer. *Cancer Lett* 2003; **191**: 229-237

82 **Bang YJ**, Kwon JH, Kang SH, Kim JW, Yang YC. Increased MAPK activity and MKP-1 overexpression in human gastric adenocarcinoma. *Biochem Biophys Res Commun* 1998; **250**: 43-47

83 **Hoshino R**, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, Shimada Y, Ari-i S, Wada H, Fujimoto J, Kohno M. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* 1999; **18**: 813-822

84 **Grammer TC**, Blenis J. Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. *Oncogene* 1997; **14**: 1635-1642

85 **Barry OP**, Mullan B, Sheehan D, Kazanietz MG, Shanahan F, Collins JK, O'Sullivan GC. Constitutive ERK1/2 activation in esophagogastric rib bone marrow micrometastatic cells is MEK-independent. *J Biol Chem* 2001; **276**: 15537-15546

86 **Frescas D**, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nat Rev Cancer* 2008; **8**: 438-449

87 **Major ML**, Lepe R, Costa RH. Forkhead box M1B transcriptional activity requires binding of Cdk-cyclin complexes for phosphorylation-dependent recruitment of p300/CBP coactivators. *Mol Cell Biol* 2004; **24**: 2649-2661

88 **Wang IC**, Chen YJ, Hughes D, Petrovic V, Major ML, Park HJ, Tan Y, Ackerson T, Costa RH. Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. *Mol Cell Biol* 2005; **25**: 10875-10894

89 **Rauhala HE**, Porkka KP, Tolonen TT, Martikainen PM, Tammela TL, Visakorpi T. Dual-specificity phosphatase 1 and serum/glucocorticoid-regulated kinase are downregulated in prostate cancer. *Int J Cancer* 2005; **117**: 738-745

90 **Shimada K**, Nakamura M, Ishida E, Higuchi T, Tanaka M, Ota I, Konishi N. c-Jun NH2 terminal kinase activation and decreased expression of mitogen-activated protein kinase phosphatase-1 play important roles in invasion and angiogenesis of urothelial carcinomas. *Am J Pathol* 2007; **171**: 1003-1012

91 **Tsujita E**, Taketomi A, Gion T, Kuroda Y, Endo K, Watanabe A, Nakashima H, Aishima S, Kohnoe S, Maehara Y. Suppressed MKP-1 is an independent predictor of outcome in patients with hepatocellular carcinoma. *Oncology* 2005; **69**: 342-347

92 **Calvisi DF**, Pinna F, Meloni F, Ladu S, Pellegrino R, Sini M, Daino L, Simile MM, De Miglio MR, Virdis P, Frau M, Tomasi ML, Seddaiu MA, Muroni MR, Feo F, Pascale RM. Dual-specificity phosphatase 1 ubiquitination in extracellular signal-regulated kinase-mediated control of growth in human hepatocellular carcinoma. *Cancer Res* 2008; **68**: 4192-4200

93 **Kim IM**, Ackerson T, Ramakrishna S, Tretiakova M, Wang IC, Kalin TV, Major ML, Gusarova GA, Yoder HM, Costa RH, Kalinichenko VV. The Forkhead Box m1 transcription factor stimulates the proliferation of tumor cells during development of lung cancer. *Cancer Res* 2006; **66**: 2153-2161

94 **Costa RH**, Kalinichenko VV, Major ML, Raychaudhuri P. New and unexpected: forkhead meets ARF. *Curr Opin Genet Dev* 2005; **15**: 42-48

95 **Wierstra I**, Alves J. FOXM1, a typical proliferation-associated transcription factor. *Biol Chem* 2007; **388**: 1257-1274

96 **Yoshida Y**, Wang IC, Yoder HM, Davidson NO, Costa RH. The forkhead box M1 transcription factor contributes to the development and growth of mouse colorectal cancer. *Gastroenterology* 2007; **132**: 1420-1431

97 **Wang Z**, Banerjee S, Kong D, Li Y, Sarkar FH. Down-regulation of Forkhead Box M1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. *Cancer Res* 2007; **67**: 8293-8300

98 **Zhao YY**, Gao XP, Zhao YD, Mirza MK, Frey RS, Kalinichenko VV, Wang IC, Costa RH, Malik AB. Endothelial cell-restricted disruption of FoxM1 impairs endothelial repair following LPS-induced vascular injury. *J Clin Invest* 2006; **116**: 2333-2343

99 **Laoukili J**, Kooistra MR, Bras A, Kauw J, Kerkhoven RM, Morrison A, Clevers H, Medema RH. FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nat Cell Biol* 2005; **7**: 126-136

100 **Wang X**, Kiyokawa H, Dennewitz MB, Costa RH. The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. *Proc Natl Acad Sci USA* 2002; **99**: 16881-16886

101 **Krupczak-Hollis K**, Wang X, Dennewitz MB, Costa RH. Growth hormone stimulates proliferation of old-aged regenerating liver through forkhead box m1b. *Hepatology* 2003; **38**: 1552-1562

102 **Kalinichenko VV**, Major ML, Wang X, Petrovic V, Kuechle J, Yoder HM, Dennewitz MB, Shin B, Datta A, Raychaudhuri P, Costa RH. Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor. *Genes Dev* 2004; **18**: 830-850

103 **Bektas N**, Haaf A, Veeck J, Wild PJ, Luscher-Firzlaff J, Hartmann A, Knuchel R, Dahl E. Tight correlation between expression of the Forkhead transcription factor FOXM1 and HER2 in human breast cancer. *BMC Cancer* 2008; **8**: 42

104 **Carter SL**, Eklund AC, Kohane IS, Harris LN, Szallasi Z. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nat Genet* 2006; **38**: 1043-1048

105 **Katoh Y**, Katoh M. Hedgehog signaling pathway and gastric cancer. *Cancer Biol Ther* 2005; **4**: 1050-1054

106 **Teh MT**, Wong ST, Neill GW, Ghali LR, Philpott MP, Quinn AG. FOXM1 is a downstream target of Gli1 in basal cell carcinomas. *Cancer Res* 2002; **62**: 4773-4780

107 **Pasca di Magliano M**, Hebrok M. Hedgehog signalling in cancer formation and maintenance. *Nat Rev Cancer* 2003; **3**: 903-911

108 **Kasper M**, Regl G, Frischauf AM, Aberger F. GLI transcription factors: mediators of oncogenic Hedgehog signalling. *Eur J Cancer* 2006; **42**: 437-445

109 **Crompton T**, Outram SV, Hager-Theodorides AL. Sonic hedgehog signalling in T-cell development and activation. *Nat Rev Immunol* 2007; **7**: 726-735

110 **Kim Y**, Yoon JW, Xiao X, Dean NM, Monia BP, Marcusson EG. Selective down-regulation of glioma-associated oncogene 2 inhibits the proliferation of hepatocellular carcinoma cells. *Cancer Res* 2007; **67**: 3583-3593

111 **Kise Y**, Takenaka K, Tezuka T, Yamamoto T, Miki H. Fused kinase is stabilized by Cdc37/Hsp90 and enhances Gli protein levels. *Biochem Biophys Res Commun* 2006; **351**: 78-84

112 **Lasagna N**, Fantappie O, Solazzo M, Morbidelli L, Marchetti S, Cipriani G, Ziche M, Mazzanti R. Hepatocyte growth factor and inducible nitric oxide synthase are involved in multidrug resistance-induced angiogenesis in hepatocellular carcinoma cell lines. *Cancer Res* 2006; **66**: 2673-2682

113 **Hussain SP**, Harris CC. Inflammation and cancer: an ancient link with novel potentials. *Int J Cancer* 2007; **121**: 2373-2380

114 **Ying L**, Hofseth AB, Browning DD, Nagarkatti M, Nagarkatti PS, Hofseth LJ. Nitric oxide inactivates the retinoblastoma pathway in chronic inflammation. *Cancer Res* 2007; **67**: 9286-9293

115 **Zingarelli B**, Hake PW, Yang Z, O'Connor M, Denenberg A, Wong HR. Absence of inducible nitric oxide synthase modulates early reperfusion-induced NF-kappaB and AP-1 activation and enhances myocardial damage. *FASEB J* 2002; **16**: 327-342

116 **Deora AA**, Hajjar DP, Lander HM. Recruitment and activation of Raf-1 kinase by nitric oxide-activated Ras. *Biochemistry* 2000; **39**: 9901-9908

117 **Ellerhorst JA**, Ekmekcioglu S, Johnson MK, Cooke CP, Johnson MM, Grimm EA. Regulation of iNOS by the p44/42 mitogen-activated protein kinase pathway in human melanoma. *Oncogene* 2006; **25**: 3956-3962

118 **Zhao Q**, Lee FS. Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase-beta. *J Biol Chem* 1999; **274**: 8355-8358

119 **Simile MM**, Pagnan G, Pastorino F, Brignole C, De Miglio MR, Muroni MR, Asara G, Frau M, Seddaiu MA, Calvisi DF, Feo F, Ponzoni M, Pascale RM. Chemopreventive N-(4-hydroxyphenyl)retinamide (fenretinide) targets deregulated NF-{kappa}B and Mat1A genes in the early stages of rat liver carcinogenesis. *Carcinogenesis* 2005; **26**: 417-427

120 **Calvisi DF**, Ladu S, Hironaka K, Factor VM, Thorgeirsson SS. Vitamin E down-modulates iNOS and NADPH oxidase in c-Myc/TGF-alpha transgenic mouse model of liver cancer. *J Hepatol* 2004; **41**: 815-822

121 **Sun MH**, Han XC, Jia MK, Jiang WD, Wang M, Zhang H, Han G, Jiang Y. Expressions of inducible nitric oxide synthase and matrix metalloproteinase-9 and their effects on angiogenesis and progression of hepatocellular carcinoma. *World J Gastroenterol* 2005; **11**: 5931-5937

122 **Ikeguchi M**, Ueta T, Yamane Y, Hirooka Y, Kaibara N. Inducible nitric oxide synthase and survivin messenger RNA expression in hepatocellular carcinoma. *Clin Cancer Res* 2002; **8**: 3131-3136

123 **Moriyama A**, Masumoto A, Nanri H, Tabaru A, Unoki H, Imoto I, Ikeda M, Otsuki M. High plasma concentrations of nitrite/nitrate in patients with hepatocellular carcinoma. *Am J Gastroenterol* 1997; **92**: 1520-1523

124 **Calvisi DF**, Pinna F, Ladu S, Pellegrino R, Muroni MR, Simile MM, Frau M, Tomasi ML, De Miglio MR, Seddaiu MA, Daino L, Sanna V, Feo F, Pascale RM. Aberrant iNOS signaling is under genetic control in rodent liver cancer and potentially prognostic for the human disease. *Carcinogenesis* 2008; **29**: 1639-1647

125 **Misko TP**, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, McDaniel ML, Williamson JR, Currie MG. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur J Pharmacol* 1993; **233**: 119-125

126 **Greten FR**, Karin M. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. *Cancer Lett* 2004; **206**: 193-199

127 **Favata MF**, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 1998; **273**: 18623-18632

128 **Briassouli P**, Chan F, Savage K, Reis-Filho JS, Linardopoulos S. Aurora-A regulation of nuclear factor-kappaB signaling by phosphorylation of IkappaBalpha. *Cancer Res* 2007; **67**: 1689-1695

129 **Li LG**, Xu HM. Inducible nitric oxide synthase, nitrotyrosine and apoptosis in gastric adenocarcinomas and their correlation with a poor survival. *World J Gastroenterol* 2005; **11**: 2539-2544

130 **Zhang J**, Peng B, Chen X. Expressions of nuclear factor kappaB, inducible nitric oxide synthase, and vascular endothelial growth factor in adenoid cystic carcinoma of salivary glands: correlations with the angiogenesis and clinical outcome. *Clin Cancer Res* 2005; **11**: 7334-7343

131 **Hoki Y**, Hiraku Y, Ma N, Murata M, Matsumine A, Nagahama M, Shintani K, Uchida A, Kawanishi S. iNOS-dependent DNA damage in patients with malignant fibrous histiocytoma in relation to prognosis. *Cancer Sci* 2007; **98**: 163-168

132 **Cianchi F**, Cortesini C, Fantappie O, Messerini L, Sardi I, Lasagna N, Perna F, Fabbroni V, Di Felice A, Perigli G, Mazzanti R, Masini E. Cyclooxygenase-2 activation mediates the proangiogenic effect of nitric oxide in colorectal cancer. *Clin Cancer Res* 2004; **10**: 2694-2704

133 **Rahman MA**, Dhar DK, Yamaguchi E, Maruyama S, Sato T, Hayashi H, Ono T, Yamanoi A, Kohno H, Nagasue N. Coexpression of inducible nitric oxide synthase and COX-2 in hepatocellular carcinoma and surrounding liver: possible involvement of COX-2 in the angiogenesis of hepatitis C virus-positive cases. *Clin Cancer Res* 2001; **7**: 1325-1332

134 **Kong G**, Kim EK, Kim WS, Lee KT, Lee YW, Lee JK, Paik SW, Rhee JC. Role of cyclooxygenase-2 and inducible nitric oxide synthase in pancreatic cancer. *J Gastroenterol Hepatol* 2002; **17**: 914-921

135 **Ozel E**, Pestereli HE, Simsek T, Erdogan G, Karaveli FS. Expression of cyclooxygenase-2 and inducible nitric oxide synthase in ovarian surface epithelial carcinomas: is there any correlation with angiogenesis or clinicopathologic parameters? *Int J Gynecol Cancer* 2006; **16**: 549-555

136 **Denda A**, Kitayama W, Kishida H, Murata N, Tamura K, Kusuoka O, Tsutsumi M, Nishikawa F, Kita E, Nakae D,

Konishi Y, Kuniyasu H. Expression of inducible nitric oxide (NO) synthase but not prevention by its gene ablation of hepatocarcinogenesis with fibrosis caused by a choline-deficient, L-amino acid-defined diet in rats and mice. *Nitric Oxide* 2007; **16**: 164-176

137 **Ghoshal AK**. New insight into the biochemical pathology of liver in choline deficiency. *Crit Rev Biochem Mol Biol* 1995; **30**: 263-273

138 **Du Q**, Park KS, Guo Z, He P, Nagashima M, Shao L, Sahai R, Geller DA, Hussain SP. Regulation of human nitric oxide synthase 2 expression by Wnt beta-catenin signaling. *Cancer Res* 2006; **66**: 7024-7031

139 **Calvisi DF**, Factor VM, Ladu S, Conner EA, Thorgeirsson SS. Disruption of beta-catenin pathway or genomic instability define two distinct categories of liver cancer in transgenic mice. *Gastroenterology* 2004; **126**: 1374-1386

140 **Epstein RJ**, Leung TW. Reversing hepatocellular carcinoma progression by using networked biological therapies. *Clin Cancer Res* 2007; **13**: 11-17

141 **Gariboldi M**, Pascale R, Manenti G, De Miglio MR, Calvisi D, Carru A, Dragani TA, Feo F. Analysis of loss of heterozygosity in neoplastic nodules induced by diethylnitrosamine in the resistant BFF1 rat strain. *Carcinogenesis* 1999; **20**: 1363-1368

142 **Teeguarden JG**, Newton MA, Dragan YP, Pitot HC. Genome-wide loss of heterozygosity analysis of chemically induced rat hepatocellular carcinomas reveals elevated frequency of allelic imbalances on chromosomes 1, 6, 8, 11, 15, 17, and 20. *Mol Carcinog* 2000; **28**: 51-61

143 **Avila MA**, Berasain C, Sangro B, Prieto J. New therapies for hepatocellular carcinoma. *Oncogene* 2006; **25**: 3866-3884

144 **Calvisi DF**, Pascale RM, Feo F. Dissection of signal transduction pathways as a tool for the development of targeted therapies of hepatocellular carcinoma. *Rev Recent Clin Trials* 2007; **2**: 217-236

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EDITORIAL

Renal elimination of organic anions in cholestasis

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Abstract

The disposition of most drugs is highly dependent on specialized transporters. OAT1 and OAT3 are two organic anion transporters expressed in the basolateral membrane of renal proximal tubule cells, identified as contributors to xenobiotic and endogenous organic anion secretion. It is well known that cholestasis may cause renal damage. Impairment of kidney function produces modifications in the renal elimination of drugs. Recent studies have demonstrated that the renal abundance of OAT1 and OAT3 plays an important role in the renal elimination of organic anions in the presence of extrahepatic cholestasis. Time elapsed after obstructive cholestasis has an important impact on the regulation of both types of organic anion transporters. The renal expression of OAT1 and OAT3 should be taken into account in order to improve pharmacotherapeutic efficacy and to prevent drug toxicity during the onset of this hepatic disease.

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INTRODUCTION

Pharmacotherapeutic efficacy and toxicity are governed by pharmacodynamic and pharmacokinetic factors. The ability of a drug to reach its site of action is dependent on absorption, distribution, metabolism and excretion, all of which are intimately related to transport mechanisms in barrier epithelia. The majority of drugs and their metabolites have a positive or negative charge in the body, rendering them somewhat polar and hydrophilic. This polarity limits passive permeation across lipophilic barrier membranes. Thus the disposition of most drugs is highly dependent on specialized transporters embedded in these barrier epithelia. Transporters mediate selective permeation of charged molecules through barrier membranes and are thought to play a pivotal role in detoxification and drug disposition[1,2]. Membrane transporters have been classified into the solute carrier (SLC) and the ATP-binding cassette (ABC) transporter families. The SLC22 family comprises organic cation transporters (OCTs), zwitterion/cation transporters (OCTNs), and organic anion transporters (OATs). These transporters contain 12 predicted alpha-helical transmembrane domains (TMDs) and one large extracellular loop between TMDs 1 and 2. Transporters of the SLC22 family function in different ways: (1) as uniporters that mediate facilitated diffusion in either direction (OCTs), (2) as anion exchangers (OAT1, OAT3 and URAT1), and (3) as Na(+)/l-carnitine cotransporters (OCTN2)[1-5].

OATs play an essential role in the elimination of numerous endogenous and exogenous organic anions from the body. Numerous compounds, such as drugs, environmental substances, plant and animal toxins, and metabolites of both foreign and endogenous origins, are classified as organic anions. As for drugs of pharmacological interest, it is possible to mention β-lactam antibiotics, diuretics, nonsteroidal anti-inflammatory drugs, and several antiviral drugs that are organic anions[1-5].

Two organic anion transporters, OAT1 (*Slc22a6*) and OAT3 (*Slc22a8*), are expressed in the basolateral membrane of renal proximal tubule cells and have been identified as contributors to xenobiotic and endogenous organic anion secretion[3-5]. This membrane localization and function position them for an active role in determining drug exposure and efficacy. The regulation of OATs expression is not well understood.

The function of OATs in renal cells under physiological and pathological conditions has not been, as yet, fully elucidated. Recent studies indicated that the expressions of OATs are affected by pathological states. Modifications in the renal expression of OAT1 and OAT3 have been described in renal diseases; for example, chronic renal failure[6], ischemic acute renal failure[7] and obstructive nephropathy[8,9] and in other diseases, such as arterial calcinosis[10,11] and extrahepatic cholestasis[12-14].

GENERAL CHARACTERISTICS OF OAT1 AND OAT3

OAT1 has been cloned from rat, mouse, flounder, human, pig, rabbit and *C. elegans*[1-5,15-17]. OAT1 is expressed predominantly in the kidneys and weakly in the brain. OAT1 couples organic anions entry to dicarboxylate exit[16]. This protein has been immunolocalized to the basolateral surface of the proximal tubule[18]. OAT1 mediates the transport of many compounds (dicarboxylates, nucleotides, prostaglandins, antivirals, loop and thiazide diuretics, β lactam antibiotics, and nonsteroidal anti-inflammatory drugs, including the prototypical substrate of the classical organic anions secretion pathway, para-aminohipurate (PAH)). For methotrexate, transport was demonstrated for rat OAT1, but not for human OAT1, suggesting species differences. This has been demonstrated *in vitro* by its heterologous expression following microinjection of OAT1 cRNA into *Xenopus* oocytes or transfection of OAT1 cDNA into epithelial cell lines[3-5,15-17]. Eraly *et al*[19] have recently generated a colony of OAT1 knockout mice, permitting elucidation of the role of OAT1 in the context of other potentially functionally redundant transporters. They found that the knockout mice manifest a large loss of organic anion transport (e.g. PAH) both *ex vivo* (in isolated renal slices) as well as *in vivo* (as indicated by loss of renal secretion). In the case of the organic anion, furosemide (FS), loss of renal secretion in knockout animals resulted in impaired diuretic responsiveness to this drug. These results indicate an important role for OAT1 in the functioning of the classical pathway. The gene for human OAT1, SLC22A6, is located on chromosome 11q12.3, being paired with the gene for OAT3. The mammalian OAT1 consist of 545-551 amino acids, and secondary structure algorithms predict 12 TMDs with the N- and C-termini located at the cytosolic side of the plasma membrane. In man, a longer splice variant with 563 amino acids and two shorter, non functional splice variants were found[1-5].

OAT3 was cloned from human, monkey, pig, rabbit, rat and mouse kidneys. The human OAT3 is mainly expressed in kidneys, and to a lesser extent in the brain. The human OAT3 gene is paired with that of OAT1 and located on chromosome 11q12.3. In rats, more message for rat OAT3 was found in liver than in kidneys and brain suggesting species differences. The mammalian OAT3 proteins consist of 536-542 amino acids, arranged in 12 TMDs. Inmunohistochemistry revealed the location of OAT3 at the basolateral membrane of human, rat and mouse renal proximal tubules[18,20]. In rats, OAT3 was also found in several other nephron segments including the thick ascending limb of Henle's loop, distal convoluted tubule and collecting ducts. OAT3 in proximal tubules is involved in organic anion secretion, but the physiological and pharmacological roles of OAT3 in deeper nephron segments are presently not clear. Based on message abundance, OAT3 is the predominant organic anion transporter in the human kidney. Besides kidneys and liver, OAT3 was also found in human choroid plexus and in rat cerebral capillaries. Rat OAT3 contains three potential PKC phosphorylation sites and human OAT3 contains eight. OAT3 recognizes a broad spectrum of substrates, and it mediates the transport of PAH, ochratoxin A, estrone sulfate (ES), cimetidine, benzylpenicillin, cephaloridine and glutarate. Although it is evident that the selectivity of OAT3 overlaps that of OAT1, affinities for several substrates appear to permit discrimination between both transporters. For example, OAT3 displays a moderately high affinity for ES, whereas OAT1 interacts little with ES[2]. So, ES has been frequently used as a test substrate in studies for OAT3 activity. The mechanisms of OAT3 activity revealed that it can operate as an organic anion/dicarboxylate exchanger[21,22]. Consistent with an important role for OAT3-mediated uptake in kidney and choroid plexus, recent experiments with tissue from OAT3 knockout mice show reduced uptake of PAH, ES, and taurocholate in renal cortical slices and nearly complete inhibition of transport of the fluorescent organic anion fluorescein in intact choroid plexus[23]. For some drugs, there are differences in the specificity between human and rodent OAT3. For example, rat OAT3 transports ranitidine, famotidine and AZT, and human OAT3 transport little ranitidine and no famotidine and AZT[5]. Nevertheless, a systematic survey of possible species differences does not exist.

CHOLESTASIS AND RENAL DAMAGE

Kidney and liver play a major role in the elimination of numerous potentially toxic xenobiotics, including drugs, toxins, and endogenous metabolites. In some cases, the loss of one route of elimination can be compensated by another[24-26]. It must also be mentioned that impairment of liver or kidney functions can cause syndromes characterized by injury of the alternative elimination organ[24,27,28]. For example, prolonged cholestasis, characterized by retention of bile compound, may cause renal damage (reduction in renal hemodynamics, impairment of renal excretion of water and salts, and sensitization of the kidney to anoxia damage), which sometimes leads to renal failure. Moreover, obstructive

jaundice predisposes the kidney to acute renal failure and oxidative stress[29-32]. Extrahepatic biliary obstruction significantly decreased the reduced and oxidated forms of glutathione, and significantly inhibited glutathione peroxidase activity in rat kidneys. Increases of thiobarbituric acid reactive substances were also observed[32]. The F2-isoprostanes formed during oxidant injury are renal vasoconstrictors acting *via* thromboxane (TX)-like receptors. Holt *et al*[30] have demonstrated that the antioxidants N-acetyl-cysteine and α-lipoid acid and a TX receptor antagonist can prevent renal dysfunction in experimental cholestasis. Endothelin, a potent renal vasoconstrictor and modulator of the tubular action of arginine vasopressin, shows an increased synthesis in kidneys from rats with extrahepatic cholestasis, which is reflected by increased urinary excretion and may reduce distal tubular water reabsorption in these rats, being also involved in the renal damage observed during this liver pathology[31]. In human beings and rats, extrahepatic cholestasis has been shown to render the kidney susceptible to a variety of nephrotoxic agents[29,30]. The pathophysiological cause of renal damage during the course of bile flow impairment is not well understood, even if several phenomena, such as increased access of various constituents into the kidney (bilirubin and bile salts), have been suggested[30-34]. Impairment of kidney function produces modifications in the renal elimination of drugs mediated by alterations in blood flow to the kidney, glomerular filtration, active tubular secretion, and passive tubular reabsorption[6-11,35]. Because ethical considerations usually preclude meaningful clinical investigation in patients with acute obstructive jaundice, most studies in this condition have been carried out in animals, usually dogs and rats[31,32,36]. Bile duct ligation in dogs and rodents serves as an experimental model of extrahepatic cholestasis[33,34].

RENAL ELIMINATION OF ORGANIC ANIONS AND EXPRESSION OF OAT1 AND OAT3 IN EXTRAHEPATIC CHOLESTASIS

Brandoni *et al*[12,13]. have studied the cortical renal expression of OAT1 and OAT3 in association with the pharmacokinetics and renal excretion of PAH and FS in rats with acute extrahepatic cholestasis. Male Wistar rats underwent bile duct ligation (BDL rats). All studies were carried out 21 h after surgery. The systemic and renal clearance of both PAH and FS increased in BDL rats. In kidneys from BDL rats, immunoblotting showed a significant increase in the abundance of both OAT1 and OAT3 in homogenates from renal cortex. In basolateral membranes from the kidney cortex of BDL rats, OAT1 abundance was also increased and OAT3 abundance was not modified. Immunohistochemical techniques confirmed these results. Acute obstructive jaundice is associated with an upregulation of OAT1 and OAT3, which might explain, at least in part, the increased systemic and renal elimination of PAH and FS.

In this connection, extrahepatic cholestasis is associated with the production of various cytokines and growth factors that may affect gene transcription[37]. Similarly, with OAT1 and OAT3, MRP2 upregulation in BDL rats has been described[38-40]. These authors found that bilirubin ditaurate, sulfate-conjugated bile acids, and some components of the human bile upregulate the expression of MRP2 in human renal tubular cells. MRP2 and MRP4 are two members of the multidrug resistance protein (MRP) family located at the apical membranes from the proximal tubule cells[3-5]. Both proteins mediate the apical renal transport of several anionic substances, such as PAH[3-5,41]. An up-regulation of renal MRP2 has been described at 1 d and 3 d after BDL[38-40]. On the contrary, MRP4 decreases at 3 d after BDL[42].

Three days of BDL is the period in which serum bile acids and bilirubin levels reach the peak of elevation[38,40,43]. Brandoni *et al*[14] have also studied OAT1 and OAT3 function and expression after 3 d of BDL. After this time, BDL rats displayed a reduction in the renal elimination of PAH. OAT1 protein expression in kidney homogenates was not modified, but it decreased in the basolateral membranes. In contrast, OAT3 abundance in both kidney cortex homogenates and in basolateral membranes increased by 3 d after the ligation. This study demonstrated the key role of OAT1 expression in the impaired elimination of PAH after 3 d of obstructive cholestasis.

OAT1, when heterologously expressed in oocytes or mammalian cells, is inhibited by more or less selective PKC activators. It was demonstrated that PKC induces human OAT1 down-regulation through carrier retrieval from the cell membrane without phosphorylation[3-5,44]. Angiotensin Ⅱ[45] modulates the renal proximal tubule function *via* activation of PKC. Although the role of the renin-angiotensin system in the BDL model remains controversial[30], some humoral factors including angiotensin Ⅱ induced by the 3 d BDL may induce the activation of PKC. Moreover, bile acids and high bilirubin levels can activate PKC[46,47]. Three days of BDL is the period in which serum bile acids and bilirubin levels reach peak elevation. So, it was postulated that the peak elevation of bile acids and bilirubin can also trigger PKC activation. This PKC activation may cause the phosphorylation of caveolin-2, which may induce internalization of caveolae with OAT1 protein anchored with caveolin, as has been recently suggested by Kwak *et al*[48]. This OAT1 downregulation (30%) was associated with a concomitant decrease of renal and systemic PAH clearance (40% and 30% respectively). The medium PAH plasma concentrations reached during the renal clearance infusion studies were 295 μmol/L and 376 μmol/L for the Sham and BDL rats respectively. The OAT1 mediated uptake of PAH is saturable with apparent Michaelis constants ranging from 15 μmol/L to 70 μmol/L for rat OAT1[6]. Therefore, PAH concentrations that we obtained in our "*in vivo*" experiments were

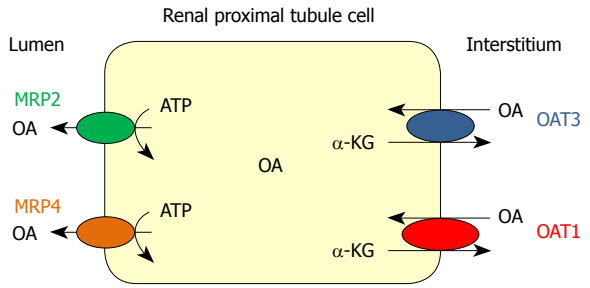

Figure 1 Schematic presentation of a renal proximal tubule cell, showing Organic Anion Transporters 1/3 (OAT1/3) and Multidrug Resistance Proteins 2/4 (MRP2/4). OA: organic anion; α-KG: α-ketoglutarate.

sufficiently higher than the reported Km of rat OAT1. The diminished secreted load of PAH measured under saturating conditions was in part accounted for by the lower number of OAT1 protein units observed in renal basolateral plasma membranes after 3 d of BDL by immunoblot technique. The opposite was observed in the early phase of extrahepatic cholestasis where an increase of 30% of OAT1 abundance was associated with a similar increase in PAH clearance[12]. The differences observed in OAT1 abundance between 21 h and 72 h of BDL remain to be explained. The increased OAT1 abundance observed in the early phase of extrahepatic cholestasis suggests a transient up-regulation similar to those described for renal OCT1 in cholestatic rats[49]. Different levels of cytokines and growth factors that may affect gene transcription might be involved in this differential response[50].

On the contrary, OAT3 expression increased both in homogenates and basolateral membranes from BDL kidneys. OAT3 is found in various cells and in all parts of the nephron, whereas OAT1 is confined to proximal tubules. The human and rat OAT3 transport PAH with relatively high affinity (87 μmol/L and 65 μmol/L respectively)[3-5,20], similarly to OAT1. On the contrary, ES, cholate, and taurocholate are substrates for OAT3 and not for OAT1 using *in vivo* and *in vitro* methodologies[3-5,19,20,23]. The over-expression of OAT3 does not compensate for the down-regulation of OAT1 regarding PAH transport because in this disease the high plasma levels of bile acids compete with PAH for OAT3 transport. Moreover, bile acids regulate the expression of several genes involved in bile salt transport[51,52]. It is possible that high bile acid levels up-regulate OAT3 expression without affecting OAT1 expression; this being another example of substrate specific regulation.

As has been mentioned above, MRP2 up-regulation was observed in the kidneys from rats with BDL of 1 d and 3 d[38-40]. On the other hand, renal MRP4 was down-regulated at 3 d after biliary obstruction[42]. Therefore, the protein expression of the luminal (MRP2 and MRP4) and basolateral organic anion renal transporters (OAT1 and OAT3) are differently regulated in extrahepatic biliary obstruction, thus indicating that different roles are played by these transporters in the pathogenesis of cholestasis. Tubular secretion is a vectorial transcellular transport system consisting of basolateral entry into the epithelial cells and secretion across the brush border membranes. Defects in either of these two processes should therefore influence the tubular secretion of anionic drugs.

Therefore, the time elapsed after obstructive cholestasis has an important impact in the regulation of OAT1 and OAT3. Studies regarding the possible role of bile acids and bilirubin, which are increased in this pathology, in the regulation of OATs are currently being performed in our laboratory.

Highly accumulated anionic drugs, as observed during cholestasis, may cause general body deterioration. The molecular mechanism(s) involved in the differential renal regulation of MRP2, MRP4, OAT3 and OAT1 expression should therefore be elucidated to prevent the occurrence of drug-induced toxicity. The renal expression of OAT1 and OAT3 should be taken into account in order to improve pharmacotherapeutic efficacy and to prevent drug toxicity during the onset of this hepatic disease.

Figure 1 shows the expression pattern of the transporters OAT1, OAT3, MRP2 and MRP4 in a cell of renal proximal tubule.

REFERENCES

1 **Shitara Y**, Horie T, Sugiyama Y. Transporters as a determinant of drug clearance and tissue distribution. *Eur J Pharm Sci* 2006; **27**: 425-446
2 **Sweet DH**. Organic anion transporter (Slc22a) family members as mediators of toxicity. *Toxicol Appl Pharmacol* 2005; **204**: 198-215
3 **Wright SH**, Dantzler WH. Molecular and cellular physiology of renal organic cation and anion transport. *Physiol Rev* 2004; **84**: 987-1049
4 **Anzai N**, Kanai Y, Endou H. Organic anion transporter family: current knowledge. *J Pharmacol Sci* 2006; **100**: 411-426
5 **Rizwan AN**, Burckhardt G. Organic anion transporters of the SLC22 family: biopharmaceutical, physiological, and pathological roles. *Pharm Res* 2007; **24**: 450-470
6 **Monica Torres A**, Mac Laughlin M, Muller A, Brandoni A, Anzai N, Endou H. Altered renal elimination of organic anions in rats with chronic renal failure. *Biochim Biophys Acta* 2005; **1740**: 29-37
7 **Di Giusto G**, Anzai N, Endou H, Torres AM. Elimination of organic anions in response to an early stage of renal ischemia-reperfusion in the rat: role of basolateral plasma membrane transporters and cortical renal blood flow. *Pharmacology* 2008; **81**: 127-136
8 **Villar SR**, Brandoni A, Quaglia NB, Torres AM. Renal elimination of organic anions in rats with bilateral ureteral obstruction. *Biochim Biophys Acta* 2004; **1688**: 204-209
9 **Villar SR**, Brandoni A, Anzai N, Endou H, Torres AM. Altered expression of rat renal cortical OAT1 and OAT3 in response to bilateral ureteral obstruction. *Kidney Int* 2005; **68**: 2704-2713
10 **Qualglia NB**, Hofer CG, Torres AM. Pharmacokinetics of organic anions in rats with arterial calcinosis. *Clin Exp Pharmacol Physiol* 2002; **29**: 48-52
11 **Quaglia NB**, Brandoni A, Ferri A, Torres AM. Early manifestation of nephropathy in rats with arterial calcinosis. *Ren Fail* 2003; **25**: 355-366
12 **Brandoni A**, Quaglia NB, Torres AM. Compensation increase in organic anion excretion in rats with acute biliary

obstruction: role of the renal organic anion transporter 1. *Pharmacology* 2003; **68**: 57-63

13 **Brandoni A**, Villar SR, Picena JC, Anzai N, Endou H, Torres AM. Expression of rat renal cortical OAT1 and OAT3 in response to acute biliary obstruction. *Hepatology* 2006; **43**: 1092-1100

14 **Brandoni A**, Anzai N, Kanai Y, Endou H, Torres AM. Renal elimination of p-aminohippurate (PAH) in response to three days of biliary obstruction in the rat. The role of OAT1 and OAT3. *Biochim Biophys Acta* 2006; **1762**: 673-682

15 **Sekine T**, Watanabe N, Hosoyamada M, Kanai Y, Endou H. Expression cloning and characterization of a novel multispecific organic anion transporter. *J Biol Chem* 1997; **272**: 18526-18529

16 **Sweet DH**, Wolff NA, Pritchard JB. Expression cloning and characterization of ROAT1. The basolateral organic anion transporter in rat kidney. *J Biol Chem* 1997; **272**: 30088-30095

17 **Wolff NA**, Werner A, Burkhardt S, Burckhardt G. Expression cloning and characterization of a renal organic anion transporter from winter flounder. *FEBS Lett* 1997; **417**: 287-291

18 **Kojima R**, Sekine T, Kawachi M, Cha SH, Suzuki Y, Endou H. Immunolocalization of multispecific organic anion transporters, OAT1, OAT2, and OAT3, in rat kidney. *J Am Soc Nephrol* 2002; **13**: 848-857

19 **Eraly SA**, Vallon V, Vaughn DA, Gangoiti JA, Richter K, Nagle M, Monte JC, Rieg T, Truong DM, Long JM, Barshop BA, Kaler G, Nigam SK. Decreased renal organic anion secretion and plasma accumulation of endogenous organic anions in OAT1 knock-out mice. *J Biol Chem* 2006; **281**: 5072-5083

20 **Cha SH**, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T, Endou H. Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* 2001; **59**: 1277-1286

21 **Sweet DH**, Chan LM, Walden R, Yang XP, Miller DS, Pritchard JB. Organic anion transporter 3 (Slc22a8) is a dicarboxylate exchanger indirectly coupled to the Na+ gradient. *Am J Physiol Renal Physiol* 2003; **284**: F763-F769

22 **Bakhiya A**, Bahn A, Burckhardt G, Wolff N. Human organic anion transporter 3 (hOAT3) can operate as an exchanger and mediate secretory urate flux. *Cell Physiol Biochem* 2003; **13**: 249-256

23 **Sweet DH**, Miller DS, Pritchard JB, Fujiwara Y, Beier DR, Nigam SK. Impaired organic anion transport in kidney and choroid plexus of organic anion transporter 3 (Oat3 (Slc22a8)) knockout mice. *J Biol Chem* 2002; **277**: 26934-26943

24 **Fleck C**, Braunlich H. Factors determining the relationship between renal and hepatic excretion of xenobiotics. *Arzneimittelforschung* 1990; **40**: 942-946

25 **Fleck C**, Braunlich H. Renal handling of drugs and amino acids after impairment of kidney or liver function--influences of maturity and protective treatment. *Pharmacol Ther* 1995; **67**: 53-77

26 **Sugi K**, Inoue M, Morino Y, Sato T. Effect of obstructive jaundice on the fate of a nephrophilic organic anion in the rat. *Biochim Biophys Acta* 1989; **987**: 217-221

27 **Kramer HJ**. Impaired renal function in obstructive jaundice: roles of the thromboxane and endothelin systems. *Nephron* 1997; **77**: 1-12

28 **Bataller R**, Sort P, Gines P, Arroyo V. Hepatorenal syndrome: definition, pathophysiology, clinical features and management. *Kidney Int Suppl* 1998; **66**: S47-S53

29 **Orellana M**, Rodrigo R, Thielemann L, Guajardo V. Bile duct ligation and oxidative stress in the rat: effects in liver and kidney. *Comp Biochem Physiol C Toxicol Pharmacol* 2000; **126**: 105-111

30 **Holt S**, Marley R, Fernando B, Harry D, Anand R, Goodier D, Moore K. Acute cholestasis-induced renal failure: effects of antioxidants and ligands for the thromboxane A2 receptor. *Kidney Int* 1999; **55**: 271-277

31 **Kramer HJ**, Schwarting K, Backer A, Meyer-Lehnert H. Renal endothelin system in obstructive jaundice: its role in impaired renal function of bile-duct ligated rats. *Clin Sci* (Lond) 1997; **92**: 579-585

32 **Gonzalez-Correa JA**, De La Cruz JP, Martin-Aurioles E, Lopez-Egea MA, Ortiz P, Sanchez de la Cuesta F. Effects of S-adenosyl-L-methionine on hepatic and renal oxidative stress in an experimental model of acute biliary obstruction in rats. *Hepatology* 1997; **26**: 121-127

33 **Koopen NR**, Muller M, Vonk RJ, Zimniak P, Kuipers F. Molecular mechanisms of cholestasis: causes and consequences of impaired bile formation. *Biochim Biophys Acta* 1998; **1408**: 1-17

34 **Reichen J**, Simon FR. Mechanisms of cholestasis. *Int Rev Exp Pathol* 1984; **26**: 231-274

35 **Talbert RL**. Drug dosing in renal insufficiency. *J Clin Pharmacol* 1994; **34**: 99-110

36 **Levy M**, Finestone H. Renal response to four hours of biliary obstruction in the dog. *Am J Physiol* 1983; **244**: F516-F525

37 **Plebani M**, Panozzo MP, Basso D, De Paoli M, Biasin R, Infantolino D. Cytokines and the progression of liver damage in experimental bile duct ligation. *Clin Exp Pharmacol Physiol* 1999; **26**: 358-363

38 **Tanaka Y**, Kobayashi Y, Gabazza EC, Higuchi K, Kamisako T, Kuroda M, Takeuchi K, Iwasa M, Kaito M, Adachi Y. Increased renal expression of bilirubin glucuronide transporters in a rat model of obstructive jaundice. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G656-G662

39 **Villanueva SS**, Ruiz ML, Soroka CJ, Cai SY, Luquita MG, Torres AM, Sanchez Pozzi EJ, Pellegrino JM, Boyer JL, Catania VA, Mottino AD. Hepatic and extrahepatic synthesis and disposition of dinitrophenyl-S-glutathione in bile duct-ligated rats. *Drug Metab Dispos* 2006; **34**: 1301-1309

40 **Lee J**, Azzaroli F, Wang L, Soroka CJ, Gigliozzi A, Setchell KD, Kramer W, Boyer JL. Adaptive regulation of bile salt transporters in kidney and liver in obstructive cholestasis in the rat. *Gastroenterology* 2001; **121**: 1473-1484

41 **Smeets PH**, van Aubel RA, Wouterse AC, van den Heuvel JJ, Russel FG. Contribution of multidrug resistance protein 2 (MRP2/ABCC2) to the renal excretion of p-aminohippurate (PAH) and identification of MRP4 (ABCC4) as a novel PAH transporter. *J Am Soc Nephrol* 2004; **15**: 2828-2835

42 **Denk GU**, Soroka CJ, Takeyama Y, Chen WS, Schuetz JD, Boyer JL. Multidrug resistance-associated protein 4 is up-regulated in liver but down-regulated in kidney in obstructive cholestasis in the rat. *J Hepatol* 2004; **40**: 585-591

43 **Pei QL**, Kobayashi Y, Tanaka Y, Taguchi Y, Higuchi K, Kaito M, Ma N, Semba R, Kamisako T, Adachi Y. Increased expression of multidrug resistance-associated protein 1 (mrp1) in hepatocyte basolateral membrane and renal tubular epithelia after bile duct ligation in rats. *Hepatol Res* 2002; **22**: 58-64

44 **Wolff NA**, Thies K, Kuhnke N, Reid G, Friedrich B, Lang F, Burckhardt G. Protein kinase C activation downregulates human organic anion transporter 1-mediated transport through carrier internalization. *J Am Soc Nephrol* 2003; **14**: 1959-1968

45 **Karim Z**, Defontaine N, Paillard M, Poggioli J. Protein kinase C isoforms in rat kidney proximal tubule: acute effect of angiotensin II. *Am J Physiol* 1995; **269**: C134-C140

46 **Rao YP**, Stravitz RT, Vlahcevic ZR, Gurley EC, Sando JJ, Hylemon PB. Activation of protein kinase C alpha and delta by bile acids: correlation with bile acid structure and diacylglycerol formation. *J Lipid Res* 1997; **38**: 2446-2454

47 **Hirohata Y**, Fujii M, Okabayashi Y, Nagashio Y, Tashiro M, Imoto I, Akiyama T, Otsuki M. Stimulatory effects of bilirubin on amylase release from isolated rat pancreatic acini. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G249-G256

48 **Kwak JO**, Kim HW, Oh KJ, Kim DS, Han KO, Cha SH. Co-localization and interaction of organic anion transporter 1 with caveolin-2 in rat kidney. *Exp Mol Med* 2005; **37**: 204-212
49 **Denk GU**, Soroka CJ, Mennone A, Koepsell H, Beuers U, Boyer JL. Down-regulation of the organic cation transporter 1 of rat liver in obstructive cholestasis. *Hepatology* 2004; **39**: 1382-1389
50 **Denson LA**, Bohan A, Held MA, Boyer JL. Organ-specific alterations in RAR alpha:RXR alpha abundance regulate rat Mrp2 (Abcc2) expression in obstructive cholestasis. *Gastroenterology* 2002; **123**: 599-607
51 **Rost D**, Herrmann T, Sauer P, Schmidts HL, Stieger B, Meier PJ, Stremmel W, Stiehl A. Regulation of rat organic anion transporters in bile salt-induced cholestatic hepatitis: effect of ursodeoxycholate. *Hepatology* 2003; **38**: 187-195
52 **Boyer JL**, Trauner M, Mennone A, Soroka CJ, Cai SY, Moustafa T, Zollner G, Lee JY, Ballatori N. Upregulation of a basolateral FXR-dependent bile acid efflux transporter OSTalpha-OSTbeta in cholestasis in humans and rodents. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1124-G1130

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EDITORIAL

Diagnosis and clinical implications of pancreatobiliary reflux

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Abstract

The sphincter of Oddi is located at the distal end of the pancreatic and bile ducts and regulates the outflow of bile and pancreatic juice. A common channel can be so long that the junction of the pancreatic and bile ducts is located outside of the duodenal wall, as occurs in pancreaticobiliary maljunction (PBM); in such cases, sphincter action does not functionally affect the junction. As the hydropressure within the pancreatic duct is usually greater than in the bile duct, pancreatic juice frequently refluxes into the biliary duct (pancreatobiliary reflux) in PBM, resulting in carcinogenetic conditions in the biliary tract. Pancreatobiliary reflux can be diagnosed from elevated amylase level in the bile, secretin-stimulated dynamic magnetic resonance cholangiop ancreatography, and pancreatography *via* the minor duodenal papilla. Recently, it has become obvious that pancreatobiliary reflux can occur in individuals without PBM. Pancreatobiliary reflux might be related to biliary carcinogenesis even in some individuals without PBM. Since few systemic studies exist with respect to clinical relevance and implications of the pancreatobiliary reflux in individuals with normal pancreaticobiliary junction, further prospective clinical studies including appropriate management should be performed.

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Key words: Pancreatobiliary reflux; Pancreaticobiliary maljunction; Biliary cancer

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INTRODUCTION

The main pancreatic duct and the common bile duct open into the duodenum either separately or *via* a common channel. The incidence of common channel formation ranges from 55%[1] to 82%[2,3]. Dowdy *et al*[4] have reported that the length of the common channel ranged from 1 mm to 12 mm, with an average length of 4.4 mm. The sphincter of Oddi, which is composed of the sphincter choledochus, the sphincter pancreaticus and the sphincter ampullae, is located at the distal end of the pancreatic and bile ducts and regulates the outflow of bile and pancreatic juice[2,3]. A common channel can be so long that the junction of the pancreatic and bile ducts is located outside of the duodenal wall, as occurs in pancreaticobiliary maljunction (PBM); in such cases, sphincter action does not functionally affect the junction, resulting in two-way regurgitation (biliopancreatic reflux: regurgitation of bile juice into the pancreatic duct, and pancreatobiliary reflux: Regurgitation of pancreatic juice into the common bile duct)[5,6].

Given that the hydropressure within the pancreatic duct is usually greater than that in the bile duct, pancreatic juice frequently refluxes into the biliary duct in PBM[7-9]. Pancreatobiliary reflux can be examined by several methods, and it has become obvious that this reflux can occur in individuals without PBM. Recent advances in diagnosis of pancreatobiliary reflux and its clinical implications were reviewed.

DIAGNOSIS OF PANCREATOBILIARY REFLUX

In PBM patients, pancreatic enzymes, especially amylase, are generally at extremely high levels in the bile within the bile duct and gallbladder obtained percutaneously or immediately after laparotomy[5,10]. It has been reported that, in PBM patients, the amylase level in the bile of the gallbladder was 123.568 ± 180.827 IU/L (mean ± SD), and the amylase level in the bile of the bile duct was 99.018 ± 162.506 IU/L[11]. Levels close to or below

the serum amylase level are rarely observed in patients with PBM. However, the normal upper limit of the bile amylase level is unknown.

Pancreaticobiliary reflux in PBM patients can be visualized radiologically using secretin-stimulated dynamic magnetic resonance cholangiopancreatography (MRCP)[12,13]. In normal pancreaticobiliary dynamics, the extrahepatic and intrahepatic bile ducts show no change following secretin injection. On the other hand, in PBM patients, the volume of the extrahepatic bile duct and the gallbladder increases due to regurgitation of pancreatic fluid secreted after secretin injection into the bile duct[12,13]. However, since bile is also secreted after secretin stimulation, enlargement of the gallbladder may imply pancreatobiliary reflux, bile secretion, or both[14].

Pancreatography *via* the minor duodenal papilla can also demonstrate pancreatobiliary reflux in PBM patients. The contrast medium injected endoscopically *via* the minor duodenal papilla refluxes into the bile duct through a long common channel without outflow into the duodenum[15].

PANCREATICOBILIARY MALJUNCTION

PBM is a congenital anomaly defined as a junction of the pancreatic and biliary ducts located outside the duodenal wall, usually forming a markedly long common channel ($\geqslant$ 15 mm) (Figure 1)[5,6]. PBM can be divided into PBM with biliary dilatation (congenital choledochal cyst) and PBM without biliary dilatation (maximal diameter of the bile duct $\leqslant$ 10 mm). In PBM patients, since the pancreatic duct and bile duct are joined outside the duodenal wall, the action of the sphincter of Oddi does not functionally affect the junction. Therefore, continuous reciprocal reflux between pancreatic juice and bile occurs, resulting in various pathological conditions in the biliary tract and pancreas[5,6,15].

Given that the hydropressure within the pancreatic duct is usually greater than that in the bile duct, pancreatic juice frequently refluxes into the biliary duct[7-9], which results in a high incidence of carcinoma in the biliary tract. According to a nationwide survey[16] performed in Japan, cancer of the biliary tract was found in 278 (17%) of 1627 of patients with PBM, which was distinctly higher than the incidence of biliary tract cancer in the general population (0.26%-1.8%). In 1239 patients with PBM with biliary dilatation, the occurrence rate of biliary cancer was 11% (131/1239), and cancer of the gallbladder was seen in 85 (65%) out of the 131 patients. In 388 patients with PBM without biliary dilatation, biliary cancer occurred in 147 patients (38%), and 93% (137/147) of the associated biliary cancers were gallbladder cancers.

The etiology of gallbladder cancer in patients with PBM has not been fully clarified. In PBM patients, gallstones do not appear to contribute to carcinogenesis, based on the low rate of gallstone detection[17]. The age of patients with gallbladder cancer associated with PBM without biliary dilatation is significantly younger than in those without PBM[17,18]. The

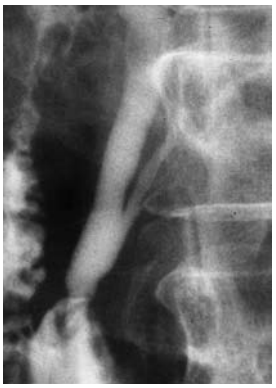

Figure 1 Endoscopic retrograde cholangiopancreatography of a patient with PBM shows a long common channel.

mechanism of carcinogenesis in PBM is considered to be related to stagnation of refluxed pancreatic juice into the biliary tract. Refluxed proteolytic pancreatic enzymes and phospholipase A2 are activated in the biliary tract. Phospholipase A2, which has a direct proliferative effect on the gallbladder mucosa, produces lysophosphatidylcholin, which has a cytotoxic effect[19]. These agents may injure the epithelium of the biliary tract and induce metaplasia or promote cancer progress.

Epithelial hyperplasia of the gallbladder has been reported to be a characteristic pathologic change in PBM patients. The incidence of epithelial hyperplasia of the gallbladder associated with PBM reportedly ranges from 39%[20] to 63%[21], and it is as high as 91%[22] to 100%[23] in PBM without biliary dilatation. Tanno *et al*[22] have reported that the Ki-67 labeling index of epithelial hyperplasia of the gallbladder with PBM was 6.1% $\pm$ 1.5% (mean $\pm$ SD), which was significantly greater than the index of 1.2% $\pm$ 1.0% in control gallbladder mucosa without PBM. K-ras mutations in the non-cancerous gallbladder epithelium have been detected in 22%[22] to 50%[24] of PBM patients. Considering that increased cell proliferation is linked to the development of cancer by means of tumor promotion and an increased rate of random mutations, the gallbladder mucosa of PBM patients can be considered to be a premalignant region.

The treatment of choice for PBM with biliary dilatation is prophylactic flow-diversion surgery (bile duct resection and bilioenteric anastomosis) before malignant changes can take place in the biliary tract[25]. Although a standard treatment protocol for PBM without biliary dilatation has not been established, prophylactic cholecystectomy is performed in many institutes, as most biliary cancers that develop in cases of PBM without biliary dilatation are gallbladder cancers[16,18].

HIGH CONFLUENCE OF PANCREATICOBILIARY DUCTS

There are some cases with a relatively long common channel that are not classified as PBM because the sphincter of Oddi includes the pancreaticobiliary ductal junction.

Sterling[1] have reported that it varies from 1.2 mm to 8.4 mm, averaging 4.4 mm. Rienhoff and Pickrell[26]

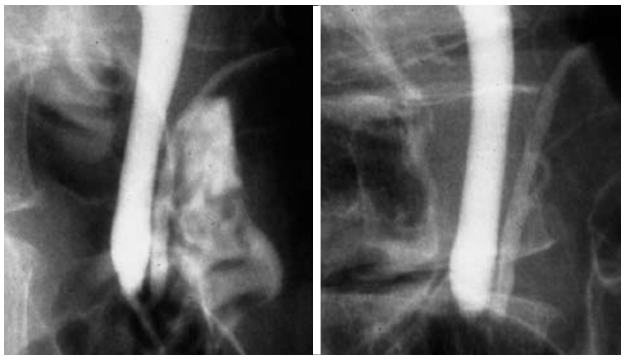

Figure 2 HCPBD. Cholangiopancreatogram of a patient with high confluence of pancreaticobiliary ducts and a common channel of 9 mm in length (left). The communication between pancreatic and bile ducts was destroyed with sphincter contraction (right).

have reported that 92 (53%) of 173 cases had a common channel length within 2 mm, 62 (36%) had a common channel length ranging from 3 mm to 5 mm, and 19 (11%) had a common channel length > 6 mm. Based on the above findings, to investigate the clinical significance of a relatively long common channel, we defined a high confluence of pancreaticobiliary ducts (HCPBD) as a common channel length ⩾ 6 mm, in which the communication was occluded when the sphincter was contracted (Figure 2)[27-29].

In a series of 3459 patients who underwent endoscopic retrograde cholangiopancreatography (ERCP) in our hospital, 74 patients (2.1%) had PBM, including 41 with biliary dilatation (congenital choledochal cyst) and 65 (1.9%) with HCPBD. Although PBM occurred predominantly in females, there was no difference between genders in patients with HCPBD. The average age at the time of diagnosis was significantly younger in PBM patients with biliary dilatation. Reflux of contrast medium into the pancreatic duct was detected in 12 (86%) of 14 patients with HCPBD who underwent postoperative T-tube cholangiography. The average bile amylase level was elevated to 47 774 IU/L in seven HCPBD patients, but it was lower than that of PBM patients. Compared to controls, the incidences of gallbladder cancer were significantly higher in PBM patients with biliary dilatation (17%) and without biliary dilatation (73%), and HCPBD patients (11%). The average age at the time of diagnosis of the gallbladder cancer patients with HCPBD (64.5 years old) was between that of the PBM patients without biliary dilatation (57.0 years old) and patients without these maljunctions (69.5 years old). The incidence of gallbladder stones in conjunction with gallbladder cancer associated with HCPBD (14%) or PBM (6%) was significantly lower than in those with gallbladder cancers without these maljunctions (62%). Similar to PBM patients without biliary dilatation, hyperplastic change of the gallbladder mucosa with increased epithelial cell proliferative activity was detected in cases of HCPBD. Furthermore, K-ras mutations of the non-cancerous epithelium of the gallbladder were detected in five (28%) of 18 HCPBD cases. A relatively long common channel, as well as a long common channel with PBM, appears to be an important risk factor for the development of gallbladder cancer. However, since there are several differences in gender, age at diagnosis, bile amylase level, and incidence of associated gallbladder cancer between HCPBD and PBM patients, HCPBM should be treated at present as an entity separate from PBM. Although further research is necessary to determine the appropriate management, including prophylactic cholecystectomy, of patients with HCPBD, clinicians should be vigilant regarding the development of gallbladder cancer in such patients.

PANCREATOBILIARY REFLUX IN INDIVIDUALS WITH A NORMAL PANCREATICOBILIARY JUNCTION

By demonstrating elevated levels of pancreatic enzymes in bile sampled from the common bile duct or gallbladder, or by MRCP evidence of biliary duct dilatation after secretin injection, it has been recently recognized that pancreatobiliary reflux can occur with normal pancreaticobiliary junction.

High bile amylase levels are found in some patients without PBM. Anderson *et al*[30] have reported that the bile amylase level obtained through an indwelling T-tube was higher than the serum amylase level in 21 (81%) of 26 patients with biliary tract disease, and that bile amylase level fluctuated considerably in the same patient. Therefore, they have suggested that intermittent reflux of pancreatic juice might initiate inflammatory changes in the gallbladder and could play a role in gallstone formation by altering the constituents that maintain cholesterol in a soluble state. A high bile trypsin level has also been reported in patients with bile duct stones[31]. Itokawa *et al*[32] have reported that the amylase level in the bile obtained during ERCP was higher than the serum amylase level in 22 (26%) of 86 patients, and the rate of a high amylase level in the bile was significantly higher in patients who were elderly, had a dilated common bile duct, and in those who had choledocholithiasis. Recently, Sai *et al*[33] have demonstrated enhanced visualization of the intrahepatic and extrahepatic bile ducts and gallbladder with increased maximal diameter of the extrahepatic bile duct and short axis of the gallbladder on secretin-stimulated dynamic MRCP in four (5%) of 74 patients who had a normal pancreaticobiliary junction on ERCP. The bile amylase level was markedly elevated in all four patients, and three of these four patients had gallbladder cancer. This would suggest that there is a relationship between pancreatobiliary reflux in individuals with a normal pancreaticobiliary junction and gallbladder cancer.

Pancreatobiliary reflux can also occur in cases of sphincter dysfunction[30,34], periampullary diverticula[35], as well as after endoscopic sphincterotomy[36] or endoscopic papillary balloon dilatation[37]. Pancreatobiliary reflux in many cases with normal pancreaticobiliary junction seems to be caused by dysfunction of the sphincter of Oddi. Furthermore, unlike in PBM, pancreatobiliary reflux in individuals with a normal pancreaticobiliary

junction occurs not continuously but transiently. Carcinogenesis in the biliary tract is strongly related to stagnation of bile intermingled with refluxed pancreatic juice. Since, in individuals with a normal pancreaticobiliary maljunction, the pancreatic juice refluxes into the common bile duct and is cleared rapidly without stasis, the occurrence of gallbladder cancer poses a problem in these cases. It seems rather obvious that pancreatobiliary reflux might be related to carcinogenesis of the gallbladder. However, the clinical relevance of pancreatobiliary reflux in individuals with normal pancreaticobiliary junction is unknown. Further prospective clinical studies are needed to determine which patients require evaluation of biliary amylase levels and prophylactic cholecystectomy.

CONCLUSION

Pancreatobiliary reflux can be examined using various methods, and it has become clear that reflux can occur in some individuals without PBM. Although the true prevalence and the mechanism of pancreatobiliary reflux in individuals without PBM are unclear, the reflux might be related to biliary carcinogenesis even in individuals with a normal pancreaticobiliary junction. More cases need to be studied in order to determine the clinical implications, including appropriate management.

REFERENCES

1 **Sterling JA**. The common channel for bile and pancreatic ducts. *Surg Gynecol Obstet* 1954; **98**: 420-424
2 **Suda K**, Miyano T, Hashimoto K. The choledocho-pancreatico-ductal junction in infantile obstructive jaundice diseases. *Acta Pathol Jpn* 1980; **30**: 187-194
3 **Suda K**, Miyano T, Konuma I, Matsumoto M. An abnormal pancreatico-choledocho-ductal junction in cases of biliary tract carcinoma. *Cancer* 1983; **52**: 2086-2088
4 **Dowdy GS**, Waldron GW, Brown WG. Surgical anatomy of the pancreatobiliary ductal system. Observations. *Arch Surg* 1962; **84**: 229-246
5 **The Japanese Study Group on Pancreaticobiliary Maljunction.** Diagnostic criteria of pancreaticobiliary maljunction. *J Hepatobiliary Pancreat Surg* 1994; **1**: 219-221
6 **Kamisawa T**, Egawa N, Nakajima H, Tsuruta K, Okamoto A, Matsukawa M. Origin of the long common channel based on pancreatographic findings in pancreaticobiliary maljunction. *Dig Liver Dis* 2005; **37**: 363-367
7 **Csendes A**, Kruse A, Funch-Jensen P, Oster MJ, Ornsholt J, Amdrup E. Pressure measurements in the biliary and pancreatic duct systems in controls and in patients with gallstones, previous cholecystectomy, or common bile duct stones. *Gastroenterology* 1979; **77**: 1203-1210
8 **Carr-Locke DL**, Gregg JA. Endoscopic manometry of pancreatic and biliary sphincter zones in man. Basal results in healthy volunteers. *Dig Dis Sci* 1981; **26**: 7-15
9 **Arendt T**, Stoffregen C, Kloehn S, Monig H, Nizze H, Folsch UR. Santorini's duct--risk factor for acute pancreatitis or protective morphologic variant? Experiments in rabbits. *Eur J Gastroenterol Hepatol* 1997; **9**: 569-573
10 **Davenport M**, Stringer MD, Howard ER. Biliary amylase and congenital choledochal dilatation. *J Pediatr Surg* 1995; **30**: 474-477
11 **Tashiro S**, Imaizumi T, Ohkawa H, Okada A, Katoh T, Kawarada Y, Shimada H, Takamatsu H, Miyake H, Todani T. Overall report on the registration study of the Japanese study group on pancreaticobiliary maljunction for the past 10 years. In: Koyanagi Y, Aoki T. Pancreaticobiliary maljunction. Tokyo: Igaku Tosho, 2002: 401-410
12 **Matos C**, Nicaise N, Deviere J, Cassart M, Metens T, Struyven J, Cremer M. Choledochal cysts: comparison of findings at MR cholangiopancreatography and endoscopic retrograde cholangiopancreatography in eight patients. *Radiology* 1998; **209**: 443-448
13 **Hosoki T**, Hasuike Y, Takeda Y, Michita T, Watanabe Y, Sakamori R, Tokuda Y, Yutani K, Sai C, Mitomo M. Visualization of pancreaticobiliary reflux in anomalous pancreaticobiliary junction by secretin-stimulated dynamic magnetic resonance cholangiopancreatography. *Acta Radiol* 2004; **45**: 375-382
14 **Motosugi U**, Ichikawa T, Araki T, Kitahara F, Sato T, Itakura J, Fujii H. Secretin-stimulating MRCP in patients with pancreatobiliary maljunction and occult pancreatobiliary reflux: direct demonstration of pancreatobiliary reflux. *Eur Radiol* 2007; **17**: 2262-2267
15 **Kamisawa T**, Okamoto A. Biliopancreatic and pancreatobiliary refluxes in cases with and without pancreaticobiliary maljunction: diagnosis and clinical implications. *Digestion* 2006; **73**: 228-236
16 **Tashiro S**, Imaizumi T, Ohkawa H, Okada A, Katoh T, Kawaharada Y, Shimada H, Takamatsu H, Miyake H, Todani T. Pancreaticobiliary maljunction: retrospective and nationwide survey in Japan. *J Hepatobiliary Pancreat Surg* 2003; **10**: 345-351
17 **Ohta T**, Nagakawa T, Ueno K, Maeda K, Ueda N, Kayahara M, Akiyama T, Kanno M, Konishi I, Izumi R. Clinical experience of biliary tract carcinoma associated with anomalous union of the pancreaticobiliary ductal system. *Jpn J Surg* 1990; **20**: 36-43
18 **Sugiyama M**, Atomi Y. Anomalous pancreaticobiliary junction without congenital choledochal cyst. *Br J Surg* 1998; **85**: 911-916
19 **Shimada K**, Yanagisawa J, Nakayama F. Increased lysophosphatidylcholine and pancreatic enzyme content in bile of patients with anomalous pancreaticobiliary ductal junction. *Hepatology* 1991; **13**: 438-444
20 **Yamamoto M**, Nakajo S, Tahara E, Ito M, Taniyama K, Shimamoto F, Miyoshi N, Hayashi Y, Akiyama H, Nakai S. Mucosal changes of the gallbladder in anomalous union with the pancreatico-biliary duct system. *Pathol Res Pract* 1991; **187**: 241-246
21 **Hanada K**, Itoh M, Fujii K, Tsuchida A, Hirata M, Ishimaru S, Iwao T, Eguchi N, Kajiyama G. Pathology and cellular kinetics of gallbladder with an anomalous junction of the pancreaticobiliary duct. *Am J Gastroenterol* 1996; **91**: 1007-1011
22 **Tanno S**, Obara T, Fujii T, Mizukami Y, Shudo R, Nishino N, Ura H, Klein-Szanto AJ, Kohgo Y. Proliferative potential and K-ras mutation in epithelial hyperplasia of the gallbladder in patients with anomalous pancreaticobiliary ductal union. *Cancer* 1998; **83**: 267-275
23 **Tsuchida A**, Itoi T, Endo M, Kitamura K, Mukaide M, Itokawa F, Ozawa T, Aoki T. Pathological features and surgical outcome of pancreaticobiliary maljunction without dilatation of the extrahepatic bile duct. *Oncol Rep* 2004; **11**: 269-276
24 **Matsubara T**, Sakurai Y, Sasayama Y, Hori H, Ochiai M, Funabiki T, Matsumoto K, Hirono I. K-ras point mutations in cancerous and noncancerous biliary epithelium in patients with pancreaticobiliary maljunction. *Cancer* 1996; **77**: 1752-1757
25 **Matsumoto Y**, Fujii H, Itakura J, Matsuda M, Nobukawa B, Suda K. Recent advances in pancreaticobiliary maljunction. *J Hepatobiliary Pancreat Surg* 2002; **9**: 45-54
26 **Rienhoff WF**, Pickrell KL. Pancreatitis: an anatomic study of the pancreatic and extrahepatic biliary systems. *Arch Surg* 1945; **51**: 205-219
27 **Kamisawa T**, Amemiya K, Tu Y, Egawa N, Sakaki N,

Tsuruta K, Okamoto A, Munakata A. Clinical significance of a long common channel. *Pancreatology* 2002; **2**: 122-128

28 **Kamisawa T**, Funata N, Hayashi Y, Egawa N, Nakajima H, Tsuruta K, Okamoto A, Yamaguchi T. Pathologic changes in the non-carcinomatous epithelium of the gallbladder in patients with a relatively long common channel. *Gastrointest Endosc* 2004; **60**: 56-60

29 **Kamisawa T**, Kuwata G, Chen PY, Tu Y, Fujiwara T, Endoh J, Arakawa T, Koizumi K, Nakajima H, Egawa N. Precancerous lesions in the gallbladder of patients with a long common channel. *Dig Endosc* 2006; **18**: 192-195

30 **Anderson MC**, Hauman RL, Suriyapa C, Schiller WR. Pancreatic enzyme levels in bile of patients with extrahepatic biliary tract disease. *Am J Surg* 1979; **137**: 301-306

31 **Vracko J**, Wiechel KL. Trypsin level in gallbladder bile and ductitis and width of the cystic duct. *Hepatogastroenterology* 2000; **47**: 115-120

32 **Itokawa F**, Itoi T, Nakamura K, Sofuni A, Kakimi K, Moriyasu F, Tsuchida A, Aoki T. Assessment of occult pancreatobiliary reflux in patients with pancreaticobiliary disease by ERCP. *J Gastroenterol* 2004; **39**: 988-994

33 **Sai JK**, Suyama M, Kubokawa Y, Tadokoro H, Sato N, Maehara T, Iida Y, Kojima K. Occult pancreatobiliary reflux in patients with a normal pancreaticobiliary junction. *Gastrointest Endosc* 2003; **57**: 364-368

34 **Peveretos P**, Polydorou A, Golematis P, Golematis B. The role of the pancreatic enzymes in the pathogenesis of cholelithiasis. *Mt Sinai J Med* 1988; **55**: 369-373

35 **Sugiyama M**, Atomi Y. Periampullary diverticula cause pancreatobiliary reflux. *Scand J Gastroenterol* 2001; **36**: 994-997

36 **Sugiyama M**, Atomi Y. Does endoscopic sphincterotomy cause prolonged pancreatobiliary reflux? *Am J Gastroenterol* 1999; **94**: 795-798

37 **Sugiyama M**, Atomi Y. Endoscopic papillary balloon dilation causes transient pancreatobiliary and duodenobiliary reflux. *Gastrointest Endosc* 2004; **60**: 186-190

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REVIEW

Is pegylated interferon superior to interferon, with ribavarin, in chronic hepatitis C genotypes 2/3?

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Abstract

Over the past decade, significant improvements have been made in the treatment of chronic hepatitis C (CHC), especially with the introduction of combined therapy using both interferon and ribavarin. The optimal dose and duration of treatment is still a matter of debate and, importantly, the efficacy of this combined treatment varies with the viral genotype responsible for infection. In general, patients infected with viral genotypes 2 or 3 more readily achieve a sustained viral response than those infected with viral genotype 1. The introduction of a pegylated version of interferon in the past decade has produced better clinical outcomes in patients infected with viral genotype 1. However, the published literature shows no improvement in clinical outcomes in patients infected with viral genotypes 2 or 3 when they are treated with pegylated interferon as opposed to non-pegylated interferon, both given in combination with ribavarin. This is significant because the cost of a 24-wk treatment with pegylated interferon in less-developed countries is between six and 30 times greater than that of treatment with interferon. Thus, clinicians need to carefully consider the cost-*versus*-benefit of using pegylated interferon to treat CHC, particularly when there is no evidence for clinically measurable benefits in patients with genotypes 2 and 3 infections.

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Key words: Hepatitis C; Genotypes; Interferon

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INTRODUCTION

The hepatitis C virus (HCV) is a non-cytopathic member of the Flaviviridae family that causes acute and chronic hepatitis and can lead to the development of hepatocellular carcinoma (HCC). An estimated 3% of the world's population is infected with HCV[1]. Acute infection is usually asymptomatic, which makes early diagnosis difficult. A distinct feature of HCV infection is its proclivity towards becoming chronic in as many as 70% of acute infections. Importantly, chronic hepatitis C (CHC) is associated with the progressive development of fibrosis and cirrhosis that, if left untreated, can lead to end-stage liver disease with an estimated 5%-20% mortality rate.

PREVALENCE AND VIRAL GENOTYPE

Preliminary surveys from Pakistan suggest that the seroprevalence of HCV is > 5%, while other surveys suggest rates as high as 10%[2-6]. In one study[7] of prospective blood donors in Faisalabad, Pakistan, 78/294 individuals or 26.5% were found to be seropositive for HCV. If representative, this would be appreciably greater by far than the prevalence rate of 2.9% estimated for Egypt, a country with one of the highest HCV infection rates[8].

The predominant form of HCV infections in Pakistan is of the genotype 3 variety, and this genotype is also predominant in northern, northeastern, and central India, with infection rates as high as 71% in acute

hepatitis patients, and as high as 82% in chronic hepatitis patients[9,10]. From a global public health perspective, sizeable fractions of CHC populations outside southern Asia are also infected with viral genotypes 2 and 3. For example, 26.9% of CHC patients in Serbia and Montenegro have been reported to be infected with genotypes 2 or 3[11]. An estimated 37% of 90 consecutive liver patients from Novara, Italy, had genotype 2[12]. In Cordoba, Argentina, 55% of 96 consecutive liver patients had genotype 2 and 5% had genotype 3[13]. An estimated HCV prevalence of 31% for genotype 3 and 4.3% for genotype 2 has been reported in Brazil[14]. These data from the more densely populated areas of the world suggest that the actual number of people infected with either genotype 2 or 3 is quite substantial compared to those with genotype 1, the predominant form in North America and northern Europe, although an estimated 16% of CHC patients in the United States are also infected with genotypes 2 or 3, as compared to 72% with genotype 1 (http://www.hepatitis.va.gov/vahep?page=diag-tests-03-05).

TREATMENT OPTIONS AND CLINICAL OUTCOMES

In the past two decades, considerable progress has been made in the treatment of this disease with the introduction of interferon (IFN), and, subsequently, with the addition of the guanosine analogue, ribavarin (RBV), in 1998. Typically, patients are treated with IFN by subcutaneous injection three times per week in conjunction with a daily oral dose of RBV (800-1200 mg/day, adjusted by body weight) for between 24 and 48 wk. The therapeutic efficacy is typically measured by the following end points: (1) a sustained virologic response (SVR), defined as no detectable levels of the viral RNA in serum at least 24 wk after the end of treatment; (2) a decrease in the liver enzyme, alanine aminotransferase (ALT) to within the normal reference range; and (3) signs of histological improvement as demonstrated typically by a paired liver biopsy performed prior to the initiation of therapy and at 24 wk after the end of therapy.

Correlations between serum virus levels, serum ALT levels, and liver pathology, while generally acceptable[15,16], have not been definitively established. The primary measure of current therapy is the achievement of virtually no detectable levels of HCV in serum at least 24 wk following the end of therapy, defined as the SVR. The SVR correlates well in clinical practice with patient recovery and wellbeing.

In the past decade, it has been reported that the addition of a polyethylene glycol (Peg) moiety to IFN (termed, Peg-IFN) significantly enhances its half-life in the blood, such that patients can be dosed with the Peg-IFN once weekly as compared to three times a week for the non-pegylated IFN.

In a study[17] of 531 CHC patients (190 of whom were HCV genotype 2 or 3, and 329 were genotype 1), treated with Peg-IFN or IFN, without RBV, for 48 wk and followed up for an additional 24 wk, the overall SVR at 72 wk was 39% (95% CI, 33%-45%) for Peg-IFN and 19% (95% CI, 14%-24%) for IFN. The data were not separated by genotype. The authors noted that the rate of relapse between weeks 48 and 72 was higher among patients who had a response to Peg-IFN compared to those treated with IFN. In a randomized double-blind study[18] of 1219 patients treated with Peg-IFN alone compared to IFN, both administered without RBV, significant improvements, including SVR, were noted in patients with genotype 1 virus, with a trend towards an improved, although not significant, response in patients with genotypes 2 and 3.

Treatment of CHC patients with Peg-IFN + RBV (the RBV dose typically weight-based but sometimes flat-dosed between 800 and 1200 mg/day, per os) has been shown to result in appreciably better clinical outcomes and has been touted as the so-called gold standard in the treatment of CHC. This conclusion stems almost exclusively from two studies[19,20] in which CHC patients were treated with Peg-IFN + RBV or with IFN + RBV. The data from these two studies have shown that the single greatest advantage of the Peg-IFN over IFN, both given in combination with RBV, has been the achievement of a statistically significant SVR in CHC patients with genotype 1, the recalcitrant form of this viral disease. The studies showed no consistent advantage of Peg-IFN over IFN (either one given in combination with RBV) in patients with viral genotypes 2 or 3. In the Manns *et al* study[19], the SVR was 76% for genotypes 2 or 3 and ranged from 36 to 56% in genotype 1 patients treated with IFN + RBV or Peg-IFN + RBV. In a randomized study of 1311 CHC patients given only Peg-IFN + RBV, Hadziyannis *et al*[21] reported an SVR of approximately 80% in those infected with genotype 2 or 3 virus *versus* approximately 52% in those with genotype 1 virus. Several other studies[22-38] of CHC patients with genotype 2 or 3 virus, treated with either Peg-IFN or IFN, both administered with RBV, are summarized in Table 1 and show no better results with either IFN, when given in combination with RBV, for genotypes 2 and 3 viruses.

Notwithstanding the published clinical data[19-38] summarized in Table 1, the debate on the optimal duration of Peg-IFN treatment with RBV continues. In a recent paper, Shiffman *et al*[34] have suggested that it may be prudent to treat CHC patients with genotype 2 or 3 virus for 24 wk as opposed to 16 wk, to ensure a lower percentage of relapses. The concept of an early virological response, sometimes referred to as a rapid viral response (RVR), reported in some fraction of CHC patients within 4 wk, has been proposed as a clinically useful predictor of SVR[39]. However, while this concept is valuable in CHC patients infected with genotype 1 or 4 virus, some 97% of patients with genotypes 2 and 3 viruses show an RVR, and therefore, this concept is less useful in CHC patients infected with genotype 2 or 3[40]. Importantly, the rates of relapse can increase with therapies of shorter duration, although

Table 1 Achievement of SVR by treatment with IFN + RBV or Peg-IFN + RBV in CHC patients

No. of patients	IFN + RBV	Peg-IFN + RBV	HCV genotype	Reference
446	79	81%	2/3	Manns *et al*[19]
214	61	76%	2/3	Fried *et al*[20]
492	NT	74-88	2/3	Hadziyannis *et al*[21]
253	66	NT	2/3	McHutchinson *et al*[22]
75	73	NT	non-1	Davis *et al*[23]
100	79.5	NT	3	Khokar[24]
350	85	NT	3	Muhammad *et al*[25]
20	95	NT	3	Hazari *et al*[26]
283	NT	80	2	Mangia *et al*[27]
	NT	66	3	Mangia *et al*[27]
18	72	NT	3	Medeiros-Filho *et al*[28]
28	NT	78	2/3	Gupta *et al*[29]
142	NT	81.7	2/3	Elefsiniotis *et al*[30]
1552	NT	75.6-79.2	2/3	Jacobson *et al*[31]
397	NT	83.6	2/3	Borroni *et al*[32]
230	82	78	2	Rumi *et al*[33]
356	NT	75	2	Shiffman *et al*[34]
369	NT	66	3	Shiffman *et al*[34]
285	NT	86.3-93.2	2/3	Dalgard *et al*[35]
82	73	87	2/3	Poustchi *et al*[36]
51	66.7	NT	2	Kawamura *et al*[37]
141	NT	67.5-77.8	2/3	Ferenci *et al*[38]

NT: Not tested.

the SVR rates (27.8% and 58.8%) were inexplicably low in one particular study of genotype 3 CHC patients in Pakistan[41].

Emerging data[27,30,32,38-40] suggest differences in SVR between patients with genotype 2 or 3 HCV, with genotype 2 yielding better responses than 3. There may also be additional nuances between genotypes 2a and 2c that could be used to the benefit of patients with these sub-types of HCV infection.

NEWER SUPPLEMENTAL TREATMENT OPTIONS TO INCREASE THERAPEUTIC EFFICACY AND PREVENT RELAPSE

Two new adjuvants to existing therapies with IFN + RBV or Peg-IFN + RBV are noteworthy in their potential to vastly improve SVR and clinical outcomes, and importantly, to decrease relapse rates in CHC patients. While abnormalities in glucose metabolism in CHC patients were recognized as far back as 1994[42], the application of this knowledge in the clinical setting has been introduced in just the past few years, as the epidemic of obesity and pre-diabetes or diabetes has come into sharp focus as a matter of great public health concern.

Eighty-two CHC patients[36] with genotype 2 or 3 virus were treated with IFN + RBV or Peg-IFN + RBV. Insulin resistance was measured by the homeostasis model (HOMA-IR). Patients with HOMA-IR values < 2 had an SVR of 94%, those with HOMA-IR values of 2-4 or > 4 had an SVR of 65%. The authors concluded that SVR rates > 90% are achievable in persons with low HOMA-IR values in genotype 2 and 3 patients, but drop to the 60% level more typically seen in CHC genotype 1 patients when their HOMA-IR values are > 2. Similar results have been reported more recently by others[43-46].

In an elegant series of papers from the Siddiqui Laboratory at the University of California at San Diego, *in vitro* studies on the mechanisms of HCV-induced changes in liver cells have identified the importance of intracellular calcium and oxidative stress in activating signal transducer and activator of transcription and nuclear factor-κB which in turn, contribute to the release of pro-inflammatory cytokines and contribute to liver pathology in HCV-infected cells[47-51]. This mechanism has been confirmed by others[52,53] and summarized in a recent review, in the context of immune system derangements observed in CHC[54].

We are in the early stages of designing a pilot study to evaluate the effectiveness of drugs, such as exenatide, used to overcome insulin resistance and agents that would block calcium derangements in the liver of CHC patients infected with HCV genotype 2 or 3.

CONCLUSION

Published and unpublished clinical observations suggest that an SVR is achieved in 70%-88% of CHC patients infected with genotype 2 or 3 HCV treated with IFN + RBV. A weight-of-evidence analysis using the data cited in Table 1, suggests no greater benefit in treating genotype 2 or 3 infections with Peg-IFN, which can be as much as six to 30 times more expensive than IFN (unpublished data). While the number of patients studied (Table 1) may be smaller than optimally required for a definitive answer to the issue of whether Peg-IFN, despite its additional costs, brings any clinical benefit to CHC patients with genotype 2 or 3 infection, the similarities in SVR between the two IFNs suggest that neither offers a measurable clinical benefit over the other when administered with RBV.

The 2002 National Institutes of Health (NIH) Consensus Conference Statement[55] on Management of Hepatitis C stated, "Among patients with genotypes 2 or 3, SVRs with standard interferon and ribavarin were comparable to those with pegylated interferon and ribavarin, and thus standard interferon and ribavarin could be used in treating patients with those genotypes". The published data since the 2002 NIH Consensus Statement have not falsified this recommendation. Thus, the recommendation of duration of Peg-IFN therapy for hepatitis C with these genotypes needs to be tempered by whether or not Peg-IFN offers any clinical advantage, given its much higher cost, when used with RBV, and especially in less-developed countries.

REFERENCES

1 **Weiss U**. Hepatitis C. *Nature* 2005; **436**: 929
2 **Malik IA**, Ahmad N, Luqman M, Legters LJ, Khalil-Ullah, Zaheeruddin, Ahmed A, Bukhtiari N, Nabi S, Mubarik A. Hepatitis C as a cause of chronic liver disease in northern Pakistan. *J Pak Med Assoc* 1992; **42**: 67-68
3 **Aslam M**, Aslam J. Seroprevalence of the antibody to hepatitis C in select groups in the Punjab region of Pakistan.

J Clin Gastroenterol 2001; **33**: 407-411
4 **Khan TS**, Rizvi F, Rashid A. Hepatitis C seropositivity among chronic liver disease patients in Hazara, Pakistan. *J Ayub Med Coll Abbottabad* 2003; **15**: 53-55
5 **Khokhar N**, Gill ML, Malik GJ. General seroprevalence of hepatitis C and hepatitis B virus infections in population. *J Coll Physicians Surg Pak* 2004; **14**: 534-536
6 **Muhammad N**, Jan MA. Frequency of hepatitis "C" in Buner, NWFP. *J Coll Physicians Surg Pak* 2005; **15**: 11-14
7 **Ahmad N**, Asgher M, Shafique M, Qureshi JA. An evidence of high prevalence of Hepatitis C virus in Faisalabad, Pakistan. *Saudi Med J* 2007; **28**: 390-395
8 **Alter MJ**. Epidemiology of hepatitis C virus infection. *World J Gastroenterol* 2007; **13**: 2436-2441
9 **Chaudhuri S**, Das S, Chowdhury A, Santra A, Bhattacharya SK, Naik TN. Molecular epidemiology of HCV infection among acute and chronic liver disease patients in Kolkata, India. *J Clin Virol* 2005; **32**: 38-46
10 **Hissar SS**, Goyal A, Kumar M, Pandey C, Suneetha PV, Sood A, Midha V, Sakhuja P, Malhotra V, Sarin SK. Hepatitis C virus genotype 3 predominates in North and Central India and is associated with significant histopathologic liver disease. *J Med Virol* 2006; **78**: 452-458
11 **Svirtlih N**, Delic D, Simonovic J, Jevtovic D, Dokic L, Gvozdenovic E, Boricic I, Terzic D, Pavic S, Neskovic G, Zerjav S, Urban V. Hepatitis C virus genotypes in Serbia and Montenegro: the prevalence and clinical significance. *World J Gastroenterol* 2007; **13**: 355-360
12 **Sartori M**, Andorno S, Avogadro E, Ballare M, La Terra G, Leone F, Quaglia V, Fortina G, Aglietta M. High prevalence of hepatitis C virus (HCV) genotype 2 in Italian patients with chronic liver disease. *Ital J Gastroenterol* 1996; **28**: 452-456
13 **Re V**, Lampe E, Yoshida CF, de Oliveira JM, Lewis-Ximinez L, Spinsanti L, Elbarcha O, Contigiani M. Hepatitis C virus genotypes in Cordoba, Argentina. Unexpected high prevalence of genotype 2. *Medicina* (B Aires) 2003; **63**: 205-210
14 **Bassit L**, Ribeiro-Dos-Santos G, Da Silva LC, Takei K, Villaca P, David-Neto E, Chamone D, Saez-Alquezar A. Genotype distributions of hepatitis C virus in Sao Paulo, Brazil: rare subtype found. *Hepatology* 1999; **29**: 994-995
15 **Patel K**, McHutchison JG. Initial treatment for chronic hepatitis C: current therapies and their optimal dosing and duration. *Cleve Clin J Med* 2004; **71** Suppl 3: S8-S12
16 **Saleem N**, Mubarik A, Qureshi AH, Siddiq M, Ahmad M, Afzal S, Hussain AB, Hashmi SN. Is there a correlation between degree of viremia and liver histology in chronic hepatitis C? *J Pak Med Assoc* 2004; **54**: 476-479
17 **Zeuzem S**, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ. Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med* 2000; **343**: 1666-1672
18 **Lindsay KL**, Trepo C, Heintges T, Shiffman ML, Gordon SC, Hoefs JC, Schiff ER, Goodman ZD, Laughlin M, Yao R, Albrecht JK. A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. *Hepatology* 2001; **34**: 395-403
19 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
20 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
21 **Hadziyannis SJ**, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355
22 **McHutchison JG**, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492
23 **Davis GL**, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1493-1499
24 **Khokhar N**. Effectiveness of 48 weeks Interferon alfa-2b in combination with ribavirin as initial treatment of chronic hepatitis. *J Ayub Med Coll Abbottabad* 2002; **14**: 5-8
25 **Muhammad N**, Jan MA, Rahman N. Outcome of combined interferon-ribavirin in the treatment of chronic hepatitis C. *J Coll Physicians Surg Pak* 2004; **14**: 651-653
26 **Hazari S**, Panda SK, Gupta SD, Batra Y, Singh R, Acharya SK. Treatment of hepatitis C virus infection in patients of northern India. *J Gastroenterol Hepatol* 2004; **19**: 1058-1065
27 **Mangia A**, Santoro R, Minerva N, Ricci GL, Carretta V, Persico M, Vinelli F, Scotto G, Bacca D, Annese M, Romano M, Zechini F, Sogari F, Spirito F, Andriulli A. Peginterferon alfa-2b and ribavirin for 12 vs. 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2005; **352**: 2609-2617
28 **Medeiros-Filho JE**, de Carvalho Mello IM, Pinho JR, Neumann AU, de Mello Malta F, da Silva LC, Carrilho FJ. Differences in viral kinetics between genotypes 1 and 3 of hepatitis C virus and between cirrhotic and non-cirrhotic patients during antiviral therapy. *World J Gastroenterol* 2006; **12**: 7271-7277
29 **Gupta R**, Ramakrishna CH, Lakhtakia S, Tandan M, Banerjee R, Reddy DN. Efficacy of low dose peginterferon alpha-2b with ribavirin on chronic hepatitis C. *World J Gastroenterol* 2006; **12**: 5554-5556
30 **Elefsiniotis IS**, Pantazis KD, Dimitroulopoulos D, Koutsounas S, Moulakakis A, Paraskevas E. Impact of shorter duration of treatment on virological response rate in genotype 2 or 3 chronic hepatitis C virus infection. *Indian J Gastroenterol* 2007; **26**: 209-213
31 **Jacobson IM**, Brown RS Jr, Freilich B, Afdhal N, Kwo PY, Santoro J, Becker S, Wakil AE, Pound D, Godofsky E, Strauss R, Bernstein D, Flamm S, Pauly MP, Mukhopadhyay P, Griffel LH, Brass CA. Peginterferon alfa-2b and weight-based or flat-dose ribavirin in chronic hepatitis C patients: a randomized trial. *Hepatology* 2007; **46**: 971-981
32 **Borroni G**, Andreoletti M, Casiraghi MA, Ceriani R, Guerzoni P, Omazzi B, Terreni N, Salerno F. Effectiveness of pegylated interferon/ribavirin combination in 'real world' patients with chronic hepatitis C virus infection. *Aliment Pharmacol Ther* 2008; **27**: 790-797
33 **Rumi MG**, Aghemo A, D'Ambrosio R, Ronchi G, Del Ninno E, Gallus S, Colombo M. Lack of rapid virological response predicts interferon-alpha2b/ribavirin therapy failure in HCV genotype 2 patients: a single-centre study. *Antivir Ther* 2007; **12**: 1033-1040
34 **Shiffman ML**, Suter F, Bacon BR, Nelson D, Harley H, Sola R, Shafran SD, Barange K, Lin A, Soman A, Zeuzem S. Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2007; **357**: 124-134
35 **Dalgard O**, Bjoro K, Ring-Larsen H, Bjornsson E, Holberg-Petersen M, Skovlund E, Reichard O, Myrvang B, Sundelof B, Ritland S, Hellum K, Fryden A, Florholmen J, Verbaan H. Pegylated interferon alfa and ribavirin for 14 versus 24 weeks in patients with hepatitis C virus genotype 2 or 3 and rapid virological response. *Hepatology* 2008; **47**: 35-42

36 **Poustchi H**, Negro F, Hui J, Cua IH, Brandt LR, Kench JG, George J. Insulin resistance and response to therapy in patients infected with chronic hepatitis C virus genotypes 2 and 3. *J Hepatol* 2008; **48**: 28-34

37 **Kawamura Y**, Arase Y, Ikeda K, Suzuki F, Suzuki Y, Kobayashi M, Akuta N, Hosaka T, Sezaki H, Yatsuji H, Kobayashi M, Kumada H. The efficacy of short-term interferon-beta therapy for chronic hepatitis C patients with low virus load. *Intern Med* 2008; **47**: 355-360

38 **Ferenci P**, Brunner H, Laferl H, Scherzer TM, Maieron A, Strasser M, Fischer G, Hofer H, Bischof M, Stauber R, Gschwantler M, Steindl-Munda P, Staufer K, Loschenberger K. A randomized, prospective trial of ribavirin 400 mg/day versus 800 mg/day in combination with peginterferon alfa-2a in hepatitis C virus genotypes 2 and 3. *Hepatology* 2008; **47**: 1816-1823

39 **Yu ML**, Dai CY, Huang JF, Hou NJ, Lee LP, Hsieh MY, Chiu CF, Lin ZY, Chen SC, Hsieh MY, Wang LY, Chang WY, Chuang WL. A randomised study of peginterferon and ribavirin for 16 versus 24 weeks in patients with genotype 2 chronic hepatitis C. *Gut* 2007; **56**: 553-559

40 **Berg T**, Carosi G. Optimizing outcomes in patients with hepatitis C virus genotype 2 or 3. *Antivir Ther* 2008; **13** Suppl 1: 17-22

41 **Zuberi BF**, Zuberi FF, Memon SA, Qureshi MH, Ali SZ, Afsar S. Sustained virological response based on rapid virological response in genotype-3 chronic hepatitis C treated with standard interferon in the Pakistani population. *World J Gastroenterol* 2008; **14**: 2218-2221

42 **Ishigami Y**, Kanda T, Wada M, Shimizu Y. [Glucose intolerance during interferon therapy in patients with chronic hepatitis type C] *Nippon Rinsho* 1994; **52**: 1901-1904

43 **Simo R**, Lecube A, Genesca J, Esteban JI, Hernandez C. Sustained virological response correlates with reduction in the incidence of glucose abnormalities in patients with chronic hepatitis C virus infection. *Diabetes Care* 2006; **29**: 2462-2466

44 **Imazeki F**, Yokosuka O, Fukai K, Kanda T, Kojima H, Saisho H. Prevalence of diabetes mellitus and insulin resistance in patients with chronic hepatitis C: comparison with hepatitis B virus-infected and hepatitis C virus-cleared patients. *Liver Int* 2008; **28**: 355-362

45 **Harrison SA**. Insulin resistance among patients with chronic hepatitis C: etiology and impact on treatment. *Clin Gastroenterol Hepatol* 2008; **6**: 864-876

46 **Romero-Gomez M**, Fernandez-Rodriguez CM, Andrade RJ, Diago M, Alonso S, Planas R, Sola R, Pons JA, Salmeron J, Barcena R, Perez R, Carmona I, Duran S. Effect of sustained virological response to treatment on the incidence of abnormal glucose values in chronic hepatitis C. *J Hepatol* 2008; **48**: 721-727

47 **Gong G**, Waris G, Tanveer R, Siddiqui A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proc Natl Acad Sci USA* 2001; **98**: 9599-9604

48 **Tardif KD**, Waris G, Siddiqui A. Hepatitis C virus, ER stress, and oxidative stress. *Trends Microbiol* 2005; **13**: 159-163

49 **Waris G**, Turkson J, Hassanein T, Siddiqui A. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. *J Virol* 2005; **79**: 1569-1580

50 **Waris G**, Felmlee DJ, Negro F, Siddiqui A. Hepatitis C virus induces proteolytic cleavage of sterol regulatory element binding proteins and stimulates their phosphorylation via oxidative stress. *J Virol* 2007; **81**: 8122-8130

51 **Nasimuzzaman M**, Waris G, Mikolon D, Stupack DG, Siddiqui A. Hepatitis C virus stabilizes hypoxia-inducible factor 1alpha and stimulates the synthesis of vascular endothelial growth factor. *J Virol* 2007; **81**: 10249-10257

52 **Farinati F**, Cardin R, Bortolami M, Guido M, Rugge M. Oxidative damage, pro-inflammatory cytokines, TGF-alpha and c-myc in chronic HCV-related hepatitis and cirrhosis. *World J Gastroenterol* 2006; **12**: 2065-2069

53 **Ogata H**, Chinen T, Yoshida T, Kinjyo I, Takaesu G, Shiraishi H, Iida M, Kobayashi T, Yoshimura A. Loss of SOCS3 in the liver promotes fibrosis by enhancing STAT3-mediated TGF-beta1 production. *Oncogene* 2006; **25**: 2520-2530

54 **Mengshol JA**, Golden-Mason L, Rosen HR. Mechanisms of Disease: HCV-induced liver injury. *Nat Clin Pract Gastroenterol Hepatol* 2007; **4**: 622-634

55 **NIH Consensus Statement on Management of Hepatitis C: 2002.** *NIH Consens State Sci Statements* 2002; **19**: 1-46

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REVIEW

Azoxymethane-induced rat aberrant crypt foci: Relevance in studying chemoprevention of colon cancer

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Abstract

The pathogenesis of colon cancer involves sequential and multistep progression of epithelial cells initiated to a cancerous state with defined precancerous intermediaries. Aberrant crypt foci (ACF) represent the earliest identifiable intermediate precancerous lesions during colon carcinogenesis in both laboratory animals and humans. ACF are easily induced by colon-specific carcinogens in rodents and can be used to learn more about the process of colon carcinogenesis. For over two decades, since its first discovery, azoxymethane (AOM)-induced rodent ACF have served as surrogate biomarkers in the screening of various anticarcinogens and carcinogens. Several dietary constituents and phytochemicals have been tested for their colon cancer chemopreventive efficacy using the ACF system. There has been substantial effort in defining and refining ACF in terms of understanding their molecular make-up, and extensive research in this field is currently in progress. In chemoprevention studies, AOM-induced rat ACF have been very successful as biomarkers, and have provided several standardized analyses of data. There have been several studies that have reported that ACF data do not correlate to actual colon tumor outcome, however, and hence there has been an ambiguity about their role as biomarkers. The scope of this mini-review is to provide valuable insights and limitations of AOM-induced rat ACF as biomarkers in colon cancer chemoprevention studies. The role of the dynamics and biological heterogeneity of ACF is critical in understanding them as biomarkers in chemoprevention studies.

INTRODUCTION

Aberrant crypt foci (ACF) have been identified and defined as putative precancerous lesions of the colon in both experimental models and humans[1-4]. In animal models, ACF as a biological marker have been functional in identifying both naturally-occurring and synthetically-derived compounds, as well as foods and nutritive agents for their ability to prevent or control the process of colon carcinogenesis[5-7]. On the other hand, they have also been vital in understanding toxic effects of various compounds[8]. The use of the rodent ACF as a biomarker has been successful in chemoprevention studies mainly due to the fact that they are: (a) preneoplastic lesions; (b) morphologically distinguishable from normal crypts; (c) induced in colons of several animal models; (d) remarkable similarity between human and rodents; (e) rapidly detectable; (f) easily modified by genetic changes or drug/dietary interventions; (g) amenable in short term experimental modules (8 wk); (h) classifiable according to their progression; and (i) generally present in larger populations. Whether addressing aspects of cancer prevention/control or toxicity, studies have essentially focused on the ability to modulate the incidence of total ACF or incidence of ACF displaying advanced biological features related to size, dysplasia and crypt multiplicity. It has been widely accepted that the incidence of ACF in rodents correlates strongly with the final tumor outcome[9,10]. Several factors such as dose of carcinogen, route of administration, time of intervention during the carcinogenic state, age and sex of the animals,

location and appearance of lesions in the colon, as well as the choice of animal models play a critical role in understanding the modulation of ACF, tumor outcomes, and their incidental correlation[11].

For colon cancer chemoprevention studies, the ability of compounds to regress or inhibit the incidence of total or subtypes of ACF has been utilized using either short-term (6-8 wk) or long-term (12-54 wk) carcinogen-injected rodent studies. While interpreting data arising from these studies, especially outcome of ACF and their sub-types as well as tumors, two points need special consideration. Firstly, it has to be understood that ACF, which are dynamic in nature, are amenable to growth and expansion, and may differ in number and growth features at different end-points. Secondly, in the course of their dynamics, ACF undergo several genetic changes, plausibly acquire novel phenotypes conducive to their progression, and discard those that render them normal or make them susceptible to regression[12,13]. Detailed review of the molecular features of ACF can be found in the papers by Cheng and Lai[12] and Alrawi *et al*[14], and recent advances in human ACF and their potential as biomarkers of human colorectal cancer have been elegantly discussed by Orlando *et al*[15], Stevens *et al*[16] and Gupta *et al*[17]. Depending on their growth/developmental stage, ACF are heterogeneous in their molecular make-up[12-14]. The growth and development of ACF on one hand could be regressed or inhibited, while on the other augmented by various compounds; both depend on the ACF dynamics as well as their heterogeneity[7].

ACF DYNAMICS AND CHEMOPREVENTION

In screening compounds for their efficacy to prevent or control colon cancer, the popular choices of *in vivo* models are male rats of F344, Sprague-Dawley or Wistar strains that are approximately 5-6 wk old, at least 180-200 g in weight, and injected s.c. with two weekly doses of AOM at 15 mg/kg body[5,11]. In such protocols, the test compounds are added to the diet either at the time of the first AOM injection, or after a period of 4-8 wk. While in the former protocol, the emphasis is to determine the efficacy to prevent the appearance and growth of ACF at the initiation and post-initiation stages; the latter focuses on changes in ACF that have been established for a given period at the post-initiation and promotion stages[7]. In these models, two week after the second injection, the colonic epithelial cells are initiated, and a huge population of ACF that are primal (1-3 crypts per foci) in appearance are observed in the colons[13,18]. With time (promotion stages), the cells of the aberrant crypt within the same foci clonally grow and expand to morphologically distinctive larger aberrant crypts, or those that could be classified as either intermediate (foci with 4-6 crypts) or advanced lesions. These changes are generally seen 12-wk post-carcinogen injection. With time, a decline in the incidence of primal lesions and the gradual increase in the incidence of other ACF types such as large, intermediate or advanced are noted. Towards and beyond 24 wk post-carcinogen injection which can be categorized as the progression stage, the incidence of advanced lesions reaches a peak and the appearance of microadenomas, adenomas and adenocarcinomas is evident. Another important criterion for understanding the early stages of colon carcinogenesis is the differential growth dynamics of ACF in different regions of the colon[19,20]. Spatial distribution of ACF differs among the proximal, mid and distal sections of the colon of AOM-injected F344 rats, with more incidence of ACF in the mid sections compared to the distal colon[20]. In understanding the ability of compounds to modulate the incidence of ACF and their subtypes, several comparisons can be made. For instance, the incidence of ACF or their subtypes could be compared between an experimentally-treated group and the controls (placebo, vehicle-treated) at a single time point. Otherwise, a comparison between time-frames within a single treatment group could be made. Comparisons of ACF incidence made between different colonic sections, but within same time point or same treatment group would give regional differences in modulation. An ideal method would be detailed simultaneous comparisons between time-points, treatment groups and colonic regions on the incidence of ACF and subtypes to give a cross-sectional review of the data. Similarly, the incidences of adenomas and adenocarcinomas can be compared. The entire data such as the incidences of ACF and their subtypes, adenomas and adenocarcinomas have to be considered holistically before making conclusions on the modulating efficacy of a compound.

HETEROGENEITY OF ACF AND CHEMOPREVENTION

The initiation of a single epithelial cell within the crypt to undergo histogenetic changes and gradually proliferate to form morphologically distinct foci is the first and foremost stage in the genesis of ACF in the colon. Monoclonality in ACF, including those without dysplasia, put ACF at the earliest identifiable stage of this carcinogenic process[21]. These colon precancerous lesions progressively advance by acquiring novel genotypes and phenotypes segregating these lesions into different histological and morphological sub-types identified by: (a) size, (b) degree of dysplasia, (c) crypt multiplicity, and (d) a combination of dysplasia and crypt multiplicity. Segregation of ACF into sub-types is vital to understand the disease process and to delineate the novel changes exhibited at the microscopic level in conjunction with the given appearance and characteristics of tumors. As ACF progress from one stage to another, epithelial cells within the ACF undergo genetic changes that lead to their radical behavior to skip growth inhibition or apoptosis and favor proliferation and growth[11,12]. Their goal seems to be achieving a "neoplastic bliss". Those molecular features that result in neoplastic transformation appear sequentially, and are due to a complex interplay/crosstalk of several signal transduction pathways. Purported

chemopreventive agents retard or cease the growth and/or inhibit the development of ACF or their subtypes. Compounds are known to inhibit preneoplastic changes by blocking key molecular pathways in the colonic epithelial cells. As a result, either the progression within the preneoplastic stages, or the conversion of preneoplasia to neoplastic transformation is halted. The response of a single crypt within an ACF with multiple crypts (those with four or more crypts per foci) may differ in the modulatory influence of a compound. In such a case (within multicrypt foci), although all the crypts are proposed to have developed by clonal expansion of a single transformed cell, each crypt plausibly branches itself from others by acquiring differential genotypic changes, influenced largely by the environment. The rate at which the changes are acquired by different crypts within a focus may differ considerably owing to a biological heterogenous subtype within the ACF. Hence, within one ACF, each crypt may succumb differently to growth inhibition by a particular compound. However, signals from those crypt cells that undergo inhibition may be transferred to cells that may be avoiding the inhibitory function of the compound, making the latter initiate growth inhibition. Eventually, a complex dialogue within a single ACF is created, and the crypt eventually succumbs to the inhibitory actions of the compound. Genetic and molecular heterogeneity of crypts can also be evident as histological differences such as hyperplastic and dysplastic crypts[12].

LIMITATIONS OF THE AOM-INDUCED RAT ACF AS A BIOMARKER

Limitations to the use of ACF and their sub-types as a biomarker to identify cancer preventive agents is compromised as the disease progresses from early to late stages. Increased biological segregation from normal crypts increases the specificity to the diseased state but the ability of early lesions, such as the ACF to predict the differences between two or more experimental groups are limited by the fact that their number also declines. With time, ACF undergo the selection process; while some regress, remodel or even get eliminated; others progress forward to the next stage. For example as shown in Figure 1, consider a pool of AOM-injected rat colonic ACF with different sub-types at varied duration (8, 12 and 24 wk post-initiation). In 8 wk, ACF with 1, 2 or 3 crypts per foci appear, and at this time point the incidence of total ACF (irrespective of their crypt multiplicity) becomes a valid biomarker. In subsequent weeks, ACF with higher crypt multiplicities or those with advanced biological features appear, while those with lower crypt multiplicities regress. So, at this time point, incidences of higher crypt multiplicities or those displaying advanced features could be considered more specific. In more advanced stages of the carcinogenic process, the value or sensitivity of ACF as a biomarker to predict tumor outcome diminishes; however, their specificity as an indictor of the stage remains uncompromised. The use of ACF and its sub-types as biomarkers thus depend much on the stage of

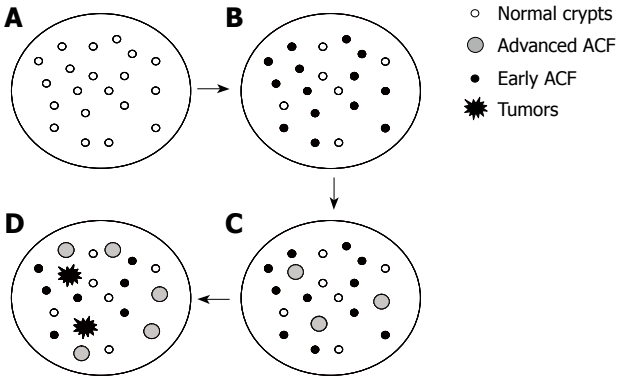

Figure 1 A pictorial representation of the dynamics of ACF during azoxymethane-induced rat colon carcinogenesis. A: Colon with normal crypts prior to AOM injection; B: Colon bearing early ACF at approximately 8-wk post AOM injections; C: Colon bearing early and advanced ACF at approximately 12-wk post AOM injections; D: Colon bearing early and advanced ACF and tumors at approximately 24-wk post AOM injections. As the population of ACF (inclusive of all sub-types) increase with time after AOM injections, a relative decline in the specificity as a biological marker is observed.

colon carcinogenesis. Only at early stages, total number of ACF and ACF displaying different biological features may be considered as a valid biomarker. At very early stages the relevance of utilizing a parameter involving advanced lesions such as aberrant crypts per ACF (AC/ACF) becomes invalid, as at early stages to begin with there may not be a significant pool of ACF with varied features. The description as 'very early', 'early' and 'advanced' stages of colon carcinogenesis have been used cautiously and have considered the AOM-induced rats as the model system (described in the former Section).

ACF with severe dysplasia have been identified as the actual precursors of colonic adenomas and adenocarcinomas[22]. The recent findings of the presence of β-catenin accumulated aberrant crypts (BCAC)[23], mucin-depleted crypts (MDF)[24], and flat-dysplastic ACF (FDACF)[25] in carcinogen-treated rat colons are significant advances in the identification of events leading to tumor development. The morphological features of these lesions resemble those of advanced ACF, as described earlier[10,13,14]; strongly suggesting that they plausibly are subtypes of advanced ACF. However, it is still unclear as to the exact evolutionary sequence of BCAC, MDF or FDACF, and their relevance to the human situation. These specific ACF-subtypes are purported to accurately predict tumor outcome[20-22]. However using them as valid biomarkers to screen chemicals/agents for potential colon cancer chemoprevention requires further scrutiny. Indeed, the simplistic methylene blue staining method in assessing ACF in whole mounts of colon remains a valuable tool in screening compounds for their colon cancer chemopreventive potential.

CONCLUSION

ACF are unique biological markers for the purpose of assessing potential colon cancer chemopreventive efficacy of compounds during the early stages of colon carcinogenesis. Interpreting the ability of compounds

to retard or inhibit the incidence of ACF is becoming evidently complex. Sometimes, ACF data seem not to correlate with that of tumor outcome; in such cases, a single variable such as ACF incidence at a given time point to that of tumor outcome may have to be taken for correlative consideration. Incorporation of the cross-sectional criteria described in the earlier sections may facilitate a realistic correlation between the incidences of ACF and tumor outcome. Thus, in interpreting ACF data, their dynamics and heterogeneity need to be considered in order to formulate stronger correlations to tumor outcomes. A recent trend in the identification of ACF subtypes are certainly useful in understanding their biology, but their use as biomarkers in efficacy studies requires caution, especially when the relation between their specificity and predictability at a given time of the carcinogenic process is taken in to account. More studies are encouraged to identify the molecular repertoire of ACF that categorize them as preneoplastic lesions of the colon. Abrogating the growth and development of preneoplastic lesions emphasizes an early strategy on cancer prevention or control. Defining the role of ACF in colon carcinogenesis is vital to understand the relevance as a biological marker in the study of chemopreventive agents. The short-term AOM-induced rat ACF thus remains an ideal early biomarker for the identification of various chemopreventive agents against colon cancer.

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REFERENCES

1 **Bird RP**. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* 1987; **37**: 147-151
2 **Roncucci L**, Stamp D, Medline A, Cullen JB, Bruce WR. Identification and quantification of aberrant crypt foci and microadenomas in the human colon. *Hum Pathol* 1991; **22**: 287-294
3 **Pretlow TP**, Barrow BJ, Ashton WS, O'Riordan MA, Pretlow TG, Jurcisek JA, Stellato TA. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. *Cancer Res* 1991; **51**: 1564-1567
4 **Kim J**, Ng J, Arozulllah A, Ewing R, Llor X, Carroll RE, Benya RV. Aberrant crypt focus size predicts distal polyp histopathology. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 1155-1162
5 **Reddy BS**. Studies with the azoxymethane-rat preclinical model for assessing colon tumor development and chemoprevention. *Environ Mol Mutagen* 2004; **44**: 26-35
6 **Corpet DE**, Tache S. Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr Cancer* 2002; **43**: 1-21
7 **Bird RP**, Good CK. The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Toxicol Lett* 2000; **112-113**: 395-402
8 **Bruce WR**. Aberrant crypt foci in the detection of colon carcinogens. *Prog Clin Biol Res* 1990; **347**: 129-137
9 **Magnuson BA**, Carr I, Bird RP. Ability of aberrant crypt foci characteristics to predict colonic tumor incidence in rats fed cholic acid. *Cancer Res* 1993; **53**: 4499-4504
10 **Pretlow TP**, O'Riordan MA, Somich GA, Amini SB, Pretlow TG. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis* 1992; **13**: 1509-1512
11 **Bird RP**. Aberrant crypt foci system to study cancer preventive agents in the colon. In: Hanausek M, Walaszek Z. Methods in molecular medicine: Tumor Marker Protocols, Totowa. New Jersey: Humana, 1998; **14**: 465-474
12 **Cheng L**, Lai MD. Aberrant crypt foci as microscopic precursors of colorectal cancer. *World J Gastroenterol* 2003; **9**: 2642-2649
13 **McLellan EA**, Medline A, Bird RP. Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. *Cancer Res* 1991; **51**: 5270-5274
14 **Alrawi SJ**, Schiff M, Carroll RE, Dayton M, Gibbs JF, Kulavlat M, Tan D, Berman K, Stoler DL, Anderson GR. Aberrant crypt foci. *Anticancer Res* 2006; **26**: 107-119
15 **Orlando FA**, Tan D, Baltodano JD, Khoury T, Gibbs JF, Hassid VJ, Ahmed BH, Alrawi SJ. Aberrant crypt foci as precursors in colorectal cancer progression. *J Surg Oncol* 2008; **98**: 207-213
16 **Stevens RG**, Swede H, Rosenberg DW. Epidemiology of colonic aberrant crypt foci: review and analysis of existing studies. *Cancer Lett* 2007; **252**: 171-183
17 **Gupta AK**, Pretlow TP, Schoen RE. Aberrant crypt foci: what we know and what we need to know. *Clin Gastroenterol Hepatol* 2007; **5**: 526-533
18 **McLellan EA**, Medline A, Bird RP. Dose response and proliferative characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. *Carcinogenesis* 1991; **12**: 2093-2098
19 **Shpitz B**, Bomstein Y, Mekori Y, Cohen R, Kaufman Z, Neufeld D, Galkin M, Bernheim J. Aberrant crypt foci in human colons: distribution and histomorphologic characteristics. *Hum Pathol* 1998; **29**: 469-475
20 **Ghirardi M**, Nascimbeni R, Villanacci V, Fontana MG, Di Betta E, Salerni B. Azoxymethane-induced aberrant crypt foci and colorectal tumors in F344 rats: sequential analysis of growth. *Eur Surg Res* 1999; **31**: 272-280
21 **Siu IM**, Robinson DR, Schwartz S, Kung HJ, Pretlow TG, Petersen RB, Pretlow TP. The identification of monoclonality in human aberrant crypt foci. *Cancer Res* 1999; **59**: 63-66
22 **Nucci MR**, Robinson CR, Longo P, Campbell P, Hamilton SR. Phenotypic and genotypic characteristics of aberrant crypt foci in human colorectal mucosa. *Hum Pathol* 1997; **28**: 1396-1407
23 **Yamada Y**, Yoshimi N, Hirose Y, Matsunaga K, Katayama M, Sakata K, Shimizu M, Kuno T, Mori H. Sequential analysis of morphological and biological properties of beta-catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. *Cancer Res* 2001; **61**: 1874-1878
24 **Caderni G**, Femia AP, Giannini A, Favuzza A, Luceri C, Salvadori M, Dolara P. Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. *Cancer Res* 2003; **63**: 2388-2392
25 **Paulsen JE**, Loberg EM, Olstorn HB, Knutsen H, Steffensen IL, Alexander J. Flat dysplastic aberrant crypt foci are related to tumorigenesis in the colon of azoxymethane-treated rat. *Cancer Res* 2005; **65**: 121-129

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REVIEW

Genetic determination of irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder. According to the Rome Ⅲ criteria, IBS is defined as recurrent abdominal pain or discomfort for at least 3 d per month during the previous 3 mo associated with two or more of the following symptoms: improvement with defecation, onset associated with a change in the frequency of stool and/or onset associated with a change in form or appearance of stool. There is growing evidence regarding the genetic contribution in IBS, however the precise etiology of IBS is still unknown. The evaluation of the genetic influence is based on twin studies, familial aggregation and genetic epidemiological investigations. Most studies showed a concordance for IBS significantly greater in monozygotic than in dizygotic twins. The majority of the studies have shown that familial aggregation may represent exposures to a similar environment, as well as the influence of genetic factors. Whereas no specific gene has been identified in association with IBS, recent studies have noticed the importance of polymorphisms in the promoter region of the serotonin reuptake transporter gene, G-protein beta 3 subunit gene (*C825T*), cholecystokinin receptor (*CCKAR* gene 779T>C), and high-producer tumor necrosis factor genotype. Further studies are necessary to determine how genetic factors influence the clinical manifestations and therapeutical response in IBS patients.

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Key words: Irritable bowel syndrome; Genetic factors; Gene polymorphisms; Twin studies; Familial aggregation

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INTRODUCTION

Irritable bowel syndrome (IBS) is defined according to Rome Ⅲ criteria as recurrent abdominal pain or discomfort for at least 3 d per month during the previous 3 mo associated with two or more of the following symptoms: improvement with defecation, onset associated with a change in the frequency of stools and/or onset associated with a change in form or appearance of stools[1,2]. There are four types of IBS: constipation-predominant IBS, diarrhea-predominant IBS, mixed IBS and unclassified IBS. Within one year, 75% of patients change subtypes. Population-based studies show prevalence of IBS to be 10%-20% and an incidence level of IBS at 1%-2% per year[3].

The precise etiology of IBS is still unknown, but there is evidence in the last decade regarding the contribution of genetic, infectious, psychosocial and dietary factors.

Although to date there has been little support found for the role of different genes in the development of IBS, growing evidence suggests that genetic factors may contribute to the etiology and clinical manifestations of IBS.

The evaluation of genetic influence is based on familial aggregation, twin studies, and genetic epidemiological studies focusing on gene polymorphisms.

FAMILIAL AGGREGATION

Although the family clustering of IBS has been noticed in medical practice for several years, Whorwell undertook

pioneering work regarding familial aggregation of IBS. He found that 33% of patients with IBS reported a family history of IBS compared with only 2% of the control group[4].

A family cluster study of 643 subjects from Olmsted County, USA, showed a significant association between having a first-degree relative with bowel problems and presenting with IBS [odds ratio, 2.3; 95% confidence interval (CI), 1.3-3.9]. Those who reported having a spouse with bowel problems were no more likely to present with IBS. The authors also concluded that familial associations may represent exposure to a similar environment as well as the influence of genetic factors[5].

A recent study that directly surveyed relatives showed a prevalence of IBS of 17% in patients' relatives *vs* 7% in spouses' relatives [odds ratio adjusted for age and sex 2.7 (95% CI, 1.2-6.3)]. When also adjusted for somatization score, the odds ratio was 2.5 (95% CI, 0.9-6.7). The authors concluded that IBS presents a familial aggregation due to genetic or intrafamilial environmental factors, but this may be partially explained by familial aggregation of somatization[6].

Some limitations of these studies were that only abdominal symptoms in first-degree relatives were assessed and that the IBS diagnosis was not confirmed by a specialist.

Some studies have shown that specific types of gastrointestinal illness behavior may be learned through modeling[7], thus biasing the data on genetic transmission.

A Japanese study showed that both patients with IBS and IBS nonconsulters were more likely than controls to present positive family history (33.9% *vs* 12.6%, $P < 0.001$, for patients; 26.1% *vs* 12.6%, $P < 0.01$, for nonconsulters). The parental history was associated with a significantly higher impact on clinical manifestations, including indigestion, diarrhea, constipation, anxiety. The authors concluded that a parental history of bowel problems represents a significant risk factor for development of IBS in Japan, as reported in USA. Patients with a family history present with more psychological distress than other patients[8].

TWINS STUDIES

The contribution of genetic factors to the development of IBS was assessed in several major twin studies.

The Australian study, conducted on 343 twin pairs, by Morris-Yates, in 1998, showed a higher concordance rate for IBS in monozygotic twins than in dizygotic twins (33.3% *vs* 13.3%). This study, revealing that 56.9% (95% CI, 40.6%-75.9%) of the variance is attributed to additive genetic factors, shows a substantial involvement of the genetic component in IBS[9].

The American study conducted by Levy and published in 2001, studied the concordance rate of IBS among 6060 twin pairs. The concordance rate in monozygotic twins was twice as high as that in dizygotic twins (17.2% *vs* 8.4%, $P = 0.03$). However, the number of dizygotic twins with IBS who have mothers with IBS was greater than the number of dizygotic twins with IBS who have co-twins with IBS (15.2% *vs* 6.7%, $P < 0.001$); data also showed that having a mother or a father with IBS are both independent predictors of irritable bowel status ($P < 0.001$) and both are stronger predictors than having a twin with IBS. Data about the other twin accounted for little additional predictive power. The study concluded that heredity contributes to development of IBS, but social learning has also an important influence[10].

In contrast with these studies, a British study published in 2005, which included 1870 twin pairs, showed an IBS prevalence of 17% in monozygotic twins and 16% in dizygotic twins. There was no significant difference regarding the concordance rates between monozygotic and dizygotic twins (28% *vs* 27%). Logistic regression analysis revealed that decreasing age and increasing psychosomatic score were independent factors associated with IBS. Somatization was shown to be moderately heritable. The study concluded that genetic factors are of little or no contribution to the development of IBS[11].

A Norwegian study published in 2006 showed a concordance for IBS significantly greater in monozygotic (22.4%) than in dizygotic (9.1%) twins ($P = 0.011$). The heritability of IBS was found to be 48.4% among females. Birth weight below 1500 g (adjusted odds ratio 2.4 (95% CI, 1.1-5.3)) influenced significantly the development of IBS, which occurred 7.7 years earlier in the low weight group than in higher weight groups[12].

Another recent twin study performed on 986 twin pairs and published in 2007 showed a polychoric correlation for monozygotic twins and IBS (0.47) larger than that for dizygotic twins (0.17). Genetic variance was 22%, but adjusting for anxiety and depression removed the statistical significance for IBS. The study concluded that genetic factors are involved in IBS, possibly mediated by the hereditability of anxiety and depression[13].

GENE POLYMORPHISMS

No single pathophysiologic mechanism explains entirely the clinical manifestations of IBS. Current evidence suggests that altered brain-gut axis is the key mechanism associated with disordered motility, visceral hypersensitivity and autonomic dysfunction[3,14]. Regulation of these connections occurs *via* numerous neurotransmitters such as CCK, VIP, substance P, serotonin (5-hydroxytryptamine, 5-HT). Recent studies have also shown the involvement of the corticotropin-releasing hormone (CRH) in stress-related pathophysiology of IBS and possibly in inflammation of the intestinal mucosa[15].

Genetic factors may influence all these mechanisms, affecting both central and peripheral levels of the brain-gut interrelations[16].

Some authors have shown that substances and genes involved in the brain-gut axis may represent the key factor solving IBS[14].

Gene polymorphisms involve the serotonergic, adrenergic and opioidergic systems, and genes encoding proteins with immunomodulatory and/or neuromodulatory features[17,18].

Serotonin polymorphisms

At the gastrointestinal level, 5-HT acts as a paracrine signalling molecule and as a transmitter released by serotonergic interneurons. Serotonin activates at least five types of receptors, influencing intestinal peristalsis, secretion and signalling in the brain-gut axis. Based on the differences in structure and function, seven types of 5-HT receptors have been described[18]. In patients with IBS, stimulation of 5-HT type 3 receptors may lead to cramps, urgency, diarrhea and colonic contractions. The serotonin removal from the sites of action is mediated by a specific protein, the serotonin reuptake transporter (SERT). Increased 5-HT release in the bowel may induce diarrhea, nausea, and vomiting[19].

A high level of 5-HT may result from exaggerated synthesis, excessive release, or inadequate uptake and inactivation. Modifications in the serotonin transporter, responsible for removing 5-HT from the interstitial space and terminating its action, may also contribute to gastrointestinal motility troubles[19].

Whereas no specific gene has been identified in association with IBS, recent studies have demonstrated the importance of polymorphisms in the promoter region of the serotonin reuptake transporter gene for motility disorders[19].

The *SERT* gene encoding the SERT protein is located on the chromosome 17q11.2-q1. A functional polymorphism, an insertion or deletion of 44 base pairs in the 5-HT-transporter-gene-linked polymorphic region (5-HTTLPR), was first reported in 1996 by Heils[20].

An association between 5-HTTLPR polymorphism and diarrhea in women with IBS has been reported[19]; however, insufficient numbers of constipation-predominant IBS patients represented a limitation of this study. Homozygous or heterozygous patients for the 5-HTTLPR deletion (the short allele) have decreased transcription of *SLC6A4*, reduced expression of the sodium-dependent serotonin transporter and, therefore, reduced reuptake of serotonin. Homozygous patients for the 5-HTTLPR insertion polymorphism (the long allele) present wth significantly slower colonic transit than heterozygous patients, whereas no difference regarding the colonic transit time is found between homozygous or heterozygous patients for the 5-HTTLPR deletion polymorphism[17,19].

The 5-HTTLPR polymorphism was also studied in various behavioral traits and psychiatric disorders; a recent study has shown that IBS patients homozygous for the short allele of 5-HTTLPR or carrying a STin2.9VNTR allele (polymorphism located in intron 2 of *SERT* gene) are significantly more likely to present a history of depression[20].

Another study has revealed that 5-HTTLPR long allele may be one pathway that activates negative emotion in females and has a contrary action in males[21].

A recent Chinese study has investigated the relationship between polymorphisms of the serotonin reuptake transporter and the IBS subtypes, and its influence on the efficacy of tegaserod treatment. This study suggests that individuals with the long allele genotype are vulnerable to develop IBS with constipation and respond poorly to tegaserod treatment[22]. Another study has shown that alosetron, a 5-HT type 3 receptor antagonist, relieves IBS-related pain and normalizes bowel function in women with diarrhea-predominant IBS. This study also suggests that this drug is more effective in homozygous patients than in heterozygous patients for 5-HTTLPR insertion. However, the small number of patients enrolled in this study does not permit a firm conclusion[23,24].

Interaction of serotonergic and adrenergic receptors

Adrenergic agents act upon the sensory and motor function of the human gastrointestinal tract, *via* α_2 adrenergic receptors (adrenoceptors). Three human α_2 adrenoceptors have been identified: the α_{2A}, α_{2B} and α_{2C} subtypes.

There is evidence that polymorphisms in the genes encoding these receptors may decrease the synaptic autoinhibitory feedback and increase presynaptic release of norepinephrine. Norepinephrine transporter modulates the synaptic level of norepinephrine. There is evidence of an interaction between serotonin and norepinephrine in the modulation of gastrointestinal function[25]. Associations have been observed between polymorphisms in *SLC6A4* or genes that encode α_{2A} and α_{2C} adrenoceptors and IBS phenotypes. A polymorphism in *ADRA2C*, the gene encoding the α_{2C} adrenoceptor, is associated with an increased likelihood of constipation in patients with gastrointestinal functional disorders ($P = 0.05$), and also with an increased likelihood of severe and frequent somatic symptoms. Polymorphisms in the genes encoding the α_{2A} adrenoceptor and the α_{2C} adrenoceptor are significantly associated with a high somatic symptom score[26].

In patients with functional dyspepsia, modified alpha-adrenoreceptor function and depression are common; features that are linked to a G-protein beta 3 (GNB3) subunit gene polymorphism (C825T), as shown in a study published in 2004. G-protein beta 3 subunit 825 *CC* genotype was significantly associated with unexplained (functional) dyspepsia; the odds ratio adjusted for age and sex for upper abdominal symptoms was 2.2 (95% CI, 1.4-3.3)[27].

Cholecystokinin

Cholecystokinin (CCK) is released by endocrine I cells within the duodenal and jejunal mucosa in response to the products of protein and fat digestion.

Patients with IBS have high fasting and postprandial plasma levels of CCK. The effects of CCK are mediated *via* CCK-A receptor and CCK-B receptor (also known as CCK1-R and CCK2-R) located in the peripheral and central nervous system. Therapeutical blockade of CCK-A receptors may stimulate gut motility and may

reduce colonic transit time in patients with constipation-predominant IBS[28].

A polymorphism in *CCKAR* gene (779T>C) in IBS patients with constipation is associated with slower gastric emptying. This suggests that, compared with the 779T variant, the 779C substitution results in an increased response to endogenous CCK, retarding the gastric emptying. Future confirmatory studies are needed, due to the small sample size in this study[29].

Cytokines

Although the definition of IBS states that no active inflammation causes symptoms, transient mucosal inflammation is considered to be an important factor for the manifestation of IBS.

Cytokines are involved in the regulation of the immune and inflammatory reaction with proinflammatory effects, such as tumor necrosis factor (TNF) and interferon, and with anti-inflammatory effects, such as interleukin 10 (IL-10) and transforming growth factor (TGF) β1.

A genetic predisposition to produce high or low amounts of a particular cytokine may modify the susceptibility to a certain disease, or affect its clinical manifestation. Some studies have shown no significant association between TGF β genotypes and IBS patients.

By contrast, a low prevalence of the high-producer genotype of IL-10 in patients with IBS has been noticed, suggesting the association between the predisposition to produce low amounts of this cytokine and IBS, or a possible protective role of high levels of IL-10. A genetic predisposition to produce low levels of anti-inflammatory cytokines may signify a compromised control of the inflammatory response[17,30].

Another study has shown that high-producer TNF-α genotype is more prevalent in IBS patients than in healthy subjects. Homozygous high-producer genotypes were rare in both groups; the heterozygous genotype was found in 41% of IBS patients *vs* only 26% of healthy controls. By contrast, no significant difference regarding the IL-10 genotypes prevalence was found in IBS patients and control group. The authors concluded that high producer TNF-α and low producer IL-10 genotypes were significantly more prevalent in IBS patients than control group (9% *vs* 3%, $P = 0.035$) and in diarrhea (20%) compared to other IBS subtypes (< 4%, $P = 0.026$)[30]. Another study showed a significantly low prevalence of the high-producer IL-10 genotype in IBS patients compared with controls (21% *vs* 32%)[31]. This difference might be related to variation in genotype frequencies according to ethnicity: the high-producer IL-10 genotype is significantly higher in the Irish population (34%) than in Africans (9.5%). A limitation of the study consisted of the assessment of only one anti-inflammatory cytokine polymorphism[17].

Post-infectious IBS

A recent study evaluated the involvement of genetic factors in post-infectious IBS; the authors capitalized on the opportunity to study these correlations after the contamination of the municipal water in a small rural town in Canada. They identified four candidates associated with post-infectious IBS: two located in Toll-like receptor 9 (the coding SNP rs352139 and the promoter SNP rs5743836), one involving the promoter SNP of E-cadherin (rs16260) and the other involving a promoter SNP of IL-6 (rs1800795). The authors concluded that after corrections for multiple comparisons, none of these associations is significant and stressed the importance of the further population studies for validations of the gene candidates in post-infectious IBS[32].

Psychological distress

Association between psychological distress and IBS may be due to common genetic factors, but this remains debatable.

The association between major depressive disorder and IBS (with a 13%-45% co-occurrence) may involve genetic and environmental common pathophysiological mechanisms[33]. A recent study has shown the lack of specificity regarding this association; chronic widespread pain related to fibromyalgia and chronic fatigue are also associated with IBS. The authors concluded that genetic and family environmental factors do not explain the association between IBS and major depressive disorder[33,34].

Another recent study has investigated the genetic compo-nent in the co-occurrence of IBS with psychological factors; the authors have shown that independent risk factors for IBS are: female gender, somatization, neuroticism, phobia; no environmental factors are significantly involved. Depression and neuroticism do not co-occur with IBS through common genetic factors, whereas somatization associated with IBS share common genetic components[35]. The authors concluded that identifying the genes involved in somatization may represent a key in understanding IBS etiology[35].

CONCLUSION

IBS is a multifactorial functional disorder, resulting from a complex interaction of genes, environment and psychosocial factors. Recent studies have shown that genes may play an important role in IBS. Thus, the environmental factors can trigger the clinical manifestations of IBS when acting on a certain genetic background.

Genetic factors may be directly linked to gastro-intestinal sensory and motor functions or cause initiation of the modifications underlying the symptoms in the presence of exogenous factors.

Further studies are necessary to develop the facts known so far about the genetic determination of the clinical features and therapeutical response in IBS.

REFERENCES

1 **Longstreth GF**, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491

2 **Drossman DA**, Dumitrascu DL. Rome III: New standard for functional gastrointestinal disorders. *J Gastrointestin Liver Dis* 2006; **15**: 237-241

3 **Drossman DA**, Morris CB, Hu Y, Toner BB, Diamant N, Leserman J, Shetzline M, Dalton C, Bangdiwala SI. A prospective assessment of bowel habit in irritable bowel syndrome in women: defining an alternator. *Gastroenterology* 2005; **128**: 580-589

4 **Whorwell PJ**, McCallum M, Creed FH, Roberts CT. Non-colonic features of irritable bowel syndrome. *Gut* 1986; **27**: 37-40

5 **Locke GR 3rd**, Zinsmeister AR, Talley NJ, Fett SL, Melton LJ 3rd. Familial association in adults with functional gastrointestinal disorders. *Mayo Clin Proc* 2000; **75**: 907-912

6 **Kalantar JS**, Locke GR 3rd, Zinsmeister AR, Beighley CM, Talley NJ. Familial aggregation of irritable bowel syndrome: a prospective study. *Gut* 2003; **52**: 1703-1707

7 **Levy RL**, Whitehead WE, Von Korff MR, Feld AD. Intergenerational transmission of gastrointestinal illness behavior. *Am J Gastroenterol* 2000; **95**: 451-456

8 **Kanazawa M**, Endo Y, Whitehead WE, Kano M, Hongo M, Fukudo S. Patients and nonconsulters with irritable bowel syndrome reporting a parental history of bowel problems have more impaired psychological distress. *Dig Dis Sci* 2004; **49**: 1046-1053

9 **Morris-Yates A**, Talley NJ, Boyce PM, Nandurkar S, Andrews G. Evidence of a genetic contribution to functional bowel disorder. *Am J Gastroenterol* 1998; **93**: 1311-1317

10 **Levy RL**, Jones KR, Whitehead WE, Feld SI, Talley NJ, Corey LA. Irritable bowel syndrome in twins: heredity and social learning both contribute to etiology. *Gastroenterology* 2001; **121**: 799-804

11 **Mohammed I**, Cherkas LF, Riley SA, Spector TD, Trudgill NJ. Genetic influences in irritable bowel syndrome: a twin study. *Am J Gastroenterol* 2005; **100**: 1340-1344

12 **Bengtson MB**, Ronning T, Vatn MH, Harris JR. Irritable bowel syndrome in twins: genes and environment. *Gut* 2006; **55**: 1754-1759

13 **Lembo A**, Zaman M, Jones M, Talley NJ. Influence of genetics on irritable bowel syndrome, gastro-oesophageal reflux and dyspepsia: a twin study. *Aliment Pharmacol Ther* 2007; **25**: 1343-1350

14 **Fukudo S**, Hongo M. On the organ choice in psychosomatic disorders. Irritable bowel syndrome: a disorder of abnormal brain-gut interactions. *Japanese Journal of Psychosomatic Medicine* 1999; **39**: 159-166

15 **Fukudo S**. Role of corticotropin-releasing hormone in irritable bowel syndrome and intestinal inflammation. *J Gastroenterol* 2007; **42** Suppl 17: 48-51

16 **Saito YA**, Petersen GM, Locke GR 3rd, Talley NJ. The genetics of irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2005; **3**: 1057-1065

17 **Adam B**, Liebregts T, Holtmann G. Mechanisms of disease: genetics of functional gastrointestinal disorders--searching the genes that matter. *Nat Clin Pract Gastroenterol Hepatol* 2007; **4**: 102-110

18 **Cervio E**, Rondanelli M, Balestra B, Dellabianca A, Agazzi A, Giacosa A, Tonini M. [Recent insights into the pathogenesis of abdominal symptoms in functional bowel disorders] *Recenti Prog Med* 2007; **98**: 69-73

19 **Yeo A**, Boyd P, Lumsden S, Saunders T, Handley A, Stubbins M, Knaggs A, Asquith S, Taylor I, Bahari B, Crocker N, Rallan R, Varsani S, Montgomery D, Alpers DH, Dukes GE, Purvis I, Hicks GA. Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women. *Gut* 2004; **53**: 1452-1458

20 **Jarrett ME**, Kohen R, Cain KC, Burr RL, Poppe A, Navaja GP, Heitkemper MM. Relationship of SERT polymorphisms to depressive and anxiety symptoms in irritable bowel syndrome. *Biol Res Nurs* 2007; **9**: 161-169

21 **Mizuno T**, Aoki M, Shimada Y, Inoue M, Nakaya K, Takahashi T, Itoyama Y, Kanazawa M, Utsumi A, Endo Y, Nomura T, Hiratsuka M, Mizugaki M, Goto J, Hongo M, Fukudo S. Gender difference in association between polymorphism of serotonin transporter gene regulatory region and anxiety. *J Psychosom Res* 2006; **60**: 91-97

22 **Li Y**, Nie Y, Xie J, Tang W, Liang P, Sha W, Yang H, Zhou Y. The association of serotonin transporter genetic polymorphisms and irritable bowel syndrome and its influence on tegaserod treatment in Chinese patients. *Dig Dis Sci* 2007; **52**: 2942-2949

23 **Camilleri M**, Northcutt AR, Kong S, Dukes GE, McSorley D, Mangel AW. Efficacy and safety of alosetron in women with irritable bowel syndrome: a randomised, placebo-controlled trial. *Lancet* 2000; **355**: 1035-1040

24 **Camilleri M**, Atanasova E, Carlson PJ, Ahmad U, Kim HJ, Viramontes BE, McKinzie S, Urrutia R. Serotonin-transporter polymorphism pharmacogenetics in diarrhea-predominant irritable bowel syndrome. *Gastroenterology* 2002; **123**: 425-432

25 **Hirafuji M**, Ogawa T, Kato K, Hamaue N, Endo T, Parvez H, Minami M. Noradrenaline stimulates 5-hydroxytryptamine release from mouse ileal tissues via alpha(2)-adrenoceptors. *Eur J Pharmacol* 2001; **432**: 149-152

26 **Kim HJ**, Camilleri M, Carlson PJ, Cremonini F, Ferber I, Stephens D, McKinzie S, Zinsmeister AR, Urrutia R. Association of distinct alpha(2) adrenoceptor and serotonin transporter polymorphisms with constipation and somatic symptoms in functional gastrointestinal disorders. *Gut* 2004; **53**: 829-837

27 **Holtmann G**, Siffert W, Haag S, Mueller N, Langkafel M, Senf W, Zotz R, Talley NJ. G-protein beta 3 subunit 825 CC genotype is associated with unexplained (functional) dyspepsia. *Gastroenterology* 2004; **126**: 971-979

28 **Cremonini F**, Camilleri M, McKinzie S, Carlson P, Camilleri CE, Burton D, Thomforde G, Urrutia R, Zinsmeister AR. Effect of CCK-1 antagonist, dexloxiglumide, in female patients with irritable bowel syndrome: a pharmacodynamic and pharmacogenomic study. *Am J Gastroenterol* 2005; **100**: 652-663

29 **D'Amato M**, Rovati LC. Cholecystokinin-A receptor antagonists: therapies for gastrointestinal disorders. *Expert Opin Investig Drugs* 1997; **6**: 819-836

30 **van der Veek PP**, van den Berg M, de Kroon YE, Verspaget HW, Masclee AA. Role of tumor necrosis factor-alpha and interleukin-10 gene polymorphisms in irritable bowel syndrome. *Am J Gastroenterol* 2005; **100**: 2510-2516

31 **Gonsalkorale WM**, Perrey C, Pravica V, Whorwell PJ, Hutchinson IV. Interleukin 10 genotypes in irritable bowel syndrome: evidence for an inflammatory component? *Gut* 2003; **52**: 91-93

32 **Villani A**, Lemire M, Thabane M, Geneau G, Belisle A, Fortin G, Collins SM, Franchimont D, Marshall JK. Genetic risk factors for post-infectious IBS in the walkerton outbreak of waterborne gastroenteritis. *Gastroenterology Suppl* 2008; **134**: A122

33 **Whitehead WE**. Twin studies used to prove that the comorbidity of major depressive disorder with IBS is NOT influenced by heredity. *Am J Gastroenterol* 2007; **102**: 2230-2231

34 **Wojczynski MK**, North KE, Pedersen NL, Sullivan PF. Irritable bowel syndrome: a co-twin control analysis. *Am J Gastroenterol* 2007; **102**: 2220-2229

35 **Talley NJ**, Jones M, Lembo A. Psychological co-morbidity with irritable bowel syndrome is influenced by heredity: A U.S. co-twin study. *Gastroenterology Suppl* 2008; **134**: A276

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TOPIC HIGHLIGHT

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How expensive is inflammatory bowel disease? A critical analysis

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Abstract

Economic analysis of chronic diseases is required for proper allocation of resources and understanding cost-effectiveness studies of new therapies. Studies on health care cost of ulcerative colitis (UC) and Crohn's disease (CD) are reviewed here. These studies were carried out in various countries with disparate health care systems. In the United States, data were often modeled or retrieved from large insurance schemes. Surgery and in-patient hospitalization accounted for over half the outlay on UC and CD. Fistulous disease in CD and parenteral nutrition were very costly. In Canada, overall charges were lower than in the United States, but there too, surgical costs were relatively high. In European studies, economic data were abstracted directly from patients' files. One pan-European study examined the outlay on UC and CD in a community-based prospective inception cohort followed for 10 years. Overall costs in Europe were lower than in the United States. Surgery, hospitalization, year of follow-up, disease phenotype in CD and ASCA-positivity impacted significantly on costs. In all studies, the cost data were right skewed, aminosalicylates were expensive drugs, and biological agents the most expensive; moreover indirect costs were not calculated. Infliximab raised costs considerably in CD, but there were no long-term follow-up studies, so that the cost-benefit of biological agents remains unknown. In conclusion, costs of managing UC and CD vary by country, surgery, genotype and several other factors. The most important question for further research is whether the biological therapies are cost-effective in the long-term.

INTRODUCTION

The science of medical economics, as a branch of welfare economics, is concerned with providing quality health care to patients while streamlining costs. In the current age of escalating health care charges and growing constraints on national health budgets, formal economic analysis of chronic diseases is essential for the proper allocation of resources and the design of cost-effectiveness studies of novel therapeutic agents and diagnostic modalities that are invariably more expensive than existing methods. The fundamental requirements of good economic studies are given in Table 1. Inflammatory bowel disease (IBD) comprises ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis (IC). These are idiopathic chronic inflammatory conditions that affect the intestines in particular, with a high rate of extraintestinal manifestations[1]. CD and UC present predominantly in young adults, whose concerns relate to their higher education, careers and establishing their families. These persons then find themselves having to cope with illnesses that have life-time morbidity. Life-long medical treatment is required, and often surgery. CD has been shown to have an increased mortality even in its early course[2], but this was not found for UC[3]. Patients spend variable time periods in transition states, which depend on the severity of disease, type of

pharmacotherapy given, need for surgery, and apparently also geographical origin[4,5]. Additionally, there is the risk of intestinal and extraintestinal malignancies, and extraintestinal manifestations in the joints and other systems. There is evidence for a rising incidence of these maladies in many countries, including in Asia and Africa, where cases were scarce until recently[6]. For all the foregoing reasons, the direct health care costs of UC and CD are expected to place a considerable and increasing burden on national health care resources. In addition, there is the large burden of indirect health costs engendered by decreased work productivity and disability[7,8]. This is quite difficult to estimate and no suitable method exists.

There have been a number of studies published on the health care costs of UC and CD and these are reviewed in this article. IC will not be discussed in detail because it is rarer and has been seldom the subject of economic analysis. A MEDLINE search was conducted using the terms ulcerative colitis, Crohn's disease, indeterminate colitis, inflammatory bowel disease, infliximab (and other biological agents), health care cost, economics, charges, cost-benefit, cost-utility, cost-effectiveness, and Markov model, to find pertinent original research articles published after 1990, excluding letters, commentaries and reviews. The studies selected for inclusion will be reviewed by country. Of note, there have been few actual investigations (as opposed to speculative models) attending to the issue of the cost-utility of modern biological agents that are becoming so widely used in CD, and now more recently, in UC. Furthermore, there are new expensive technologies for the investigation of patients with CD and UC. These matters have considerable economic importance.

Table 1 Requirements of good cost-of-disease studies

Requirements
Economic analysis is carried out from the societal perspective
The cohort is community-based
The study provides knowledge of
Total direct cost of a disease
Inclusion of diagnostic costs and treatments until diagnosis
Inpatient and outpatient costs
Resources that drive costs
The analysis
Accounts for the skew of cost data
Provides for time-based discounted downstream costs
The result
Is applicable to patient cohorts from other countries
Provides a basis for cost–utility analysis

COST STUDIES

United States

In the 1990s, the initial cost studies in IBD were carried out in the United States. Hay *et al*[9,10] produced the first reports. They described an economic evaluation of UC and CD using a medical decision algorithm costing methodology, based on examination of 1988-1989 claims data from a major United States commercial medical insurer. They calculated a mean annual direct cost per patient with CD as $6561 (1990 US dollars), and for UC $1488. Surgery accounted for the majority of these sums. The distribution of annual medical expenses was right-skewed. The top 2% of CD patients accounted for 28.9% of total charges and 34.3% of the total amount paid; for UC, the corresponding estimates were 36.2% and 39.0%. Surgery and inpatient costs accounted for some 56% of costs; outpatient care 3.0%-7.1%; initial diagnostic workup 1.5%-7.8%, and medications 10%.

Feagan *et al*[11] used a commercial claims database serving employees of about 50 large companies in the US, to determine the payer costs of CD. Charge claims from 607 patients were analyzed and the results expressed in 1995 US values. The mean annual cost of medical care per patient was determined to be $12417 (95% CI, $10226-$14607). In a sub-set of 117 hospitalized CD patients, the mean annual cost per patient was $37135 (95% CI, $28227-$46043). Pharmacy charges amounted to about 4% of this outlay. The cost data were again right-skewed, with 25% of patients accounting for 80% of total costs. Thus, in 607 CD patients, the median annual cost was $3668 (95% CI, $1417-$12107), and in the sub-set of hospitalized patients the median annual cost per patient was $21671 (95% CI, $11738-$35535). The major determinants of cost, in descending order of magnitude, were inpatient hospitalization, outpatient services, physician office visits and prescription medications. Twenty-seven of the CD patients had a fistula, and these individuals encountered 2.5-fold higher mean total medical care costs, $31370 per patient per year, of which amount, inpatient services contributed 71%.

Silverstein *et al*[4] performed a Markov model analysis of the costs of health care; the utilization/time profile in this study was based largely on assumed conditions. They examined direct health care costs in a population-based inception CD cohort (174 patients, time period 1970-1993), who were residing in Olmsted County, southeastern Minnesota. Clinical information was abstracted retrospectively and sometimes from original medical records, and data on direct medical charges (the payer's perspective) were obtained from a county database. Medical expenses of matched non-CD subjects were subtracted from the health care expenses of the CD patients to derive charges that were then attributed solely to their CD illness. Results were given in 1995 US prices. The estimated life-time direct cost of health care in the CD cohort (with a 5% annual discount rate applied) was $125404. Taking the projected follow-up period of the disease to be 42.6 years (mean age at diagnosis 32.1 years plus assumed 42.6 years of life after diagnosis in a representative patient), the mean cost of health care can be calculated to be $2944 per patient per year of disease. In appreciation of the skew of health care cost data, calculations using median values can be performed instead. The predicted median life-time cost of health care was $39906. Again, using the authors' assumed median follow-up period of 46.4 years (28.1 median age at diagnosis plus 46.4 projected life years),

the median cost can be calculated at $860 per patient per year of disease. Alternately, using the figure of 10 years median follow-up time of the cohort, as stated by the authors, the cost of disease becomes $3991 per patient per follow-up year (or roughly $12 504 mean cost per patient per disease year). Surgery and aminosalicylate therapy accounted for 44% and 30% of the total cost, respectively. The mean cost of surgery was $9109 per patient per month, compared with medical costs up to $1000 per patient per month for cases in remission or with mild disease, or up to $1500 per patient per month for those on steroids and immunosuppression. Charges for physician services constituted the largest component of services for non-surgical patients. CD patients in remission after medical or surgical treatment had health care expenses that were substantially higher than those of the matched controls.

Cohen *et al*[12] looked at the cost of hospitalization in CD patients hospitalized in Chicago. There were 147 patients with 175 hospitalizations, mostly surgical, in a 1-year period ending in June 1997. Duration of hospitalization was: overall, 8.7 d; medical, 7.5 d; and surgical, 9.6 d. The mean overall hospitalization charges, excluding physicians' fees, totaled $12 528, with medical and surgical admissions being $10 020 and $14 409, respectively. The mean charges including physicians' fees amounted to: overall, $35 378; medical, $20 744; and surgical, $46 354. High-cost items were surgery and total parenteral nutrition.

Canada

Bernstein *et al*[13] investigated the direct hospital costs from the payer perspective for a cohort of 187 CD and 115 UC patients at a tertiary care hospital in Manitoba, in 1994-1995. Charts were reviewed to validate that the admissions were for CD or UC only. There were 275 hospital admissions entered in the analysis. Resources used were documented and priced (Canadian $, 1994-1995) according to the hospital billing database. Cases were classified as medical or surgical admissions. Data are shown here for the costs that were related to CD or UC and not non-digestive disease admissions. The mean cost of hospital admission per medical case was C$2571 (95% CI, C$1801-C$3340) for CD and C$2186 (95% CI, C$1449-C$2922) for UC. The mean cost per hospitalized surgical case was higher, with C$3427 (95% CI, C$2728-C$4126) for CD and C$4635 (95% CI, C$3549-C$5726) for UC. Using the median values per hospitalized patient, the medical cost was C$1664 for CD and C$1262 for UC; the surgical cost was C$2546 for CD and C$3341 for UC. Surgery accounted for 50% of all hospital admissions, 58% of all hospital days, and 61% of all costs. Patients with multiple admissions were more costly than those with only a single admission. Patients receiving total parenteral nutrition demonstrated escalated costs. IC was more costly for surgical hospitalization (mean C$6898) than CD or UC.

Europe

Cost studies were reported from Europe in the present decade. Bassi *et al*[14] reported retrospectively the inpatient and outpatient costs, from the payer perspective (UK National Health service), of patients with CD (160 prevalent cases, 12 incident cases), UC (253, 31) and IC (20, three) at a university hospital in north-west England, over a 6-mo period, ending in December 2000. Charges for hospitalization, surgery, outpatient services, investigations and medications were documented. IC was included with UC in the paper. Values were expressed in 2000-2001 values. Costs are re-calculated here to represent a period of a calendar year. The cost for treating CD was £3416 per patient per year, and for UC, £3021 per patient per year. Fourteen percent of the patients were hospitalized during the study period. Inpatient and surgery costs accounted for over half of the costs for CD and UC. Patients not requiring hospitalization had less than 10% of the costs of patients who were admitted. The outlay on oral 5-aminosalicylates exceeded that of all other medications. Cost data were highly skewed: the 10% most costly patients in CD and UC accounted for 59% and 62% of total costs. Variables driving up the cost of disease care were a diagnosis of CD, hospitalization, and severity of illness, but not age, sex or disease extent.

Ebinger *et al*[15] assessed retrospectively the cost of outpatient care in CD (390 patients) and UC (158 patients) in a German university hospital in Ulm from 1997 to 2000, using the payer perspective and the current hospital fee schedule. The mean annual cost for outpatient care was €3171 in all 548 patients, with drugs accounting for 85% of this outlay. The mean cost for one outpatient visit was €162, of which diagnostic procedures accounted for 82% of charges. Costs were higher in patients with disease duration of less than one year. Variables driving the cost of care were frequent visits to outpatient clinics, complications and corticosteroid therapy. Costs were no different between patients with CD or UC.

Odes *et al*[16] carried out a cost analysis in a community-based inception cohort with 10 years of follow up. There were 425 CD patients and 896 UC patients from eight European countries and Israel (the European Collaborative Study Group of Inflammatory Bowel Disease (EC-IBD) inception cohort, established 1991-1993) followed through to 2004. Extensive measures were taken to assure the highest rate of case ascertainment. All patients met the exacting diagnostic criteria of Lennard-Jones[1]. Data on consumption of resources were obtained retrospectively from electronic patient questionnaires (in nine languages) and electronic physician-per-patient follow-up questionnaires. The mean annual expenditure (2004 monetary values) on total health care (outpatient care, diagnostics, hospitalization, surgery, medication) in IBD was €1871 (SD €4884) per patient per year over the decade of follow up. The mean outlay on total health care for CD (€2548 per patient-year) was appreciably higher than that for UC (€1524 per patient-year). Mean costs varied considerably between countries, being highest in Denmark at €3705 per patient-year and lowest in Norway at €888 per patient-

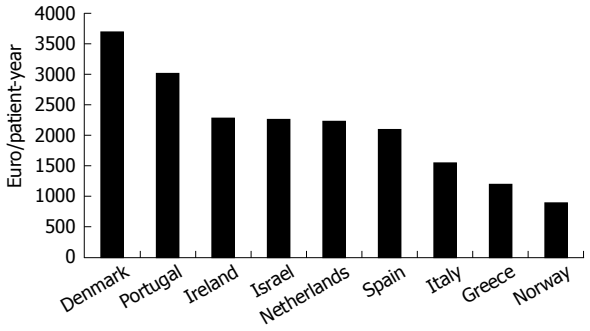

Figure 1 Mean annual cost of health care for IBD patients in the EC-IBD countries with 10-year follow-up. Data provided by EC-IBD.

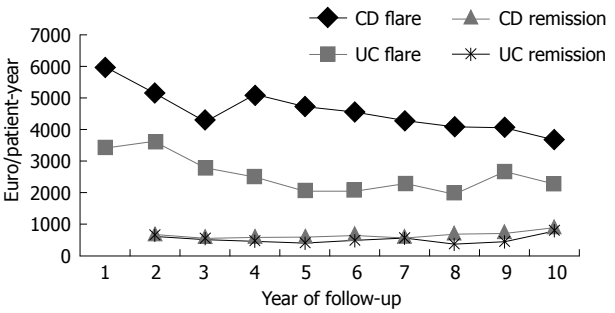

Figure 2 Mean annual expenditure in CD and UC in remission and with flares in the EC-IBD countries during 10 year follow-up. Data provided by EC-IBD.

year (Figure 1). Median costs were lower than mean costs, which confirmed the right skew of the mean cost data. The most expensive resources were hospitalization, which accounted for 63% of the cost in CD and 45% in UC. Total and hospitalization costs were much higher in the first year after diagnosis than in the follow-up period. The significant variables driving the cost of care were country, year of follow-up [year 1: odds ratio (OR) = 3] and diagnosis (CD: OR 1.5); sex and age had no incremental effect on cost. Patients with flares were more expensive than those without during the entire follow-up period (Figure 2). 5-Aminosalicylate was the most expensive drug category; this was the result of a high price and long duration of use. There were too few patients receiving infliximab in this study to greatly affect cost. The extent of disease was found to affect the cost significantly in UC but not CD patients; the cost in CD varied significantly according to the Vienna classification phenotypes. Of importance in this context is the finding that the extent of UC is known to be changeable over time in about half of patients, with extension as well as regression being noted[17].

Juan *et al*[18] reported a cost study on CD in Spain in 2003. It was based on questionnaires to gastroenterologists to obtain disease-related information, and phone interviews to patients to obtain drug utilization data. The annual cost per patient was estimated at between €2104 to €6808 for direct medical costs, and €4704 for indirect costs. Hospitalization and loss of productivity were the items driving these respective costs.

INFLIXIMAB AND COST

Infliximab entered widely into the management of CD over the last decade or so, and since 2006 for UC as well. Recently, other biological therapies have entered the market. Infliximab is an antibody to tumor necrosis factor α which has revolutionized therapy in over 50% of patients who receive this treatment, and who were non-responders to standard medical therapies[19]. There is a developing trend to introduce infliximab earlier into the therapeutic regimen, in the hope that prolonged unresponsive illness, steroid side-effects and surgery may be avoided, and health-related quality of life improved. The use of maintenance therapy with biological agents is gaining acceptance, as it reduces hospitalization and surgery[20]. However, these are expensive agents, and assessment of their impact on short- and long-term health care costs is required. Such studies lead to disease activity scores and outcomes of biological treatments as reported in clinical trials. Not all reports fulfill these criteria. Modeling can only approximate real patient documentation but does provide a useful indicator of costs. To date, there have been few reports published in this area.

Arseneau *et al*[21] were the first to determine the cost-utility of treatment with infliximab in CD with perianal fistulae. Using a Markov model, and with a given cost of a three-course infusion of infliximab of $6420 (2000 prices), the cost of achieving one quality-adjusted life-year exceeded US $350 000. This result was highly dependent on the cost of the medication. Since 2001, the cost of infliximab has decreased somewhat, and several of the disease-course assumptions have changed. A more recent retrospective audit by Jewel *et al*[22], based on 205 patients who received infliximab in seven hospitals in the UK, showed that the mean total health care costs in the 6 mo before the initial infliximab infusion (£2883 per patient) exceeded the mean costs in the 6 mo following the infusion (£2744 per patient), by an average of £139 per patient (a saving of 5%), which suggests that this biological agent is potentially cost-effective. There was a 76% reduction in the number of hospitalization days and 79% of patients reduced or stopped taking corticosteroids. Lindsay *et al*[23] used this study as a basis for determining the cost-effectiveness of scheduled maintenance treatment with infliximab for moderate/severe luminal and fistulizing CD in hypothetical patients in a commercially funded study. Markov models were constructed to simulate the progression of adult CD patients, using transition states estimated from published clinical trials of infliximab, particularly the ACCENT trial. The respective incremental costs per quality-adjusted life year gained were £26 128 and £29 752 in severe luminal and fistulizing CD at a 5-year time horizon. The body weight of patients had the most important impact on the incremental cost-effectiveness ratio, since dosing of infliximab is weight-based. A 10% increase in body weight raises the cost per quality-adjusted life year by some £7000. The reduction in surgery predicted in this

study was similar to that documented by Saro *et al*[24] and the time of follow up was similar. Dose-escalation in patients losing their response to infliximab was very expensive[25].

Jaisson-Hot *et al*[26] carried out a life-time cost-utility analysis with an analytic Markov decision model in non-fistulizing resistant CD patients treated with infliximab (episodic re-infusions for relapse, or scheduled maintenance therapy), compared with conventional surgery and medical treatment. The perspective was that of the third-party payer system. Utility measurement using Standard Gamble was employed to adjust the survival time for each transition state of disease. The incremental cost-utility ratio per quality-adjusted life years saved varied from €63 701 (episodic re-infusions) to over €762 245 (scheduled infusions). The analysis suggested that infliximab could be cost-effective only in the case of treatment for flares of disease.

Saro *et al*[24] reported the impact of infliximab on health care costs in CD patients in northern Spain. There were 34 patients in the study with a mean disease duration of 13.6 years. The mean follow-up time was 9.8 years before and 4.3 years after the first infliximab treatment. About 53% of the patients had penetrating and/or perianal disease, and 41% had extraintestinal manifestations. Following the introduction of infliximab (which was always given as 5 mg/kg body weight) to the treatment regimen, mean hospitalization costs were reduced from €2783 to €679 per patient-year, and the mean surgical cost dropped from €139 to €79 per patient-year. However, the cost of infliximab treatment amounted to a mean €7996 per patient-year. Therefore, the total annual cost of health care increased considerably, from a mean €4464 per patient-year before to a mean €10 594 per patient-year after the introduction of infliximab. This study contradicts that of Jewell *et al*[22]. It must be stressed that all the foregoing studies did not account for the innate fluctuations of disease activity and the so-called placebo-response of the disease, so that future studies need to take these factors into consideration in the interpretation of outcomes and costs after therapeutic interventions.

GENOTYPES AND PHENOTYPES DRIVING COSTS

It would be very useful to identify patients whose genotypes and phenotypes predicted higher health care costs, since aggressive treatment might reduce morbidity as well as cost. Alternatively, genotypes and phenotypes with a better prognosis would be a reassuring finding. It was reported recently that the expenditure on patients with CD was greatly influenced by the disease phenotypes. In a cohort of 418 patients with CD and 10 years of follow-up, the total cost of health care for the Montreal classification[27] behavior phenotypes was €1690 for non-stricturing/non-penetrating, €2081 for stricturing, €3133 for penetrating, and €3356 for penetrating-with-perianal-fistula ($P < 0.001$); all values given as means per phenotype-patient-year[28]. Taking these phenotypes in the same order, the cost of surgical hospitalization was €215, €751, €1293 and €1275 per patient-phenotype-year ($P < 0.001$). Surgical hospitalization costs differed significantly by the location phenotypes: ileum, €558, €209, €492 and €542, expressed as means per patient-phenotype-year ($P < 0.001$). Younger age at diagnosis predicted greater surgical expenses. Estimates of the proportion of CD patients with the various phenotypes of disease vary in different reports[28]. Notably, there is a change in phenotype with length of follow-up, with structuring and penetrating disease becoming more frequent[29]. Unlike behavior, location of disease was shown to be relatively stable over time[29]. Additionally, smoking appears to influence disease location in CD, with current smokers having a lower rate of colonic disease, fewer strictures and fistulae, and a lower rate of surgery[30]. CD patients with stricturing and penetrating disease were more likely to be positive for anti-*Saccharomyces cerevisiae* antibody[31]. CD patients with the NOD2/CARD15 mutation Gly908Arg or positive serology to ASCA had higher health care costs, in particular for surgery, and prolonged hospitalization[32]. However, the mutants Leu1007fsinsC and Arg702Trp had little effect on disease course in that study. ASCA-positive patients were significantly younger at diagnosis than ASCA-negative patients, and Gly908Arg-positive patients showed a trend towards younger age at diagnosis of CD. In another study, NOD2/CARD15 variants in CD patients aged under 16 years were strongly associated with jejunal and ileal involvement, stricturing disease and early recourse to surgery[33]. ASCA is an established serological marker for CD. Moreover, ASCA is frequently detected in CD patients with NOD2/CARD15 allele mutations[34]. NOD2/CARD15 variants constitute a risk factor for ileal site of disease, development of intestinal strictures and fistulae, occurrence of more severe disease, and an increased requirement for surgery[35]. It is possible that measurements of Gly908Arg and ASCA at onset can be used to foretell increased health care costs in CD patients.

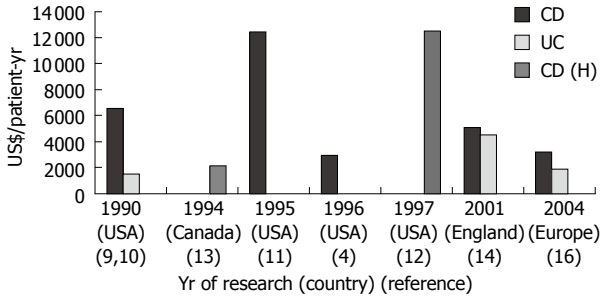

Figure 3 Mean annual total direct cost of health care in selected countries, discounted[36] to year 2004, and expressed as US$ (currency conversions from http://www.oanda.com/convert/fxhistory, accessed 10 May 2008). H: Hospital costs only.

CONCLUSION

CD and UC are serious diseases with considerable health care costs. The studies reviewed above however

displayed considerable disparity of outcomes (Figure 3). These differences clearly cannot be resolved by simply applying a discount rate (currently reckoned to be 2.5%-3.0%[36]) to the costs derived at different time periods. Some of these discrepancies can be attributed to variations of methodology, selection of cohorts, locality, and whether the health care system is private or public. In these studies, the costs were computed from the payer (third party) perspective or from economic data abstracted directly from patients' files, according to the nature of the research. Differences of medical practice would seem to play a role as well. There are also wide differences in the health care price structure within and between countries[9,16]. This could explain some of the differences in health care expenditure between the various European countries that participated in the EC-IBD study. In all the studies, the charges for hospitalization, surgery and biological therapy comprised a large percentage of costs. Despite the trend to do as much care as possible on an outpatient basis, it appears that the rate of hospitalization is increasing[37]. The benefits of keeping patients in remission include a significant drop in the rate of medical as well as surgical hospitalization[38], and therefore a reduction in cost. A new issue in recent years is the welcome development of biological therapies for CD and UC; these drugs have profoundly improved the treatment of these diseases, but with a large price tag. Available economic modeling exercises and the study from Spain imply that infliximab has a relatively high incremental cost per quality-adjusted life year compared with standard care, but this requires further investigation, preferably with real patient data. Comparison of the costs of biological agents with established treatments in trials is complicated by the need to assess the downstream effects of an intervention. In those situations, modeling is a quicker method to obtain cost data, but has its limitations. More data of health cost in CD and UC are certainly required.

REFERENCES

1 **Lennard-Jones JE**. Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl* 1989; **170**: 2-6; discussion 16-19

2 **Wolters FL**, Russel MG, Sijbrandij J, Schouten LJ, Odes S, Riis L, Munkholm P, Bodini P, O'Morain C, Mouzas IA, Tsianos E, Vermeire S, Monteiro E, Limonard C, Vatn M, Fornaciari G, Pereira S, Moum B, Stockbrugger RW. Crohn's disease: increased mortality 10 years after diagnosis in a Europe-wide population based cohort. *Gut* 2006; **55**: 510-518

3 **Hoie O**, Schouten LJ, Wolters FL, Solberg IC, Riis L, Mouzas IA, Politi P, Odes S, Langholz E, Vatn M, Stockbrugger RW, Moum B. Ulcerative colitis: no rise in mortality in a European-wide population based cohort 10 years after diagnosis. *Gut* 2007; **56**: 497-503

4 **Silverstein MD**, Loftus EV, Sandborn WJ, Tremaine WJ, Feagan BG, Nietert PJ, Harmsen WS, Zinsmeister AR. Clinical course and costs of care for Crohn's disease: Markov model analysis of a population-based cohort. *Gastroenterology* 1999; **117**: 49-57

5 **Wolters FL**, Joling C, Russel MG, Sijbrandij J, De Bruin M, Odes S, Riis L, Munkholm P, Bodini P, Ryan B, O'Morain C, Mouzas IA, Tsianos E, Vermeire S, Monteiro E, Limonard C, Vatn M, Fornaciari G, Rodriguez D, Groot W, Moum B, Stockbrugger RW. Treatment inferred disease severity in Crohn's disease: evidence for a European gradient of disease course. *Scand J Gastroenterol* 2007; **42**: 333-344

6 **Russel MG**, Stockbrugger RW. Epidemiology of inflammatory bowel disease: an update. *Scand J Gastroenterol* 1996; **31**: 417-427

7 **Blomqvist P**, Ekbom A. Inflammatory bowel diseases: health care and costs in Sweden in 1994. *Scand J Gastroenterol* 1997; **32**: 1134-1139

8 **Sculher M**. The role and estimation of productivity costs in economic evaluation. In: Drummond M, McGuire A. Economic evaluation in health care. New York: Oxford University Press, 2001: 94-112

9 **Hay JW**, Hay AR. Inflammatory bowel disease: costs-of-illness. *J Clin Gastroenterol* 1992; **14**: 309-317

10 **Hay AR**, Hay JW. Inflammatory bowel disease: medical cost algorithms. *J Clin Gastroenterol* 1992; **14**: 318-327

11 **Feagan BG**, Vreeland MG, Larson LR, Bala MV. Annual cost of care for Crohn's disease: a payor perspective. *Am J Gastroenterol* 2000; **95**: 1955-1960

12 **Cohen RD**, Larson LR, Roth JM, Becker RV, Mummert LL. The cost of hospitalization in Crohn's disease. *Am J Gastroenterol* 2000; **95**: 524-530

13 **Bernstein CN**, Papineau N, Zajaczkowski J, Rawsthorne P, Okrusko G, Blanchard JF. Direct hospital costs for patients with inflammatory bowel disease in a Canadian tertiary care university hospital. *Am J Gastroenterol* 2000; **95**: 677-683

14 **Bassi A**, Dodd S, Williamson P, Bodger K. Cost of illness of inflammatory bowel disease in the UK: a single centre retrospective study. *Gut* 2004; **53**: 1471-1478

15 **Ebinger M**, Leidl R, Thomas S, Von Tirpitz C, Reinshagen M, Adler G, Konig HH. Cost of outpatient care in patients with inflammatory bowel disease in a German University Hospital. *J Gastroenterol Hepatol* 2004; **19**: 192-199

16 **Odes S**, Vardi H, Friger M, Wolters F, Russel MG, Riis L, Munkholm P, Politi P, Tsianos E, Clofent J, Vermeire S, Monteiro E, Mouzas I, Fornaciari G, Sijbrandij J, Limonard C, Van Zeijl G, O'morain C, Moum B, Vatn M, Stockbrugger R. Cost analysis and cost determinants in a European inflammatory bowel disease inception cohort with 10 years of follow-up evaluation. *Gastroenterology* 2006; **131**: 719-728

17 **Langholz E**, Munkholm P, Davidsen M, Nielsen OH, Binder V. Changes in extent of ulcerative colitis: a study on the course and prognostic factors. *Scand J Gastroenterol* 1996; **31**: 260-266

18 **Juan J**, Estiarte R, Colome E, Artes M, Jimenez FJ, Alonso J. Burden of illness of Crohn's disease in Spain. *Dig Liver Dis* 2003; **35**: 853-861

19 **Rutgeerts P**, Van Assche G, Vermeire S. Review article: Infliximab therapy for inflammatory bowel disease--seven years on. *Aliment Pharmacol Ther* 2006; **23**: 451-463

20 **Lichtenstein GR**, Yan S, Bala M, Blank M, Sands BE. Infliximab maintenance treatment reduces hospitalizations, surgeries, and procedures in fistulizing Crohn's disease. *Gastroenterology* 2005; **128**: 862-869

21 **Arseneau KO**, Cohn SM, Cominelli F, Connors AF Jr. Cost-utility of initial medical management for Crohn's disease perianal fistulae. *Gastroenterology* 2001; **120**: 1640-1656

22 **Jewell DP**, Satsangi J, Lobo A, Probert C, Forbes A, Ghosh S, Shaffer J, Frenz M, Drummond H, Troy G, Turner S, Younge L, Evans L, Moosa M, Rodgers-Gray B, Buchan S. Infliximab use in Crohn's disease: impact on health care resources in the UK. *Eur J Gastroenterol Hepatol* 2005; **17**: 1047-1052

23 **Lindsay J**, Punekar YS, Morris J, Chung-Faye G. Health-economic analysis: cost-effectiveness of scheduled maintenance treatment with infliximab for Crohn's disease--modelling outcomes in active luminal and fistulizing disease in adults. *Aliment Pharmacol Ther* 2008; **28**: 76-87

24 **Saro C**, da la Coba C, Casado MA, Morales JM, Otero B. Resource use in patients with Crohn's disease treated with infliximab. *Aliment Pharmacol Ther* 2007; **26**: 1313-1323

25 **Kaplan GG**, Hur C, Korzenik J, Sands BE. Infliximab dose

escalation vs. initiation of adalimumab for loss of response in Crohn's disease: a cost-effectiveness analysis. *Aliment Pharmacol Ther* 2007; **26**: 1509-1520

26 **Jaisson-Hot I**, Flourie B, Descos L, Colin C. Management for severe Crohn's disease: a lifetime cost-utility analysis. *Int J Technol Assess Health Care* 2004; **20**: 274-279

27 **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus Jr EV, Pena AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5-36

28 **Odes S**, Vardi H, Friger M, Wolters F, Hoie O, BMoum B, Bernklev T, Yona H, Russel M, Munkholm P, Langholz E, Riis L, Politi P, Bondini P, Tsianos E, Katsanos K, Clofent J, Vermeire S, Freitas J, Mouzas I, Limonard C, O'Morain C, Monteiro E, Fornaciari G, Vatn M, Stockbrugger R. Effect of phenotype on health care costs in Crohn's disease: A European study using the Montreal classification. *J Crohn's Colitis* 2007; **1**: 87-96

29 **Louis E**, Collard A, Oger AF, Degroote E, Aboul Nasr El Yafi FA, Belaiche J. Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. *Gut* 2001; **49**: 777-782

30 **Aldhous MC**, Drummond HE, Anderson N, Smith LA, Arnott ID, Satsangi J. Does cigarette smoking influence the phenotype of Crohn's disease? Analysis using the Montreal classification. *Am J Gastroenterol* 2007; **102**: 577-588

31 **Riis L**, Vind I, Vermeire S, Wolters F, Katsanos K, Politi P, Freitas J, Mouzas IA, O'Morain C, Ruiz-Ochoa V, Odes S, Binder V, Munkholm P, Moum B, Stockbrugger R, Langholz E. The prevalence of genetic and serologic markers in an unselected European population-based cohort of IBD patients. *Inflamm Bowel Dis* 2007; **13**: 24-32

32 **Odes S**, Friger M, Vardi H, Claessens G, Bossuyt X, Riis L, Munkholm P, Wolters F, Yona H, Hoie O, Beltrami M, Tsianos E, Katsanos K, Mouzas I, Clofent J, Monteiro E, Messori A, Politi P, O'Morain C, Limonard C, Russel M, Vatn M, Moum B, Stockbrugger R, Vermeire S. Role of ASCA and the NOD2/CARD15 mutation Gly908Arg in predicting increased surgical costs in Crohn's disease patients: a project of the European Collaborative Study Group on Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2007; **13**: 874-881

33 **Russell RK**, Drummond HE, Nimmo EE, Anderson N, Smith L, Wilson DC, Gillett PM, McGrogan P, Hassan K, Weaver LT, Bisset M, Mahdi G, Satsangi J. Genotype-phenotype analysis in childhood-onset Crohn's disease: NOD2/CARD15 variants consistently predict phenotypic characteristics of severe disease. *Inflamm Bowel Dis* 2005; **11**: 955-964

34 **Cruyssen BV**, Peeters H, Hoffman IE, Laukens D, Coucke P, Marichal D, Cuvelier C, Remaut E, Veys EM, Mielants H, De Vos M, De Keyser F. CARD15 polymorphisms are associated with anti-Saccharomyces cerevisiae antibodies in caucasian Crohn's disease patients. *Clin Exp Immunol* 2005; **140**: 354-359

35 **Economou M**, Trikalinos TA, Loizou KT, Tsianos EV, Ioannidis JP. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. *Am J Gastroenterol* 2004; **99**: 2393-2404

36 **Lipscomb J**, Weinstein MC, Torrance GW. Time preference. In: Gold MR, Siegel JE, Russell LB, Weinstein MC. Cost-effectiveness in health and medicine. New York: Oxford, 1996: 214-246

37 **Nguyen GC**, Tuskey A, Dassopoulos T, Harris ML, Brant SR. Rising hospitalization rates for inflammatory bowel disease in the United States between 1998 and 2004. *Inflamm Bowel Dis* 2007; **13**: 1529-1535

38 **Lichtenstein GR**, Yan S, Bala M, Hanauer S. Remission in patients with Crohn's disease is associated with improvement in employment and quality of life and a decrease in hospitalizations and surgeries. *Am J Gastroenterol* 2004; **99**: 91-96

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CLINICAL RESEARCH

Distinguishing between parenchymal and anastomotic leakage at duct-to-mucosa pancreatic reconstruction in pancreaticoduodenectomy

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Abstract

AIM: To distinguish anastomotic from parenchymal leakage at duct-to-mucosa reconstruction of the pancreatic remnant.

METHODS: We reviewed the charts of 68 pancreaticoduodenectomies performed between 5/2000 and 12/2005 with end-to-side duct-to-mucosa pancreatojejunostomy (PJ). The results of pancreatography, as well as peripancreatic drain volumes, and amylase levels were analyzed.

RESULTS: Of 68 pancreatojejunostomies, 48 had no leak by pancreatography and had low-drain amylase (normal); eight had no pancreatographic leak but had elevated drain amylase (parenchymal leak); and 12 had pancreatographic leak and elevated drain amylase (anastomotic leak). Although drain volumes in the parenchymal leak group were significantly elevated at postoperative day (POD) 4, no difference was found at POD 7. Drain amylase level was not significantly different at POD 4. In contrast, at POD 7, the anastomotic-leak group had significantly elevated drain amylase level compared with normal and parenchymal-leak groups (14158 ± 24083 IU/L *vs* 89 ± 139 IU/L and 1707 ± 1515 IU/L, respectively, $P = 0.012$).

CONCLUSION: For pancreatic remnant reconstruction after pancreaticoduodenectomy, a combination of pancreatogram and peripancreatic drain amylase levels can be used to distinguish between parenchymal and anastomotic leakage at pancreatic remnant reconstruction.

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Key words: Anastomotic leak; Pancreatic leak; Pancreaticoduodenectomy; Pancreatogram; Whipple procedure

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INTRODUCTION

Pancreaticoduodenectomy remains the surgical resection of choice for cancer of the head of the pancreas. Surgical techniques for reconstructing the pancreatic remnant after pancreaticoduodenectomy include end-to-end pancreatojejunostomy (PJ) with invagination, end-to-side PJ with[1,2] or without pancreas invagination[3], pancreatic duct invagination[4], pancreaticocutaneous fistula[5], and external drainage of the pancreatic duct without pancreaticoenteric reconstruction[6]. However, pancreatic leakage remains a significant complication, occurring in 0%-37% of cases[1,7,8]. An unrecognized leak at the PJ anastomosis can result in lethal complications, in particular, intra-abdominal abscess and hemorrhage[9-12].

Pancreatic leaks have traditionally been defined by amylase levels measured in the peripancreatic fluid obtained by placing a drain immediately around the PJ anastomosis[13]. However, pancreatic juice can originate either from the main pancreatic duct or from the cut surface of the pancreatic parenchyma. Thus, an elevated level of amylase in the fluid obtained from the peripancreatic drain suggesting pancreatic leakage does not indicate whether the leak is at the duct-to-mucosa anastomosis (an anastomotic leak) or from the resection parenchyma surface (a parenchymal leak). The existence

of parenchymal leakage was previously shown to be from an accessory small duct in the parenchyma, separate from the duct-to-mucosa anastomosis[14]. However, it is difficult to distinguish between an anastomotic and a parenchymal pancreatic leak at end-to-side duct-to-mucosa PJ reconstruction. The aim of this study was to assess the PJ following pancreaticoduodenectomy to characterize the nature of PJ leaks.

In the cases studied, the pancreatic remnant following pancreaticoduodenectomy was reconstructed with end-to-side duct-to-mucosa PJ, as described previously[15]. The PJ anastomosis was critically evaluated postoperatively with pancreatography *via* an externalized pancreatic stent placed at the time of surgery. We found that duct-to-mucosa anastomotic leakage could be distinguished from parenchymal leakage at the PJ reconstruction after pancreaticoduodenectomy by using the combined radiographic findings on pancreatography and amylase levels in the peripancreatic drain fluid.

MATERIALS AND METHODS

Medical records of patients who underwent abdominal exploration for pancreatic mass between May 2000 and December 2005 were reviewed. The review of patient records was approved by the Institutional Review Board at the Mayo Clinic in Jacksonville, Florida. Classical pancreaticoduodenectomy was performed as previously described[3]. The pancreas was divided left of the portal vein[14]. The pancreatic remnant was reconstructed with end-to-side duct-to-mucosa PJ using 6-0 PDS sutures as previously described[15]. The anterior and posterior parenchyma-to-serosa layers were completed with interrupted 3-0 silks. End-to-side choledochojejunostomy and antecolic gastrojejunostomy were performed.

Across the PJ anastomosis, a stent was placed through the jejunal limb as shown in Figure 1, similar to previous reports[11,16-18]. The exit site of the stent at the jejunal serosa was secured with a Witzel's tunnel using 3-0 silks and a 5-0 Vicryl™ suture. A stent was similarly placed across the biliary reconstruction. This stenting technique was adopted from the same technique used routinely in liver transplantation at our center since 1998. A 6-mm round Blake drain was placed beneath the PJ reconstruction and brought through the left abdominal wall. A nasogastric tube was positioned just above the gastrojejunal anastomosis. The biliary and pancreatic stents were brought through the right abdominal wall.

On postoperative day (POD) 4, a pancreatogram and a cholangiogram were performed by interventional radiologists, who gently injected 1 to 3 mL of water-soluble contrast through the respective externalized pancreatic and biliary stents. The biliary and pancreatic anastomoses were evaluated by fluoroscopy. Serum and drain amylase levels were measured. Drain amylase level was considered significant when it was at least 3 times the upper normal level of serum amylase[13]. After several days of oral food intake, normal pancreatogram results, and low or normal drain amylase level, the drain was removed and the patient was discharged. The patient returned to the outpatient clinic on POD 28, and a repeat pancreatogram and cholangiogram were performed. If there was no evidence of leakage at either anastomosis, the stents were removed.

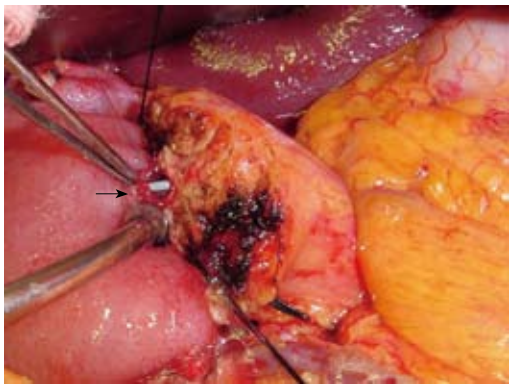

Figure 1 PJ reconstruction of pancreatic remnant after pancreaticoduodenectomy. Posterior layer with interrupted 3-0 silk sutures approximating pancreatic parenchyma to jejunal serosa. Arrow shows ongoing duct-to-mucosa anastomosis with stent inside lumen. Once the duct-to-mucosa anastomosis is completed, anterior of parenchyma to jejunal serosa approximation is done.

When a leak was identified at the PJ anastomosis, the peripancreatic drain was maintained until the leak resolved. Patients were started on a fat-free diet and subcutaneous octreotide (Novartis, USA). A pancreatogram was repeated weekly until the PJ leak resolved. Once the leak was resolved, as confirmed by repeat pancreatography, the stents and drain were removed and a regular diet was begun. Surgical re-exploration was performed when peripancreatic abscess was not amenable to percutaneous drainage, and/or when a widely disrupted PJ anastomosis was visualized on pancreatogram.

RESULTS

From May 2000 to December 2005, of the 105 cases of surgical exploration for pancreatic mass, there were seven palliative bypasses due to unresectable metastatic disease. Seventeen cases had distal pancreatectomy, two transduodenal ampullectomy, 11 pancreaticoduodenectomy with total pancreatectomy, and 66 classical pancreaticoduodenectomy. The demographic data are summarized in Table 1.

Of the 66 patients who had pancreaticoduodenectomy, two required revision of the PJ anastomosis. One patient had afferent loop obstruction and necrosis due to radiation-induced stricture 2 years after the pancreaticoduodenectomy. The afferent loop was revised with a new end-to-side duct-to-mucosa PJ anastomosis and biliojejunostomy. The other patient developed a large peripancreatic abscess and PJ anastomotic leak that did not resolve with conservative management. After re-exploration, the PJ was reconstructed. Since these revisions required complete reconstruction of the pancreatic remnant, the two revised PJ anastomoses were considered independent anastomoses. The outcomes in these 68 PJ anastomoses formed the basis of this study.

Table 1 Clinical parameters of the three groups of patients who had duct-to-mucosa PJ reconstruction after resection of the pancreatic head

	Normal	Parenchymal leak	Anastomotic leak
n	48	8	12
Age (yr[1])	67.5 (29-81)	56.0 (51-79)	65.5 (51-82)
Diagnosis			
Ductal adenocarcinoma	28	1	4
Duodenal adenomas	3	0	1
Neuroendocrine	2	2	1
FAP	0	1	0
Cystadenoma	2	0	1
Benign stricture	2	0	0
Chronic pancreatitis	4	1	1
IPMN	0	2	3
Others	7	1	1
RBC transfused (U[1])	2 (0-39)	2 (0-18)	2.5 (0-88)
OR time (h[1])	5.94 (4.18-11.04)	5.55 (3.40-14.55)	5.04 (4.93-10.65)
Biliary leak	1	0	0
Gastric leak	2	0	0
Intraabdominal abscess	0	0	1

The normal group had no evidence of any pancreatic leakage. Parenchymal leak indicates no extravasation at the duct-to-mucosa anastomosis in the presence of elevated amylase levels in the peripancreatic drain. Anastomotic leak indicates active contrast extravasation at the PJ reconstruction during pancreatography in the postoperative period after pancreaticoduodenectomy. FAP: Familial adenomatous polyposis; IPMN: Intraductal papillary mucinous neoplasm. Other diagnoses included pseudoinflammatory tumor, hereditary pancreatitis, metastatic renal cell carcinoma, solid pseudopapillary tumor, cystic lymphangioma, microcystic adenoma, recurrent duodenal perforation s/p liver transplant; GIST: Gastric efferent stricture with GJ efferent stricture. [1]Age, RBC transfused, and OR time are expressed as median (range).

Table 2 Outcome patterns of PJ anastomosis after pancreaticoduodenectomy based on pancreatography findings and amylase levels in peripancreatic drain fluid

	Normal	Anastomotic leakage	Parenchymal leakage
Pancreatography	No extravasation	Extravasation present	Normal
Peripancreatic drain	Normal	Elevated	Elevated
Occurrence, *n* (%)	48 (72.7)	8 (12.1)	12 (18.2)

Evaluating duct-to-mucosa anastomosis of PJ reconstruction via pancreatography

The integrity of the PJ anastomosis was visualized by fluoroscopy. Of the 68 PJ anastomoses, eight pancreatic anastomotic stents migrated out of the pancreatic remnant. Of those eight, three had successful reflux of the injected contrast into the pancreatic ductal system, demonstrating no leak. In the remaining five, there was no reflux of contrast into the pancreatic ductal system, nor was extravasation seen at the PJ anastomosis. Of the 60 cases with successful pancreatograms, 48 showed no PJ extravasation, i.e., no anastomotic leak, and 12 showed the presence of an anastomotic leak.

A typical pancreatogram and cholangiogram are shown in Figure 2, demonstrating intact PJ and choledochojejunostomy anastomoses. Three patients had mild abdominal discomfort during the pancreatogram injections without increased serum amylase levels. The discomfort resolved spontaneously within a few hours, and there were no other complications directly associated with the pancreatic stents and pancreatograms. Although not shown, in all cases serum amylase levels did not change the day before or the day after the pancreatograms. Serum amylase levels were normal by POD 4.

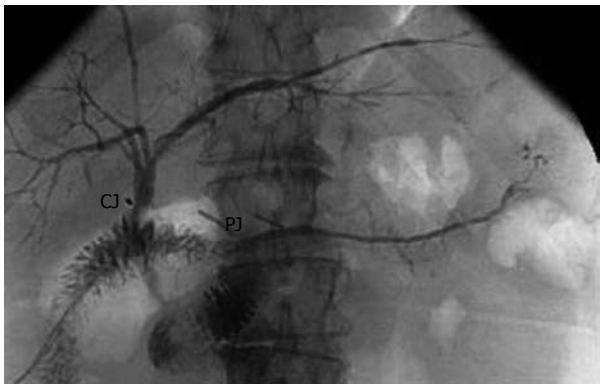

Figure 2 Typical normal pancreatogram and cholangiogram. CJ indicates bilioenteric anastomosis, and PJ indicates PJ reconstruction.

Characteristics of three different outcomes of the PJ anastomosis

As shown in Table 2, we noted three outcomes at the PJ anastomosis after pancreaticoduodenectomy.

Normal: This pattern was associated with a normal pancreatogram, demonstrating an intact PJ anastomosis and low amylase levels from the drain. Forty-eight cases had this normal pattern, including both PJ revisions. The eight cases in which the pancreatic stent dislodged into the lumen of the jejunum had normal drain amylase levels and were included in this normal group. As shown in Figure 3, the peripancreatic PJ drain amylase levels and volumes in the normal group were lower than those in patients with a pancreatic leakage.

Parenchymal leak: This pattern was recognized when there was a normal PJ anastomosis without contrast extravasation on pancreatograms but with persistently high amylase levels in the peripancreatic drains. Eight patients had this pattern. On POD 4 and 7, drain amylase levels in the parenchymal leak group were elevated at 1885 ± 1129 IU/L *vs* 428 ± 1063 IU/L ($P = 0.062$) and 1707 ± 151 IU/L *vs* 89 ± 139 IU/L ($P = 0.012$), respectively. The drain amylase levels became normal between POD 18 and POD 101 after parenchymal pancreatic leak. Most parenchymal leaks resolved by POD 48. In one patient, a repeat study showed the presence of a parenchymal side-branch duct that appeared to be responsible for the high amylase levels detected in the drain (Figure 4). Tessel fibrin sealant was injected, and the drain was removed. Follow-up study showed no fluid collection at the immediate

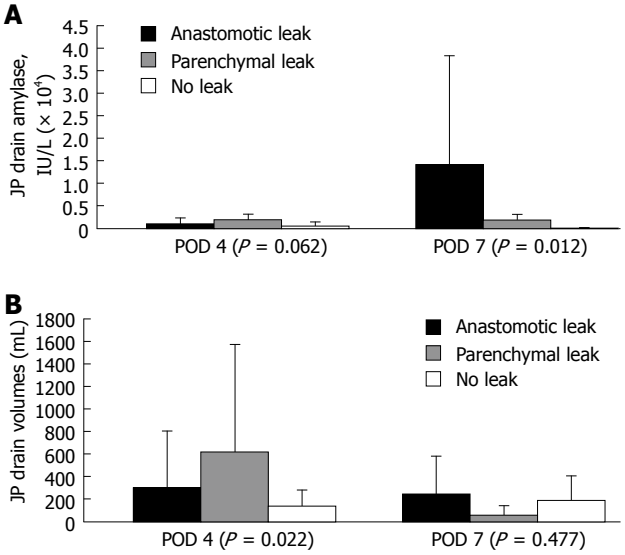

Figure 3 Fluids collected from the peripancreatic drain are analyzed for amylase content and volumes at postoperative day (POD) 4 and 7. A: Amylase levels are not different among the 3 groups at POD 4 (P = 0.062); however, at POD 7, the anastomotic leak group has the significantly highest level, then the parenchymal leak group as compared to the normal group (P = 0.012). B: Volume of peripancreatic drain of the parenchymal leak group was significantly increased above the anastomotic leak and normal group at POD 4 (P = 0.022); on the other hand, there was no difference among the three groups at POD 7 (P = 0.477).

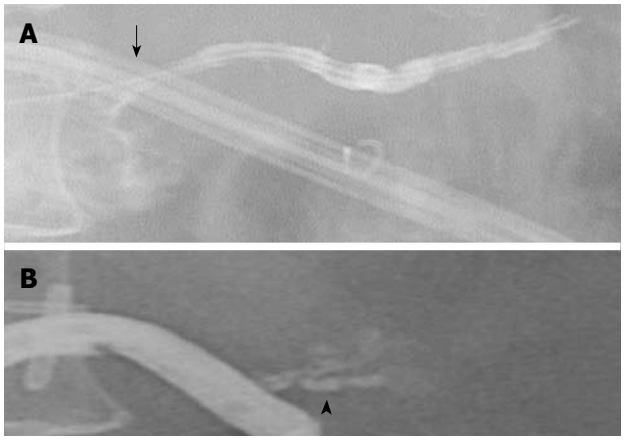

Figure 4 Parenchymal leaks due to a side branch in the pancreatic remnant surface. A: Normal pancreatogram shows intact PJ anastomosis (arrow); B: Sinogram shows a side-branch duct of the pancreas remnant (arrowhead).

or surrounding areas of the pancreatic anastomosis, suggesting complete resolution of the pancreatic leakage.

Anastomotic leak: This pattern was defined by the presence of contrast extravasation at the duct-to-mucosa PJ anastomosis on pancreatogram. These pancreatographic findings were similar to those in a previous report[11]. There were 12 anastomotic leaks. Eleven of the 12 PJ leaks were diagnosed on POD 7 *via* pancreatogram. The highest drain amylase level was 90 000 IU/L. Most elevated drain amylase levels occurred between POD 5 and POD 9, when the anastomotic leaks were also documented radiographically. In fact,

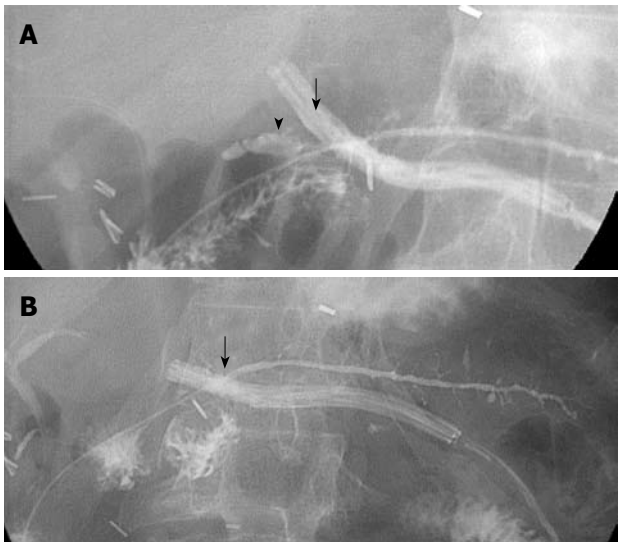

Figure 5 Anastomotic leak is visualized at the initial diagnosis and its resolution is confirmed. A: Contrast extravasation into the surrounding (arrowhead) from the PJ anastomotic (arrow); B: Complete resolution of PJ anastomotic leak (arrow).

the drain amylase levels in this group were significantly elevated at 14158 ± 24083 IU/L *vs* 1707 ± 1515 IU/L and 89 ± 139 IU/L for the parenchymal leak and normal groups at POD 7, respectively (Figure 3A). There was no significant difference among the three groups at POD 4.

The volumes of the peripancreatic drains did not correlate with the leaks seen on pancreatography or the drain amylase levels. As shown in Figure 3B, the volumes of the peripancreatic drains in the anastomotic group were not different from the other two groups at POD 7, although the parenchymal leak group had significantly higher drain volumes at POD 4.

The results of serial pancreatograms provide insight into the natural progression of anastomotic leaks at an end-to-side duct-to-mucosa anastomosis after pancreaticoduodenectomy. Figure 5 shows an example of an anastomotic leak at the duct-to-mucosa PJ reconstruction. The first pancreatogram, done on POD 4, did not show a leak. With a high level of drain amylase on POD 7, repeat pancreatogram showed a significant leak at the duct-to-mucosa anastomosis with extravasation into the surrounding space (Figure 5A). Subsequent pancreatograms showed gradual and complete resolution of the PJ leak (Figure 5B).

Of the 12 pancreatic anastomotic leaks, eight showed radiographic evidence of resolution in conjunction with drain amylase levels tapering toward normal levels by POD 42. In two cases, the anastomotic leak resolved by POD 123 and 189. None of the pancreatic anastomotic leaks resolved without therapeutic intervention, such as replacement of the peripancreatic drains if the primary drains were displaced and/or replacement of pancreatic anastomotic stents if they became dislodged from the lumen of the pancreatic remnant. One patient had a small leak at the PJ anastomosis on pancreatogram on POD 4. The drain amylase level was 1184 IU/L on POD 2. No subsequent amylase levels were obtained.

This patient had portal vein thrombosis, intracranial infarction, and multiorgan failure, and died on POD 31. Autopsy did not show a pancreatic leak. The pancreatic leak had resolved and was not the cause of death.

Among the 12 patients with pancreatic anastomotic leaks, only one required surgical re-exploration due to refractory abscess. On the day of surgical re-exploration at POD 37, the drain amylase level was 1919 IU/L. Yet, the pancreatogram showed a large leak at the PJ anastomosis. During re-exploration, the PJ anastomosis was found to be almost completely disrupted. A new end-to-side duct-to-mucosa PJ anastomosis was performed. No PJ anastomotic leak recurred.

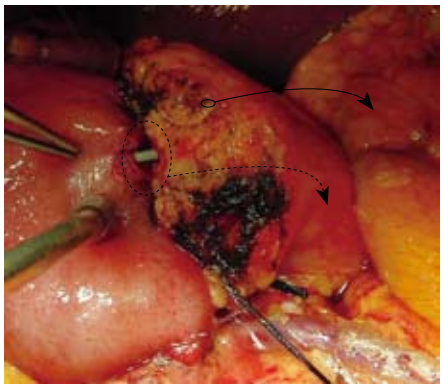

Figure 6 At completed PJ anastomosis, pancreatic leakage can occur either at anastomosis (dotted circle and arrow) or at a small peripheral duct opening or tear in the parenchyma (black circle and arrow).

DISCUSSION

Pancreatic leakage results in major morbidity and mortality after pancreaticoduodenectomy. In this study, we critically examined the integrity of the duct-to-mucosa PJ anastomosis. By analyzing both pancreatography findings and drain amylase levels, we found a way to distinguish parenchymal from anastomotic leakage at the PJ anastomosis. Of the 20 pancreatic leaks at the PJ anastomosis, eight (40%) originated from the parenchymal or small side branch duct, and 12 (60%) originated from the duct-to-mucosa anastomosis. These findings suggest that in duct-to-mucosa PJ anastomosis, parenchymal leakage is almost as common as anastomotic leakage.

In parenchymal pancreatic leakage, pancreatography consistently showed no extravasation at the pancreatic anastomosis. Nevertheless, drain amylase levels were elevated. In one of the eight cases, a small side-branch ductal leak was found along the parenchymal surface of the pancreatic remnant. Although only one case, it represented a potential and likely source of pancreatic leakage in the absence of true anastomotic leakage. It also appears that these parenchymal non-anastomotic leaks tend to resolve spontaneously. These findings are consistent with a previous report demonstrating that most pancreatic leaks do not require surgical exploration[19]. In another case, a 1.5-mm side branch in the cut surface of the pancreatic remnant was observed intraoperatively. This side branch duct was approximate 1 cm from the main pancreatic duct and was only 1 cm in length. Fibrin sealant was injected, and the PJ anastomosis was completed in routine fashion. Postoperatively, there was no evidence of pancreatic leakage.

In the cases studied, pancreatic anastomotic leakage was confirmed by the presence of contrast extravasation at the duct-to-mucosa anastomosis on pancreatogram. The levels of drain amylase continued to increase until the leak started to resolve. In contrast to parenchymal leaks, anastomotic leaks tend not to resolve spontaneously. Appropriate intervention, either replacement of the indwelling anastomotic stent or surgical exploration for large anastomotic disruption, is required. In this series, after re-establishing the dislodged pancreatic stents by interventional radiology, the two anastomotic leaks resolved at 123 and 189 d, compared with 14 wk (or 98 d) in a previous report[20]. Eight of the 12 PJ anastomotic leaks resolved within 10 wk in this series, with a range of 15 to 76 d. The patients whose PJ leak took the longest to heal had a dislodged pancreatic anastomotic stent. Once the percutaneous stent was successfully replaced, the PJ leak healed within 3 wk. These findings support the concept that mucosa-to-ductal epithelial healing is probably promoted by stent placement across the PJ anastomosis[21]. In addition, the externalized stent allows for continuous external drainage of luminal pancreatic juice and promotes healing of the PJ anastomosis. However, our results do not directly suggest that a pancreatic stent is required. Pancreatic stents are not commonly used, therefore, PJ anastomoses might heal without a stent. Rather, the use of pancreatic stents in this study may provide a means to understand the exact source and nature of pancreatic leaks after pancreaticoduodenectomy.

We have found that most pancreatic leaks can be managed conservatively, which is consistent with the findings of others[16,19,20]. The indications for surgical re-exploration are intra-abdominal refractory abscess or hemorrhage. In this series, no hemorrhage occurred. However, hemorrhage secondary to a pancreatic leak remains a lethal complication. Accurate diagnosis of a pancreatic anastomotic leak is thus essential. In our series, only one of the 12 anastomotic leaks needed surgical correction.

Other studies have indicated different factors related to increased risk of a pancreatic leak after pancreatectomy, such as soft pancreatic texture and small duct size[4,11,22]. It seems probable that pancreatic leaks may result from parenchymal tears by sutures, resulting in a leak *via* a parenchymal side-branch duct. However, without objective evidence, such as a pancreatogram, it would be impossible to define the exact nature of the leak.

Although they did not demonstrate their findings radiographically, Matsusue and colleagues suggested a similar distinction for peripancreatic sepsis as the result of parenchymal or non-anastomotic seepage of pancreatic juice versus a pancreatic fistula from

pancreatic anastomotic failure[19]. Our findings for parenchymal leakage correspond to their description of peripancreatic sepsis, and our anastomotic leak corresponds to their pancreatic fistula. Furthermore, without radiographic evidence, a pancreatic leak can be classified by using the volume and amylase level of the fluid in the peripancreatic drain and the requirement for therapeutic intervention[13]. However, the most important difference lies within our capability to directly visualize radiographically the pancreatic anastomosis. Together, the results from drain amylase levels and the pancreatogram can clearly distinguish a true anastomotic leak from a parenchymal non-anastomotic leak as illustrated in Figure 6. These findings are limited, however, by the small number of parenchymal leaks (n = 8). Larger numbers in future prospective studies will be required to further understand the exact nature of pancreatic leaks at PJ reconstruction after pancreaticoduodenectomy.

One can argue for the need for pancreatic and biliary stenting. From our experience with liver transplantation, biliary stenting is not uniformly used. This is because the placement of the biliary tube is usually performed *via* a T-tube through a surgically created opening in the common bile duct, which leads to persistent biliary leakage after removal of the biliary T-tube. In contrast, since the beginning of the liver transplantation program at our center in 1998, we insert the biliary tube *via* the donor cystic duct and secure it with a rubber band. For bilioenteric anastomosis, the biliary tube is inserted through the jejunal limb and secured with a Witzel's tunnel. The biliary tube and the pancreatic stent are each kept in place with a 5-O Vicryl™ suture, which will dissolve by 4 wk, allowing for safe removal of the biliary tube at 28 d. When this method is used, biliary stenting and pancreatic stenting have been safe. No complications have occurred as a result of a stent.

Although it was not a stated aim of this study, we have found that the technique of placing an externalized PJ anastomotic stent at the time of pancreaticoduodenectomy is helpful for postoperative management. Such stenting allows access to the pancreatic ductal system for assessing anastomotic integrity by pancreatography and control of drainage, should a leak be found. None of our patients with leaks had significant hemorrhage, peripancreatic inflammation, or completion pancreatectomy[10]. In addition, the presence of this stent allows ongoing assessment of the anastomosis and facilitates decisions about timing of drain removal and progression of diet. For these reasons, we continue to use this technique in our practice.

In conclusion, duct-to-mucosa anastomotic leakage can be distinguished from parenchymal (non-anastomotic) leakage after duct-to-mucosa PJ. Both parenchymal and anastomotic pancreatic leaks contribute almost equally to pancreatic leaks after pancreaticoduodenectomy. Knowledge of the exact nature of the leak is important for optimal treatment and management of this complication.

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COMMENTS

Background

Pancreatic leakage after pancreaticoduodenectomy is a significant source of morbidity and mortality, with anastomotic leaks being more refractory than parenchymal leaks. It is important to be able to distinguish one from the other so that appropriate treatment can be given.

Research frontiers

Pancreatic surgeons need new ways to monitor pancreatic leakage after pancreaticoduodenectomy to know whether conservative treatment is sufficient or more aggressive treatment should be undertaken.

Innovations and breakthroughs

By analyzing pancreatographic findings, drainage volumes, and amylase levels, author found patterns among cases of pancreatic leakage that enable us to distinguish parenchymal leakage from anastomotic leakage. Specifically, in parenchymal pancreatic leakage, pancreatography showed no extravasation at the pancreatic anastomosis, yet drain amylase levels were elevated, whereas in anastomic pancreatic leakage, contrast extravasation is present and the levels of drain amylase increased until the leak started to resolve. In contrast to parenchymal leaks, anastomotic leaks tend not to resolve spontaneously.

Applications

This retrospective study identified distinguishing characteristics of parenchymal *versus* anastomotic leakage. These distinctions should be confirmed prospectively and can be used clinically to provide better treatment after pancreatic surgery.

Terminology

Pancreaticoduodenectomy is a procedure that is used to treat tumors located in the head of the pancreas. Parenchymal leakage refers to leakage that occurs from the resection surface of the pancreas remnant, and anastomotic leakage refers to a specific leakage that occurs due to a failure of the reconstructed duct-to-mucosa anastomosis between the pancreas remnant and the intestine.

Peer review

The author evaluated the incidence of anastomotic versus parenchymal leakage following duct-to-mucosa pancreatic reconstruction in pancreaticoduodenectomy. This is a well-written paper. The findings provided great inspiration about how to consider and manage pancreatic leakage after pancreaticoduodenectomy.

REFERENCES

1 **Bartoli FG**, Arnone GB, Ravera G, Bachi V. Pancreatic fistula and relative mortality in malignant disease after pancreaticoduodenectomy. Review and statistical meta-analysis regarding 15 years of literature. *Anticancer Res* 1991; **11**: 1831-1848

2 **Z'graggen K**, Uhl W, Friess H, Buchler MW. How to do a safe pancreatic anastomosis. *J Hepatobiliary Pancreat Surg* 2002; **9**: 733-737

3 **Evans DB**, Lee JE, Pisters PWT. Pancreaticoduodenectomy (Whipple Operation) and total pancreatectomy for cancer. In: Baker RJ, Fischer JE. Mastery of surgery, 4th ed. New York: Lippincott Williams and Wilkins, 2001: 1299-1318

4 **Suzuki Y**, Fujino Y, Tanioka Y, Hiraoka K, Takada M, Ajiki T, Takeyama Y, Ku Y, Kuroda Y. Selection of pancreaticojejunostomy techniques according to pancreatic texture and duct size. *Arch Surg* 2002; **137**: 1044-1047; discussion 1048

5 **Reissman P**, Perry Y, Cuenca A, Bloom A, Eid A, Shiloni E, Rivkind A, Durst A. Pancreaticojejunostomy versus controlled pancreaticocutaneous fistula in pancreaticoduodenectomy for periampullary carcinoma. *Am J Surg* 1995; **169**: 585-588

6 **Katsaragakis S**, Antonakis P, Konstadoulakis MM,

Androulakis G. Reconstruction of the pancreatic duct after pancreaticoduodenectomy: a modification of the Whipple procedure. *J Surg Oncol* 2001; **77**: 26-29; discussion 30
7 **Grobmyer SR**, Rivadeneira DE, Goodman CA, Mackrell P, Lieberman MD, Daly JM. Pancreatic anastomotic failure after pancreaticoduodenectomy. *Am J Surg* 2000; **180**: 117-120
8 **Lau ST**, Simchuk EJ, Kozarek RA, Traverso LW. A pancreatic ductal leak should be sought to direct treatment in patients with acute pancreatitis. *Am J Surg* 2001; **181**: 411-415
9 **Brodsky JT**, Turnbull AD. Arterial hemorrhage after pancreatoduodenectomy. The 'sentinel bleed'. *Arch Surg* 1991; **126**: 1037-1040
10 **Smith CD**, Sarr MG, vanHeerden JA. Completion pancreatectomy following pancreaticoduodenectomy: clinical experience. *World J Surg* 1992; **16**: 521-524
11 **van Berge Henegouwen MI**, De Wit LT, Van Gulik TM, Obertop H, Gouma DJ. Incidence, risk factors, and treatment of pancreatic leakage after pancreaticoduodenectomy: drainage versus resection of the pancreatic remnant. *J Am Coll Surg* 1997; **185**: 18-24
12 **Choi SH**, Moon HJ, Heo JS, Joh JW, Kim YI. Delayed hemorrhage after pancreaticoduodenectomy. *J Am Coll Surg* 2004; **199**: 186-191
13 **Bassi C**, Dervenis C, Butturini G, Fingerhut A, Yeo C, Izbicki J, Neoptolemos J, Sarr M, Traverso W, Buchler M. Postoperative pancreatic fistula: an international study group (ISGPF) definition. *Surgery* 2005; **138**: 8-13
14 **Matsumoto Y**, Fujii H, Miura K, Inoue S, Sekikawa T, Aoyama H, Ohnishi N, Sakai K, Suda K. Successful pancreatojejunal anastomosis for pancreatoduodenectomy. *Surg Gynecol Obstet* 1992; **175**: 555-562
15 **Ohwada S**, Ogawa T, Kawate S, Tanahashi Y, Iwazaki S, Tomizawa N, Yamada T, Ohya T, Morishita Y. Results of duct-to-mucosa pancreaticojejunostomy for pancreaticoduodenectomy Billroth I type reconstruction in 100 consecutive patients. *J Am Coll Surg* 2001; **193**: 29-35
16 **Cullen JJ**, Sarr MG, Ilstrup DM. Pancreatic anastomotic leak after pancreaticoduodenectomy: incidence, significance, and management. *Am J Surg* 1994; **168**: 295-298
17 **Yeh TS**, Jan YY, Jeng LB, Hwang TL, Wang CS, Chen SC, Chao TC, Chen MF. Pancreaticojejunal anastomotic leak after pancreaticoduodenectomy--multivariate analysis of perioperative risk factors. *J Surg Res* 1997; **67**: 119-125
18 **Howard JM**. Pancreatojejunostomy: leakage is a preventable complication of the Whipple resection. *J Am Coll Surg* 1997; **184**: 454-457
19 **Matsusue S**, Takeda H, Nakamura Y, Nishimura S, Koizumi S. A prospective analysis of the factors influencing pancreaticojejunostomy performed using a single method, in 100 consecutive pancreaticoduodenectomies. *Surg Today* 1998; **28**: 719-726
20 **Kazanjian KK**, Hines OJ, Eibl G, Reber HA. Management of pancreatic fistulas after pancreaticoduodenectomy: results in 437 consecutive patients. *Arch Surg* 2005; **140**: 849-854; discussion 854-856
21 **Biehl T**, Traverso LW. Is stenting necessary for a successful pancreatic anastomosis? *Am J Surg* 1992; **163**: 530-532
22 **Yang YM**, Tian XD, Zhuang Y, Wang WM, Wan YL, Huang YT. Risk factors of pancreatic leakage after pancreaticoduodenectomy. *World J Gastroenterol* 2005; **11**: 2456-2461

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BASIC RESEARCH

Innate immune reactivity of the liver in rats fed a choline-deficient L-amino-acid-defined diet

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Abstract

AIM: To investigate the innate immune reactivity of tumor necrosis factor-alpha (TNF-α), Toll-like receptor 4 (TLR4), and CD14 in the liver of non-alcoholic steatohepatitis (NASH) model rats.

METHODS: Male F344 rats were fed a choline-deficient L-amino-acid-defined (CDAA) diet. The rats were killed after 4 or 8 wk of the diet, and their livers were removed for immunohistochemical investigation and RNA extraction. The liver specimens were immunostained for TNF-α, TLR4, and CD14. The gene expressions of *TNF-α*, *TLR4*, and *CD14* were determined by reverse-transcriptase polymerase chain reaction (RT-PCR). Kupffer cells were isolated from the liver by Percoll gradient centrifugation, and were then cultured to measure TNF-α production.

RESULTS: The serum and liver levels of TNF-α in the CDAA-fed rats increased significantly as compared with the control group, as did the immunohistochemical values and gene expressions of TNF-α, TLR4, and CD14 with the progression of steatohepatitis. TNF-α production from the isolated Kupffer cells of the CDAA-fed rats was elevated by lipopolysaccharide stimulation.

CONCLUSION: The expressions of TNF-α, TLR4, and CD14 increased in the NASH model, suggesting that TLR4 and CD14-mediated endotoxin liver damage may also occur in NASH.

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Key words: Nonalcoholic steatohepatitis; Kupffer cells; Toll-like receptor 4; CD14; Endotoxins; Tumor necrosis factor-alpha

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INTRODUCTION

Some patients who are not regular drinkers of alcohol have hepatic histological features that mimic alcoholic hepatitis and can progress to liver cirrhosis[1]. This condition has been named non-alcoholic steatohepatitis (NASH) and is thought to begin with excessive fat accumulation in the liver (first hit), followed by aggravating factors such as oxidative stress, inflammatory cytokines, and endotoxins (second hit)[2].

Kupffer cells, the resident macrophages in the liver, are localized in the non-parenchymal tissue and involved in the organ's innate immunity. Activation of Kupffer cells induces progression of NASH *via* tumor necrosis factor-alpha (TNF-α)[3]. The recent discovery of Toll-like receptor 4 (TLR4), a functional receptor on the surface of macrophages and various other types of cells that transmit Et signals, has led to recognition of molecules, including CD14, that bind to lipopolysaccharides (LPSs) as the mainstay of the mechanism that initiates signal transduction leading to LPS recognition and cellular activation[4].

NASH is characterized by hepatic steatosis, inflammation, and fibrosis, with increased risk of developing cirrhosis and hepatocellular carcinoma (HCC)[5]. It is

known that the choline-deficient L-amino-acid-defined (CDAA) fed rat model shows fatty accumulation and fibrosis, and after 16 wk the liver shows cirrhotic changes and nodules appear like HCC[6]. This model is sometimes used as a hepatocarcinogenesis model. In this study, we used a CDAA diet to produce one of the experimental animal models of NASH[7], to investigate the innate immune reactivity of the liver in NASH.

MATERIALS AND METHODS

Animal model

Male F344 rats (6-wk-old, weighing 180-200 g) were purchased from Japan SLC (Hamamatsu, Japan), and fed a CDAA diet for up to 8 wk to induce NASH. At 1, 4, and 8 wk, the control rats (fed a standard rat chow), and the CDAA-fed rats were killed and bled from the inferior vena cava (IVC). Liver tissues were excised after perfusion with PBS into the portal vein. All procedures were approved by our institutional animal care committee and conducted in accordance with Nara Medical University Guidelines for the Care and Use of Laboratory Animals.

Serum alanine aminotransferase (ALT)

Serum samples from the control and CDAA-fed rats were used to measure the serum ALT levels. The serum ALT levels were determined using a 7170 Clinical Analyzer (Hitachi High-Technologies, Tokyo, Japan).

Serum TNF-α

Serum samples (50 μL) from the control and CDAA-fed rats were used to measure the TNF-α concentration by enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA, USA).

Liver TNF-α

Liver tissues were frozen in liquid nitrogen, powdered, and 30 mg were homogenized in 1 mL of lysis buffer. The supernatant (50 μL) was used to measure the TNF-α levels by ELISA kit (BioSource International).

Histology

The liver tissues were fixed in 40 g/L formalin, embedded in paraffin, and 5-μm thick sections were cut from the paraffin blocks for staining with hematoxylin-eosin (HE) and Azan stains. Histological grading and staging were performed using a modified scoring system based on the classification of either Matteoni *et al*[8] or Brunt *et al*[9] proposed NAFLD types 1-4 based on long-term outcome studies, while Brunt *et al*[9] proposed a system of grading and staging for NASH that follows methods of separate assessment for necroinflammatory lesions (grading) and fibrosis (staging) accepted in other forms of non-biliary chronic liver diseases.

Immunohistochemistry

The TNF-α immunohistochemical staining was performed on the paraffin sections using an anti-rat TNF-α/TNFSF1A antibody (R&D Systems, Minneapolis, MN, USA), and the TLR4 and CD14 immunohistochemical staining was performed on frozen sections using an anti-rat TLR4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and an anti-rat CD14 antibody (Santa Cruz Biotechnology Inc.), respectively. After that, a Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA), and DAB peroxidase substrate solution (Vector Laboratories Inc.), with counterstaining by Hematoxylin Mayer were used. The stained areas were analyzed by NIH image software (Version 1.61, U.S. National Institute of Health, Bethesda, MD, USA).

Kupffer cell isolation and culture

Kupffer cells were isolated from the control and CDAA-fed rat livers according to a previously described procedure[10,11]. In brief, the liver was perfused through the portal vein with 100 mL of PBS containing 0.5 mmol/L EGTA (Research Organics, Cleveland, OH, USA) solution. The perfusion was carried out at 37℃ at a flow rate of 10 mL/min. Subsequent perfusion with Hanks' balanced salt solution containing 0.5 g/L collagenase type Ⅳ (Wako, Osaka, Japan) (collagenase solution) was carried out at 37℃ at a flow rate of 10 mL/min for 20 min. Then, the liver was excised and minced in the collagenase solution.

The liver slurry was filtered through gauze mesh, then washed with collagenase solution. Next, Percoll (Sigma-Aldrich Corp, St. Louis, MO, USA) was used for density gradient centrifugation of cells. Kupffer cells were collected from the layer between 1.04 and 1.06. They were then cultured in RPMI 1640 supplemented with 10% fetal bovine serum and SAG (50 μg/mL streptomycin, 50 μg/mL ampicillin, 0.3 g/L L-glutamine). The viability of the cultured Kupffer cells was determined by trypan blue exclusion. Kupffer cells were seeded at a density of 5×10^5 cells/mL on a 12-well plate with RPMI 1640. For LPS-stimulated group, Kupffer cells were then exposed to 1 μg/mL of LPS (Sigma-Aldrich Corp.) and incubated for 4.5 h at 37℃ in a 50 mL/L CO_2-humidified air atmosphere. The cells untreated with LPS were used as control. The supernatant was analyzed by ELISA kit (BioSource International) to measure the TNF-α concentration.

Reverse transcriptase-PCR (RT-PCR) analysis

The total RNA was extracted from the powdered frozen liver samples using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Briefly, 2 μg of the RNA sample were used for cDNA synthesis using Moloney Murine Leukemia Virus (M-MLV), Reverse Transcriptase RNaseH Minus (Toyobo, Osaka, Japan), and RNase Inhibitor (Toyobo) to investigate the expressions of *TNF-α* mRNA, *TLR4* mRNA, and *CD14* mRNA. PCR procedure was performed as follows: the samples were heated at 94℃, subjected to 30 cycles of 45 s at 94℃ for denaturing, 45 s at 60℃ for annealing,

Table 1 Primers for the target genes used in RT-PCR

Target gene	Primer
TNF-α	5′-TACTGAACTTCGGGGTGATTGGTCC-3′ (F)
	5′-CAGCCTTGTCCCTTGAAGAGAACC-3′ (R)
TLR4	5′-CGCTTTCAGCTTTGCCTTCATTAC-3′ (F)
	5′-TGCTACTTCCTTGTGCCCTGTGAG-3′ (R)
CD14	5′-GTGCTCCTGCCCAGTGAAAGAT-3′ (F)
	5′-GATCTGTCTGACAACCCTGAGT-3′ (R)
β-actin	5′-ACCACAGCTGAGAGGGAAATCG-3′ (F)
	5′-AGAGGTCTTTACGGATGTCAAAG-3′ (R)

F: Sense; R: Antisense.

and 2 min at 72℃ for extension. Following RT-PCR, 10-μL samples of the amplified products were resolved by electrophoresis on 15 g/L agarose gel, and stained with ethidium bromide. Each PCR product was analyzed by NIH image software. The specific primers for *TNF-α*, *TLR4*, *CD14*, and *β-actin* used are described in Table 1.

Statistical analysis

All results are expressed as mean ± SD. The analysis was carried out using Statview software (version 5.0, SAS Institute, Cary, NC, USA). Statistical differences between groups were evaluated using the paired *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Serum ALT

The mean serum ALT level in the control rats was 41.7 ± 7.4 IU/L, as compared with 524.6 ± 101.7 IU/L, 267.9 ± 47.5 IU/L, and 251.4 ± 81.6 IU/L in the 1-, 4-, and 8-wk CDAA-fed rats, respectively. All CDAA-fed groups showed a statistically significant elevation in the serum ALT level as compared with the control group ($P < 0.01$).

Serum TNF-α

As compared with the control rats (5.2 ± 4.4 pg/mL), the serum TNF-α level in the 4-wk CDAA-fed rats was elevated (55.9 ± 1.7 pg/mL, $P < 0.01$), and that in the 8-wk CDAA-fed rats (48.2 ± 5.3 pg/mL, $P < 0.01$) was elevated (Figure 1A).

Liver TNF-α

The liver TNF-α was 15.8 ± 3.6 pg/μg protein in the control rats, 30.3 ± 5.4 pg/μg protein in the 4-wk CDAA-fed rats, and 29.6 ± 4.4 pg/μg protein in the 8-wk CDAA-fed rats. The CDAA-fed groups showed a statistically significant elevation in the liver TNF-α level as compared with the control group ($P < 0.05$) (Figure 1B).

Histological findings

Histologically, the livers of the 1-wk CDAA-fed rats showed inflammation and fat deposits, but no fibrosis, and were classified as Matteoni's type 2. The livers of the 4-wk CDAA-fed rats showed more inflammation, fat deposits, and fibrosis, equivalent to Matteoni's type 3 and grade 2/stage 2 of Brunt's NASH classification. The histological findings in the livers of the 8-wk CDAA-fed rats were equivalent to Matteoni's type 4 and Brunt's grade 2/stage 3 (Figure 2).

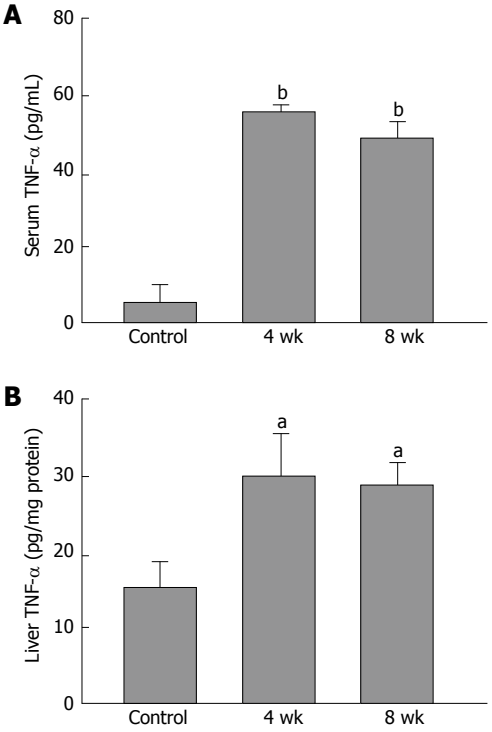

Figure 1 Serum TNF-α levels and liver TNF-α (ELISA method) compared with the control group ($n = 5$). A: Serum TNF-α levels were significantly elevated in the livers of the 4- and 8-wk CDAA-fed rats; B: Liver TNF-α was significantly elevated in the livers of the 4- and 8-wk CDAA-fed rats. [a]$P < 0.05$ *vs* the control; [b]$P < 0.01$ *vs* the control.

Immunohistochemical findings

The TNF-α immunoreactivities in the tissue samples from the livers of the 4- and 8-wk CDAA-fed rats were increased as compared with those in the control rats. The TLR4 and CD14 immunohistochemical findings in the 4- and 8-wk CDAA-fed rats were also increased as compared with those in the control rats. Moreover, the immunopositive areas in those animals were localized either in the periphery of fatty spots or non-parenchymal cells. The semiquantitative analysis of TNF-α, TLR4, and CD14 staining revealed significantly increased immunoreactivity in the livers of the 4-wk (14600 ± 5700, 26400 ± 2300, 17400 ± 6000, respectively) and 8-wk (10600 ± 1800, 21600 ± 4700, 21300 ± 3900, respectively) CDAA-fed rats as compared with the control rats (1560 ± 1080, 3160 ± 1000, 4290 ± 1110, respectively) ($P < 0.01$) (Figure 3).

Kupffer cell isolation and culture

The viability of the cultured Kupffer cells was over 98% as determined by trypan blue exclusion. The basic TNF-α production of the Kupffer cells isolated from 4- (26.4 ± 2.3 pg/mL) and 8-wk CDAA-fed rats (26.6 ± 1.4 pg/mL) was equal to that of the control rats (23.4 ± 1.8 pg/mL). After LPS stimulation, the TNF-α

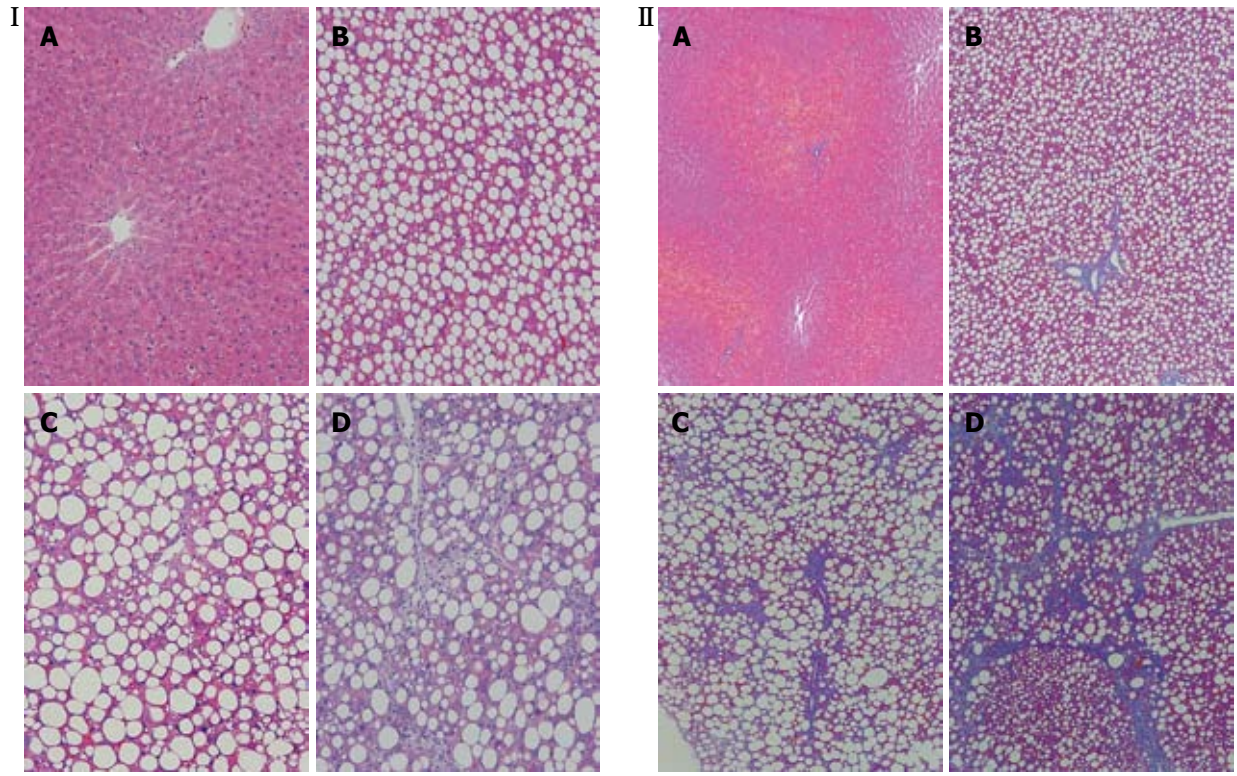

Figure 2 Inflammation and fibrosis in the rat liver. I: HE, x 200; II: Azan, x 100. A: Control rat; B: 1-wk CDAA-fed rat; C: 4-wk CDAA-fed rat; D: 8-wk CDAA-fed rat.

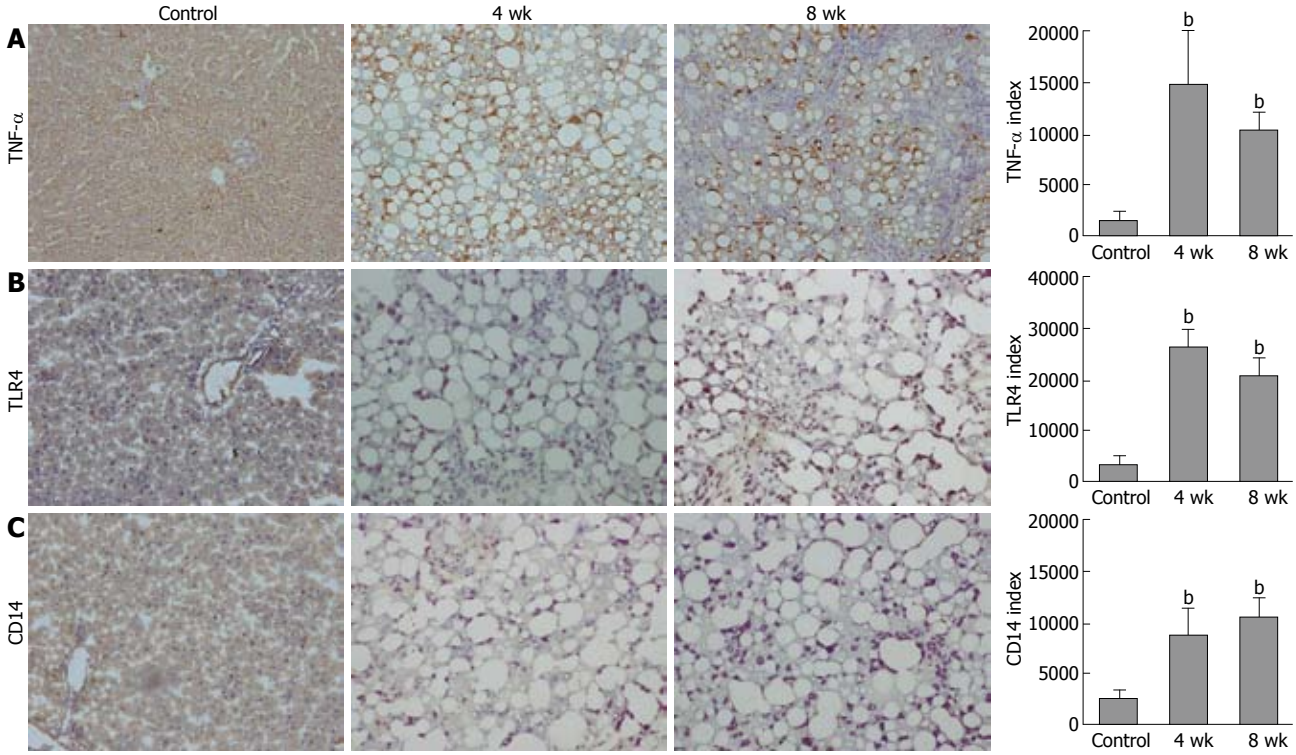

Figure 3 Immunohistochemical staining and semiquantification in the rat liver (x 200). A: For TNF-α; B: For TLR4; C: For CD14. $^{b}P < 0.01$ *vs* the control.

production in the control rats (48.9 ± 7.5 pg/mL) was significantly higher than the basic control levels, and that in the 4-wk (85.8 ± 16.8 pg/mL) and 8-wk (73.8 ± 8.4 pg/mL) CDAA-fed rats was significantly higher than in the control rats ($P < 0.01$) (Figure 4).

RT-PCR analysis

The expression of both *TNF-α* mRNA and *TLR4* mRNA was significantly increased in the livers of the 4- and 8-wk CDAA-fed rats as compared with those in the control group ($P < 0.01$). The expression of *CD14*

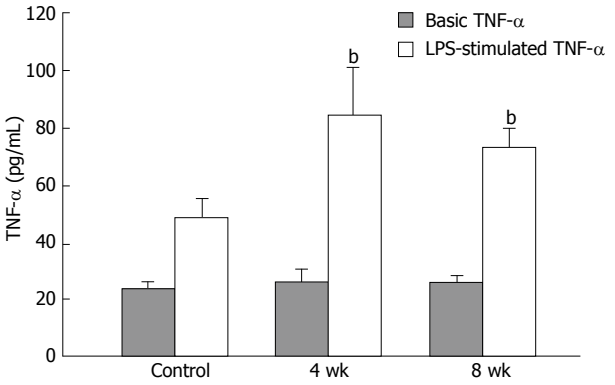

Figure 4 TNF-α production of Kupffer cells *in vitro*. The basic TNF-α production of Kupffer cells isolated from 4- and 8-wk CDAA-fed rats was equal to that of the control rats. After LPS stimulation, the TNF-α production of the control rats was significantly higher than the basic control levels, and that of the 4- and 8-wk CDAA-fed rats was significantly higher than that of the control rats. $^{b}P < 0.01$ *vs* the control.

mRNA was increased in the livers of the 8-wk CDAA-fed rats as compared with that in the control group ($P < 0.05$) (Figure 5).

DISCUSSION

Kupffer cells secrete inflammatory cytokines[12] and reactive oxide[13], which affect the hepatocytes, stellate cells, endothelial cells, *etc*[14]. In alcoholic hepatitis, inflammatory cytokines, such as TNF-α, induce the liver injury[15]. After chronic ethanol feeding, Kupffer cells exhibit enhanced sensitivity to LPS-stimulated TNF-α production[16]. Increased serum levels of TNF-α have been noticed in patients with NASH[17]. The mechanism of the latter may be secretion from adipocytes, as a result of obesity, or secretion from Kupffer cells activated by endogenous endotoxin originating from enteric bacteria. The activated Kupffer cells induce increases in the levels of cytokines, such as TNF-α or reactive oxide, which may be involved in the progression of NASH. Rats fed a high-fat diet become obese and show insulin resistance. In addition, the activities of both nuclear factor kappa enhancer binding protein (NF-κB) and the inhibitor of NF-κB kinase beta (IKKβ) significantly increase in the liver, and the expression of inflammatory cytokines such as TNF-α becomes excessive[18]. Our results showed that the livers of CDAA-fed rats developed inflammation in the early stage, and this was sustained by chronic feeding of the diet. After 4 wk or more, the livers of the CDAA-fed rats developed progressive steatohepatitis. In the present study, we confirmed by ELISA, immunohistochemistry, and RT-PCR that both the serum level of TNF-α and the liver TNF-α expression were increased, suggesting involvement of TNF-α in the development and progression of NASH similar to that in alcoholic hepatitis. Moreover, the TNF-α production of the isolated Kupffer cells was equal in the NASH model. Kupffer cells from the control rats were sensitive to LPS stimulation. Furthermore, those from NASH were more sensitive to LPS stimulation, suggesting that the continuous inflammation leads to up-regulation of the LPS-

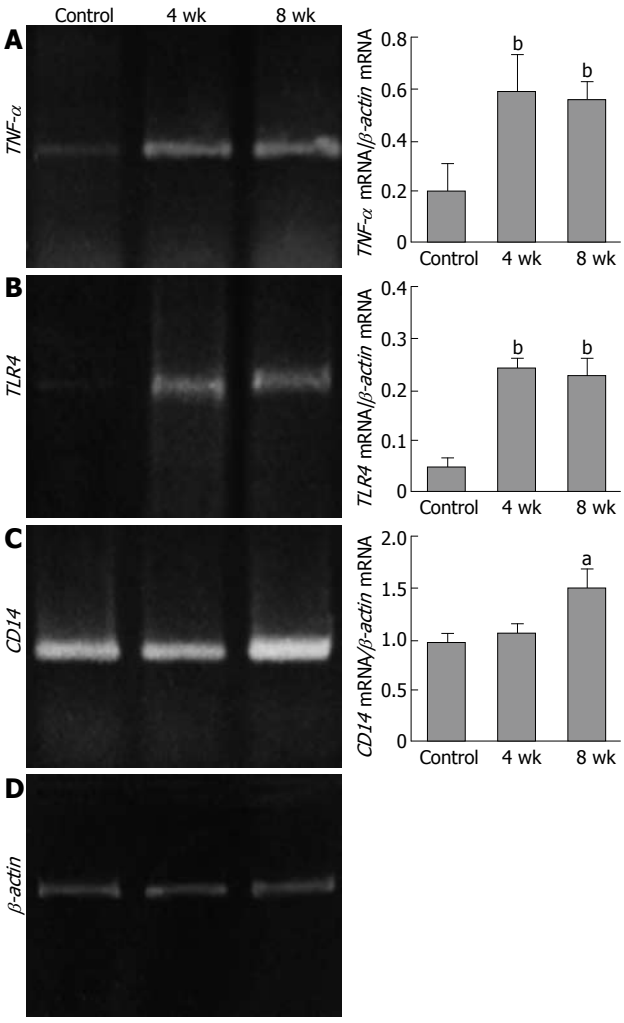

Figure 5 RT-PCR and semiquantification ($n = 5$). A: *TNF-α* mRNA; B: *TLR4* mRNA; C: *CD14* mRNA; D: *β-actin* mRNA. $^{a}P < 0.05$ *vs* the control, $^{b}P < 0.01$ *vs* the control.

stimulated TNF-α production by the activated Kupffer cells.

To date, 13 TLRs have been described in mice and 11 in humans. Recently, TLR4 has been highlighted as an important membrane protein[19,20] and is now established as the receptor for LPS. CD14 binds LPS and subsequently presents it to TLR4 and MD-2, which activate the intracellular signaling pathway *via* myeloid differentiation factor 88 (MyD 88), resulting in NF-κB activation[21]. Experiments using TLR4 knockout (KO) mice or CD14 KO mice have revealed a reduced reaction to LPS and a reduced expression of TNF-α[22,23], indicating that LPS signaling induces TNF-α production *via* TLR4 or CD14. The intake of alcohol facilitates the permeability of endotoxin from the gut to the portal vein, and the increased concentration of endotoxin in the portal vein leads to Kupffer cell activation *via* TLR4, with consequent increases in the liver expression of TNF-α, progression of inflammation, and fibrosis of the liver[24]. In the experimental chronic liver injury model, the expression of TLR4 in isolated Kupffer cells increases with progression of liver injury[25], suggesting a correlation between inflammation and expression of

TLR4. The present study showed increased expression of mRNA levels and immunohistochemical values of TNF-α, TLR4, and CD14 in the livers of rats with NASH induced by CDAA diet, suggesting that TLR4-mediated endotoxin liver damage may also occur in NASH. Rivera *et al*[26] also demonstrated enhanced TLR4 expression together with steatohepatitis in the liver of mice fed a methionine choline-deficient (MCD) diet. They further reported that the liver injury was attenuated in the TLR4 mutant mice and in Kupffer cell-depleted mice, and pointed out the importance of TLR4 signaling and Kupffer cells in the pathogenesis of steatohepatitis. Although these models lack obesity and insulin resistance, two major characteristics of NASH in humans, the possible relation of Kupffer cells with inflammation, steatosis, and fibrosis may resemble the human NASH. We also demonstrated decreased Kupffer cell phagocytic activity in this model[27]. The possible Kupffer cell dysfunction characterized by decreased phagocytic activity and increased cytokine production should be investigated in future studies.

Although the pathogenic mechanism of NASH is still unclear, its histopathology is similar to that of alcoholic hepatitis. Enhanced activation of the TLR4 dependent signaling pathways was also observed after chronic ethanol feeding[28]. There is a possibility that TLR4-dependent innate immunity induced by intestinal bacterial endotoxin may work as a common pathway in these two types of steatohepatitis. Further studies are needed to confirm this hypothesis.

In conclusion, our study focused on the innate immune reactivity of livers in the rat model of NASH, and we found overexpressions of TNF-α, TLR4, and CD14 in association with liver fibrosis and inflammation. These results suggest that TLR4 and CD14-mediated endotoxin liver damage may also occur in NASH.

COMMENTS

Background

Recently, non-alcoholic steatohepatitis (NASH) has become a common disease in Japan. The two-hit theory has been widely accepted to explain the progression from simple steatosis to NASH. Moreover, NASH is known as a disease progressing to liver cirrhosis or hepatocellular carcinoma (HCC). The mechanism of NASH progression has not been clarified yet. Authors investigated the engagement of Toll-like receptor 4 (TLR4) or CD14 in the rat NASH model.

Research frontiers

This is the first report showing the innate immune response in the rat NASH model. Authors found overexpression of tumor necrosis factor-alpha (TNF-α), TLR4, and CD14 in association with liver fibrosis and inflammation. These results suggest that TLR4 and CD14-mediated endotoxin liver damage may occur in NASH.

Innovations and breakthroughs

The present study demonstrated the overexpression of TNF-α, TLR4, and CD14 in the rat NASH model. This result suggests that the involvement of endotoxin-induced TLR4 activation led to the NASH progression.

Applications

The present study is an experimental research using the rat NASH model, which was made by feeding a choline-deficient L-amino-acid-defined diet to rats, to examine their innate immunity. For understanding NASH progression, further evaluation of the cytokine production from the Kupffer cells and the expressions of TLR4 and CD14 from the isolated Kupffer cells should be employed in the subsequent experiments.

Terminology

TLR4 is a transmembrane receptor for lipopolysaccharides (LPS) on the surface of macrophages. LPS stimulation activates TLR4 by binding with CD14, resulting in pro-inflammatory cytokine production, like TNF-α.

Peer review

Authors described the importance of innate immune responses to the pathogenesis of rat model of NASH. The findings are important.

REFERENCES

1 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438
2 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845
3 **Tomita K**, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, Kitamura N, Toda K, Kaneko T, Horie Y, Han JY, Kato S, Shimoda M, Oike Y, Tomizawa M, Makino S, Ohkura T, Saito H, Kumagai N, Nagata H, Ishii H, Hibi T. Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. *Gut* 2006; **55**: 415-424
4 **Su GL**. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G256-G265
5 **Bugianesi E**, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M, Rizzetto M. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2002; **123**: 134-140
6 **Sakaida I**, Kubota M, Kayano K, Takenaka K, Mori K, Okita K. Prevention of fibrosis reduces enzyme-altered lesions in the rat liver. *Carcinogenesis* 1994; **15**: 2201-2206
7 **Koteish A**, Diehl AM. Animal models of steatosis. *Semin Liver Dis* 2001; **21**: 89-104
8 **Matteoni CA**, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413-1419
9 **Brunt EM**, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467-2474
10 **Olynyk JK**, Clarke SL. Isolation and primary culture of rat Kupffer cells. *J Gastroenterol Hepatol* 1998; **13**: 842-845
11 **Seglen PO**. Preparation of rat liver cells. 3. Enzymatic requirements for tissue dispersion. *Exp Cell Res* 1973; **82**: 391-398
12 **Tsujimoto T**, Kuriyama S, Yamazaki M, Nakatani Y, Okuda H, Yoshiji H, Fukui H. Augmented hepatocellular carcinoma progression and depressed Kupffer cell activity in rat cirrhotic livers. *Int J Oncol* 2001; **18**: 41-47
13 **Bilzer M**, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. *Liver Int* 2006; **26**: 1175-1186
14 **Diehl AM**. Recent events in alcoholic liver disease V. effects of ethanol on liver regeneration. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G1-G6
15 **Hansen J**, Cherwitz DL, Allen JI. The role of tumor necrosis factor-alpha in acute endotoxin-induced hepatotoxicity in ethanol-fed rats. *Hepatology* 1994; **20**: 461-474
16 **Aldred A**, Nagy LE. Ethanol dissociates hormone-stimulated cAMP production from inhibition of TNF-alpha production in rat Kupffer cells. *Am J Physiol* 1999; **276**: G98-G106
17 **Crespo J**, Cayon A, Fernandez-Gil P, Hernandez-Guerra M, Mayorga M, Dominguez-Diez A, Fernandez-Escalante JC, Pons-Romero F. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* 2001; **34**: 1158-1163
18 **Cai D**, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J,

Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005; **11**: 183-190
19 **Aderem A**, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000; **406**: 782-787
20 **Chow JC**, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 1999; **274**: 10689-10692
21 **Medzhitov R**, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997; **388**: 394-397
22 **Haziot A**, Ferrero E, Kontgen F, Hijiya N, Yamamoto S, Silver J, Stewart CL, Goyert SM. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* 1996; **4**: 407-414
23 **Hoshino K**, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999; **162**: 3749-3752
24 **Uesugi T**, Froh M, Arteel GE, Bradford BU, Thurman RG. Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. *Hepatology* 2001; **34**: 101-108
25 **Hua J**, Qiu de K, Li JQ, Li EL, Chen XY, Peng YS. Expression of Toll-like receptor 4 in rat liver during the course of carbon tetrachloride-induced liver injury. *J Gastroenterol Hepatol* 2007; **22**: 862-869
26 **Rivera CA**, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol* 2007; **47**: 571-579
27 **Tsujimoto T**, Kawaratani H, Kitazawa T, Hirai T, Ohishi H, Kitade M, Yoshiji H, Uemura M, Fukui H. Decreased phagocytic activity of Kupffer cells in a rat nonalcoholic steatohepatitis model. *World J Gastroenterol* 2008; **14**: 6036-6043
28 **Nagy LE**. Recent insights into the role of the innate immune system in the development of alcoholic liver disease. *Exp Biol Med* (Maywood) 2003; **228**: 882-890

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BASIC RESEARCH

Gene expression profiling: Canonical molecular changes and clinicopathological features in sporadic colorectal cancers

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Abstract

AIM: To investigate alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth, possibly determining at-risk populations and predicting responses to treatment.

METHODS: Using microarray gene-expression analysis, we analyzed patterns of gene expression relative to canonical molecular changes and clinicopathological features in 84 sporadic colorectal cancer patients, standardized by tumor location. Subsets of differentially expressed genes were confirmed by real-time reverse-transcript polymerase chain reaction (RT-PCR).

RESULTS: The largest number of genes identified as being differentially expressed was by tumor location, and the next largest number by lymphovascular or neural invasion of tumor cells and by mismatch repair (MMR) defects. Amongst biological processes, the immune response was significantly implicated in entire molecular changes observed during colorectal tumorigenesis ($P < 0.001$). Amongst 47 differentially expressed genes, seven (*PISD*, *NIBP*, *BAI2*, *STOML1*, *MRPL21*, *MRPL16*, and *MKKS*) were newly found to correlate with tumorigenesis and tumor growth. Most location-associated molecular changes had distinct effects on gene expression, but the effects of the latter were sometimes contradictory.

CONCLUSION: We show that several differentially expressed genes were associated with canonical molecular changes in sporadic colorectal cancers, possibly constituting alternative or subordinate pathways of tumorigenesis. As tumor location was the dominant factor influencing differential gene expression, location-specific analysis may identify location-associated pathways and enhance the accuracy of class prediction.

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Key words: Colorectal adenocarcinomas; Sporadic; Gene expression; Profiling; Tumorigenesis

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INTRODUCTION

Analysis of genetic alterations in hereditary colorectal cancers have identified several molecular changes, including those involving APC-Wnt signaling, mismatch repair (MMR) defects, RAF cascades, and p53 alterations[1-3]. The pattern of molecular changes observed in hereditary colon cancers suggested a stepwise model for colorectal tumorigenesis. About 80% of colorectal cancers, however, are sporadic, and the pattern of genetic alterations observed in hereditary tumors has been consistently observed in only a small number of sporadic tumors[1]. These findings suggest the existence of alternative or subordinate and crossover pathways of colorectal tumorigenesis.

The APC protein is thought to contribute to all processes governing tumor tissues, including proliferation, migration, apoptosis, and differentiation[4]. Loss of APC

function leads to intracellular β-catenin stabilization, the key component of canonical Wnt signaling, and constitutive signaling of β-catenin within the nucleus[5,6]. The current model of colon tumorigenesis suggests that MMR defects cause tumors primarily through two mechanisms, mutations in tumor suppressor gene pathways and inappropriate apoptosis[7]. Sporadic colorectal cancers with MMR defects, including almost all those with BRAF mutations, are thought to arise through the CpG island methylator phenotype (CIMP) associated with methylation of MLH1[3]. These alterations initiate cellular processes directed towards either proliferation or differentiation, depending on signal intensity and duration[8]. Alternatively, RAS mutations may be early events in the adenoma-carcinoma sequence, and *RAF* alterations may be related to the progression and development of de novo colorectal cancer[9].

The p53 pathway is ubiquitously lost in human cancers, either by *p53* mutations, observed in 60% of tumors, or by loss of cell signaling upstream and downstream of p53 in the 40% of cancers expressing wild-type p53[10]. Following disruption of $p21^{WAF1}$, p53 expression is enhanced because of p53 stabilization, which correlates with the increased expression of the tumor suppressor $p14^{ARF}$, an inhibitor of the ubiquitin ligase activity of MDM2[11]. Apart from these molecular changes, however, little is known about crossover pathways between APC-Wnt signaling and MMR or RAF alterations. *APC* and *RAS* mutations have been shown to be synergistic in promoting β-catenin nuclear translocation, thus enhancing canonical Wnt signal transduction[12]. Moreover, APC was shown to regulate cellular proliferation and transformation induced by the activation of both RAS and β-catenin signaling[13].

To identify alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth, we assessed gene expression patterns, relative to canonical molecular changes and clinicopathological features in patients with colorectal tumors. Individual steps and pathways were sorted into various biological processes. We also performed location-specific analysis to determine whether this exercise might improve the accuracy of class prediction. Our results may also be used to determine at-risk populations and to predict responses to treatment.

MATERIALS AND METHODS

Patients and tissue samples

We prospectively enrolled 84 consecutive patients with sporadic colorectal cancer scheduled to undergo curative resection between 2006 and 2007 at the Asan Medical Center (Seoul, Korea) (Table 1). Tumors were standardized by location, and samples of tumor and normal colonic mucosa, taken at least 5 cm from the tumor borders, were obtained at the time of surgery. The tissue samples were snap-frozen in liquid nitrogen. Total RNA was extracted using RNeasy RNA extraction kits (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions, and DNA was extracted from lymphocytes and tumors using standard methods. Cancer staging was determined by imaging studies and operative findings with histological diagnosis according to the American Joint Committee on Cancer (6th ed., 2001). Our sample size was determined for competent cluster analysis using an efficient annealing algorithm with error rates of < 10%. All patients provided written informed consent, and the study protocol was approved by the Institutional Review Board for Human Genetic and Genomic Research, in accordance with the Declaration of Helsinki.

Clinicopathological features and molecular changes in colorectal tumorigenesis

Methods of representative molecular changes in tumor tissues, including *APC* mutations, Wnt-activated alterations, MMR defects, RAF-mediated changes, and p53 alterations have been described using different samples[14]. Briefly, *APC* mutations were assessed throughout all exons and introns, whereas Wnt-activated alterations were assessed by immune staining for β-catenin, Axin2, GSK3β, and E-cadherin. The search for MMR alterations included microsatellite instability (MSI) assays using the Bethesda panel, assays of methylation status at the 5'-promoter site and the 3'-small site of *hMLH1*, and immune staining for hMLH1 and hMSH2. We assessed RAF-mediated alterations by determining *BRAF* codon 600 mutations, mutations in *KRAS* exons 12 and 13, and immune staining for MEK. Alterations in *p53* were assessed by immune staining for altered p53. Crossover was defined when a tumor carried both APC/Wnt-activated changes and MMR defects or RAF-mediated alterations.

cDNA microarray and data analyses

The 21k cDNA microarray chips were prepared using Korean Unigene Information (KUGI) cDNA clones (http://kugi.kribb.re.kr/) and Incyte Human 10k cDNA clones. The PCR products of each clone were spotted on type-7 glass slides using an Array Spotter Generation Ⅲ (Amersham Pharmacia, Piscataway, NJ, USA). Aliquots of tumor and non-tumor RNAs (20 mg respectively) were used as templates for the synthesis of cDNA, labeled with Cy5 or Cy3, respectively, using SuperScript Ⅱ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 2 h at 42℃. The two labeled cDNAs were mixed, filtered through Microcon YM-30 filters (Millipore, Bedford, MA, USA) to exclude unincorporated dNTPs, and hybridized to the microarray slides at 50℃ overnight using a 3DNA Array 50 kit (Genisphere Inc., Hatfield, PA, USA). After hybridization, each microarray was washed twice with 2 × SSC with 0.2% (w/v) SDS at room temperature for 5 min, and finally with 95% (v/v) ethanol at room temperature for 1 min. The slides were scanned using a ScanArray 5000 Scanner (Axon Instruments, Union City, CA, USA), and scanned images were analyzed using the GenePix Pro 4.0 program (Axon Instruments). The raw data were normalized using the print-tip Lowess method available in the OLIN package

Table 1 Clinicopathological features relative to location of sporadic colorectal cancers

Clinicopathologic features	Tumor location[1] (No. of patients)			*P*
	R (*n* = 27)	L (*n* = 29)	P (*n* = 28)	
Male/Female	18/9	15/14	20/8	0.273
Age	62 ± 7	60 ± 12	62 ± 10	0.646
AJCC stage[2], Ⅰ/Ⅱ/Ⅲ/Ⅳ	4/13/6/4	4/15/6/4	4/10/9/5	0.926
Tumor differentiation, WD +	22/5	29/0	24/4	0.021 (R *vs* L)
MD/PD + muc				0.052 (L *vs* P)
Synchronous adenoma, -/+	18/9	23/6	14/14	0.052 (L *vs* P)
LVN invasion, -/+	15/12	22/7	20/8	0.236

[1]R: Cecum-splenic flexure of transverse colon; L: Splenic flexure of transverse colon-sigmoid colon; P: Rectum. [2]According to the American Joint Committee on Cancer (6th ed., 2001). WD, MD, PD, and muc, well-, moderately-, poorly-differentiated, and mucinous. LVN: Lymphovascular or neural invasion of tumor cells.

of the Bioconductor project (http://www.bioconductor.org)[15]. Missing values were imputed using the k-nearest neighbor method (available at the GEPAS web service: http://gepas.bioinfo.cipf.es/cgi-bin/preprocess/). The raw data have been deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/projects/geo/) under the accession number GSE10982.

Quantitative reverse-transcript polymerase chain reaction (RT-PCR)

Total cellular RNA (5 μg) was reverse transcribed into cDNA using SuperScript Ⅱ (Invitrogen). Real-time (RT)-PCR was performed using the Exicycler Quantitative Thermal Block (Bioneer, Daejeon, Korea). The RT-PCR reaction product (100 ng) was amplified in a 15 μL reaction volume with 2 × SYBR Premix EX *Taq* (Takara, Shiga, Japan). Primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Following an initial denaturation at 95℃ for 1 min, the amplification protocol consisted of 45 cycles of denaturation at 95℃ for 30 s, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s, followed by a final extension step of 72℃ for 10 min. The β-actin protein was used as an internal control. Relative quantification of each mRNA was analyzed by the comparative threshold cycle (TC) method.

Parametric analysis of gene set enrichment (PAGE)

We applied the PAGE method to identify significant changes in expression of gene sets[16]. Diverse categories of gene sets included molecular changes associated with colorectal tumorigenesis, namely cell cycle and apoptosis pathways, receptor protein tyrosine kinase signaling, Wnt/cadherin signaling, DNA MMR, and TGF-β signaling pathway. They were prepared from Affymetrix annotation files (http://www.affymetrix.com/netaffy) and annotation files were downloaded from the Source web service (http://genome-www5.stanford.edu/cgi-bin/source/sourceBatchSearch). Gene sets identified by gene ontology (GO) protocols included those involved in various biological processes, genes responsible for cellular components and molecular functions, genes defined by chromosomal locations, genes related by InterPro domains, and genes involved in distinct metabolic pathways. Pathway information was obtained from the BioCarta (http://www.biocarta.com) and KEGG (http://www.genome.ad.jp/kegg/) databases. Publications on differentially expressed genes were accessed to understand gene effects on biological functions and tumorigenesis, using PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez).

Statistical analysis

The associations of molecular changes and clinicopathologic features with tumor location were examined by cross-table analysis using Fisher's exact or Pearson's χ^2 tests with their significance level at 5%. The statistical significance of between-group comparisons was analyzed using Student's *t*-test and *Q*-values were calculated from corresponding *P*-values to control the false discovery rate (FDR) that may occur when testing multiple hypotheses[17]. Differential gene expression between tumors and normal epithelia were deemed to be significant at $P < 0.01$ for initial screening and $P < 0.001$ for individual gene candidates. Class prediction was examined using the BRB-Array Tools package (version 3.6) available at http://linus.nci.nih.gov/BRB-ArrayTools.html. All computations were performed using R statistical programming language (http://cran.r-project.org/) and the Bioconductor packages.

RESULTS

Differentially expressed genes relative to molecular changes and clinicopathological features

Assays for genes differentially expressed relative to molecular changes and clinicopathological features (Tables 2 and 3) showed that tumor location was associated with the highest numbers of differentially expressed genes. When we compared the right colon with the left colon and rectum taken together, we found that 1628 genes were differentially expressed, and when we compared the right colon with the left colon and rectum considered separately, we found that 1263 genes were differentially expressed. The next greatest extent of differential gene expression was seen when lymphovascular or neural invasion (LVI) of tumor cells occurred, and an analysis by defects in MMR yielded the next largest differentially expressed gene set. The differentially expressed genes significantly associated with canonical tumorigenesis and tumor progression are

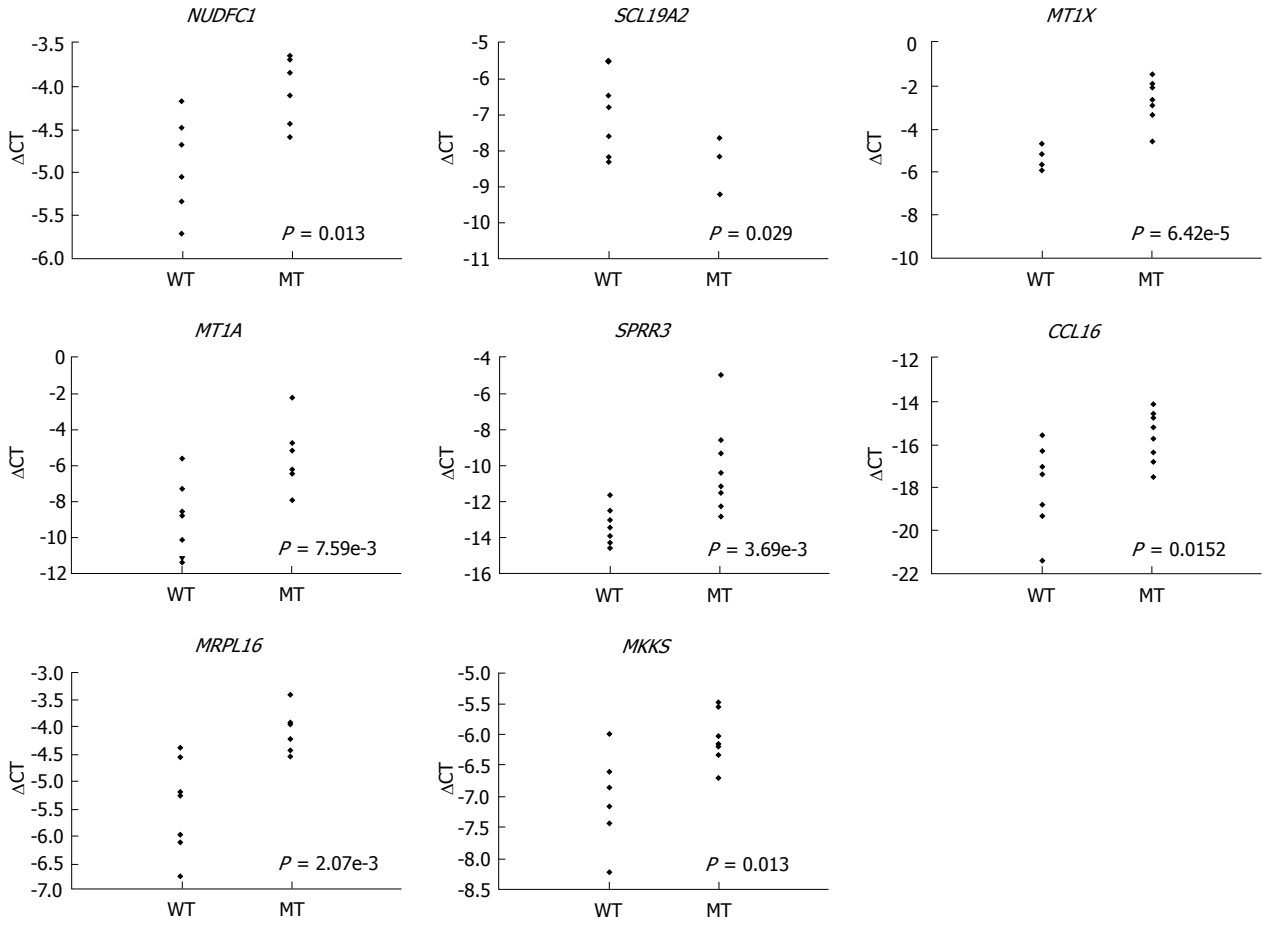

Figure 1 Quantitative RT-PCR of selected genes associated with molecular changes and clinicopathological features from microarray gene expression data. These were *NUDFC1* and *SCL19A2* (with *APC* mutations), *MT1X* and *MT1A* (with MMR defects), *SPRR3* (with crossover), *CCL16* (with lymphovascular or neural invasion), and *MRPL16* and *MKKS* (with synchronous adenoma). Genes differentially expressed between two groups were selected and their expression patterns measured using RT-PCR. WT: Without molecular or clinicopathological changes; MT: Molecular or clinicopathological changes. *P*-values from unpaired *t*-tests are shown.

collectively shown in Table 3. The differential expression of several candidate and novel genes was confirmed by real time RT-PCR (Figure 1).

Gene sets associated with APC and Wnt pathways

APC mutations are related to expression of constituents of the extracellular matrix (ECM) and to formation of the axonemal dynein complex, whereas Wnt-associated alterations are associated with the immune response, ECM formation, and filopodium expression. In addition, changes in pyruvate and arginine/proline metabolism have been associated with *APC* mutations; whereas alterations in G-protein receptor binding, the activities of various chemokines, phosphatase binding efficiency, and glycolysis/gluconeogenesis rates are associated with mutations in *Wnt*. We found that upregulation of three genes (*CDH7*, *DYRK1A*, *PISD*) and downregulation of one (*SLC19A2*) were associated with APC mutations, whereas upregulation of two genes (*PRAF2*, *CD99L2*) and downregulation of one (*FOXF1*) were associated with Wnt-activated changes ($P < 0.001$).

Gene set alterations associated with the MMR and RAF pathways

Biologically, MMR defects affect the immune response (including antigen processing), chromosome functions, and cytoskeleton structure, whereas RAF-mediated alterations are related to thyroid hormone generation and cytoplasmic effects. Cadmium and copper ion binding, MHC class Ⅱ receptor activity, and fatty acid metabolism have been associated with MMR defects, and protein dimerization activity with RAF-mediated alterations. We found that four upregulated genes (*MT1X*, *MT1A*, *SST*, *TDG*) and three downregulated genes (*HMGB1*, *SUGT1*, *VTI1B*) were associated with MMR defects, and that two were upregulated (*PPP1R13L*, *CAST*) and one was downregulated (*RAB22A*) in association with RAF-mediated alterations ($P < 0.001$).

Gene set alterations associated with p53 and crossover pathways

Alterations in p53 have been associated with the immune response (including antigen processing), ECM structure, and sensory perception, whereas crossover was related to cell cycle stage and protein localization. MHC class I receptor activity, oxidoreductase activity, and glycolysis were associated with p53 alterations, and protein kinase binding and renyltransferase activity were associated with crossover. No upregulated but three downregulated genes (*HLA-F*, *XRCC3*, *CCDC24*)

Table 2 Number of differentially expressed genes in terms of molecular changes and clinicopathological features

Parameters	No. of patients (missing)	No. of differentially expressed genes (P < 0.01), total (up/down)
Molecular changes[1], -/+		
APC mutations	55/27 (2)	83 (41/42)
Wnt-activated	45/38 (1)	82 (37/45)
MMR defects	70/14	238 (122/116)
RAF-mediated	58/26	108 (59/49)
Altered p53 expression	24/59 (1)	125 (57/68)
Crossover	64/19 (1)	92 (44/48)
Clinicopathologic features		
Tumor location[2], R/L + P	27/57	1628 (936/692)
R/L/P	27/29/28	1263
AJCC stage[3], Ⅰ + Ⅱ/Ⅲ + Ⅳ	50/34	195 (103/92)
Tumor differentiation, WD + MD/PD + muc	75/9	151 (69/82)
Synchronous adenoma, -/+	55/29	152 (92/60)
LVN invasion, -/+	57/27	279 (147/132)

[1]Wnt-activated: explored by β-catenin assay, AXIN2 measurement, and GSK-3β immune staining; MMR defect: analyzed by MSI assay, MLH1 5'-promoter measurement or 3'-methylation, and MLH1 or MSH2 immune staining; RAF-mediated alterations: assayed by detection of mutations in *BRAF* V600E and *KRAS* exons 12 and 13, and MEK immune staining; Crossover: When a tumor carried both APC/Wnt-mediated alterations and MMR defects or RAF-mediated alterations. [2]R: Caecum-splenic flexure of transverse colon; L: Splenic flexure of transverse colon-sigmoid colon; P: Rectum. [3]According to the American Joint Committee on Cancer (6th ed., 2001). WD, MD, PD, and muc, well-, moderately-, poorly-differentiated, and mucinous; MMR: Mismatch repair.

were associated with p53 alterations, whereas four upregulated genes (*NID2*, *EGLN3*, *NIBP*, *SPRR3*) and three downregulated genes (*ITIH1*, *CFH*, *ABI3BP*), were associated with crossover ($P < 0.001$).

Tumor location-specific analysis shows distinct patterns of gene expression

As colon tumor location had a very marked effect on differential gene expression, genes differentially expressed as a result of particular molecular changes and clinicopathological features may be concealed by tumor location. Interestingly, the number of genes differentially expressed as a result of tumor location increased slightly in association with several clinicopathological variables, although the sample size was much smaller (about one-third, data not shown) than those of other tumor sets. More importantly, most molecular changes had distinct location-associated effects on gene expression, but these were sometimes accompanied by contradictory effects. For example, p53 alterations inhibited the expression of antigen presentation-related genes only in right colon cancers, but had no effect in left colon or rectal cancers (Figure 2). After separation into classes showing various molecular and clinicopathological characteristics, the accuracy of binary outcome prediction was estimated using seven different machine learning algorithms available at BRBArrayTools. As expected, the best prediction accuracy (85%-94%) was achieved by tumor location (Table 4), followed by MMR defects (76%-83%). Most of the other molecular and clinicopathological variables had prediction accuracies < 75%. The prediction accuracies for each variable were

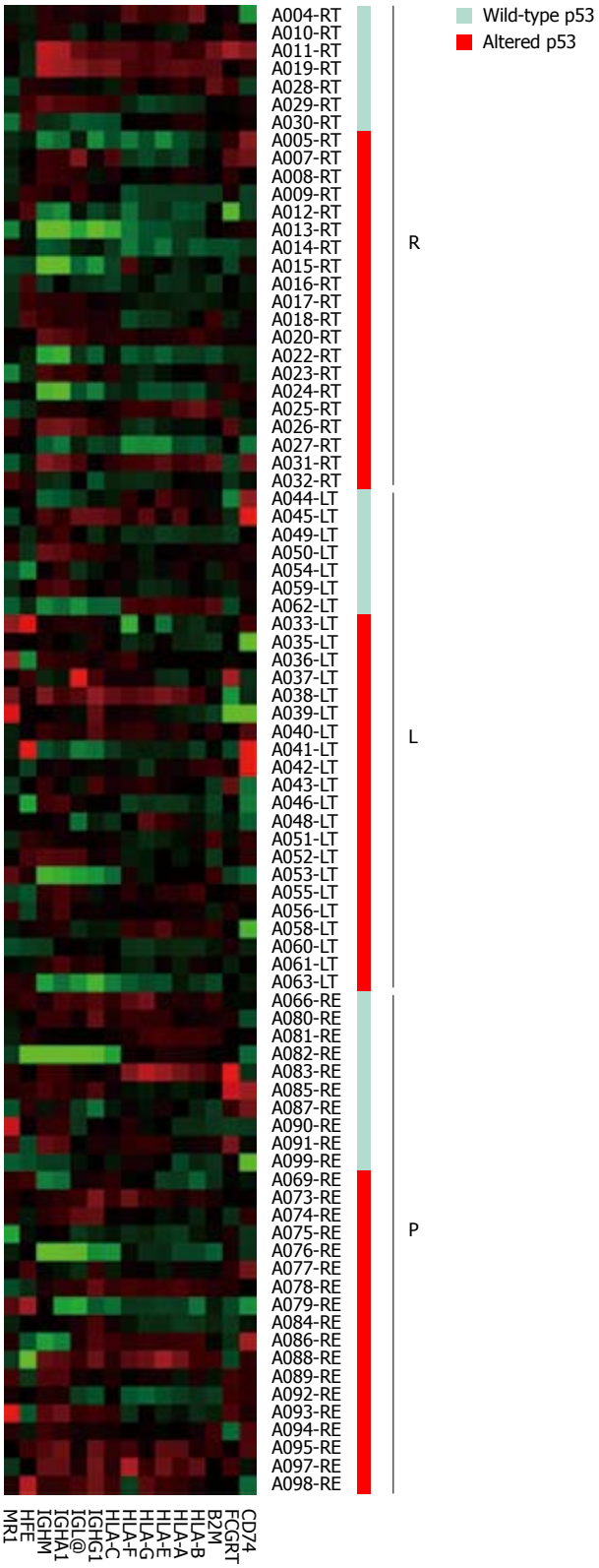

Figure 2 Pattern of expression of genes in the "antigen presentation, endogenous antigen" gene set as distinguished by tumor location and p53 status. Protein p53 alterations in the ascending colon coordinately decreased the expression of genes in the gene set whereas p53 alterations in the descending colon or rectum had no effect on gene expression. R: Cecum-splenic flexure of transverse colon; L: Splenic flexure of transverse colon-sigmoid colon; P: Rectum.

Table 3 Differential gene expression associated with molecular changes and clinicopathological features[1]

Parameters	Symbol	Name	Log_2 fold changes	Unadjusted *P*
APC mutations	*CDH7*	Cadherin 7, type 2	0.419885	0.00033
	DYRK1A	DS tyr-(Y)-phosphorylation regulated kinase 1A	0.326912	0.000343
	SLC19A2	Solute carrier family 19 member 2	-0.48574	0.000427
	PISD	Phosphatidylserine decarboxylase	0.272127	0.000545
	NDUFC1	NADH dehydrogenase 1, subcomplex unknown	0.539581	0.000572
Wnt-activated alterations	*PRAF2*	PRA1 domain family, member 2	0.620619	0.000205
	FOXF1	Forkhead box F1	-0.93359	0.000524
	CD99L2	CD99 molecule-like 2	0.753489	0.000772
MMR defects	*HMGB1*	High-mobility group box 1	-0.38141	3.74E-06
	MT1X	Metallothionein 1X	0.965429	0.000252
	MT1A	Metallothionein 1A	1.17894	0.000351
	SUGT1	SGT1, G2 allele of SKP1 (S. cerevisiae)	-0.34799	0.00039
	VTI1B	Vesicle transport with t-SNAREs homolog 1B (yeast)	-0.28513	0.000435
	SST	Somatostatin	1.24407	0.000564
	TDG	Thymine-DNA glycosylase	0.479829	0.000946
RAF-mediated alterations	*RAB22A*	RAB22A, member RAS oncogene family	-0.36017	0.000289
	PPP1R13L	Protein phosphatase 1, regulatory subunit 13 like	0.823379	0.000614
	CAST	Calpastatin	0.285773	0.000872
Altered p53 expression	*HLA-F*	Major histocompatibility complex, class I, F	-0.55645	0.000429
	XRCC3	XRCC in Chinese hamster cells 3	-0.26678	0.000588
	CCDC24	Coiled-coil domain containing 24	-0.28995	0.000996
Crossover[2]	*NID2*	Nidogen 2 (osteonidogen)	0.938223	0.000214
	EGLN3	egl nine homolog 3 (C. elegans)	0.740933	0.000375
	ITIH1	Inter-alpha (globulin) inhibitor H1	-0.34777	0.0004
	CFH	Complement factor H	-0.33826	0.000542
	ABI3BP	ABI gene family, member 3 binding protein	-0.82558	0.000637
	NIBP	NIK and IKK binding protein	0.649949	0.000688
	SPRR3	Small praline-rich protein 3	0.99738	0.000946
AJCC stage[3]	*PNPT1*	Polyribonucleotide nucleotidyltransferase 1	0.716381	4.94E-06
	BAI2	Brain-specific angiogenesis inhibitor 2	0.545369	1.57E-05
	ADCY1	Adenylate cyclase 1 (brain)	-0.43437	1.97E-05
	VEGFC	Vascular endothelial growth factor C	0.329341	9.11E-05
	ATAD3B	ATPase family, AAA domain containing 3B	-0.40978	0.000108
	CAP1	CAP, adenylate cyclase-associated protein 1 (yeast)	-0.66905	0.000405
	RPS6KA6	Ribosomal protein S6 kinase, 90kDa, polypeptide 6	0.301665	0.000586
	FGF5	Fibroblast growth factor 5	0.464162	0.000701
LVN invasion	*MMP12*	Matrix metallopeptidase 12 (macrophage elastase)	-0.69015	7.76E-05
	RAP1GDS1	RAP1, GTP-GDP dissociation stimulator 1	-0.60949	9.63E-05
	STOML1	Stomatin (EPB72)-like 1	-0.39407	0.000255
	CCL16	Chemokine (C-C motif) ligand 16	0.475838	0.000542
	NOTCH3	Notch homolog 3 (Drosophila)	0.592567	0.000679
	DHPS	Deoxyhypusine synthase	-0.33131	0.000965
Synchronous adenoma	*PARP2*	Poly (ADP-ribose) polymerase family, member 2	0.573461	6.00E-05
	MRPL21	Mitochondrial ribosomal protein L21	0.243204	0.000149
	MRPL16	Mitochondrial ribosomal protein L16	0.267004	0.000432
	MKKS	McKusick-Kaufman syndrome	0.439584	0.000447
	LHX2	LIM homeobox 2	0.663108	0.000782

[1]Differential gene expression in tumor tissues compared to normal epithelia was examined in terms of molecular changes and clinicopathological features, as was considered to be significant when $P < 0.001$; [2]When a tumor carried both APC/Wnt-mediated alterations and MMR defects or RAF-mediated alterations; [3]According to the American Joint Committee on Cancer (6th ed., 2001).

also examined after restricting the analysis by the three tumor locations mentioned above. As expected, location-specific analysis generally increased the prediction accuracy significantly (Figure 3), especially in the case of variables associated with synchronous adenoma, tumor stage, RAF-mediated changes, and Wnt-activated alterations. Accuracy, however, decreased when variables associated with *APC* mutations, p53 alterations, and crossover, were analyzed.

Clinicopathological features correlated with genomic alterations

Biologically, we found that tumor stage was related to antigen presentation, cell adhesion and migration, bone mineralization, and epithelial cell differentiation, and that lymphovascular or neural invasion was related to cell adhesion, immune response, and sensory perception. We also found that serine-type enzyme activities, high-density lipoprotein binding, pancreatic RNase activity, and glycolysis/gluconeogenesis were related to tumor stage, and that various structural molecules, hormones, serine-type enzyme activities, phosphate transport, the metabolism of the ECM and related molecules, and high density lipoprotein binding were related to lymphovascular or neural invasion. Synchronous adenoma was related to protein biosynthesis, ribosomal

Table 4 Class prediction accuracies (%) relative to molecular changes and clinicopathological features

Parameters	Genes[1]	CCP	DLDA	1-NN	3-NN	NC	SVM	BCCP
Tumor location	620	85	86	92	90	86	94	94
Synchronous adenoma	12	58	60	64	63	57	67	65
Tumor stage	22	62	58	58	60	61	54	60
APC mutations	12	72	72	57	72	67	73	67
LVN invasion	27	70	69	57	60	68	64	68
MMR defects	44	76	77	82	82	76	81	83
p53 alterations	8	65	70	61	73	66	72	79
RAF-mediated alterations	3	36	35	43	51	36	42	69
Wnt-activated alterations	9	54	54	61	61	58	49	54

[1]Number of classifier genes. CCP: Compound covariate predictor; DLDA: Diagonal linear discriminant analysis; 1-NN: One nearest-neighbor; 3-NN: Three nearest-neighbor; NC: Nearest centroid; SVM: Support vector machine; BCCP: Bayesian compound covariate predictor; LVN: Lymphovascular or neural.

proteins, and MHC class I receptor activity. We found that five genes (*PNPT1*, *BAI2*, *VEGFC*, *RPS6K6A*, *FGF5*) were upregulated and three (*ADCY1*, *ATAD3B*, *CAP1*) were downregulated in association with tumor stages; that two genes (*CCL16*, *NOTCH3*) and four genes (*MMP12*, *RAP1GDS1*, *STOML1*, *DHPS*) were up- or down-regulated, respectively, in association with lymphovascular or neural invasion, and that five genes (*PARP2*, *MRPL21*, *MRPL16*, *MKKS*, *LHX2*) were upregulated but no gene was downregulated in association with synchronous adenoma ($P < 0.001$).

DISCUSSION

Distinctive molecular changes, such as *APC* mutations and MMR defects, are respectively associated with two types of hereditary colorectal cancers, familial adenomatous polyposis and hereditary non-polyposis colorectal cancer. Although these hereditary tumors constitute fewer than 5%-8% of all colorectal cancers, the molecular changes identified in hereditary tumors are important in sporadic colorectal cancers[14,18,19]. The tumor suppressor APC is the major regulator of canonical Wnt signaling; these two proteins form a multi-protein complex encompassing kinases such as GSK-3β, CK1, and Axins, to prevent colorectal tumorigenesis[5]. Mutations in the oncogenes *RAF* and *RAS* are closely associated with MMR defects, and may act as alternative tumor-initiating steps that synergize with DNA methylation and occur within the context of serrated polyps[19,20]. The p53 protein, which normally induces G1 cell cycle arrest to facilitate DNA repair during replication, cannot induce cell cycle arrest when mutated in later stages of the adenoma-carcinoma sequence, thus leading to cell proliferation[21].

In our study, ECM interactions and the immune response were down- and up-regulated in tumors with *APC* mutations and Wnt-activated alterations, respectively. Gene set analysis earlier showed that the structural motif of osteopontin mediated critical cell-matrix and cell-cell signaling whose transcriptional regulation involves multiple pathways including Wnt/β-catenin/APC/GSK-3β/Tcf-4[22]. Expression of the E-cadherin β-catenin was observed in dendritic cells and loss of E-cadherin adhesion triggered a functionally distinct pathway of maturation linked more closely to the maintenance of tolerance than to the initiation of immunity[23]. In $Apc^{Min/+}$ mice, in which *APC* mutations are upregulated, dietary arginine increased colon tumorigenesis[24]. Amongst the eight genes we identified that were associated with *APC* mutations and Wnt activation, we found that one, *PISD*, was a novel gene upregulated in tumor cells with these alterations. Phosphatidylserine decarboxylation may provide a functionally important source of phosphatidylethanolamine in mitochondria[25].

We found that MMR defects correlated positively with an enhanced immune response and metal ion binding, whereas RAF alterations correlated with activation of cellular processes and thyroid hormone generation. Many tumor-infiltrating lymphocytes are present in MSI+ tumors, along with activated CD8+ cytotoxic T cells[26,27]. Furthermore, tumor-specific peptides generated by MSI may be involved in anti-tumor immune responses and may be useful in the diagnosis and treatment of patients with MSI+ colorectal cancers[27]. The deleterious effects of Cd2+ reported to date include generation of reactive oxygen species, inhibition of DNA repair, depletion of glutathione, and alteration of apoptosis[28]. In contrast, RAS/RAF/MEK/ERK-transduced signals can initiate cellular processes directed towards either proliferation or differentiation, depending on signal intensity and duration[29]. *RAF* mutations are associated with advanced clinical stages and early recurrence in patients with papillary thyroid cancer[30]. Amongst the genes up-regulated by MMR defects are the metallothionein genes, including *MT1X* and *MT1A*, which are expressed differentially in various tissues, during several developmental stages, and in response to metals, steroids, and stress[30]. Several of these genes, including *MT1X*, were overexpressed in MSI+ colorectal and gastric cancers[31].

We found that alterations in p53 downregulated immune responses and ascorbic acid binding. Anti-p53 IgG has been detected in the sera of subjects with various types of cancer, indicating induction of anti-p53 CD4+ Th cells[32]. Ascorbic acid can block the effects of TNF-α on endothelial cell proliferation and apoptosis

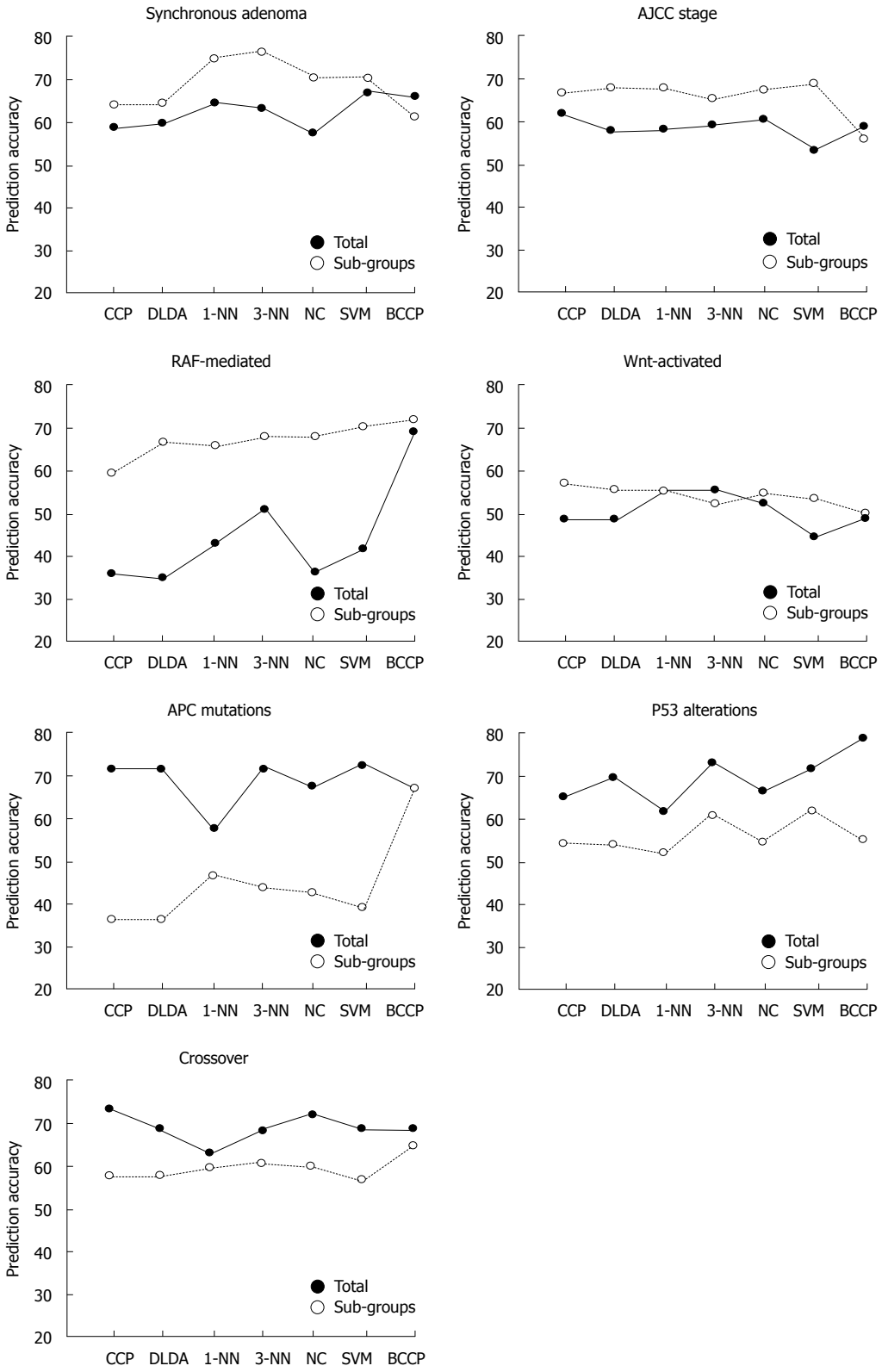

Figure 3 Accuracy of class prediction increases with tumor location-specific analysis. Samples were divided into three subgroups corresponding to three tumor locations (right colon, left colon, and rectum). Class prediction was performed using either all samples or samples within each subgroup. For tumor location-specific analysis, the results of class prediction (true or false) from each of the three locations were combined to calculate the overall prediction accuracy. Ten genetic or clinicopathological parameters were analyzed.

by inhibiting TNF-α-induced p53 expression and Rb hypophosphorylation, as well as by promoting collagen IV production[33]. We also observed that the crossover pathway between APC/Wnt-activated and MMR defects or RAF-mediated alterations, which has rarely been observed in human colorectal cancers, was associated with cell cycle and protein localization. Recently, mice carrying compound *Apc* and *Ras* mutations were characterized as having a striking increase in intestinal tumor multiplicity and progression, compared with *Apc*-only mutant animals[12]. Amongst the seven genes we identified as associated with the crossover pathway, one, *NIBP*, was a novel gene upregulated in tumor cells with these alterations. NIBP has been reported to enhance the cytokine-induced NF-κB signaling pathway by interacting with NIK and IKKβ[34], which may activate the TNF-induced invasive activity of tumor cells.

Embryologically, the right and left colon has different

origins, the midgut and hindgut, respectively, and is supplied by different circulation and innervation[35]. We found that tumor location was the dominant factor for differential gene expression in colorectal cancers. Thus, location-specific analysis may more precisely discriminate between alterations in gene expression caused by canonical molecular changes. The dependence of gene expression differences on tumor location has been reported previously[36-39]. The dominant expression pattern has been shown to be consistent with different embryonic origins and a second pattern reveals a gradual change from the caecum to the rectum[39]. We found that the prediction accuracy by tumor location-specific analysis was increased using analyses by synchronous adenoma, tumor stage, and RAF-mediated and Wnt-activated alterations, but decreased by analyses using *APC* mutations and p53 alterations. These findings suggest that *APC* mutations and p53 alterations may affect tumorigenesis as initiators and terminators, respectively, along the entire colon. In the absence of *APC* mutations and p53 alterations, however, synchronous adenoma, tumor stage, RAF-mediated changes, and Wnt-activated alterations may determine tumorigenesis at different locations. In addition, we found that several biological processes were affected differently by tumor location, often in opposite senses. One of the most significantly altered biological processes was the immune response. We observed that genes involved in the immune response were coordinately downregulated in left colon cancers with p53 alterations but not in right colon or rectal cancers. The same trend was observed with *APC* mutations, but the opposite trend was observed with MMR defects. Location-specific analysis also allowed the prediction of gene class by expression profiling in 6 of 10 parameters in our analysis, and in agreement with previous findings[38]. Gene expression profiling has been used to predict metastasis or recurrence in patients with stage Ⅱ colon cancer, thus enhancing the selection of chemosensitive patients for adjuvant chemotherapy[40,41]. Our finding that distinct molecular pathways of tumorigenesis occur in right and left colon cancers suggests that prediction of responsiveness to adjuvant therapy will benefit from location stratification.

In our study, both tumor stage and lymphovascular or neural invasion were associated with antigen presentation, ECM metabolism, and cellular and extracellular processes that determine tumor initiation and progression. Amongst the 14 differentially expressed genes associated with these biological functions, one encodes CCL16, a chemoattractant for monocytes and lymphocytes that can increase tumor rejection, antigen presentation by macrophages, T cell cytotoxicity, and the angiogenic activity of vascular endothelial cells[42]. *BAI2* and *STOML1* are novel genes, upregulated in advanced cancers and downregulated in lymphovascular and neural tumor invasion, respectively. Human BAI2, probably a G-protein-coupled receptor in the brain, participates in the early stages of neovascularization of the cerebral cortex after ischemia[43]. The stomatin homolog (UNC-24) of *C. elegans*, a protein similar to the human stomatin homolog STOML1 (SLP-1), is required for normal locomotor response to volatile anesthetics and contains a region of sequence homologous to the nonspecific lipid transfer protein[44].

As the traditional adenoma-carcinoma sequence, which is instigated in adenomas (or aberrant crypt foci) by the APC-Wnt signaling pathway, accounts for more than two-thirds of all colorectal cancers[3], we examined the molecular association of tumorigenesis in patients with synchronous adenoma. We found that three novel genes, *MRPL21*, *MRPL16*, and *MKKS*, were upregulated in tumors with synchronous adenoma. A mitochondrial ribosomal protein, MRPL21, arrests the cell cycle by increasing p21WAF1/CIP1 and p27Kip1 levels under growth inhibitory conditions[45]. The *MRPL16* gene originated *via* duplication of a pre-existing mitochondrial ribosomal protein gene as well as by recruitment of some DNA sequence from outside of the mitoribosomal genome[46]. McKusick-Kaufman syndrome (MKKS) is a human developmental anomaly syndrome featuring hydrometrocolpos, postaxial polydactyly, and congenital heart disease[47]. In protein biosynthesis, MKKS is similar in function to type Ⅱ chaperonins, which are responsible for folding a wide range of proteins[48].

In conclusion, we found that the differential expression of 47 genes was associated with canonical molecular changes and clinicopathological characteristics of sporadic colorectal cancers, possibly constituting alternative or subordinate pathways of tumorigenesis and tumor growth. Currently, the seven novel genes of our study that correlate with tumorigenesis and tumor growth, are functionally assessed to be possible candidates as diagnostic or therapeutic targets for colorectal cancers. Amongst these biological processes, the immune response was uniformly involved in all molecular changes, that is, APC/Wnt-activated alterations, changes arising from MMR defects, RAF-mediated changes, and p53-caused alterations. As tumor location was the dominant factor for differential gene expression in colorectal cancers, location-specific analysis may precisely discriminate particular gene expression profiles and enhance the accuracy of tumor class prediction.

COMMENTS

Background

Although various molecular changes have been identified in colorectal cancers, a clear pattern is detected in only 6.6% of these tumors, indicating the need to identify alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth.

Research frontiers

To identify alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth, this study assessed gene expression patterns, relative to canonical molecular changes and clinicopathological features, in patients with colorectal tumors. Individual steps and pathways were sorted into various biological processes.

Innovations and breakthroughs

The largest number of genes identified as differentially expressed was by tumor location, and the next largest number by lymphovascular or neural invasion of tumor cells and by mismatch repair (MMR) defects. Amongst biological

processes, the immune response was significantly implicated in entire molecular changes observed during colorectal tumorigenesis ($P < 0.001$). Amongst 47 differentially expressed genes, seven (*PISD*, *NIBP*, *BAI2*, *STOML1*, *MRPL21*, *MRPL16*, and *MKKS*) were newly found to correlate with tumorigenesis and tumor growth. Most location-associated molecular changes had distinct effects on gene expression, but the effects of the latter were sometimes contradictory.

Applications

This study found that the differential expression of 47 genes was associated with canonical molecular changes and clinicopathological characteristics of sporadic colorectal cancers, possibly constituting alternative or subordinate pathways of tumorigenesis and tumor growth. The seven novel genes of this study correlate with tumorigenesis and tumor growth and can functionally be assessed as possible candidates for diagnostic or therapeutic targets of colorectal cancers.

Terminology

The cDNA microarray becomes a fundamental tool to gain direct molecular insight into tumorigenesis. Additionally, as phenotypic diversities of cancer occur from genetic alterations, genomic expression profiling might have been recognized as the first step to find useful therapeutic targets.

Peer review

This paper describes alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth, constituting an individual geno-pathogenesis map for colorectal cancer. As the study strengthened tumor location as a dominant factor for differential gene expression in colorectal cancers, location-specific analysis precisely discriminate particular gene expression profiles, possibly providing individual responses to respective regimen. It's an interesting paper.

REFERENCES

1 **Smith G**, Carey FA, Beattie J, Wilkie MJ, Lightfoot TJ, Coxhead J, Garner RC, Steele RJ, Wolf CR. Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. *Proc Natl Acad Sci USA* 2002; **99**: 9433-9438

2 **Lynch HT**, de la Chapelle A. Genetic susceptibility to non-polyposis colorectal cancer. *J Med Genet* 1999; **36**: 801-818

3 **Weisenberger DJ**, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006; **38**: 787-793

4 **Sansom OJ**, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, Winton DJ. Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* 2004; **18**: 1385-1390

5 **Fodde R**, Brabletz T. Wnt/beta-catenin signaling in cancer stemness and malignant behavior. *Curr Opin Cell Biol* 2007; **19**: 150-158

6 **Thorstensen L**, Lind GE, Løvig T, Diep CB, Meling GI, Rognum TO, Lothe RA. Genetic and epigenetic changes of components affecting the WNT pathway in colorectal carcinomas stratified by microsatellite instability. *Neoplasia* 2005; **7**: 99-108

7 **Chao EC**, Lipkin SM. Molecular models for the tissue specificity of DNA mismatch repair-deficient carcinogenesis. *Nucleic Acids Res* 2006; **34**: 840-852

8 **Fang JY**, Richardson BC. The MAPK signalling pathways and colorectal cancer. *Lancet Oncol* 2005; **6**: 322-327

9 **Ikehara N**, Semba S, Sakashita M, Aoyama N, Kasuga M, Yokozaki H. BRAF mutation associated with dysregulation of apoptosis in human colorectal neoplasms. *Int J Cancer* 2005; **115**: 943-950

10 **Bourdon JC**. p53 and its isoforms in cancer. *Br J Cancer* 2007; **97**: 277-282

11 **Javelaud D**, Besancon F. Inactivation of p21WAF1 sensitizes cells to apoptosis via an increase of both p14ARF and p53 levels and an alteration of the Bax/Bcl-2 ratio. *J Biol Chem* 2002; **277**: 37949-37954

12 **Janssen KP**, Alberici P, Fsihi H, Gaspar C, Breukel C, Franken P, Rosty C, Abal M, El Marjou F, Smits R, Louvard D, Fodde R, Robine S. APC and oncogenic KRAS are synergistic in enhancing Wnt signaling in intestinal tumor formation and progression. *Gastroenterology* 2006; **131**: 1096-1109

13 **Park KS**, Jeon SH, Kim SE, Bahk YY, Holmen SL, Williams BO, Chung KC, Surh YJ, Choi KY. APC inhibits ERK pathway activation and cellular proliferation induced by RAS. *J Cell Sci* 2006; **119**: 819-827

14 **Kim JC**, Cho YK, Roh SA, Yu CS, Gong G, Jang SJ, Kim SY, Kim YS. Individual tumorigenesis pathways of sporadic colorectal adenocarcinomas are associated with the biological behavior of tumors. *Cancer Sci* 2008; **99**: 1348-1354

15 **Futschik ME**, Crompton T. OLIN: optimized normalization, visualization and quality testing of two-channel microarray data. *Bioinformatics* 2005; **21**: 1724-1726

16 **Kim SY**, Volsky DJ. PAGE: parametric analysis of gene set enrichment. *BMC Bioinformatics* 2005; **6**: 144

17 **Storey JD**, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci USA* 2003; **100**: 9440-9445

18 **Early DS**, Fontana L, Davidson NO. Translational approaches to addressing complex genetic pathways in colorectal cancer. *Transl Res* 2008; **151**: 10-16

19 **Jass JR**. Colorectal cancer: a multipathway disease. *Crit Rev Oncog* 2006; **12**: 273-287

20 **Fujiwara T**, Stolker JM, Watanabe T, Rashid A, Longo P, Eshleman JR, Booker S, Lynch HT, Jass JR, Green JS, Kim H, Jen J, Vogelstein B, Hamilton SR. Accumulated clonal genetic alterations in familial and sporadic colorectal carcinomas with widespread instability in microsatellite sequences. *Am J Pathol* 1998; **153**: 1063-1078

21 **Ryan KM**, Vousden KH. Cancer: pinning a change on p53. *Nature* 2002; **419**: 795, 797

22 **Wai PY**, Kuo PC. Osteopontin: regulation in tumor metastasis. *Cancer Metastasis Rev* 2008; **27**: 103-118

23 **Jiang A**, Bloom O, Ono S, Cui W, Unternaehrer J, Jiang S, Whitney JA, Connolly J, Banchereau J, Mellman I. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 2007; **27**: 610-624

24 **Yerushalmi HF**, Besselsen DG, Ignatenko NA, Blohm-Mangone KA, Padilla-Torres JL, Stringer DE, Guillen JM, Holubec H, Payne CM, Gerner EW. Role of polyamines in arginine-dependent colon carcinogenesis in Apc(Min) (/+) mice. *Mol Carcinog* 2006; **45**: 764-773

25 **Steenbergen R**, Nanowski TS, Beigneux A, Kulinski A, Young SG, Vance JE. Disruption of the phosphatidylserine decarboxylase gene in mice causes embryonic lethality and mitochondrial defects. *J Biol Chem* 2005; **280**: 40032-40040

26 **Michael-Robinson JM**, Biemer-Hüttmann A, Purdie DM, Walsh MD, Simms LA, Biden KG, Young JP, Leggett BA, Jass JR, Radford-Smith GL. Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status. *Gut* 2001; **48**: 360-366

27 **Houston AM**, Michael-Robinson JM, Walsh MD, Cummings MC, Ryan AE, Lincoln D, Pandeya N, Jass JR, Radford-Smith GL, O'Connell J. The "Fas counterattack" is not an active mode of tumor immune evasion in colorectal cancer with high-level microsatellite instability. *Hum Pathol* 2008; **39**: 243-250

28 **Waalkes MP**. Cadmium carcinogenesis. *Mutat Res* 2003; **533**: 107-120

29 **Xing M**. BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. *Endocr Rev* 2007; **28**: 742-762

30 **Mayo KE**, Warren R, Palmiter RD. The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells.

Cell 1982; **29**: 99-108
31 **Giacomini CP**, Leung SY, Chen X, Yuen ST, Kim YH, Bair E, Pollack JR. A gene expression signature of genetic instability in colon cancer. *Cancer Res* 2005; **65**: 9200-9205
32 **Ito D**, Albers A, Zhao YX, Visus C, Appella E, Whiteside TL, DeLeo AB. The wild-type sequence (wt) p53(25-35) peptide induces HLA-DR7 and HLA-DR11-restricted CD4+ Th cells capable of enhancing the ex vivo expansion and function of anti-wt p53(264-272) peptide CD8+ T cells. *J Immunol* 2006; **177**: 6795-6803
33 **Saeed RW**, Peng T, Metz CN. Ascorbic acid blocks the growth inhibitory effect of tumor necrosis factor-alpha on endothelial cells. *Exp Biol Med* (Maywood) 2003; **228**: 855-865
34 **Hu WH**, Pendergast JS, Mo XM, Brambilla R, Bracchi-Ricard V, Li F, Walters WM, Blits B, He L, Schaal SM, Bethea JR. NIBP, a novel NIK and IKK(beta)-binding protein that enhances NF-(kappa)B activation. *J Biol Chem* 2005; **280**: 29233-29241
35 **Wexner SD**, Jorge JM. Colon and rectal surgery. 5th ed. Corman ML, editor. Philadelphia: Lippincott Williams & Wilkins, 2005: 1-29
36 **Glebov OK**, Rodriguez LM, Nakahara K, Jenkins J, Cliatt J, Humbyrd CJ, DeNobile J, Soballe P, Simon R, Wright G, Lynch P, Patterson S, Lynch H, Gallinger S, Buchbinder A, Gordon G, Hawk E, Kirsch IR. Distinguishing right from left colon by the pattern of gene expression. *Cancer Epidemiol Biomarkers Prev* 2003; **12**: 755-762
37 **Birkenkamp-Demtroder K**, Olesen SH, Sørensen FB, Laurberg S, Laiho P, Aaltonen LA, Orntoft TF. Differential gene expression in colon cancer of the caecum versus the sigmoid and rectosigmoid. *Gut* 2005; **54**: 374-384
38 **Komuro K**, Tada M, Tamoto E, Kawakami A, Matsunaga A, Teramoto K, Shindoh G, Takada M, Murakawa K, Kanai M, Kobayashi N, Fujiwara Y, Nishimura N, Hamada J, Ishizu A, Ikeda H, Kondo S, Katoh H, Moriuchi T, Yoshiki T. Right- and left-sided colorectal cancers display distinct expression profiles and the anatomical stratification allows a high accuracy prediction of lymph node metastasis. *J Surg Res* 2005; **124**: 216-224
39 **LaPointe LC**, Dunne R, Brown GS, Worthley DL, Molloy PL, Wattchow D, Young GP. Map of differential transcript expression in the normal human large intestine. *Physiol Genomics* 2008; **33**: 50-64
40 **Wang Y**, Jatkoe T, Zhang Y, Mutch MG, Talantov D, Jiang J, McLeod HL, Atkins D. Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. *J Clin Oncol* 2004; **22**: 1564-1571
41 **Barrier A**, Boelle PY, Roser F, Gregg J, Tse C, Brault D, Lacaine F, Houry S, Huguier M, Franc B, Flahault A, Lemoine A, Dudoit S. Stage II colon cancer prognosis prediction by tumor gene expression profiling. *J Clin Oncol* 2006; **24**: 4685-4691
42 **Strasly M**, Doronzo G, Cappello P, Valdembri D, Arese M, Mitola S, Moore P, Alessandri G, Giovarelli M, Bussolino F. CCL16 activates an angiogenic program in vascular endothelial cells. *Blood* 2004; **103**: 40-49
43 **Kee HJ**, Koh JT, Kim MY, Ahn KY, Kim JK, Bae CS, Park SS, Kim KK. Expression of brain-specific angiogenesis inhibitor 2 (BAI2) in normal and ischemic brain: involvement of BAI2 in the ischemia-induced brain angiogenesis. *J Cereb Blood Flow Metab* 2002; **22**: 1054-1067
44 **Barnes TM**, Jin Y, Horvitz HR, Ruvkun G, Hekimi S. The Caenorhabditis elegans behavioral gene unc-24 encodes a novel bipartite protein similar to both erythrocyte band 7.2 (stomatin) and nonspecific lipid transfer protein. *J Neurochem* 1996; **67**: 46-57
45 **Kim MJ**, Yoo YA, Kim HJ, Kang S, Kim YG, Kim JS, Yoo YD. Mitochondrial ribosomal protein L41 mediates serum starvation-induced cell-cycle arrest through an increase of p21(WAF1/CIP1). *Biochem Biophys Res Commun* 2005; **338**: 1179-1184
46 **Smits P**, Smeitink JA, van den Heuvel LP, Huynen MA, Ettema TJ. Reconstructing the evolution of the mitochondrial ribosomal proteome. *Nucleic Acids Res* 2007; **35**: 4686-4703
47 **Stone DL**, Slavotinek A, Bouffard GG, Banerjee-Basu S, Baxevanis AD, Barr M, Biesecker LG. Mutation of a gene encoding a putative chaperonin causes McKusick-Kaufman syndrome. *Nat Genet* 2000; **25**: 79-82
48 **Agashe VR**, Hartl FU. Roles of molecular chaperones in cytoplasmic protein folding. *Semin Cell Dev Biol* 2000; **11**: 15-25

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BASIC RESEARCH

Activation of canonical Wnt signaling pathway promotes proliferation and self-renewal of rat hepatic oval cell line WB-F344 *in vitro*

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Abstract

AIM: To investigate the effect of activation of canonical Wnt signaling pathway on the proliferation and differentiation of hepatic oval cells *in vitro*.

METHODS: WB-F344 cells were treated with recombinant Wnt3a (20, 40, 80, 160, 200 ng/mL) in serum-free medium for 24 h. Cell proliferation was measured by Brdu incorporation analysis; untreated WB-F344 cells were taken as controls. After treatment with Wnt3a (160 ng/mL) for 24 h, subcellular localization and protein expression of β-catenin in WB-F344 cells treated and untreated with Wnt3a were examined by immunofluorescence staining and Western blot analysis. *CyclinD1* mRNA expression was determined by semi-quantitative reverse-transcript polymerase chain reaction (RT-PCR). The mRNA levels of some phenotypic markers (*AFP*, *CK-19*, *ALB*) and two hepatic nuclear factors (*HNF-4*, *HNF-6*) were measured by RT-PCR. Expressions of CK-19 and AFP protein were detected by Western blot analysis.

RESULTS: Wnt3a promoted proliferation of WB-F344 cells. Stimulation of WB-F344 cells with recombinant Wnt3a resulted in accumulation of the transcriptional activator β-catenin, together with its translocation into the nuclei, and up-regulated typical Wnt target gene *CyclinD1*. After 3 d of Wnt3a treatment in the absence of serum, WB-F344 cells retained their bipotential to express several specific phenotypic markers of hepatocytes and cholangiocytes, such as AFP and CK-19, following activation of the canonical Wnt signaling pathway.

CONCLUSION: The canonical Wnt signaling pathway promotes proliferation and self-renewal of rat hepatic oval cells.

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Key words: Canonical Wnt signaling pathway; Oval cells; Cell proliferation; Self-renewal of cells

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INTRODUCTION

The liver is an organ with a remarkable regenerative capacity, provided by preexisting hepatocytes and cholangiocytes under normal conditions[1]. However, viral infection, alcohol, some drugs and self-immunity may cause liver inflammation and fibrosis. When the regenerative ability of hepatocytes to divide and replace the damaged tissue is compromised, oval cells are activated, and then may give rise to both hepatocytes and biliary epithelial cells[2,3]. Oval cell activation and proliferation serve as a source of cell replenishment and tissue repair as they differentiate into functional mature hepatocytes, thus aiding in the process of liver regeneration[4,5]. Therefore, oval cells are also designated as facultative hepatic stem cells because they proliferate only in response to chronic regenerative stimuli and have bipotential differentiation capabilities for both hepatocytes and cholangiocytes[6,7]. Although oval cells are usually found in normal human fetal livers, they are also observed in numerous hepatic pathologies[8,9]. Oval cell activation in malignancies such as hepatocellular carcinoma (HCC) and cholangiocarcinoma is of special relevance in light of the theories supporting the existence of cancer stem cells, which may serve as a tumor source, or as a mechanism

of cancer recurrence or metastasis[10,11]. However, little is known about the signaling mechanisms involved in oval cell proliferation and differentiation.

The canonical Wnt signaling pathway is highly conserved throughout animal development, during which it exerts pleiotropic effects on cell proliferation, differentiation, and polarity or migration[12]. The Wnt signaling pathway, identified recently, critically regulates various postnatal stem cell compartments, including the hematopoietic, skin, and enteric systems. In this respect, it has been demonstrated that hematopoietic stem cells maintain an undifferentiated self-renewing state through constitutive activation of the canonical Wnt signaling pathway with Wnt3a, a prominent member of the Wnt family[13,14]. In the liver, there is accumulating evidence that Wnt/β-catenin signaling plays a central role in various aspects of hepatic biology, including liver development, regeneration, growth, and oncogenesis. During liver development, β-catenin critically regulates hepatic progenitor cell proliferation, and over-expression or inhibition of β-catenin either increases or decreases the overall liver size, respectively[15-19]. Studies of pathological specimens and rodent models of liver diseases have demonstrated aberrations in the Wnt/β-catenin signaling pathway in conditions ranging from hepatitis to HCC[20]. However, no studies have definitively addressed the role of canonical Wnt signaling in proliferation and differentiation of hepatic oval cells. Therefore, we hypothesize that Wnt signaling can regulate the proliferation and differentiation of oval cells.

WB-F344, a rat hepatic stem-like epithelial cell line, isolated from the liver of an adult male Fischer-344 rat, can express a phenotypic repertory of both hepatocytes and bile duct epithelial cells compared with those of normal hepatocytes, biliary epithelial (ductular) and "oval" cells isolated from liver treated with chemical carcinogens. The phenotypic properties of cultured liver epithelial cell line most resemble those of the "oval" cells. Thus, it is considered to be an *in vitro* model of bipotent oval cells as it shares their phenotype[21,22]. After transplantation into livers of adult syngeneic German-strain Fischer-344 rats that are deficient in bile canalicular enzyme dipeptidyl peptidase Ⅳ (DPP-Ⅳ), WB-F344 cells integrate into hepatic plates and differentiate into mature hepatocytes[23]. Moreover, when treated with sodium butyrate and cultured on Matrigel, WB-F344 cells can differentiate along the biliary phenotype *in vitro*[24]. In the present study, we provided direct evidence for the activation of canonical Wnt signal transduction in WB-F344 cells in response to Wnt ligands, and showed that activation of canonical Wnt signaling regulates the proliferation and bipotential of WB-F344 cells. A better understanding of its role in hepatic stem cell proliferation and differentiation can lead to the successful manipulation of liver biology for therapeutic purposes.

MATERIALS AND METHODS

Cell line culture

The rat hepatic oval cell line (WB-F344) was obtained from Academy of Military Medical Sciences. The cells were cultured in Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Gibco BRL, USA), 10 mL of 200 mmol/L L-glutamine and 0.5 mL of penicillin-streptomycin mixture. All cultures were maintained at 37℃ in 50 mL/L CO_2.

Proliferation test (Brdu incorporation assays)

Cell proliferation was detected by Brdu incorporation assays. A total of 6000 WB-F344 cells were seeded into 96-well culture plates. Cells were brought to 90% confluence and then fasted overnight in serum-free media before addition of Brdu to a final concentration of 10 μmol/L. Two hours later, the cells were stimulated with escalating doses of Wnt3a (R&D, Systems, Inc) (20, 40, 80, 160, and 200 ng/mL) for 24 h, washed free of BrdU and harvested. The cells were fixed with 4% paraformaldehyde for 30 min, blocked with 1% BSA in phosphate-buffered saline containing 0.2% Triton X-100 for 30 min, incubated with a HRP-Brdu antibody (1:200) for 2 h at 37℃, and washed. A TMB substrate solution was added to the wells and color developed in proportion to the amount of Brdu bound. The stop solution changed the color from blue to yellow, and intensity of the color was measured at 450 nm with a spectrophotometer.

Immunofluorescence staining

For subcellular localization of β-catenin by immunocytochemistry, WB-F344 cells were plated onto chamber slides resulting in 90% confluence, and then fasted overnight in serum-free media before the addition of 160 ng/mL Wnt3a (R&D, Systems, Inc). The cells were fixed with 4% paraformaldehyde for 30 min at 37℃. Permeabilization of the cells was achieved after incubation for 30 min at 37℃ with PBS containing 0.2% Triton X-100. To minimize nonspecific binding of the antibody, blocking was carried out with a buffer containing 1% bovine serum albumin for 1 h. β-catenin antibody (R&D, Systems, Inc) was applied at a 1:25 dilution for 90 min at 37℃. As a negative control, PBS was used instead of the primary antibody to exclude the unspecific binding of the secondary antibody. No fluorescent labeling was observed in the negative control. After repeated washing with PBS, the cells were incubated with a goat-anti-mouse antibody labeled with fluorescein isothiocyanate (1:10) for an additional 30 min. Finally, cell nuclei were counterstained with Hoechst 33258. Images were obtained using a confocal laser scanning microscope.

RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR)

WB-F344 cells were brought to 90% confluence and then fasted overnight in serum-free media. After stimulation with or without Wnt3a, cells were cultivated for 1 and 3 d, respectively. Total RNA was extracted from WB-F344 cells treated with Wnt3a with Trizol according to the manufacture's instructions. RNA (1 μg) was reverse

Table 1 Primer sequences used for RT-PCR

Gene	Primer sequences	Annealing temp (℃)	Cycles	Ampliconsize (bp)
CyclinD1	5′-ATTGAAGCCCTTCTGGAGTCAAGCC-3′ 5′-TCTATTTTTGTAGCACCCCCCCGTC-3′	56	26	420
AFP	5′-GCTGAACCCAGAGTACTG AC-3′ 5′-GACACGTCGTAGATGAACGTG-3′	60	34	472
ALB	5′-AAGGCACCCCGATTACTCCG-3′ 5′-TGCGAAGTCACCCATCACCG-3′	56	36	608
CK-19	5′-ATGACTTCCTATAGCTATCG-3′ 5′-CACCTCCAGCTCGCCATTAG-3′	64	34	340
HNF-6	5′-GACAAATGGCAGGACGAGGG-3′ 5′-AGCGTACTGGTTTAGGTGCC-3′	62	36	781
HNF-4α	5′-CTTCCTTCTTCATGCCAG-3′ 5′-ACACGTCCCCATCTGAAG-3′	62	34	269
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	56	24	469

transcribed. For semi-quantitative PCR, the number of cycles corresponded to the mid-logarithmic phase. Primers were designed using GenBank sequences (Table 1). PCR amplification was performed using PCR Master Mix (Taqman) according to the manufacturer's instructions. PCR products were analyzed by electrophoresis on a 2% agarose gel.

Western blot analysis

After stimulation with Wnt3a, cells were cultivated for 1 and 3 d, respectively. Trypsinized protein was isolated using a lysis buffer (50 mmol/L Na_2HPO_4, 50 mmol/L NaH_2PO_4, 0.2 mol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, pH 6.0). After incubation on ice for 30 min, samples were centrifuged at 13000 r/min for 20 min at 4℃. Then, a 2 × dithiothreitol (DTT) loading buffer containing 0.4 mol/L Tris (pH 6.8), 4% SDS, 20% glycerol, and 10% DTT was added to the sample supernatants, and incubated for 5 min at 95℃. Following electrophoretic separation by 10% SDS-polyacrylamide gel electrophoresis, proteins were electroblotted onto nitrocellulose membranes. The membranes were blocked in a NET buffer (150 mmol/L NaCl; 5 mmol/L EDTA, pH 8.0; 50 mmol/L Tris/HCl, pH 7.5; 0.05% Triton X-100) containing 2.5% gelatin (Merck) for 1 h at room temperature. Polyclonal antibodies against β-catenin (R&D, Systems, Inc), cytokeratin-19 (CK-19) (Santa Cruz Biotechnology Inc), AFP (Santa Cruz Biotechnology Inc) were used at a dilution of 1:25 (β-catenin), 1:200 (AFP, CK-19) and 1:400 (β-actin), respectively, and incubated for 1 h at room temperature. Thereafter, the membranes were washed in a NET buffer, and further incubated with a peroxidase-conjugated antibody at a dilution of 1:20000. Antibody binding was visualized by DAB. The bands were semi-quantitatively evaluated by densitometric analysis. Protein expression levels of β-catenin were thereby normalized to those of the housekeeping gene β-actin.

Differentiation assay

WB-F344 cells grown in a Wnt3a-containing medium in the absence of serum were cultured with or without Wnt3a for 3 d. The medium was changed every day. After 3 d of coculture, total Mrna and protein were extracted and analyzed for expression of CK-19, ALB, AFP by RT-PCR and Western blot analysis.

Statistical analysis

All results were expressed as mean ± SD. Measurement data were analyzed using one-way analysis of variances (ANOVA, SPSS 11.5). $P < 0.05$ was considered statistically significant.

RESULTS

Activation of the Wnt pathway, accumulation of β-catenin, its translocation into nuclei, and transcriptional activation of Wnt target genes

To investigate whether treatment of WB-F344 cells with recombinant Wnt3a (160 ng/mL) influences β-catenin in WB-F344 cells at the protein level, we monitored the expression of β-catenin by semi-quantitative Western blot analysis. Accumulation of β-catenin protein (1.2-fold) was observed 1 d after addition of 160 ng/mL Wnt3a (Figure 1A). To confirm the data obtained by Western blot analysis and prove whether Wnt3a stimulation has any influence on the subcellular localization of β-catenin, immunocytochemistry was performed. Clear nuclear staining for β-catenin was observed after treatment with 160 ng/mL Wnt3a for 1 d, suggesting that Wnt3a stimulation is responsible for the accumulation of β-catenin and its translocation from cytoplasm into nuclei (Figure 1B). To evaluate whether Wnt3a-mediated accumulation of β-catenin would result in the activation of typical Wnt target genes, the mRNA expression levels of *CyclinD1* was semi-quantified by RT-PCR 1 d after Wnt3a stimulation (160 ng/mL). A significant induction in CyclinD1 was observed (Figure 2). These findings indicate that activation of the Wnt pathway in WB-F344 cells can result in accumulation of β-catenin, its translocation into nuclei, and enhanced expression of Wnt target genes.

Effect of Wnt3a on WB-F344 cell proliferation

To determine whether WB-F344 cells respond to Wnt ligand stimulation with activation of the canonical

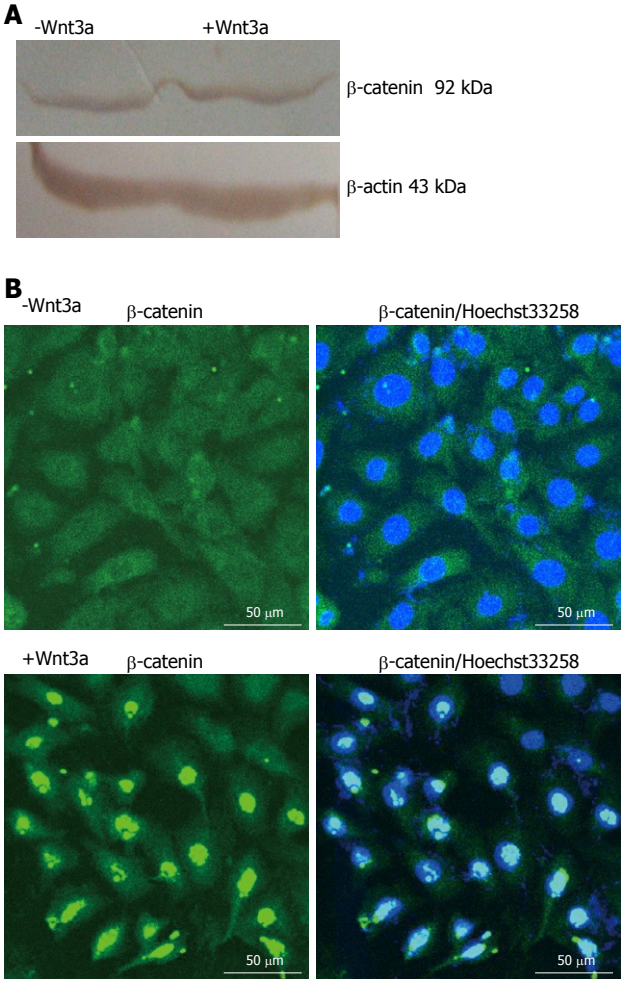

Figure 1 Effects of Wnt3a on β-catenin expression, its subcellular localization, and induction of typical Wnt target genes. A: Stimulation of WB-F344 cells with 160 ng/mL Wnt3a for 1 d revealing a slight increase in β-catenin protein level as shown by Western blot analysis and densitometric analysis; B: Immunocytochemistry analysis of β-catenin exhibiting perinuclear staining for β-catenin in unstimulated WB-F344 cells (upper panels), whereas addition of 160 ng/mL Wnt3a for 1 d (lower panels) showing clear nuclear staining for β-catenin. Immunofluorescence was performed using a polyclonal antibody against β-catenin (left panels). In addition, nuclei of WB-F344 cells were stained with Hoechst33258 (right panels). Scale bars: 50 μm.

Wnt pathway, WB-F344cells in culture were stimulated by purified Wnt3a protein. Brdu incorporation assays were performed to determine whether Wnt stimulates the proliferation of WB-F344 cells. After serum starvation, Wnt3a stimulation of WB-F344 cells resulted in significantly more uptake of Brdu compared with controls in the absence of serum. The proliferation of WB-F344 cells increased after the addition of Wnt3a, and reached its peak at 160 ng/mL Wnt3a (Figure 3). To evaluate whether the expression of Wnt target gene CyclinD1 is directly correlated with the proliferation of WB-F344 cells, the mRNA expression level of *CyclinD1* was determined by semi-quantitative RT-PCR 1 d after Wnt3a stimulation (160 ng/mL). A significant up-regulation of CyclinD1 expression was observed, suggesting that CyclinD1 has a major impact on the proliferation of WB-F344cells. These findings indicate that proliferation of WB-F344 cells is stimulated by

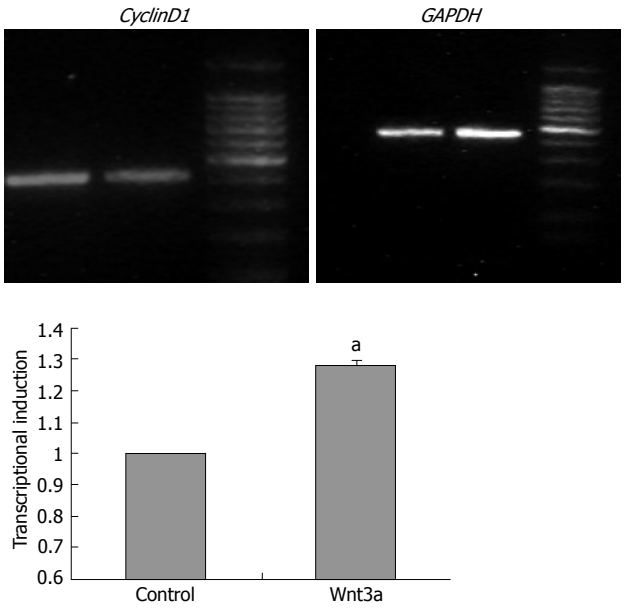

Figure 2 The mRNA expression levels of the known Wnt target genes *CyclinD1* was semi-quantified at day 1 during stimulation with Wnt3a (160 ng/mL) and normalized to the expression levels in untreated WB-F344 cells (set as 100%). The mRNA expression level of *CyclinD1*, one of the known Wnt target genes in WB-F344 cells after stimulation by Wnt3a (160 ng/mL) for 1 d was semi-quantified by RT-PCR and the results was normalized to the expression levels in untreated WB-F344 cells (set as 1). *CyclinD1* was upregulated under stimulating conditions on day 1. For quantification, *CyclinD1* mRNA was scanned by densitometric analysis and normalized to GAPDH. Data are presented as mean ± SD of triplicate experiments. $^{a}P < 0.05$ in comparison with un-treated cells.

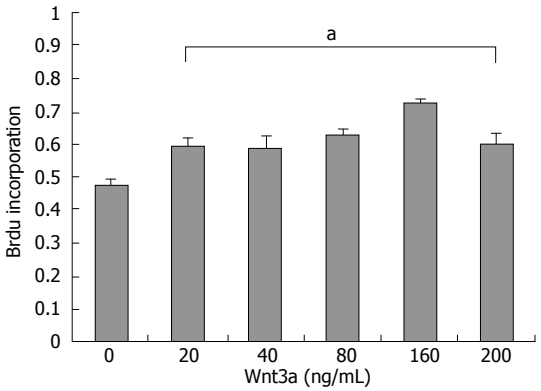

Figure 3 Proliferation of WB-F344 cells upon treatment with Wnt3a measured by Brdu incorporation assay. The proliferation of WB-F344 cells was significantly enhanced by stimulation with recombinant Wnt3a for 1 d Data are presented as mean ± SD. $^{a}P < 0.05$ *vs* untreated WB-F344 cells.

the canonical Wnt signaling pathway, which may be the mechanism underlying the up-regulated CyclinD1 expression.

Activation of the canonical Wnt signaling pathway promoted self-renewal of WB-F344 cells

Although Wnt3a can activate the canonical Wnt signaling pathway, it remains unclear whether activation of the canonical Wnt signaling pathway results in differentiation of WB-F344 cells. If Wnt3a can promote cell differentiation, WB-F344 cells treated with Wnt3a

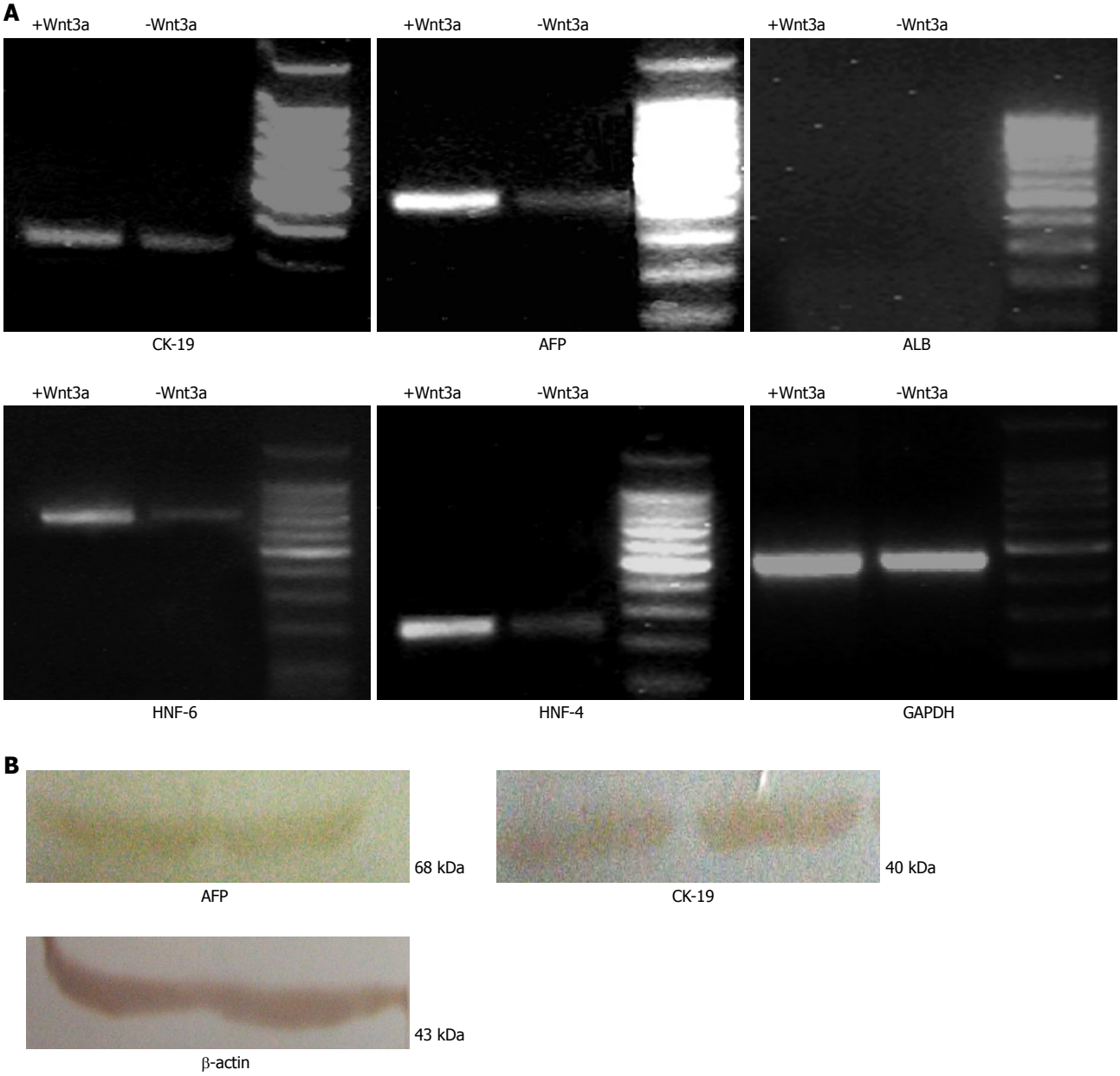

Figure 4 RT-PCR and Western blot analysis of differentiated WB-F344 cells treated or untreated with Wnt3a. A: Wnt3a-treated WB-F344 cells expressing two phenotypic markers (CK-19 and AFP) and two hepatic nuclear factors (HNF4α and HNF-6) at mRNA level with untreated WB-F344 cells as controls; B: Wnt3a-treated cells expressing two phenotypic markers (CK-19 and AFP) at protein level.

should express specific markers after activation of the canonical Wnt signaling pathway. To test this hypothesis, WB-F344 cells were cultured in the presence or absence of Wnt3a-containing medium for 3 d under serum-free conditions. RT-PCR and Western blot were performed for the expression of markers in WB-F344 cells treated or untreated with Wnt3a. Two cell–specific markers, AFP and CK-19 and two hepatocyte nuclear factors, HNF-4α, HNF-6 were expressed in both Wnt3a-treated and untreated WB-F344 cells. In respect of these markers, Wnt3a-treated WB-F344 cells did not show any marked deviation compared with untreated WB-F344 cells (Figure 4). The transcription factors, known to play a key role in differentiation of hepatocytes and cholangiocytes, were expressed in both Wnt3a-treated and untreated WB-F344 cells. These findings suggest that self-renewal of WB-F344 cells is stimulated by the canonical Wnt signal transduction.

DISCUSSION

Oval cell activation occurs in the majority of chronic liver diseases and increases with the severity of the disease. In moderate and severe degrees of inflammation, intermediate hepatocytes occur, having a phenotype intermediate between progenitor cells/ductular cells and mature hepatocytes. The number of these intermediate hepatocytes gradually increases with higher degrees of inflammation and necrosis in necrotizing hepatitis or with more advanced stages of (non) alcoholic steatohepatitis[25-27]. However, little is known about the signaling pathways involved in controlling hepatic oval

cell proliferation and differentiation. The present study demonstrated that Wnt/β-catenin signaling plays an important role in the proliferation and renewal of oval cells *in vitro*.

Although multiple Wnts are now known to play a role in the proliferation and renewal of stem cells via the canonical or non-canonical pathway, Wnt-1 and -3 have a similar mode of action leading to β-catenin accumulation[28,29]. Taking advantage of this redundancy and availability of biologically active Wnt3a, we tested the impact of Wnt enrichment on WB-F344 cells, at beginning of cultures. Thus, this study directly addressed the effect of Wnt3a on the proliferation and differentiation of WB-F344 cells.

We performed this study in serum-free conditions to minimize other confounding factors. Our initial results demonstrate that Wnt proteins, showing high transforming activity in WB-F344 cells, could activate the canonical Wnt signaling pathway. To activate the canonical Wnt signaling pathway, WB-F344 cells were stimulated with recombinant Wnt3a (160 ng/mL) for 24 h. β-catenin, upon treatment with Wnt3a, translocated into the nuclei of WB-F344 cells where it promotes TCF/LEF-dependent transcription, with a slightly increased accumulation of β-catenin at protein level, suggesting that degradation of β-catenin diminished by the destructive complex is the underlying mechanism[30]. Nuclear accumulation of this protein indicates that activity of the Wnt signal pathway is increased[31]. Furthermore, Wnt3a-mediated nuclear translocation of β-catenin results in the up-regulation of CyclinD1, suggesting that the canonical Wnt target gene, originally identified in colon carcinoma cells[32], can be induced in WB-F344 cells. Accumulated β-catenin is translocated into the nuclei of WB-F344 cells where it binds to the transcription factors, T cell factor (Tcf)/lymphoid enhancer factor (Lef), thereby stimulates expression of target genes[33]. Therefore, activation of the Wnt signaling pathway in WB-F344 cells results in accumulation of β-catenin, its translocation into nuclei, and enhanced expression of Wnt target genes.

We also investigated the effect of recombinant Wnt3a on proliferation of WB-F344 cells in serum-free environment, showing that recombinant Wnt3a could stimulate the proliferation of WB-F344 cells. This observation is consistent with the reported findings that β-catenin plays a central role in regulating the proliferation and regeneration of hepatocytes[34-36]. To investigate whether the expression of CyclinD1 is directly correlated with the proliferation of WB-F344 cells, mRNA expression in *CyclinD1*, which plays a fundamental role in cell cycle at the G1-S phase transition, was semi-quantified by RT-PCR 1 d after Wnt3a stimulation (160 ng/mL). A significant mRNA expression was induced in *CyclinD1*. This effect was even more pronounced under Wnt stimulatory conditions. Moreover, it was reported that proliferation of WB-F344 cells is severely impaired in the absence of β-catenin, secondary to the decreased expression of downstream targets such as CyclinD1, which are critical in proliferation[37]. It has also been shown that WB-F344 cells proliferate in postnatal liver development, *ex vivo* embryonic liver development, and in facultative liver stem and oval cells[38-41]. These findings further support the idea that the Wnt signaling pathway is largely involved in controlling the proliferation of WB-F344 cells.

Oval cells are known to be tumorigenic[42]. The Wnt/β-catenin signaling pathway plays an important role in the pathogenesis of hepatic adenoma and its progression to HCC[43] and also in self-renewal of stem cells in several tissue types[44]. β-catenin is a mediator of cancer stem cells[43] and plays an important role in early liver development at the stage of ongoing hepatic progenitor proliferation[39,45]. In the present study, the hepatobiliary phenotype was confirmed by RT-PCR and Western blot analysis. Wnt3a-treated and untreated WB-F344 cells cells were positive for AFP and CK-19 but negative for ALB, one of the final maturation phase markers, indicating that they were immature cells. The bipotential phenotype of WB-F344 cells was observed in oval cells, presumed precursors of hepatocytes and biliary cells. HNF4α and HNF6 are known to play a key role in differentiation of hepatocytes and cholangiocytes[46-50]. Our results also demonstrate that WB-F344 cells treated or untreated with Wnt3a expressed HNF4α and HNF6. Studies in knockout mice showed that hepatocyte nuclear factor HNF4α regulates transcription of genes essential for hepatocytic cell lineage[51,52], whereas HNF6 is involved in the development of gallbladder and bile ducts[53,54]. HNF4α and HNF6 are expressed in fetal hepatoblasts and show different expression patterns in adult liver. HNF4α is exclusively expressed in fetal and adult hepatocytes. No HNF4α expression has been observed in fetal ductal plate or in bile duct epithelium or in normal adult liver. HNF6 is also expressed in fetal and adult hepatocytes and in fetal BEC. HNF6 is completely lost from the BEC with mature biliary phenotype[55]. It was reported more recently that the Wnt/β-catenin signaling pathway plays a critical role in oval cell activation[40]. HNF4α and HNF6 are expressed in oval cells of the liver activated by 2-AAF/PH[56]. Our results show that activation of the Wnt/β-catenin signaling pathway could promote self-renewal of WB-F344 cells, indicating that some molecules involved in the canonical Wnt pathway may be therapeutic targets. β-catenin targeting might be of essence in preneoplastic and early or late HCC as a chemopreventive or chemotherapeutic measure.

In conclusion, the canonical Wnt signaling pathway plays a key role in regulating the proliferation and self-renewal of hepatic oval cells. The detailed mechanism of Wnt3a underlying the differentiation of WB-F344 cells and whether β-catenin can directly control the expression of HNF4α and HNF6 need further study.

COMMENTS

Background

Oval cell activation occurs in the majority of chronic liver diseases and increases with the severity of the disease. However, little is known about the signaling pathways involved in controlling hepatic oval cell proliferation

and differentiation. The canonical Wnt signaling pathway is highly conserved throughout animal development during which it exerts pleiotropic effects on cell proliferation, differentiation, and polarity or migration. In the present study, the authors aimed to investigate the effect of activation of canonical Wnt signaling pathway on the proliferation and differentiation of heaptic oval cells *in vitro*.

Research frontiers

There is accumulating evidence that Wnt/β-catenin signaling in the liver plays a central role in various aspects of hepatic biology, including liver development, regeneration, growth, and oncogenesis. Studies of pathological specimens and rodent models of liver diseases have demonstrated aberrations in the Wnt/β-catenin signaling pathway in conditions ranging from hepatitis to hepatocellular carcinoma (HCC). However, no studies have definitively addressed the role of canonical Wnt signaling in proliferation and differentiation of hepatic oval cells.

Innovations and breakthroughs

This is the first study addressing the role of canonical Wnt signaling in proliferation and differentiation of hepatic oval cells. The canonical Wnt signaling pathway plays a key role in regulating the proliferation and self-renewal of hepatic oval cells.

Applications

Oval cells are known to be tumorigenic. The Wnt/β-catenin signaling pathway plays an important role in the pathogenesis of hepatic adenoma and its progression to HCC, and also in self-renewal of stem cells in several tissue types. β-catenin is a mediator of cancer stem cells. Activation of the Wnt/β-catenin signaling pathway could promote self-renewal of oval cells, indicating that some molecules involved in the canonical Wnt pathway may be therapeutic targets. β-catenin targeting might be of essence in preneoplastic and early or late HCC as a chemopreventive or chemotherapeutic measure.

Terminology

The Wnt signaling pathway, identified recently, critically regulates various postnatal stem cell compartments, including the hematopoietic, skin, and enteric systems. In this respect, it has been demonstrated that hematopoietic stem cells maintain an undifferentiated self-renewing state through constitutive activation of the canonical Wnt signaling pathway with Wnt3a, a prominent member of the Wnt family.

Peer review

In this study, the authors demonstrated the role of canonical Wnt signaling in proliferation and differentiation of hepatic oval cells. This work adds significant information that activation of the Wnt/β-catenin signaling pathway could promote proliferation and self-renewal of oval cells, indicating that some molecules involved in the canonical Wnt pathway may be therapeutic targets.

REFERENCES

1 **Taub R**. Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol* 2004; **5**: 836-847
2 **Lowes KN**, Croager EJ, Olynyk JK, Abraham LJ, Yeoh GC. Oval cell-mediated liver regeneration: Role of cytokines and growth factors. *J Gastroenterol Hepatol* 2003; **18**: 4-12
3 **Walkup MH**, Gerber DA. Hepatic stem cells: in search of. *Stem Cells* 2006; **24**: 1833-1840
4 **Fausto N**, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech Dev* 2003; **120**: 117-130
5 **Petersen BE**. Hepatic "stem" cells: coming full circle. *Blood Cells Mol Dis* 2001; **27**: 590-600
6 **Fausto N**. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* 2004; **39**: 1477-1487
7 **Newsome PN**, Hussain MA, Theise ND. Hepatic oval cells: helping redefine a paradigm in stem cell biology. *Curr Top Dev Biol* 2004; **61**: 1-28
8 **Masson NM**, Currie IS, Terrace JD, Garden OJ, Parks RW, Ross JA. Hepatic progenitor cells in human fetal liver express the oval cell marker Thy-1. *Am J Physiol Gastrointest Liver Physiol* 2006; **291**: G45-G54
9 **Roskams T**, De Vos R, Van Eyken P, Myazaki H, Van Damme B, Desmet V. Hepatic OV-6 expression in human liver disease and rat experiments: evidence for hepatic progenitor cells in man. *J Hepatol* 1998; **29**: 455-463
10 **Knight B**, Matthews VB, Olynyk JK, Yeoh GC. Jekyll and Hyde: evolving perspectives on the function and potential of the adult liver progenitor (oval) cell. *Bioessays* 2005; **27**: 1192-1202
11 **Lee JS**, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 2006; **12**: 410-416
12 **Logan CY**, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004; **20**: 781-810
13 **Lowry WE**, Blanpain C, Nowak JA, Guasch G, Lewis L, Fuchs E. Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. *Genes Dev* 2005; **19**: 1596-1611
14 **Wodarz A**, Nusse R. Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol* 1998; **14**: 59-88
15 **Devereux TR**, Stern MC, Flake GP, Yu MC, Zhang ZQ, London SJ, Taylor JA. CTNNB1 mutations and beta-catenin protein accumulation in human hepatocellular carcinomas associated with high exposure to aflatoxin B1. *Mol Carcinog* 2001; **31**: 68-73
16 **Micsenyi A**, Tan X, Sneddon T, Luo JH, Michalopoulos GK, Monga SP. Beta-catenin is temporally regulated during normal liver development. *Gastroenterology* 2004; **126**: 1134-1146
17 **Monga SP**, Monga HK, Tan X, Mule K, Pediaditakis P, Michalopoulos GK. Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification. *Gastroenterology* 2003; **124**: 202-216
18 **Monga SP**, Pediaditakis P, Mule K, Stolz DB, Michalopoulos GK. Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration. *Hepatology* 2001; **33**: 1098-1109
19 **Taniguchi K**, Roberts LR, Aderca IN, Dong X, Qian C, Murphy LM, Nagorney DM, Burgart LJ, Roche PC, Smith DI, Ross JA, Liu W. Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. *Oncogene* 2002; **21**: 4863-4871
20 **Thompson MD**, Monga SP. WNT/beta-catenin signaling in liver health and disease. *Hepatology* 2007; **45**: 1298-1305
21 **Thorgeirsson SS**, Grisham JW. Overview of recent experimental studies on liver stem cells. *Semin Liver Dis* 2003; **23**: 303-312
22 **Tsao MS**, Smith JD, Nelson KG, Grisham JW. A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells. *Exp Cell Res* 1984; **154**: 38-52
23 **Coleman WB**, McCullough KD, Esch GL, Faris RA, Hixson DC, Smith GJ, Grisham JW. Evaluation of the differentiation potential of WB-F344 rat liver epithelial stem-like cells in vivo. Differentiation to hepatocytes after transplantation into dipeptidylpeptidase-IV-deficient rat liver. *Am J Pathol* 1997; **151**: 353-359
24 **Couchie D**, Holic N, Chobert MN, Corlu A, Laperche Y. In vitro differentiation of WB-F344 rat liver epithelial cells into the biliary lineage. *Differentiation* 2002; **69**: 209-215
25 **Roskams T**, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, Achten R, Verslype C, Diehl AM. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *Am J Pathol* 2003; **163**: 1301-1311
26 **Lowes KN**, Brennan BA, Yeoh GC, Olynyk JK. Oval cell numbers in human chronic liver diseases are directly related to disease severity. *Am J Pathol* 1999; **154**: 537-541
27 **Libbrecht L**, Desmet V, Van Damme B, Roskams T. Deep intralobular extension of human hepatic 'progenitor cells' correlates with parenchymal inflammation in chronic viral hepatitis: can 'progenitor cells' migrate? *J Pathol* 2000; **192**: 373-378
28 **Kispert A**, Vainio S, McMahon AP. Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric

mesenchyme in the developing kidney. *Development* 1998; **125**: 4225-4234
29 **Ikeya M**, Lee SM, Johnson JE, McMahon AP, Takada S. Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* 1997; **389**: 966-970
30 **Nelson WJ**, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004; **303**: 1483-1487
31 **Kikuchi A**, Yamamoto H, Kishida S. Multiplicity of the interactions of Wnt proteins and their receptors. *Cell Signal* 2007; **19**: 659-671
32 **Tetsu O**, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999; **398**: 422-426
33 **Cadigan KM**, Nusse R. Wnt signaling: a common theme in animal development. *Genes Dev* 1997; **11**: 3286-3305
34 **Suksaweang S**, Lin CM, Jiang TX, Hughes MW, Widelitz RB, Chuong CM. Morphogenesis of chicken liver: identification of localized growth zones and the role of beta-catenin/Wnt in size regulation. *Dev Biol* 2004; **266**: 109-122
35 **Monga SP**, Pediaditakis P, Mule K, Stolz DB, Michalopoulos GK. Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration. *Hepatology* 2001; **33**: 1098-1109
36 **Tan X**, Behari J, Cieply B, Michalopoulos GK, Monga SP. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. *Gastroenterology* 2006; **131**: 1561-1572
37 **Shtutman M**, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci USA* 1999; **96**: 5522-5527
38 **Sekine S**, Gutierrez PJ, Lan BY, Feng S, Hebrok M. Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy. *Hepatology* 2007; **45**: 361-368
39 **Tan X**, Apte U, Micsenyi A, Kotsagrelos E, Luo JH, Ranganathan S, Monga DK, Bell A, Michalopoulos GK, Monga SP. Epidermal growth factor receptor: a novel target of the Wnt/beta-catenin pathway in liver. *Gastroenterology* 2005; **129**: 285-302
40 **Apte U**, Thompson MD, Cui S, Liu B, Cieply B, Monga SP. Wnt/beta-catenin signaling mediates oval cell response in rodents. *Hepatology* 2008; **47**: 288-295
41 **Hu M**, Kurobe M, Jeong YJ, Fuerer C, Ghole S, Nusse R, Sylvester KG. Wnt/beta-catenin signaling in murine hepatic transit amplifying progenitor cells. *Gastroenterology* 2007; **133**: 1579-1591
42 **Roskams T**. Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. *Oncogene* 2006; **25**: 3818-3822
43 **Zucman-Rossi J**, Jeannot E, Nhieu JT, Scoazec JY, Guettier C, Rebouissou S, Bacq Y, Leteurtre E, Paradis V, Michalak S, Wendum D, Chiche L, Fabre M, Mellottee L, Laurent C, Partensky C, Castaing D, Zafrani ES, Laurent-Puig P, Balabaud C, Bioulac-Sage P. Genotype-phenotype correlation in hepatocellular adenoma: new classification and relationship with HCC. *Hepatology* 2006; **43**: 515-524
44 **Moon RT**, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet* 2004; **5**: 691-701
45 **Hussain SZ**, Sneddon T, Tan X, Micsenyi A, Michalopoulos GK, Monga SP. Wnt impacts growth and differentiation in ex vivo liver development. *Exp Cell Res* 2004; **292**: 157-169
46 **Duncan SA**. Transcriptional regulation of liver development. *Dev Dyn* 2000; **219**: 131-142
47 **Lee CS**, Friedman JR, Fulmer JT, Kaestner KH. The initiation of liver development is dependent on Foxa transcription factors. *Nature* 2005; **435**: 944-947
48 **Zaret KS**. Liver specification and early morphogenesis. *Mech Dev* 2000; **92**: 83-88
49 **Zhou H**, Rogler LE, Teperman L, Morgan G, Rogler CE. Identification of hepatocytic and bile ductular cell lineages and candidate stem cells in bipolar ductular reactions in cirrhotic human liver. *Hepatology* 2007; **45**: 716-724
50 **Lemaigre FP**. Development of the biliary tract. *Mech Dev* 2003; **120**: 81-87
51 **Li J**, Ning G, Duncan SA. Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. *Genes Dev* 2000; **14**: 464-474
52 **Hayhurst GP**, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* 2001; **21**: 1393-1403
53 **Clotman F**, Lannoy VJ, Reber M, Cereghini S, Cassiman D, Jacquemin P, Roskams T, Rousseau GG, Lemaigre FP. The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* 2002; **129**: 1819-1828
54 **Yamasaki H**, Sada A, Iwata T, Niwa T, Tomizawa M, Xanthopoulos KG, Koike T, Shiojiri N. Suppression of C/EBPalpha expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased Hnf6 and Hnf1b expression. *Development* 2006; **133**: 4233-4243
55 **Limaye PB**, Alarcon G, Walls AL, Nalesnik MA, Michalopoulos GK, Demetris AJ, Ochoa ER. Expression of specific hepatocyte and cholangiocyte transcription factors in human liver disease and embryonic development. *Lab Invest* 2008; **88**: 865-872
56 **Shafritz DA**, Oertel M, Menthena A, Nierhoff D, Dabeva MD. Liver stem cells and prospects for liver reconstitution by transplanted cells. *Hepatology* 2006; **43**: S89-S98

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BASIC RESEARCH

Portal venous arterialization resulting in increased portal inflow and portal vein wall thickness in rats

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Abstract

AIM: To explore the influence of portal vein hemodynamic changes after portal venous arterialization (PVA) on peribiliary vascular plexus (PVP) morphological structure and hepatic pathology, and to establish a theoretical basis for the clinical application of PVA.

METHODS: Sprague-Dawley rats were randomly divided into control and PVA groups. After PVA, hemodynamic changes of the portal vein and morphological structure of hepatohilar PVP were observed using Doppler ultrasound, liver function tests, ink perfusion transparency management and three-dimensional reconstruction of computer microvisualization, and pathological examination was performed on tissue from the bile duct wall and the liver.

RESULTS: After PVA, the cross-sectional area and blood flow of the portal vein were increased, and the increase became more significant over time, in a certain range. If the measure to limit the flow in PVA was not adopted, the high blood flow would lead to dilatation of intrahepatic portal vein and its branches, increase in collagen and fiber degeneration in tunica intima. Except glutamic pyruvic transaminase (GPT), other liver function tests were normal.

CONCLUSION: Blood with a certain flow and oxygen content is important for filling the PVP and meeting the oxygen requirement of the bile duct wall. After PVA, It is the anatomic basis to maintain normal morphology of hepatohilar bile duct wall that the blood with high oxygen content and high flow in arterialized portal vein may fill PVP by collateral vessel reflux. A adequate measure to limit blood flow is necessary in PVA.

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Key words: Peribiliary vascular plexus; Portal venous arterialization; Liver transplantation; Bile duct neoplasms; Three-dimensional reconstruction; Hemodynamics

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INTRODUCTION

In the clinical practice of enlarged radical operation and liver transplantation, revascularization of the liver is often needed. For this reason, portal venous arterialization (PVA) has been extensively studied and carried out in clinical practice[1-5]. PVA has been used in more than 10 cases during liver transplantation and some transplant centers have achieved good outcomes[6,7]. Blood flow and oxygen content in the peribiliary vascular plexus (PVP) play an important role in ensuring the normal physiological function of bile-duct epithelial cells[8]. After PVA, the influence of portal vein hemodynamic changes on hepatohilar PVP is important for the incidence of bile duct complications and is directly associated with clinical application of PVA.

Therefore, the purpose of our study was to explore the influence of portal vein hemodynamic changes after PVA on PVP morphological structure, and liver function and pathology, and to establish a theoretical basis for the clinical application of PVA.

MATERIALS AND METHODS

All study methods were approved by the ethics committee of Xiamen First Hospital.

Materials

Healthy, adult, male, clean Sprague-Dawley rats weighing 250-300 g were provided from the Laboratory Animal Center, General Hospital of PLA, Beijing. All rats were maintained under specific pathogen-free conditions. The CK40 inverted phase contrast microscope and BX51TF system microscope were provided by Olympus. Perfusion ink was from China.

Establishment of PVA model

Rats were randomly divided into control and PVA groups (each group with 17 rats). Rats were fasted 12 h prior to operation, then anesthetized with 3% chloral hydrate (1 mL/100 g). Models were made under BX51TF system microscope. In the control group, after opening the abdomen, the porta hepatis was isolated, and then the abdomen was closed. In the PVA group, after opening the abdomen, the inferior vena cava, portal vein and common hepatic artery were isolated, and then double ligations were performed with 0 silk thread on the common hepatic artery and it was cut in the middle, the proximal broken end with silk thread and small round pin for further use. The portal vein and the celiac artery were blocked with vascular clamps, the stump of the common hepatic artery was connected to the portal vein, and then the celiac artery clamp was removed, and tremors from the portal vein were felt (Figure 1A). The portal vein clamp was removed and the blood flow was temporarily restored (cross-clamp for 5-7 min). Subsequently, the side-to-side anastomosis (cross-clamp for 10-12 min) of the portal vein and the inferior vena cava was performed, with the portal vein diameter the same as the anastomotic stoma diameter (Figure 1B). The anastomotic stoma was confirmed to be unobstructed. Finally, ligation was performed between the anastomotic stoma of the hepatic artery and the portal vein, and the right gastric vein branch of the portal vein. Therefore, only arterial blood flowed into the intrahepatic portal vein. All rats in the PVA and control groups were observed for 6 mo.

Hemodynamic changes of arterialized portal vein

Color Doppler ultrasound was used to monitor the cross-sectional area and blood flow of the portal vein at the mid-point of the main portal vein in seven rats from the two groups, at 1 and 6 mo after PVA, which was carried out in the Department of Ultrasound, PLA General Hospital. The included angle between the ultrasound probe and the main portal vein was < 60° and the frequency of the probe was 5-13 MHz.

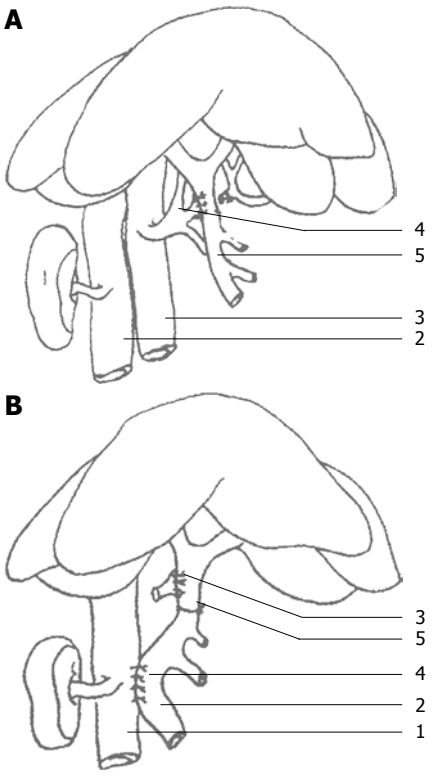

Figure 1 Animal PVA model establishment. A: Side-to-side anastomotic stoma of the hepatic artery and the portal vein. (1) kidney; (2) inferior vena cava; (3) abdominal aorta; (4) hepatic artery; (5) portal vein. B: Side-to-side anastomotic stoma of the portal vein and the venae cavae. (1) inferior vena cava; (2) portal vein; (3) arteriovenous anastomotic stoma; (4) anastomotic stoma of the portal vein and the venae cavae; (5) ligation of the portal vein.

Liver functions in rats 6 mo after PVA

Two milliliters of blood were taken from the abdominal aorta in randomly selected 8 rats from the two groups respectively for determining liver functions. Then ink perfusion was performed in the 8 rats respectively (as follow).

Capillary morphology in hepatohilar bile duct wall after PVA

Ink perfusion: Six months after PVA, the portal vein and abdominal aorta were intubated in 10 rats (remained 2 rats and 8 rats above) in the control group, and only the abdominal aorta was intubated in 10 rats (remained 2 rats and 8 rats above) from the PVA group. In the 20 rats, the thoracic aorta was ligated, then a cut was made on the vena cava of the midriff, 1% heparin physiological saline was infused till clear efflux from thoracic cavity. Ink had not been equably and slowly infused under pressure of 16.0 kPa into the abdominal aorta until ink exuded from inferior vena cava of thoracic cavity, The perfusion state of the bile duct wall capillaries was closely observed with a dissecting microscope. The liver and extrahepatic bile ducts were removed, and fixed with 10% formaldehyde solution over 72 h.

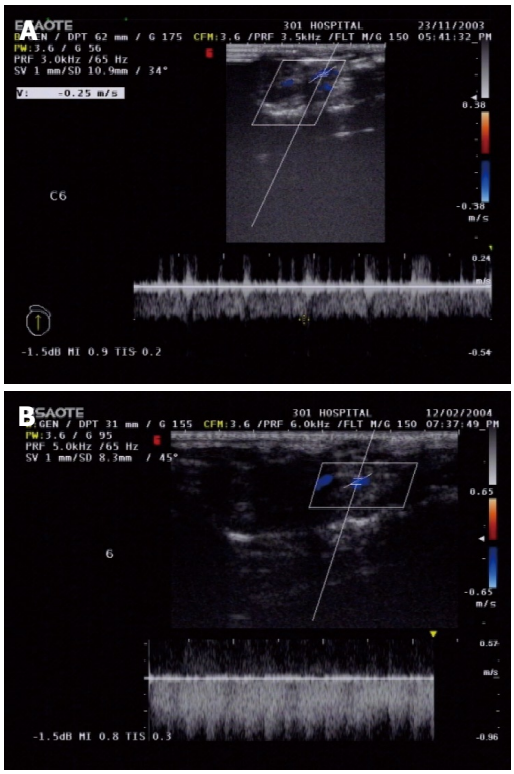

Figure 2 Frequency spectrums in the portal vein. A: Venous frequency spectrums in the portal vein of control group; B: Arterial frequency spectrums in the portal vein 6 mo after PVA.

Tissue dehydration, transparency and embedding: Five hepatohilar bile ducts from both groups were washed with lotic water, dehydrated in graded alcohol, and placed in dimethylbenzene for transparence over 24 h. The distribution of the hepatohilar bile duct wall capillaries was observed using an inverted phase contrast microscope. Another five hepatohilar bile ducts from the two groups were dehydrated in graded alcohol, placed in dimethylbenzene for transparence, embedded in paraffin, and continuously sectioned at a thickness of 20 μm. After adhering on, sections were slightly raised and heated until the paraffin disappeared, then they were incubated at 60℃ for 24 h in a thermostat. The sections were again put in dimethylbenzene for transparence over 24 h, during which the transparence liquid was changed three times. After transparence, the sections were mounted in gum, and pictures were collected with Motic 0.65 times light microscope for three-dimensional reconstruction of computer microvisualization.

Three-dimensional reconstruction of computer microvisualization: Firstly, primitive three dimensional data field was constructed by registering the sequence of two-dimensional section images according to feature points of ink perfusion blood vessel on bile duct thick sections, then the wave filter of the primitive three dimensional data was performed with median filter to inhibit noises and to enhance image characteristics, improving the ratio of signal to noise. Lastly the isosurfaces of cube voxel were extracted from three dimensional data field with Marching Cubes algorithm to construct the three-dimensional structure of sequence sections. Three-dimensional reconstruction was completed with PIV 2.53 GHz, 512M RAM microcomputer and algorithm was finished with language C++. The resolution of each section was 686 × 548 × 8 bits.

Pathological changes in hepatohilar bile duct and liver 6 mo after PVA using hematoxylin and eosin (HE) and Masson staining

The rats that underwent ultrasonography were killed 6 mo after PVA. Samples of hepatohilar bile ducts and liver were removed, fixed with 10% neutral formalin for 48-72 h, embedded in paraffin, continuously sectioned at a thickness of 5 μm, and stained with HE and Masson stain.

The steps for Masson staining were as follows. Samples were fixed with neutral formalin, sectioned, deparaffinized in water, stained with Masson composite staining solution for 5 min, washed with 0.2% acetic acid solution, washed with 5% phosphotungstic acid for 5-10 min, washed twice with 0.2% acetic acid solution, stained with bright green staining solution for 5 min, washed twice with 0.2% acetic acid solution, dehydrated in absolute alcohol, put in xylene for transparency, and mounted with neutral gum. Collagen fibers were bluish green in color and red blood cells were orange.

Statistical treatment

Data were expressed as mean ± SD. The *t* test was used for comparison between the two groups. Statistical analysis was performed with Stata software.

RESULTS

General status of experimental animals

In all rats, normal diet was restored, and fur was shiny. Body weight showed an increasing trend 3 d after operation, and then returned to normal 1 mo after PVA. There was no significant difference in body weight between the two groups at 6 mo after PVA.

Hemodynamic changes of the portal vein after PVA

Spectral changes of the portal vein and the status of vascular anastomosis 6 mo after PVA: In the portal vein, the continuous venous frequency spectrum was changed into a pulsatory arterial frequency spectrum 6 mo after PVA (Figure 2). Six months after PVA, the status of the vascular anastomosis of the portal vein with hepatic artery and vena cava is shown in Figure 3. The hepatic artery and the portal vein were obviously enlarged 6 mo after PVA.

Cross-sectional area of the portal vein in the two groups after operation: Compared with the control group, the cross-sectional area of the portal vein in the PVA group was significantly increased ($P < 0.01$) (Table 1).

Volume of blood flow of the portal vein in the two groups after operation: Compared with the control group, the volume of blood flow in the portal vein 6 mo after PVA was obviously increased ($P < 0.01$) (Table 1).

Table 1 Comparison of cross section area and volume of blood flow of the portal vein between the two groups (mean ± SD, n = 7)

Group	1 mo	6 mo
Cross section area of the portal vein (mm^2)		
Control group	0.0317 ± 0.0098	0.0333 ± 0.0103
PVA group	0.0433 ± 0.0242	0.0657 ± 0.0151[1,2]
Volume of blood flow of the portal vein (mL/min)		
Control group	37.6 ± 11.36	37.72 ± 9.09
PVA group	74.70 ± 42.76	132.01 ± 78.72[3]

[1]Compared with control $P < 0.01$; [2]Compared with cross section area of the portal vein one month after PVA $P < 0.05$; [3]Compared with control group $P < 0.01$.

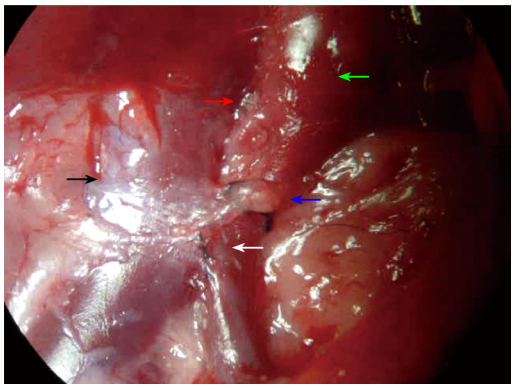

Figure 3 The status of the vascular anastomosis of the portal vein with hepatic artery and vena cava. The red arrow indicates the anastomotic site of the hepatic artery and portal vein. The white arrow indicates the side-to-side anastomotic stoma of the portal vein and the venae cavae. The green arrow indicates thickening of the portal vein. The black arrow indicates the venae cavae. The blue arrow indicates a thread blocking the portal vein.

Liver functions in rats 6 mo after PVA

Total bilirubin (TBIL), direct bilirubin (DBIL) and total bile acid (TBA) in the PVA group were greater than those in the control group, but there was no significant difference between the two groups ($P > 0.05$). Serum albumin (ALB) and alkaline phosphatase (AKP) in the PVA group were lower than those in the control group, but there was no significant significance between the two groups ($P > 0.05$). Glutamic pyruvic transaminase (GPT) in the PVA group was significantly greater than that in control group ($P < 0.01$) (Table 2).

Morphological changes of hepatohilar capillaries after PVA

Ink perfusion sections showed that, compared with the control group, the capillaries of the outside layer of the PVP were obviously thickened and dilated, and capillary density of the inside layer was increased, and capillary diameter of the inside layer was enlarged in the PVA group (Figure 4). Ink perfusion gross transparent specimens clearly showed the capillary structure in the forked site of the hepatohilar bile duct in the two groups. Capillaries in the forked site of the bile duct were obviously thickened in the PVA group (Figure 5). Three-dimensional reconstruction clearly showed the

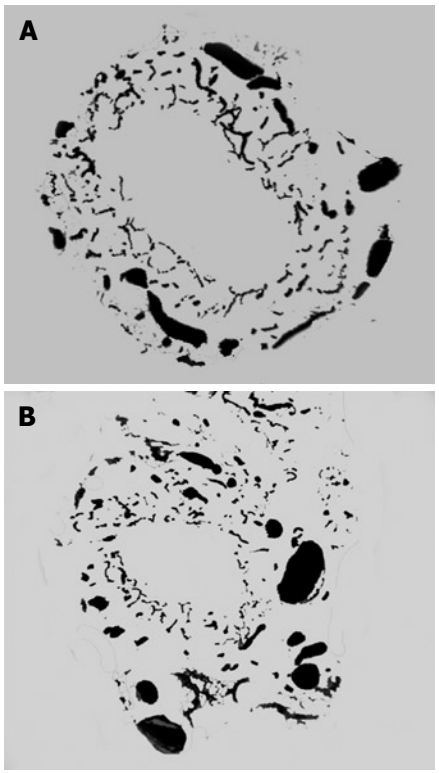

Figure 4 PVP plane structure of hepatohilar bile duct. A: Control group (20 μm, x 100); B: PVA group (20 μm, x 100).

PVP stereochemical structure of the hepatohilar bile ducts in the two groups. Peripheral vessels of the PVP were obviously thickened in the PVA group (Figure 6).

Histopathological changes in the hepatohilar bile duct wall

Figure 7A and B shows that, in both groups, the epithelial cell morphology of the bile ducts was normal, the thickness of bile duct was not increased, and there was no inflammatory cell infiltration.

Masson staining of the liver

In the PVA group, Figure 7C-F shows the dilated portal vein and its branches, thickened blood vessel wall, increased collagen fiber and fiber degeneration in the tunica intima. Hepatic lobule structure was intact. Pseudolobule formation was not present. Hepatic sinusoids were widened. The morphology of the hepatic cells was not obviously abnormal.

DISCUSSION

Since the incidence of hepatic encephalopathy is increased because of the decrease in hepatic blood perfusion after portosystemic shunting in portal hypertension, Cohn & Herrod[9] and Fisher *et al*[10] originally thought that portal vein blood was replaced with arterial blood in hepatic perfusion. Subsequently, many researchers have carried out a large number of animal experiments and confirmed that when the portal vein is perfused with arterial blood at an appropriate

Table 2 Liver Functions in the two groups (mean ± SD, *n* = 8)

Group	GPT	ALB	TBIL	DBIL	AKP	TBA
Control	62.81 ± 10.98	31.46 ± 3.97	2.53 ± 1.95	1.56 ± 1.49	144.43 ± 73.86	46.97 ± 27.89
PVA	178.96 ± 62.80[1]	29.71 ± 3.98	3.11 ± 0.95	2.19 ± 0.45	117.76 ± 53.41	72.68 ± 35.03

[1]Compared with control group $P < 0.01$.

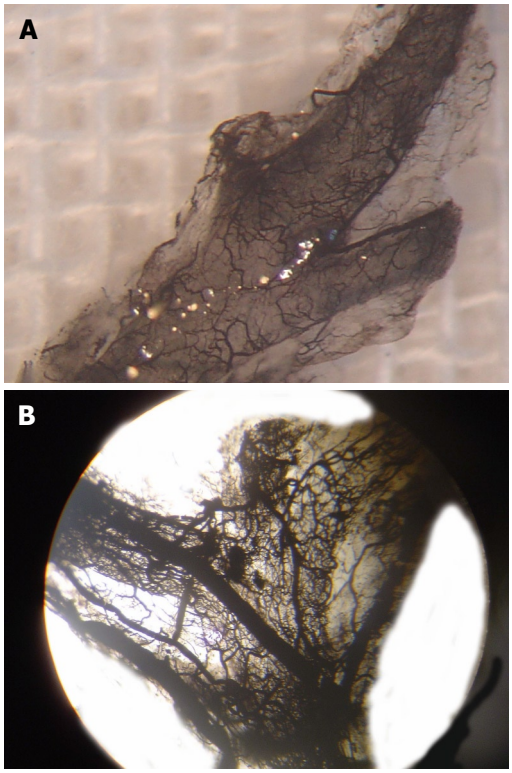

Figure 5 Microvascular distribution in forked site of hepatohilar bile ducts. A: Control group (x 100); B: PVA group (x 100).

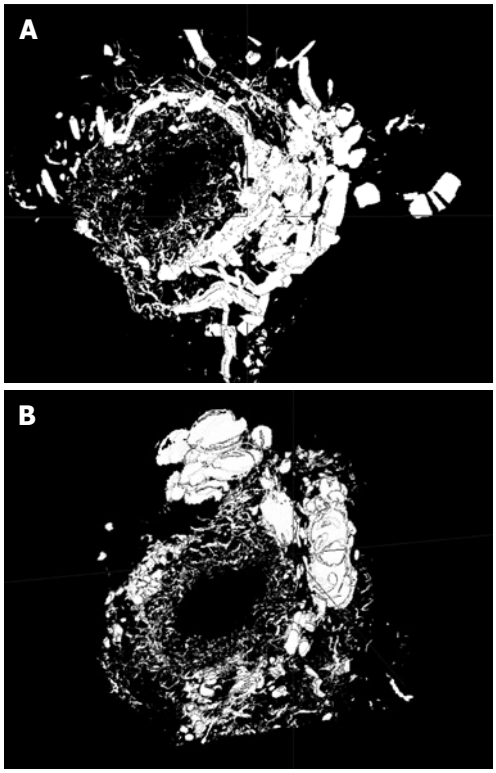

Figure 6 PVP three-dimensional reconstruction of hepatohilar bile ducts. A: Control group (12 x 20 μm); B: PVA group (12 x 20 μm).

flow and pressure, animals are able to maintain normal liver blood flow and levels of blood ammonia and blood colalin, to ensure normal liver detoxification and cell morphology. Since 1960, PVA has attracted extensive attention because of its various clinical applications[11-13], especially in the enlarged radical operation for hepatohilar cancer that invades the hepatic artery and liver transplantation. PVA has been used in reconstructing hepatic vascular access because of the presence of thrombosis of the portal and other veins, or the anatomical variation of the portal and mesenteric veins[14,15]. Morphological changes in PVP after PVA have attracted the attention of many workers. Hepatohilar bile ducts have become a concern because of their unique anatomical factors, including less collateral circulation and easily-damaged blood supply. The purpose of this study was to explore the influence of PVA on the morphology of hepatohilar bile ducts and PVP, epithelial cells of bile ducts, and to provide a theoretical basis for the clinical application of PVA.

Within 6 mo after PVA, rats were well and did not have jaundice. Their body weights showed an increasing trend. If the measure to limit blood flow was not taken within 1 mo after PVA, the cross-sectional area and blood flow of the portal vein were increased, and the increasing trend became more significant over time in a certain range. Hemodynamic changes after PVA will have an influence on hepatic structure. In our study, Masson staining 6 mo after PVA showed that, when measures to limit blood flow were not adopted, high blood flow in the portal vein led to dilation of the intrahepatic portal vein and its branches, increased collagen fibers in the tunica intima, widening of the hepatic sinusoids, and collagen fiber hyperplasia in the sinusoidal walls. However, the structure of hepatic lobule was not obviously disordered and pseudolobule formation was not present. Liver function tests showed that compared with the control group, except for GPT (significant increase $P < 0.01$), ALB in the PVA group was slightly lower, and bile acid and BIL were slightly increased, but there was no significant difference between the two groups. This demonstrated that PVA had no significant influence on ALB synthesis, bile acid and BIL metabolism and bile salt enterohepatic circulation. The increase in GPT may have been associated with high blood flow after PVA.

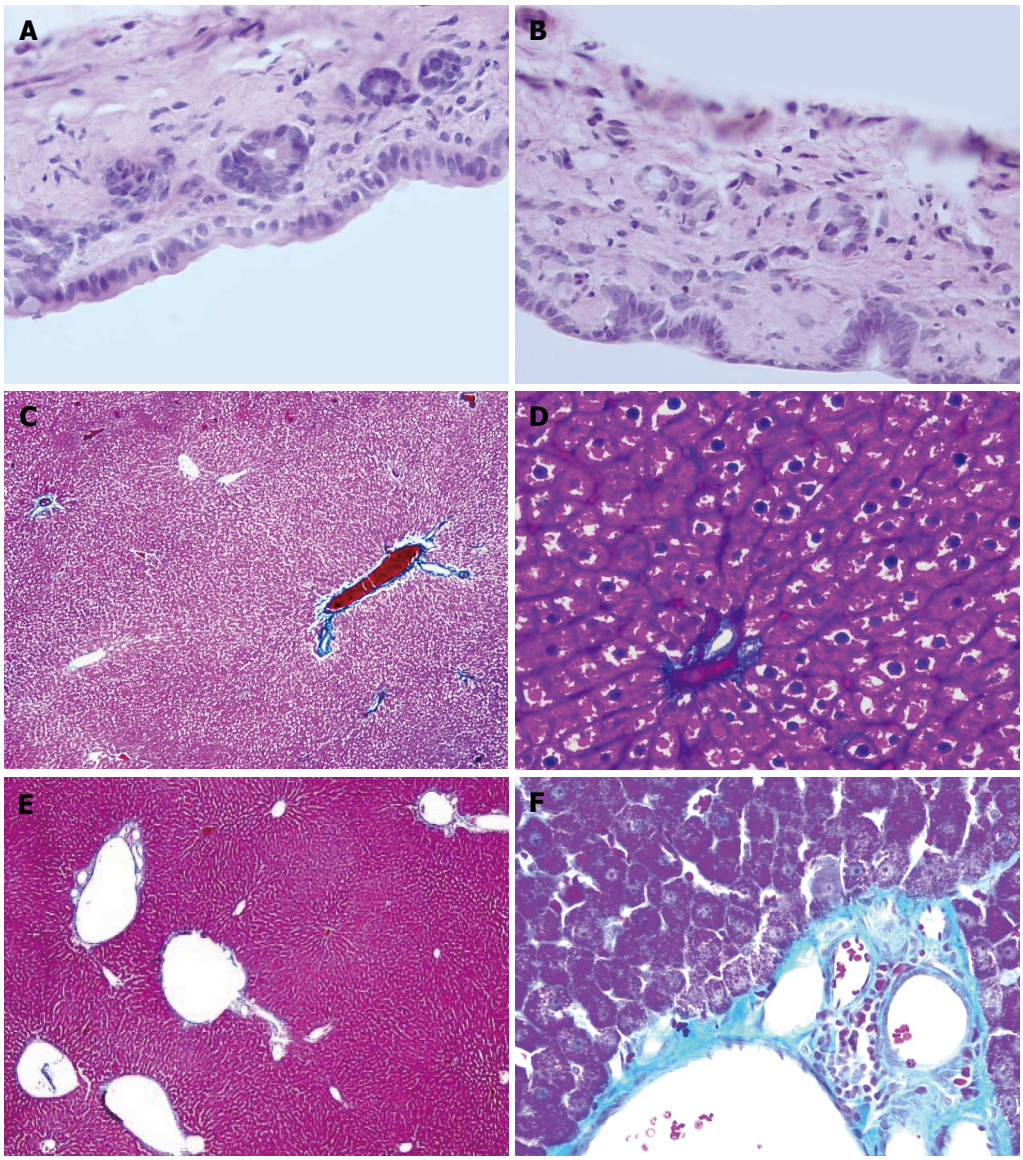

Figure 7 The pathological changes of hepatohilar bile duct and the liver in rats 6 mo after PVA using HE staining and Masson staining. A: Hepatohilar bile ducts in control group (HE, x 400); B: Hepatohilar bile ducts in PVA group (HE, x 400). C: Masson staining of the liver 6 mo after operation in control group (x 40); D: Masson staining of the liver 6 mo after operation in control group (x 400). E: Masson staining of the liver 6 mo after PVA in PVA group (x 40); F: Masson staining of the liver 6 mo after PVA in PVA group (x 400).

The PVP is common in mammals and its structure differs with various parts. It has been reported that the PVP of the large bile ducts consists of three layers of capillaries. The capillaries of the inside layer are arranged in an orderly manner and are open to capillaries beneath the epithelial lining. The middle and outside layer consist of a few capillaries, venules and arterioles in the bile duct wall or in peribiliary tissue[16]. It has been reported that different diseases will lead to different pathological features of the bile ducts, and PVP morphology will also show different pathological changes[17]. Blood flow and oxygen content in the PVP play important roles in ensuring normal physiological function of bile duct epithelial cells.

Ink perfusion thick sections and transparent specimens indicated that after PVA, capillaries of the outside layer of the PVP were obviously thickened, the number of blood vessels in the outside layer was not reduced, and capillary morphology in the hepatohilar bile duct walls was not disordered. This demonstrates that after the PVP loses its arterial blood supply, the blood in the arterialized portal vein can meet fully the requirement of the PVP, by portal vein collateral branch reflux, which may be the theoretical basis for clinical application of PVA. A semi-quantitative study of the PVP with normal portal pressure and portal hypertension has been performed by Terada *et al*[18] using immunohistochemistry. Results indicated that intrahepatic peribiliary vascular density was obviously increased in congenital portal hypertension and other forms of portal hypertension, because of extrahepatic portal vein thrombus and portal vein cancer embolus, but was only slightly increased in hepatic cirrhosis. PVP density is associated with portal vein pressure. It may be that the blood of the portal vein flows back into the PVP by internal routes, or flows directly into the PVP via arteries, which leads to PVP hyperplasia. This further indicates there are collateral vessels between the portal vein and PVP.

The three-dimensional reconstruction of computer microvisualization realistically illustrated PVP three-dimensional structure. Peribiliary capillaries after PVA were obviously thickened, which may have been caused by the increases in blood flow and blood flow rate of

the arterialized portal vein. MoticBuaa3DVol three-dimensional reconstruction software used in this study established a foundation for further research on capillary structure of hepatohilar bile ducts.

To maintain normal physical activities of bile duct tissue, adequate blood flow and a certain degree of blood oxygen content are required. Early study has shown that, since it possesses low blood flow and low oxygen content, the blood in the portal vein cannot completely fill the PVP, and local tissue is in a state of chronic hypoxia. Chronic inflammatory hyperplasia will occur in the hepatohilar bile duct administered by portal vein blood[19]. After PVA, blood flow and blood oxygen content were able to meet the requirements of the local tissues of the bile duct wall, and no ischemic lesion occurred in the bile ducts. HE staining results of this experiment disclosed that after PVA, there was no pathological change in bile duct tissue, which further verified this inference. At the same time, there were no significant differences in BIL and ALP between the two groups, which further confirmed the HE staining results.

In short, blood with a certain flow and oxygen content has important significance for filling the PVP and meeting the oxygen requirement of the bile duct wall. After PVA, it is the anatomic basis to maintain normal morphology of hepatohilar bile duct wall that the blood with high oxygen content and high flow in arterialized portal vein may fill PVP by collateral vessel reflux. The high blood flow in the portal vein after PVA is the main reason for the obvious dilation of the intrahepatic portal vein and collagen fibroplasia. Therefore, certain measures to limit blood flow in PVA are necessary.

COMMENTS

Background

During the enlarged radical operation for hepatohilar cancer that invades the hepatic artery and liver transplantation, the presence of portal vein and internal organ vein thrombosis or the congenital absence of the portal and mesenteric veins often require liver revascularization. For this reason, many researchers have carried out a large number of experiments and have applied portal venous arterialization (PVA) in clinical practice. Blood flow and oxygen content in the peribiliary vascular plexus (PVP) play an important role in ensuring normal physiological functions of bile duct epithelial cells. After PVA, the influence of portal vein hemodynamic changes on hepatohilar PVP has an important effect on the incidence of bile duct complications, and is directly associated with the clinical application of PVA. Therefore, to establish a theoretical basis for clinical application of PVA, we explored the influence of portal vein hemodynamic changes after PVA on PVP morphological structure and hepatic pathological structure.

Research frontiers

Liver revascularization is important in enlarged radical operation and liver transplantation. PVA is applied in clinical practice to resolve this problem, but some complications occur after PVA. In order to make clinical application of PVA better and reduce incidence of complications, it is necessary to study some subjects related to PVA.

Innovations and breakthroughs

In this study, we observed hemodynamic changes in the portal vein, morphological structure of hepatohilar PVP, and bile duct and liver pathology after PVA. We found that after PVA, the cross-sectional area and blood flow of the portal vein were increased and this trend became more significant over time in a certain range. If measures to limit the flow in PVA are not adopted, the high blood flow will lead to dilatation of the intrahepatic portal vein and its branches, and increased collagen and fiber degeneration in the tunica intima. Therefore, it is necessary to limit blood flow in PVA.

Applications

Measures to limit blood flow are likely to reduce the incidence of complications after PVA.

Terminology

PVA: portal venous arterialization; PVP: peribiliary vascular plexus.

Peer review

The paper addresses an important issue in hepatobiliary and liver transplantation practice, particularly in the cases of diffuse mesenteroportal venous thrombosis. In the current study, the authors used a model with a portacaval shunt in conjunction with complete arterialization of the portal inflow of the liver. The results are interesting.

REFERENCES

1 **Sheil AG**, Halliday JP, Drummond JM, Bookallil MJ, Gaudry PL, Yezerski SD. A modified technique for orthotopic liver transplantation. *Arch Surg* 1972; **104**: 720-724
2 **Blumhardt G**, Ringe B, Lauchart W, Burdelski M, Bechstein WO, Pichlmayr R. Vascular problems in liver transplantation. *Transplant Proc* 1987; **19**: 2412
3 **Erhard J**, Lange R, Giebler R, Rauen U, de Groot H, Eigler FW. Arterialization of the portal vein in orthotopic and auxiliary liver transplantation. A report of three cases. *Transplantation* 1995; **60**: 877-879
4 **Tsivian M**, Neri F, Prezzi D, Puviani L, Pacile V, Bertelli R, Cavallari G, Mattioli B, Bianchi E, Piras GL, Pariali M, Nardo B. Portal vein arterialization in hepatobiliary surgery and liver transplantation. *Transplant Proc* 2007; **39**: 1877-1878
5 **Schleimer K**, Stippel DL, Kasper HU, Tawadros S, Suer C, Schomäcker K, Hölscher A, Beckurts KT. Auxiliary liver transplantation with flow-regulated portal vein arterialization offers a successful therapeutic option in acute hepatic failure--investigations in heterotopic auxiliary rat liver transplantation. *Transpl Int* 2006; **19**: 581-588
6 **Ott R**, Böhner C, Müller S, Aigner T, Bussenius-Kammerer M, Yedibela S, Kissler H, Hohenberger W, Reck T, Müller V. Outcome of patients with pre-existing portal vein thrombosis undergoing arterialization of the portal vein during liver transplantation. *Transpl Int* 2003; **16**: 15-20
7 **Nivatvongs S**, Sirijindakul B, Nontasoot B. Portal vein arterialization for liver transplantation with extensive portomesenteric vein thrombosis: a case report. *Transplant Proc* 2004; **36**: 2267-2268
8 **Takasaki S**, Hano H. Three-dimensional observations of the human hepatic artery (Arterial system in the liver). *J Hepatol* 2001; **34**: 455-466
9 **Cohn R**, Herrod C. Some effects upon the liver of complete arterialization of its blood supply. *Surgery* 1952; **32**: 214-218
10 **Fisher B**, Russ C, Updegraff H. A suitable technique for total arterialization of the dog liver. *Surgery* 1954; **35**: 879-884
11 **Hulten O**. Arterialization of the liver in cirrhosis. *Scand J Clin Lab Invest Suppl* 1966; **18**: 43-43
12 **Maillard JN**, Benhamou JP, Rueff B. Arterialization of the liver wh portacaval shunt in the treatment of portal hypertension due to intrahepatic block. *Surgery* 1970; **67**: 883-890
13 **Otte JB**, Reynaert M, De Hemptinne B, Geubel A, Carlier M, Jamart J, Lambotte L, Kestens PJ. Arterialization of the portal vein in conjunction with a therapeutic portacaval shunt. Hemodynamic investigations and results in 75 patients. *Ann Surg* 1982; **196**: 656-663
14 **Kashfi A**, Mehrabi A, Pahlavan PS, Schemmer P, Gutt CN, Friess H, Gebhard MM, Schmidt J, Büchler MW, Kraus TW. A review of various techniques of orthotopic liver transplantation in the rat. *Transplant Proc* 2005; **37**: 185-188

15 **Orlando G**, De Luca L, Toti L, Zazza S, Angelico M, Casciani CU, Tisone G. Liver transplantation in the presence of portal vein thrombosis: report from a single center. *Transplant Proc* 2004; **36**: 199-202
16 **Kobayashi S**, Nakanuma Y, Matsui O. Intrahepatic peribiliary vascular plexus in various hepatobiliary diseases: a histological survey. *Hum Pathol* 1994; **25**: 940-946
17 **Qian YB**, Liu CL, Lo CM, Fan ST. Risk factors for biliary complications after liver transplantation. *Arch Surg* 2004; **139**: 1101-1105
18 **Terada T**, Ishida F, Nakanuma Y. Vascular plexus around intrahepatic large bile ducts in normal livers and portal hypertension. *J Gastroenterol Hepatol* 1989; **4** Suppl 1: 276-278
19 **Li WG**, Hu SX, Xue BD, Jiang ZG, Huang ZQ. Observation of hepatohilar peribiliary vascular plexus with complete absence of hepatic artery blood supply in rats. *Transplant Proc* 2007; **39**: 3424-3428

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RAPID COMMUNICATION

Co-infection of hepatitis B and hepatitis C virus in human immunodeficiency virus-infected patients in New York City, United States

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Abstract

AIM: To study the prevalence and risk factors associated with triple infection with human immunodeficiency virus (HIV)/hepatitis B virus (HBV)/hepatitis C virus (HCV) in an urban clinic population.

METHODS: Retrospective chart review of 5639 patients followed at St. Luke's-Roosevelt Hospital HIV Clinic (Center for Comprehensive Care) in New York City, USA from January 1999 to May 2007. The following demographic characteristics were analyzed: age, sex, race and HIV risk factors. A multiple logistic regression analysis was performed to evaluate the influence of demographic factors on acquisition of these viruses.

RESULTS: HIV/HBV, HIV/HCV and HIV/HBV/HCV infections were detected in 252/5639 (4.47%), 1411/5639 (25.02%) and 89/5639 (1.58%) patients, respectively. HIV/HBV co-infections were associated with male gender (OR 1.711; $P = 0.005$), black race (OR 2.091; $P < 0.001$), men having sex with men (MSM) (OR 1.747; $P = 0.001$), intravenous drug use (IDU) (OR 0.114; $P < 0.001$), IDU and heterosexual activity (OR 0.247; $P = 0.018$), or unknown (OR 1.984; $P = 0.004$). HIV/HCV co-infections were associated with male gender (OR 1.241; $P = 0.011$), black race (OR 0.788; $P = 0.036$), MSM (OR 0.565; $P < 0.001$), IDU (OR 8.956; $P < 0.001$), IDU and heterosexual activity (OR 9.106; $P < 0.001$), IDU and MSM (OR 9.179; $P < 0.001$), or transfusion (OR 3.224; $P < 0.001$). HIV/HBV/HCV co-infections were associated with male gender (OR 2.156; $P = 0.015$), IDU (OR 6.345; $P < 0.001$), IDU and heterosexual activity (OR 9.731; $P < 0.001$), IDU and MSM (OR 9.228; $P < 0.001$), or unknown (OR 4.219; $P = 0.007$).

CONCLUSION: Our study demonstrates that co-infection with HBV/HCV/HIV is significantly associated with IDU. These results highlight the need to intensify education and optimal models of integrated care, particularly for populations with IDU, to reduce the risk of viral transmission.

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Key words: Prevalence; Demographics; Human immunodeficiency virus; Hepatitis B; Hepatitis C

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INTRODUCTION

Co-infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) in patients infected with human immunodeficiency virus (HIV) is common and well-recognized worldwide, as they are blood-borne pathogens that share similar routes of transmission, such as intravenous drug use (IDU), sexual contact, percutaneous exposure, or from mother to child during pregnancy or birth[1]. The Joint United Nations Programme on HIV/

AIDS (UNAIDS) and the World Health Organization estimate that 1.2 million people live with HIV in the United States of America (US)[2]. Among HIV-infected patients studied from Western Europe and the US, chronic HBV infection has been found in 6%-14%, while chronic HCV has been found in approximately 33%[3,4]. Co-infections of HBV or HCV with HIV have been associated with increased risk of antiretroviral-therapy-related hepatotoxicity and increased risk of progression to liver disease, which is a major cause of morbidity and mortality in HIV-infected patients[5-8]. It is well known that in the US and Europe, HIV/HBV co-infection is linked most often to sexual intercourse [both heterosexual and men who have sex with men (MSM)], followed by IDU, while HIV/HCV co-infection has predominantly been associated with a non-sexual parenteral route of transmission of blood or blood products, particularly IDU[9-13]. Although the rate of HBV and/or HCV co-infection in HIV patients varies according to geographic region and various risk groups, there is limited data on the prevalence and risk factors associated with triple infections with HIV/HBV/HCV in an urban clinic population. Therefore, we investigated the co-infection patterns of HBV and HCV among HIV-infected patients coming to a HIV clinic located in New York City, to determine the prevalence and risk factors associated with triple infections with HIV/HBV/HCV.

MATERIALS AND METHODS

Study population

We conducted a retrospective chart review of 5639 patients followed in two HIV/AIDS clinics that comprise the Center for Comprehensive Care at St. Luke's–Roosevelt Hospital in New York City. The study period was from January 1999 to May 2007. All patients were HIV-infected and were analyzed for hepatitis B surface antigen (HBsAg) and HCV antibody, along with demographic characteristics and risk factors for HIV acquisition.

Virological assays

HIV infection was defined by positivity on an enzyme-linked immunosorbent assay (ELISA) (HIVABTM HIV-1/HIV-2 (rDNA) EIA, Abbott Laboratories, Abbott Park, IL, USA) and confirmatory Western Blot test (HIV 1/HIV 2 WESTERN BLOT/IMMUNOBLOT, Quest Diagnostics, USA) with viral detection of HIV RNA by polymerase chain reaction (PCR) (Roche HIV-1 Amplicor Monitor , version 1.5, Roche Diagnostics, Basel, Switzerland).

HCV serology was performed using an ELISA (VITROS^{R} Immunodiagnostics, Ortho-Clinical Diagnostics, USA). Positive HCV serology results were confirmed by PCR test [VERSANT^{R} HCV RNA 3.0 Assay (bDNA); Siemens Healthcare Diagnostics, USA]. HBV serology was performed using ELISA (VITROS^{R} Immunodiagnostics, Ortho-Clinical Diagnostics).

HIV/HBV, HIV/HCV, and HIV/HBV/HCV co-infections were defined as positive HIV and HBV serology, positive HIV and HCV serology, and positive HIV and HBV and HCV serology results, respectively.

Statistical analysis

The tables describe the distribution of co-infections among HIV-infected patients according to several characteristics. The χ^2 test was performed in order to investigate the association between the presence of HIV/HBV/HCV co-infections and demographic variables. The Fisher exact test was used where applicable. In order to identify factors associated with HIV/HBV, HIV/HCV and HIV/HBV/HCV co-infections, three multiple logistic regression analyses were performed to determine odd ratio (OR) and 95% confidence interval (CI). The level of statistical significance was fixed at $P = 0.05$. The statistical analysis was performed using the statistical software SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Epidemiological characteristics

Of the 5639 HIV-infected patients investigated, 1752 (31.07%) were co-infected with hepatitis viruses [252/5639 (4.47%) HIV/HBV, 1411/5639 (25.02%) HIV/HCV, and 89/5639 (1.58%) HIV/HBV/HCV]. The main epidemiological demographic characteristics are presented in Table 1.

HIV and HBV co-infection

Of the 252 HIV/HBV co-infected patients, the majority was male (203/252, 80.6%). Mean age was 44.2 years old. Black race was dominant (169/252, 67.1%). Risk factors for acquisition of HIV included: heterosexual contact (35.7%), MSM (46.4%) and IDU (2.4%). The results of the multiple regression analyses are presented in Table 2. HIV/HBV co-infections were associated with male gender (OR 1.711; 95% CI 1.179-2.482; $P = 0.005$), Black race (OR 2.091; 95% CI 1.404-3.114; $P < 0.001$), MSM (OR 1.747; 95% CI 1.247-2.448; $P = 0.001$), IDU (OR 0.114; 95% CI 0.049-0.262; $P < 0.001$), IDU and heterosexual activity (OR 0.247; 95% CI 0.077-0.789; $P = 0.018$), or unknown (OR 1.984; 95% CI 1.249-3.153; $P = 0.004$).

HIV and HCV co-infection

The majority of patients were male (1005/1411, 71.2%). Mean age was 50.4 years old. Hispanic and black ethnic group constituted the majority (553/1411 39.2%, 630/1411 44.6%, respectively). Concerning the risk factors of acquiring HIV, 702 patients (49.8%) reported IDU and 148 patients (10.5%) reported MSM.

Table 2 illustrates the factors associated with HIV/HCV co-infection, which show that co-infections were significantly associated with male gender (OR 1.241; 95% CI 1.052-1.464; $P = 0.011$), Black race (OR 0.788; 95% CI 0.631-0.984; $P = 0.036$), MSM (OR 0.565; 95% CI 0.446-0.715; $P < 0.001$), IDU (OR 8.956; 95% CI 7.502-10.693; $P < 0.001$), IDU and heterosexual activity

Table 1 Epidemiological demographic characteristics of HIV, HIV/HBV, HIV/HCV and HIV/HBV/HCV *n* (%)

	HIV	HIV/HBV	HIV/HCV	HIV/HBV/HCV
Number of patients and prevalence	3887 (68.93)	252 (4.47)	1411 (25.02)	89 (1.58)
Mean age (yr)	45.0	44.2	50.4	47.8
Gender				
Female	1266 (32.6)	48 (19.0)	392 (27.8)	13 (14.6)
Male	2583 (66.5)	203 (80.6)	1005 (71.2)	75 (84.3)
Male->Female or Female->Male	38 (1.0)	1 (0.4)	14 (1.0)	1 (1.1)
Ethnic group				
White	551 (14.2)	34 (13.5)	203 (14.4)	16 (18.0)
Hispanic	1267 (32.6)	44 (17.5)	553 (39.2)	34 (38.2)
African American	1951 (50.2)	169 (67.1)	630 (44.6)	39 (43.8)
Other	118 (3.0)	5 (2.0)	25 (1.8)	0 (0.0)
Risk factor of HIV acquisition				
Hetero	1683 (43.3)	90 (35.7)	285 (20.2)	9 (10.1)
MSM	1233 (31.7)	117 (46.4)	148 (10.5)	17 (19.1)
Hetero and MSM	38 (1.0)	3 (1.2)	0 (0.0)	0 (0.0)
IDU	407 (10.5)	6 (2.4)	702 (49.8)	38 (42.7)
IDU and hetero	90 (2.3)	3 (1.2)	165 (11.7)	13 (14.6)
IDU and MSM	24 (0.6)	3 (1.2)	57 (4.0)	5 (5.6)
IDU and hetero and MSM	6 (0.2)	0 (0.0)	1 (0.1)	0 (0.0)
Transfusion	36 (0.9)	3 (1.2)	21 (1.5)	0 (0.0)
Perinatal	101 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)
Other	31 (0.8)	1 (0.4)	0 (0.0)	1 (1.1)
Unknown	238 (6.1)	26 (10.3)	32 (2.3)	6 (6.7)

Data are number (%) of patients, unless otherwise indicated. Hetero, heterosexual; Other, including sexually abused, transfusion, needle stick injury.

Table 2 Multiple logistic regression analysis of factors associated with HIV/HBV, HIV/HCV, and HIV/HBV/HCV co-infection

	HIV/HBV		HIV/HCV		HIV/HBV/HCV	
Characteristics	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Gender						
Female (Ref)						
Male	1.711 (1.179-2.482)	0.005	1.241 (1.052-1.464)	0.011	2.156 (1.159-4.011)	0.015
Male->Female or Female->Male	0.399 (0.053-3.020)	0.374	1.978 (0.939-4.167)	0.073	2.091 (0.250-17.529)	0.496
Ethnic group						
White (Ref)						
Hispanic	0.766 (0.480-1.222)	0.263	0.956 (0.761-1.201)	0.699	0.915 (0.492-1.702)	0.779
Black	2.091 (1.404-3.114)	< 0.001	0.788 0.631-0.984)	0.036	0.851 (0.464-1.561)	0.602
Other	0.811 (0.309-2.125)	0.670	0.827 (0.493-1.388)	0.473	0.000 (0.000- .)	0.996
Risk factor of HIV aquisition						
Hetero (Ref)						
MSM	1.747 (1.247-2.448)	0.001	0.565 (0.446-0.715)	< 0.001	1.798 (0.763-4.238)	0.180
Hetero and MSM	1.371 (0.408-4.606)	0.610	0.000 (0.000- .)	0.998	0.000 (0.000- .)	0.998
IDU	0.114 (0.049-0.262)	< 0.001	8.956 (7.502-10.693)	< 0.001	6.345 (3.015-13.351)	< 0.001
IDU and hetero	0.247 (0.077-0.789)	0.018	9.106 (6.905-12.007)	< 0.001	9.731 (4.082-23.196)	< 0.001
IDU and MSM	0.714 (0.217-2.344)	0.578	9.179 (5.767-14.609)	< 0.001	9.228 (2.897-29.391)	< 0.001
IDU and hetero and MSM	0.000 (0.000- .)	0.999	0.900 (0.107-7.550)	0.923	0.000 (0.000- .)	0.999
Transfusion	1.161 (0.354-3.807)	0.806	3.224 (1.866-5.572)	< 0.001	0.000 (0.000- .)	0.998
Perinatal	0.000 (0.000- .)	0.996	0.000 (0.000- .)	0.996	0.000 (0.000- .)	0.997
Other	0.594 (0.080-4.437)	0.612	0.000 (0.000- .)	0.998	5.857 (0.715-47.994)	0.100
Unknown	1.984 (1.249-3.153)	0.004	0.703 (0.476-1.039)	0.077	4.219 (1.478-12.044)	0.007

(OR 9.106; 95% CI 6.905-12.007; $P < 0.001$), IDU and MSM (OR 9.179; 95% CI 5.767-14.609; $P < 0.001$), transfusion (OR 3.224; 95% CI 1.866-5.572; $P < 0.001$).

HIV and HBV/HCV co-infection

The prevalence of triple co-infection was found to be 1.58% (89/5639). Mean age was 47.8 years old and the majority was male (75/89, 84.3%). Hispanic and black ethnic group constituted the majority (34/89 38.2%, 39/89 43.8%). Regarding the risk factors of HIV acquisition, 38 patients (42.7%) reported IDU and 17 (19.1%) reported MSM.

The multiple regression analysis showed that factors associated with triple infections with HIV/HBV/HCV, were male gender (OR 2.156; 95% CI 1.159-4.011; $P = 0.015$), IDU (OR 6.345; 95% CI 3.015-13.351; $P < 0.001$), IDU and heterosexual activity (OR 9.731; 95% CI 4.082-23.196; $P < 0.001$), IDU and MSM (OR 9.228; 95% CI 2.897-29.391; $P < 0.001$), or unknown (OR 4.219; 95% CI 1.478-12.044; $P = 0.007$).

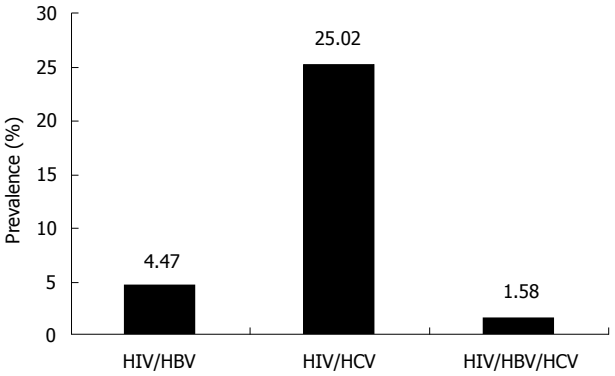

Figure 1 Prevalence of HIV and HBV and/or HCV co-infections (%).

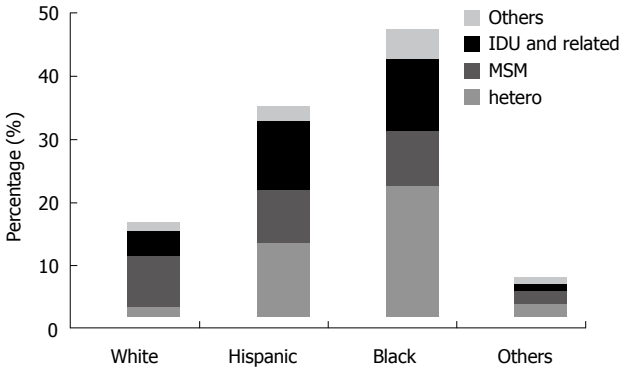

Figure 2 Ethnicity-related distribution of HIV acquisition risk factors of HIV and HBV and/or HCV co-infected patients.

DISCUSSION

Our clinics serve a large number of diverse HIV-infected populations in New York City. Most of our patients are men, black and Hispanic, which reflects the national epidemic of HIV infection[2]. In our study period of 8.5 years and the review of 5639 HIV-infected patients, as Figure 1 shows, the prevalence of HIV/HCV co-infection was remarkably high, at 25.02%. Co-infection with HBV was not uncommon, at 4.47%, and triple co-infected patients with HIV/HBV/HCV were not rare either, at 1.58%.

The results of the present study are consistent with previous studies conducted in urban HIV/HBV or HCV co-infected populations in the US and Western Europe[14,15].

In our study, HIV/HBV co-infection was more likely associated with male gender, black race and MSM, but less likely associated with IDU, and IDU plus heterosexual activity, than with heterosexual reference. These results are consistent with previous studies, demonstrating that HIV/HBV co-infection is highly linked to sexual intercourse, including MSM.

In our study, HIV/HCV co-infection was significantly associated with male gender, IDU, IDU plus heterosexual activity, IDU plus MSM, and transfusion, but less likely associated with black race and MSM than heterosexual reference. These results are in agreement with previous reports that HCV is not efficiently transmitted by perinatal or sexual exposure, which are major modes of transmission for HBV and HIV. HCV is predominantly found in persons who have had percutaneous exposure to blood products and IDU in particular[16]. Even though we did not analyze patients for unsafe sexual practices, recent evidence shows increased incidence of HCV infection in MSM population who do not use condoms, especially young individuals, and in the heterosexual population who report multiple sexual partners[17]. 10.5% and 20.2% of our HIV/HCV co-infected patients are MSM and heterosexual, respectively. Not counting for other possible risk factors, sexual acquisition of HCV is likely very important in HIV-infected MSM and heterosexual individuals with multiple partners.

The prevalence of triple infection with HIV/HBV/HCV in our cohort was 1.58% and significantly associated with male gender, IDU, IDU plus heterosexual activity, and IDU plus MSM. Our results demonstrate that IDU is the most important factor associated with triple infections with HIV/HBV/HCV in urban HIV-infected populations. Therefore, the study results highlight the need for special attention to populations with IDU for screening viral co-infections with HIV and HBV/HCV.

Interestingly, among intravenous drug users, we found that the prevalence of IDU in heterosexuals was significantly higher than that of MSM with a history of IDU in HIV/HBV/HCV and HIV/HCV co-infected patients (14.6% *vs* 5.6% $P < 0.001$, 11.7% *vs* 4% $P < 0.001$), which is consistent with previous studies conducted in the US[18,19]. This could be explained by fewer high-risk IDU practices, such as needle sharing, among MSM in our cohort, although IDU behavior was not assessed in our study. As Figure 2 illustrates, overall IDU-related prevalence in our cohort was 26.49%, 32.14%, 24.31% in white, Hispanic and black ethnic groups, respectively. Our findings contrast with recent data, in which blacks were more likely to inject than whites, while Hispanics and whites had similar injecting rates[20]. This indicates another example of considerable variations in disparities of IDU in ethnic groups in large urban US cities.

The HIV/AIDS epidemic in the US affects ethnic and racial groups disproportionally. This was clearly depicted in our study. Co-infection with hepatotropic viruses shows similar trends. Physicians who care for patients with HIV/AIDS should be vigilant to frequently screen for these infections and vaccinate for hepatitis A and B when appropriate. Also, our study demonstrates that co-infection with all three viruses, HIV/HBV/HCV, is significantly associated with IDU. These results highlight the need to intensify risk reduction education, such as needle exchange programs, safe sex programs, and optimal models of integrated care, particularly for populations with IDU, to reduce the risk of viral transmission of HIV and hepatotropic viruses.

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COMMENTS

Background

Co-infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) in human immunodeficiency virus (HIV)-infected patients has been recognized worldwide. HIV and hepatitis co-infections have considerable impact, and result in increased morbidity and mortality in these co-infected patients. However, there are limited data on prevalence and risk factors associated with triple co-infections with HIV/HBV/HCV in an urban clinic population.

Research frontiers

To investigate epidemiological characteristics of triple co-infected patients with HIV/HBV/HCV.

Innovations and breakthroughs

In this study, the prevalence of triple co-infected patients was 1.58% and it was significantly associated with male gender and intravenous drug use (IDU). The prevalence of HIV and HCV co-infected patients was 25.02%, and it was associated with male gender and IDU. Whereas, the prevalence of HIV and HBV co-infected patients was 4.47% and was associated with male gender, black race, and MSM, but not likely associated with IDU than heterosexual reference.

Applications

This study demonstrates that triple co-infections with HIV/HBV/HCV are significantly associated with IDU. Therefore, education and integrated care, particularly for populations with IDU, would help reduce the risk of viral infections.

Peer review

This is an interesting paper. The authors demonstrated that co-infection with all three viruses, HBV/HCV/HIV, is significantly associated with IDU. These results highlight the need to intensify education and optimal models of integrated care, particularly for populations with IDU, to reduce the risk of viral transmission.

REFERENCES

1 **Koziel MJ**, Peters MG. Viral hepatitis in HIV infection. *N Engl J Med* 2007; **356**: 1445-1454

2 **Joint United Nations Programme on HIV/AIDS (UNAIDS).** AIDS Epidemic Update Regional Summary: North America, Western and Central Europe. Genena, UNAIDS/08.14E, 2008. Available from: URL: http://www.unaids.org

3 **Alter MJ**. Epidemiology of viral hepatitis and HIV co-infection. *J Hepatol* 2006; **44**: S6-S9

4 **Sulkowski MS**. Viral hepatitis and HIV coinfection. *J Hepatol* 2008; **48**: 353-367

5 **Weber R**, Sabin CA, Friis-Møller N, Reiss P, El-Sadr WM, Kirk O, Dabis F, Law MG, Pradier C, De Wit S, Akerlund B, Calvo G, Monforte A, Rickenbach M, Ledergerber B, Phillips AN, Lundgren JD. Liver-related deaths in persons infected with the human immunodeficiency virus: the D:A:D study. *Arch Intern Med* 2006; **166**: 1632-1641

6 **Gebo KA**, Diener-West M, Moore RD. Hospitalization rates differ by hepatitis C satus in an urban HIV cohort. *J Acquir Immune Defic Syndr* 2003; **34**: 165-173

7 **Graham CS**, Baden LR, Yu E, Mrus JM, Carnie J, Heeren T, Koziel MJ. Influence of human immunodeficiency virus infection on the course of hepatitis C virus infection: a meta-analysis. *Clin Infect Dis* 2001; **33**: 562-569

8 **Sulkowski MS**, Thomas DL, Chaisson RE, Moore RD. Hepatotoxicity associated with antiretroviral therapy in adults infected with human immunodeficiency virus and the role of hepatitis C or B virus infection. *JAMA* 2000; **283**: 74-80

9 **Thomas DL**, Cannon RO, Shapiro CN, Hook EW 3rd, Alter MJ, Quinn TC. Hepatitis C, hepatitis B, and human immunodeficiency virus infections among non-intravenous drug-using patients attending clinics for sexually transmitted diseases. *J Infect Dis* 1994; **169**: 990-995

10 **Gilson RJ**, Hawkins AE, Beecham MR, Ross E, Waite J, Briggs M, McNally T, Kelly GE, Tedder RS, Weller IV. Interactions between HIV and hepatitis B virus in homosexual men: effects on the natural history of infection. *AIDS* 1997; **11**: 597-606

11 **Kellerman SE**, Hanson DL, McNaghten AD, Fleming PL. Prevalence of chronic hepatitis B and incidence of acute hepatitis B infection in human immunodeficiency virus-infected subjects. *J Infect Dis* 2003; **188**: 571-577

12 **Rodríguez-Méndez ML**, González-Quintela A, Aguilera A, Barrio E. Prevalence, patterns, and course of past hepatitis B virus infection in intravenous drug users with HIV-1 infection. *Am J Gastroenterol* 2000; **95**: 1316-1322

13 **Sherman KE**, Rouster SD, Chung RT, Rajicic N. Hepatitis C Virus prevalence among patients infected with Human Immunodeficiency Virus: a cross-sectional analysis of the US adult AIDS Clinical Trials Group. *Clin Infect Dis* 2002; **34**: 831-837

14 **Thio CL**. Hepatitis B in the human immunodeficiency virus-infected patient: epidemiology, natural history, and treatment. *Semin Liver Dis* 2003; **23**: 125-136

15 **Rockstroh JK**, Mocroft A, Soriano V, Tural C, Losso MH, Horban A, Kirk O, Phillips A, Ledergerber B, Lundgren J. Influence of hepatitis C virus infection on HIV-1 disease progression and response to highly active antiretroviral therapy. *J Infect Dis* 2005; **192**: 992-1002

16 **Wasley A**, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 2000; **20**: 1-16

17 **Alter MJ**, Hadler SC, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Moyer LA, Fields HA, Bradley DW. Risk factors for acute non-A, non-B hepatitis in the United States and association with hepatitis C virus infection. *JAMA* 1990; **264**: 2231-2235

18 **Vogt RL**, Richmond-Crum S, Diwan A. Hepatitis C virus infection in a human immunodeficiency virus-positive cohort in Hawaii. *J Infect Dis* 1997; **176**: 542-543

19 **Tedaldi EM**, Hullsiek KH, Malvestutto CD, Arduino RC, Fisher EJ, Gaglio PJ, Jenny-Avital ER, McGowan JP, Perez G. Prevalence and characteristics of hepatitis C virus coinfection in a human immunodeficiency virus clinical trials group: the Terry Beirn Community Programs for Clinical Research on AIDS. *Clin Infect Dis* 2003; **36**: 1313-1317

20 **Cooper H**, Friedman SR, Tempalski B, Friedman R, Keem M. Racial/ethnic disparities in injection drug use in large US metropolitan areas. *Ann Epidemiol* 2005; **15**: 326-334

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RAPID COMMUNICATION

Barium meal follow through with pneumocolon: Screening test for chronic bowel pain

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Abstract

AIM: To study the sensitivity, specificity and cost effectiveness of barium meal follow through with pneumocolon (BMFTP) used as a screening modality for patients with chronic abdominal pain of luminal origin in developing countries.

METHODS: Fifty patients attending the Gastroenterology Unit, SMS Hospital, whose clinical evaluation revealed chronic abdominal pain of bowel origin were included in the study. After routine testing, BMFT, BMFTP, contrast enhanced computed tomography (CECT) of the abdomen, barium enema and colonoscopy were performed. The sensitivity, specificity and cost effectiveness of these imaging modalities in the detection of small and/or large bowel lesions were compared.

RESULTS: Out of fifty patients, structural pathology was found in ten. Nine out of these ten patients had small bowel involvement while seven had colonic involvement alone or in combination with small bowel involvement. The sensitivity of BMFTP was 100% compared to 88.89% with BMFT when detecting small bowel involvement (BMFTP detected one additional patient with ileocecal involvement). The sensitivity and specificity of BMFTP for the detection of colonic pathology were 85.71% and 95.35% (41/43), respectively. Screening a patient with chronic abdominal pain (bowel origin) using a combination of BMFT and barium enema cost significantly more than BMFTP while their sensitivity was almost comparable.

CONCLUSION: BMFTP should be included in the investigative workup of patients with chronic abdominal pain of luminal origin, where either multiple sites (small and large intestine) of involvement are suspected or the site is unclear on clinical grounds. BMFTP is an economical, quick and comfortable procedure which obviates the need for colonoscopy in the majority of patients.

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Key words: Abdominal pain; Barium meal follow through; Cost effectiveness; Pneumocolon; Screening method

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INTRODUCTION

Abdominal pain lasting more than 6 mo duration is defined as chronic abdominal pain. Some of these patients have colicky pain or have other associated features in the form of abdominal distension, constipation or vomiting which suggests bowel involvement. The majority of these patients turn out to have functional disorders such as irritable bowel syndrome, functional dyspepsia and functional abdominal pain syndrome. Of these patients, only a few will definitely have organic lesions.

The evaluation of chronic abdominal pain of luminal etiology is a challenging problem for primary care physicians and gastroenterologists. The exact localization of lesions in either the small or large bowel remains difficult in many subjects. In tropical countries, where most of the population is of low socioeconomic status, an imag-

ing modality which screens small and large bowel lesions simultaneously at a reasonable cost and with good sensitivity and specificity is needed. Small bowel evaluation by barium meal follow through (BMFT) and colonic evaluation by double contrast barium enema (DCBE) are the standard norms[1].

The technique of pneumocolon has been used previously to evaluate the terminal ileum and caecum[2-5]. Performing pneumocolon along with BMFT at the same time evaluates both the small as well as the large bowel and is more economical than the combination of BMFT and barium enema. However, its potential in identifying colonic lesions is still controversial[6,7]. We evaluated BMFT with pneumocolon (BMFTP) as a single test for the assessment of chronic abdominal pain of bowel origin, screening both the small and large bowel simultaneously.

MATERIALS AND METHODS

One hundred and fifty consecutive patients with chronic abdominal pain attending the Gastroenterology Unit of SMS Medical College and Hospital, Jaipur were evaluated. A detailed history and thorough physical examination were carried out. Fifty patients in whom clinical evaluation suggested the involvement of the small bowel, large bowel or both were enrolled in the study (Figure 1A). Complete blood count, ESR, chest X-ray, liver function tests, renal function tests, serum amylase and complete examination of stools were performed. BMFT, DCBE, BMFTP and colonoscopy were carried out as per standard protocol. Contrast enhanced computed tomography (CECT) of the whole abdomen was also done. Subjects underwent overnight fasting and were asked to drink 100 mL of oral contrast dissolved in one liter of water around one and a half hours before the procedure. When subjects were taken for CECT, around 1 mL/kg of non-ionic contrast was given intravenously on the CT table. CT cuts were subsequently taken. We compared the sensitivity of these tests in detecting lesions as well as their cost effectiveness.

BMFTP was carried out in a similar way to BMFT. Microbar (Eskay fine chemicals) containing barium sulphate (92%) was used. 175 mg of Microbar powder was stirred in 150 mL of water to make a homogeneous solution. Patients were prepared with laxatives 1 d before the procedure. Preliminary plain abdominal films were taken prior to the procedure to rule out significant obstruction and perforation and to evaluate the patient's bowel preparation status. Preliminary films of the gastroesophageal junction, stomach and duodenum under IITV control were taken in the prone, supine and oblique positions to rule out diaphragmatic hernia, stomach and duodenum lesions and to assess the position of the duodeno-jejunal flexure. The patient then lay on his right side so that a continuous single column of barium was delivered into the small intestine. Prone postero-anterior (PA) films of the abdomen were taken every 20 min during the first hour and subsequently every 30 min to 45 min until the barium reached the colon[8]. A light dry meal was allowed after the barium had reached the ileum to speed up the examination if transit time was slow. Spot films of the ileocecal junction were also taken if required. Spot films with compression were taken of suspected abnormal bowel loops and strictures if required. When the barium was seen to cross the splenic flexure and the small bowel was almost devoid of barium on fluoroscopy, sufficient air was insufflated rectally using a sphygmomanometer cuff to achieve adequate distention of the colon.

About 300 mL to 450 mL of air was insufflated. Standard antero-posterior (AP), PA and oblique views of the colon were taken to observe the proximal and distal large intestine.

Table 1 Comparison of sensitivity and specificity in detecting colonic abnormalities using various imaging modalities and colonoscopy (%)

Imaging	Sensitivity	Specificity
Barium enema	7/7 (100)	42/43 (97.67)
BMFT with pneumocolon	6/7 (85.71)	41/43 (95.35)
CT abdomen	3/7 (42.87)	41/43 (95.35)
Colonoscopy	7/7 (100)	43/43 (100)

BMFT: Barium meal follow through.

RESULTS

One hundred and fifty patients presented with chronic abdominal pain, of which fifty patients (28 males, 22 females) had bowel symptoms. The mean age of these 50 patients was 35 years (range 25-55 years). Structural pathology was found in only 10 patients (Figure 1A). Caecal deformity along with distal ileal narrowing was seen in 2 patients, multiple ileal strictures in 3 patients, ileal stricture and stricture of the descending colon in 1 patient, multiple small bowel strictures with loss of haustration in most of the large bowel in 1 patient, jejunal and transverse colonic stricture in 2 patients (Figure 2) and stricture of the sigmoid colon in 1 patient. Nine of these ten patients had small bowel involvement while seven had colonic involvement.

BMFT detected 8 of 9 patients (sensitivity = 88.89%) who had small bowel involvement while BMFTP detected one additional patient with ileocecal involvement which was missed on BMFT (sensitivity = 100%).

In 7 patients where colonic pathology was found, BMFTP detected lesions in six patients while DCBE detected lesions in all seven patients. BMFTP gave false-positive results in 2 patients while DCBE was false-positive in one patient. Colonoscopy was normal in these false-positive cases. The sensitivity of BMFTP in the detection of colonic pathology was 85.71% (6/7) and was 100% (7/7) for DCBE. The specificity of BMFTP was 95.35% (41/43) and was 97.67% (42/43) for DCBE (Table 1).

CT scanning of the abdomen revealed small bowel involvement in only four of the nine patients (sensitivity

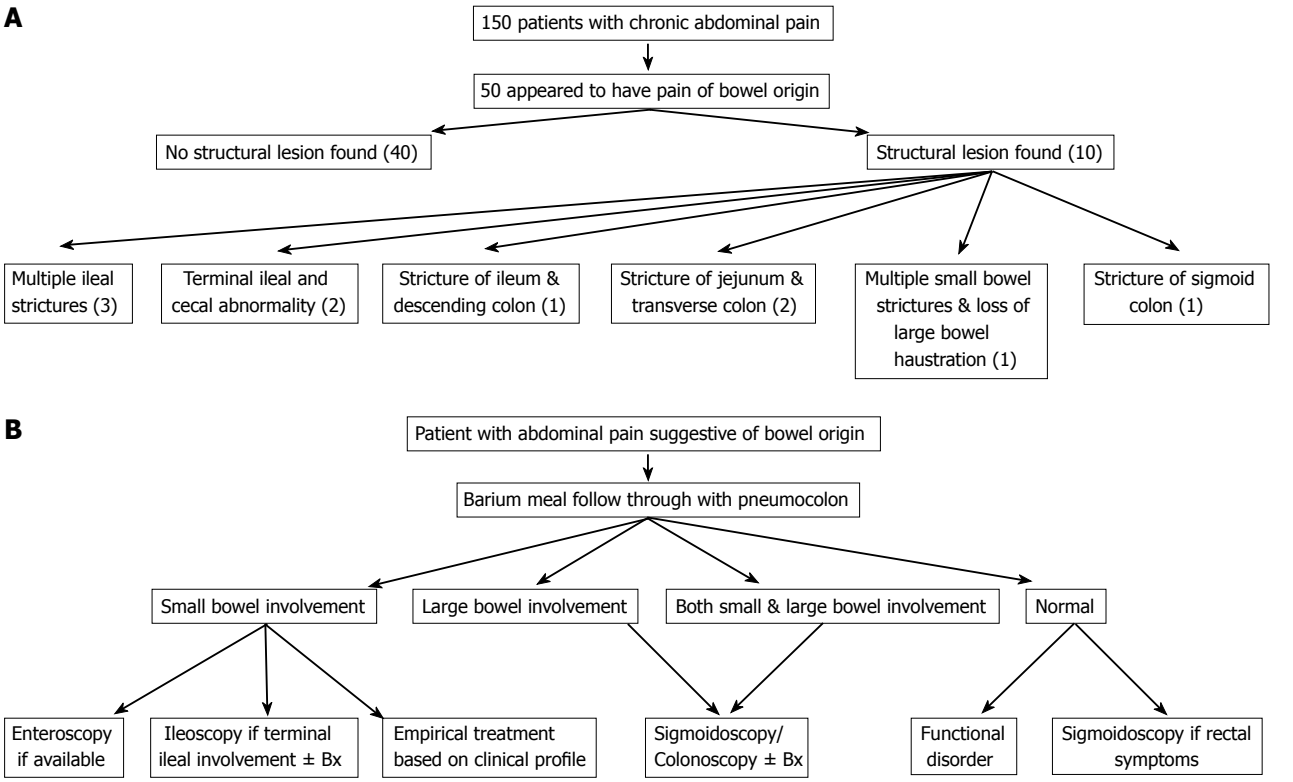

Figure 1 Study design. A: Study design revealing the various structural lesions observed in subjects; B: Step-up approach for investigating patients with chronic abdominal pain suggestive of bowel origin.

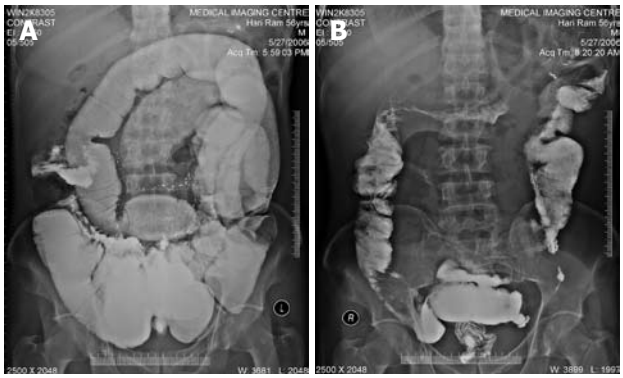

Figure 2 BMFTP. A: Showing multiple small bowel strictures; B: Adding pneumocolon to barium meal follow through revealed transverse colonic stricture in the same patient.

= 44.45%) and colonic lesions in three of seven patients (sensitivity = 42.87%), although CT revealed additional findings including abdominal lymph nodes (4 patients) and a small amount of free fluid in the abdomen (2 patients).

Colonoscopy performed in all fifty patients revealed colonic lesions in seven patients and terminal ileal involvement in two patients. Biopsies taken from various lesions revealed tuberculosis in four patients, two patients had features of Crohn's disease and one had colonic malignancy.

Screening a patient with abdominal pain suggestive of bowel origin using a combination of BMFT and DCBE cost three thousand rupees (71.43 $) while BMFTP cost eighteen hundred rupees (42.86 $) and the sensitivities were almost comparable. Screening with BMFTP instead of BMFT and DCBE obviates the need for colonoscopy in around 80% of patients.

DISCUSSION

This study revealed that screening patients with abdominal pain of luminal origin using BMFTP alone was cheaper compared to the combination of BMFT with DCBE and was almost as sensitive and specific. BMFTP obviates the need for colonoscopy in a number of patients as it also screens patients with colonic involvement.

BMFTP was better than BMFT alone in identifying terminal ileal and cecal lesions[9-12]. In a study by Marshall *et al*[13], BMFTP was found to be as accurate as ileoscopy in diagnosing terminal ileal disease. Minordi *et al*[14] showed that combining pneumocolon with BMFT improved the identification of terminal ileal and cecal lesions. Taves *et al*[7] reported two cases of cecal carcinoma which were initially described as Crohn's disease on the basis of BMFT, but were later diagnosed as malignant by the addition of pneumocolon and further confirmed by colonoscopy and pathology. In this study, we also found that the two patients who had terminal ileum and cecal involvement were both identified by BMFTP while one was missed on BMFT alone.

Adding pneumocolon to BMFT helped in the detection of colonic lesions[15-17]. Previous studies have revealed that BMFTP was better at detecting proximal colonic lesions compared to distal lesions. Chou *et al*[6]

showed that a routine overhead radiograph following the use of the pneumocolon technique was a useful adjunct to small bowel meal examination as it could yield unsuspected and clinically significant colonic findings. Colonic abnormalities such as ascending colonic cancer, acute or chronic colitis, diverticulosis, cecal polyps and ileosigmoid fistula were diagnosed using this pneumocolon technique in their study of 151 patients. Various studies which have combined contrast CT of the abdomen with pneumocolon have been used to evaluate various colonic lesions[18-22]. Some of these studies have shown CT pneumocolon to be a reliable alternative to barium enema where colonoscopy is incomplete, with the added advantage of extra luminal screening, and examination of the proximal bowel[23-25].

In this study, we were able to detect 6 of 7 colonic lesions by adding pneumocolon to BMFT. One of the patients who had sigmoid colonic stricture was missed using this imaging method and was detected by subsequent colonoscopy. Although there is little data available on the role of BMFTP in the detection of colonic lesions, most of the colonic lesions observed in this study were detected by BMFTP.

The findings from this study showed that in a patient with abdominal pain which clinically appeared to be arising from the bowel, that by performing BMFTP, both the small and large bowel were screened at the same time, and that the technique showed an efficacy comparable to that of BMFT combined with DCBE. The BMFTP technique was found to be simple and easy and obtained excellent double contrast visualization of the distal ileum and colon. The rectal and sigmoid areas were difficult to interpret on BMFTP. These patients usually have clinical symptoms suggestive of distal colonic or rectal involvement and they can be investigated with the added procedure of sigmoidoscopy.

In addition, BMFTP obviated the need for colonoscopy in the majority of patients as most of these patients were ultimately found to have functional disorders. Colonoscopy should only be performed in those patients who have positive findings on BMFTP, thereby decreasing the cost significantly. Colonoscopy is an invasive technique with significant procedure-associated discomfort.

Common luminal diseases of the gut such as tuberculosis and Crohn's disease involve more than one site in the bowel. Imaging requires the screening of both the small and large bowel for these diseases and effects therapeutic decisions. BMFTP helped in the investigation of these diseases by screening the whole lumen at one time. This study revealed that many patients who had strictures at multiple sites were successfully detected by BMFTP.

Based on our findings, we proposed a step-up approach for investigating patients with chronic abdominal pain of bowel origin (Figure 1B). Patients presenting with chronic abdominal pain of luminal nature in whom involvement of both the small and large bowel is suspected or where differentiation between the two is not possible, BMFTP should be performed after all baseline investigations. If BMFTP reveals only small bowel involvement, then depending on whether the terminal ileum is involved, patients should undergo ileo-colonoscopy with biopsy or enteroscopy. When terminal ileum is not involved and enteroscopy is unavailable, then patients should undergo empirical treatment based on their clinical profile. On the other hand, if BMFTP shows associated or isolated large bowel involvement, colonoscopy with biopsy of the lesion should be performed. By following this algorithm, one can investigate these patients in a cost effective manner which carries great significance for poor developing nations.

CONCLUSION

In tropical countries where the majority of the population is of low socio-economic status, BMFTP should be included in the investigative workup of patients with chronic abdominal pain of luminal origin, where either multiple sites (small and large intestine) of involvement are suspected or the site is unclear on clinical grounds. BMFTP is an economical, quick and comfortable procedure and obviates the need for colonoscopy in the majority of patients.

COMMENTS

Background

Few studies have tried to evaluate barium meal follow through (BMFT) with pneumocolon as a screening modality in subjects with chronic abdominal pain of luminal origin in developing countries where an inexpensive and cost effective screening tool is needed. Only a few studies have compared BMFT with pneumocolon *vs* plain BMFT for imaging ileocecal areas and the results of these studies have been varied.

Research frontiers

Evaluating small and large bowel simultaneously with a cost effective screening modality in developing countries is needed, as costly invasive modalities like double balloon enteroscopy and colonoscopy can be used only in selected subjects.

Innovations and breakthroughs

This is the first study to evaluate BMFT with pneumocolon as a screening tool for the evaluation of chronic abdominal pain of bowel origin. Adding pneumocolon to BMFT not only helped in detecting ileocecal lesions but also helped to detect colonic lesions with a sensitivity of 88.89% and a specificity of 95.35%.

Applications

This study showed that including BMFTP in the investigative workup of patients with chronic abdominal pain of luminal origin, where either multiple sites (small and large intestine) of involvement are suspected or the site is unclear on clinical grounds, helps in screening both the small and large bowel simultaneously and effectively without adding much to the total cost. This can be of great value in tropical countries where most of the population is of poor socioeconomic status.

Peer review

This study from India demonstrates the effectiveness and cost-effectiveness of BMFTP compared to other methods in screening patients with chronic abdominal pain. It's an interesting paper.

REFERENCES

1 **Maglinte DD**, Kelvin FM, O'Connor K, Lappas JC, Chernish SM. Current status of small bowel radiography. *Abdom Imaging* 1996; **21**: 247-257

2 **Kressel HY**, Evers KA, Glick SN, Laufer I, Herlinger H.

The peroral pneumocolon examination. *Radiology* 1982; **144**: 414-416
3 **Kelvin FM**, Gedgaudas RK, Thompson WM, Rice RP. The peroral pneumocolon: its role in evaluating the terminal ileum. *AJR Am J Roentgenol* 1982; **139**: 115-121
4 **Fitzgerald EJ**, Thompson GT, Somers SS, Franic SF. Pneumocolon as an aid to small-bowel studies. *Clin Radiol* 1985; **36**: 633-637
5 **Stringer DA**, Sherman P, Liu P, Daneman A. Value of the peroral pneumocolon in children. *AJR Am J Roentgenol* 1986; **146**: 763-766
6 **Chou S**, Skehan SJ, Brown AL, Rawlinson J, Somers S. Detection of unsuspected colonic abnormalities using the pneumocolon technique during small bowel meal examination. *Clin Radiol* 2000; **55**: 459-464
7 **Taves DH**, Probyn L. Cecal carcinoma: initially diagnosed as Crohn's disease on small bowel follow-through. *Can J Gastroenterol* 2001; **15**: 337-340
8 **Yong AA**, Harris JE, Shorvon PJ. The value of prone imaging in CT pneumocolon. *Clin Radiol* 2000; **55**: 959-963
9 **Cozzi G**, Bellomi M, Balzarini L, Severini A. [Oral pneumocolon in the study of the last ileal loops and the ileocecal region] *Radiol Med* 1984; **70**: 204-207
10 **Wolf KJ**, Goldberg HI, Wall SD, Rieth T, Walter EA. Feasibility of the peroral pneumocolon in evaluating the ileocecal region. *AJR Am J Roentgenol* 1985; **145**: 1019-1024
11 **Marshall JK**, Hewak J, Farrow R, Wright C, Riddell RH, Somers S, Irvine EJ. Terminal ileal imaging with ileoscopy versus small-bowel meal with pneumocolon. *J Clin Gastroenterol* 1998; **27**: 217-222
12 **Tolan DJ**, Armstrong EM, Bloor C, Chapman AH. Re: the value of the per oral pneumocolon in the study of the distal ileal loops. *Clin Radiol* 2007; **62**: 603; author reply 604
13 **Marshall JK**, Cawdron R, Zealley I, Riddell RH, Somers S, Irvine EJ. Prospective comparison of small bowel meal with pneumocolon versus ileo-colonoscopy for the diagnosis of ileal Crohn's disease. *Am J Gastroenterol* 2004; **99**: 1321-1329
14 **Minordi LM**, Vecchioli A, Dinardo G, Bonomo L. The value of the per oral pneumocolon in the study of the distal ileal loops. *Clin Radiol* 2006; **61**: 1016-1022
15 **Mittal A**, Saha MM, Pandey KK. Peroral pneumo colon--a double contrast technique to evaluate distal ileum and proximal colon. *Australas Radiol* 1990; **34**: 72-74
16 **Kellett MJ**, Zboralske FF, Margulis AR. Per oral pneumocolon examination of the ileocecal region. *Gastrointest Radiol* 1977; **1**: 361-365
17 **Calenoff L**. Rare ileocecal lesions. *Am J Roentgenol Radium Ther Nucl Med* 1970; **110**: 343-351
18 **Coakley FV**, Entwisle JJ. Spiral CT pneumocolon for suspected colonic neoplasms. *Clin Radiol* 1996; **51**: 666
19 **Amin Z**, Boulos PB, Lees WR. Technical report: spiral CT pneumocolon for suspected colonic neoplasms. *Clin Radiol* 1996; **51**: 56-61
20 **Harvey CJ**, Amin Z, Hare CM, Gillams AR, Novelli MR, Boulos PB, Lees WR. Helical CT pneumocolon to assess colonic tumors: radiologic-pathologic correlation. *AJR Am J Roentgenol* 1998; **170**: 1439-1443
21 **Miao YM**, Amin Z, Healy J, Burn P, Murugan N, Westaby D, Allen-Mersh TG. A prospective single centre study comparing computed tomography pneumocolon against colonoscopy in the detection of colorectal neoplasms. *Gut* 2000; **47**: 832-837
22 **Britton I**, Dover S, Vallance R. Immediate CT pneumocolon for failed colonoscopy; comparison with routine pneumocolon. *Clin Radiol* 2001; **56**: 89-93
23 **Harvey CJ**, Renfrew I, Taylor S, Gillams AR, Lees WR. Spiral CT pneumocolon: applications, status and limitations. *Eur Radiol* 2001; **11**: 1612-1625
24 **Sun CH**, Li ZP, Meng QF, Yu SP, Xu DS. Assessment of spiral CT pneumocolon in preoperative colorectal carcinoma. *World J Gastroenterol* 2005; **11**: 3866-3870
25 **Low VH**, Howard MH, Sheafor DH. Air insufflation: a useful adjunct to the single contrast barium enema for the evaluation of the rectum. *Int J Colorectal Dis* 2001; **16**: 46-50

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RAPID COMMUNICATION

Peripheral and mesenteric serum levels of CEA and cytokeratins, staging and histopathological variables in colorectal adenocarcinoma

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Abstract

AIM: To evaluate the differences that exist between peripheral and mesenteric serum levels of carcinoembryonic antigen (CEA) and cytokeratins in patients with colorectal adenocarcinoma.

METHODS: One hundred and thirty-eight patients with colorectal adenocarcinoma who underwent surgery at Hospital São Paulo (Discipline of Surgical Gastroenterology of UNIFESP-EPM) between December 1993 and March 2000 were retrospectively analyzed. Differences between CEA and cytokeratin (TPA-M) levels in peripheral blood (P) and in mesenteric blood (M) were studied. Associations were investigated between peripheral and mesenteric levels and the staging and histopathological variables (degree of cell differentiation, macroscopic appearance, tumor dimensions and presence of lymphatic and venous invasion).

RESULTS: Differences were observed in the numerical values of the marker levels: CEA (M) (39.10 mg/L ± 121.19 mg/L) *vs* CEA (P) (38.5 mg/L ± 122.55 mg/L), $P < 0.05$; TPA-M (M) (325.06 U/L ± 527.29 U/L) *vs* TPA-M (P) (279.48 U/L ± 455.81 U/L), $P < 0.01$. The mesenteric CEA levels were higher in more advanced tumors ($P < 0.01$), in vegetating lesions (34.44 mg/L ± 93.07 mg/L) ($P < 0.01$) and with venous invasion (48.41 mg/L ± 129.86 mg/L) ($P < 0.05$). Peripheral CEA was higher with more advanced staging ($P < 0.01$) and in lesions with venous invasion (53.23 mg/L ± 158.57 mg/L) ($P < 0.05$). The patients demonstrated increased mesenteric and peripheral TPA-M levels with more advanced tumors ($P < 0.01$ and $P < 0.01$) and in non-ulcerated lesions [530.45 U/L ± 997.46 U/L ($P < 0.05$) and 457.95 U/L ± 811.36 U/L ($P < 0.01$)].

CONCLUSION: The mesenteric levels of the tumor markers CEA and cytokeratins were higher than the peripheral levels in these colorectal adenocarcinoma patients. Higher levels of these biologic tumor markers are associated with an advanced state of cancerous dissemination.

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Key words: Colonic neoplasms; Rectal neoplasms; Biological tumor markers; Carcinoembryonic antigen; Cytokeratins

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INTRODUCTION

The estimates of cancer incidence in Brazil for the year 2006, published by INCA, indicate that colorectal cancer is the fifth most common malignant tumor type among men (11 390 new cases) and the fourth among women (13 970 new cases). The greatest incidence of cases occurs in the age group between 50 and 70 years old, but the possibility of developing this disease is already increasing after the age of 40 years is reached[1-3].

In 2004, in a study carried out in the 25 member countries of the European Union, 2 886 800 new cases of cancer and 1 711 000 deaths were recorded. The most common type was lung cancer (13.3%) followed

by colorectal cancer (13.2%) and breast cancer (13%). Lung cancer was also the greatest cause of death (341 800 cases) followed by colorectal cancer (203 700 cases)[4].

When colorectal cancer is detected in its initial stage, it may even be curable. However, the overall survival of patients with colorectal cancer does not exceed 40%. The mean five-year survival for patients with early diagnosis (stage Ⅰ) is approximately 70%, while it is 6% for advanced cases of the disease (stage Ⅳ)[1,2].

Tumor markers are substances produced by the neoplasia than can be identified in the neoplastic tissue itself and in patients' biological fluids. Many studies have been conducted to evaluate serum tumor markers at different stages of diagnosis and follow-up of colorectal carcinoma cases. Carcinoembryonic antigen (CEA) is distinguished as the most important marker[1,2,5-9].

CEA was first identified in 1965, and is a high-molecular-weight glycoprotein that is found in the cytoplasmic membrane of digestive system cells in the fetal phase and in neoplastic cells[10]. Cytokeratins form part of the microtubules of the cellular cytoskeleton, and they are released into the bloodstream during processes in which there is intense cell proliferation or apoptosis[11].

There is controversy regarding whether or not there are differences in the serum levels of the markers according to the location of the blood sample collection: from peripheral veins or from blood flowing directly out of the lesions. If there were a difference between the mesenteric and peripheral serum levels of such markers, the former might more accurately reflect the real levels produced by the tumors than would the latter. Some authors have found a significant difference between the mesenteric and peripheral levels of CEA, while others have not reproduced these results. Some studies have also demonstrated relationships between high marker levels in mesenteric serum and the histopathological variables of colorectal tumors[12-16].

The objective of the present investigation was to analyze the mesenteric and peripheral levels of CEA and cytokeratins in patients with colorectal adenocarcinoma and observe their correlation with the staging and certain histopathological variables.

MATERIALS AND METHODS

The patients were volunteers and were treated in accordance with a protocol approved by the Research Ethics Committee of the institution. In this study, 138 patients with colorectal adenocarcinoma were retrospectively analyzed. These patients were attended to and surgically treated by the Coloproctology Group, Discipline of Surgical Gastroenterology, Department of Surgery, Federal University of São Paulo-Escola Paulista de Medicina (UNIFESP-EPM). The operations were performed at Hospital São Paulo between December 1993 and March 2000. Surgical resection was performed on 124 patients, while the tumors were considered irresectable in 14 patients.

Patients who had had some other benign or malignant neoplasia at some previous time, and those for whom it was not possible to collect the data needed for the proposed analysis, were not included in the study.

With regard to the ethnic group to which the patients belonged, 68.1% were white, 22.5% brown, 6.5% yellow and 2.8% black. With regard to gender, 57.2% were female. The patients' ages ranged from 19 to 87 years, with a mean of 61.7 years.

The variables analyzed in the present investigation were: staging of the colorectal neoplasia by means of the TNM classification, degree of cell differentiation, diameter of the neoplasia, presence or absence of venous invasion and presence or absence of lymphatic invasion.

According to the TNM classification, 34 patients were in stage Ⅰ, 21 in Ⅱ, 34 in Ⅲ and 49 in Ⅳ. With regard to the degree of cell differentiation, there were 55 patients with well-differentiated tumors, 66 with moderately-differentiated tumors and three with poorly-differentiated tumors. Regarding the diameter of the neoplasia, 16 patients had tumors of $\leqslant$ 3.9 cm; 76 of 4.0-7.9 cm and 32 $\geqslant$ 8.0 cm. The presence of venous invasion was identified in the lesions of 23 patients, while lymphatic invasion was identified in 41 patients.

The collection of peripheral venous blood was done by means of direct puncture in the arm that was free of endovenous hydration, while anesthesia was being induced. Samples of 10 mL of blood were collected in dry tubes. These were centrifuged to obtain the serum from the sample, and this was stored at -20℃.

The mesenteric blood for assaying the marker levels was collected by dissection, sectioning and catheterization of the inferior mesenteric vein, when the tumor was located in the left colon or the rectum. For tumors in the right colon, collection was *via* the wide tributary vein of the superior mesenteric vein, and for tumors in the transverse colon, collection was *via* the middle colic vein. The corresponding vein was dissected and repaired with 00 cotton thread; the vein was sectioned obliquely and the catheter was introduced towards the tumor, with the collection of 10 mL of blood. This procedure was carried out before any manipulation of the tumor. The blood was centrifuged, with separation of the serum and storage in the same way as done for the peripheral blood samples.

The method utilized for assaying the CEA levels was DELFIA®. The CEA levels were considered to be normal when they were less than the limit of 5.0 mg/L.

For assaying the cytokeratin (TPA-M) levels, the LIA-mat® TPA-M Prolifigen® method was utilized, (AB Sangtec Medical®), which utilizes a reference value of 72 U/L as the cutoff point between normal and abnormal values, and this point was taken for the present investigation. The apparatus utilized for carrying out the serum assays was the Lumat LB 9501® luminometer, (EG&G Berthold).

Statistical analysis

For the statistical analysis of the data obtained in this study, *t* test and the marginal homogeneity test were

Table 1 Descriptive measurements of the tumor markers and the histopathological variables and staging of the colorectal adenocarcinoma (mean ± SD)

Variables	CEA (M) ug/L	CEA (P) ug/L	TPA-M (M) U/L	TPA-M (P) U/L
TNM				
Ⅰ	14.96 ± 43.02	15.82 ± 52.37	178.91 ± 116.08	168.44 ± 137.84
Ⅱ	3.71 ± 3.23	3.10 ± 2.63	148.56 ± 79.25	126.13 ± 82.13
Ⅲ	11.33 ± 21.21	8.00 ± 16.71	214.94 ± 164.20	151.55 ± 131.75
Ⅳ	90.28 ± 190.14	90.69 ± 190.88	578.52 ± 812.53	511.02 ± 692.70
P	0.001	0.001	0.001	0.001
Cell differentiation				
PD	22.06 ± 72.07	22.40 ± 72.24	227.35 ± 214.74	197.28 ± 234.32
MD	40.95 ± 116.87	40.52 ± 127.30	392.67 ± 695.62	338.50 ± 589.94
BD	11.40 ± 17.84	9.97 ± 14.84	111.67 ± 46.50	72.53 ± 43.22
P	0.816	0.632	0.212	0.164
Diameter (cm)				
Up to 3.9	44.80 ± 155.57	53.88 ± 190.73	194.84 ± 139.81	193.03 ± 230.85
4.0 to 7.9	27.96 ± 86.94	27.18 ± 85.14	331.94 ± 645.83	294.35 ± 561.49
⩾ 8.0	34.63 ± 89.26	31.51 ± 89.48	325.34 ± 314.19	248.44 ± 234.79
P	0.106	0.186	0.104	0.197
Macroscopic ulcerated				
No	38.21 ± 103.39	36.82 ± 108.08	530.45 ± 997.46	457.95 ± 811.36
Yes	30.01 ± 96.84	30.26 ± 104.16	248.99 ± 257.75	214.44 ± 276.23
P	0.433	0.736	0.014	0.009
Vegetating				
No	29.19 ± 103.53	30.94 ± 114.16	233.59 ± 229.94	217.40 ± 284.20
Yes	34.44 ± 93.07	32.51 ± 95.45	388.99 ± 706.77	319.81 ± 583.88
P	0.035	0.197	0.057	0.18
Infiltrative				
No	15.71 ± 41.64	15.22 ± 45.76	281.19 ± 394.86	244.83 ± 376.19
Yes	47.00 ± 129.00	47.23 ± 137.53	341.94 ± 637.18	292.49 ± 532.86
P	0.132	0.07	0.415	0.321
Venous invasion				
Present	48.41 ± 129.86	53.23 ± 158.57	347.10 ± 282.17	255.56 ± 477.84
Absent	28.09 ± 89.57	26.85 ± 88.36	304.68 ± 575.348	330.35 ± 391.40
P	0.034	0.029	0.163	0.094
Lymp. invasion				
Present	49.76 ± 129.89	46.75 ± 125.35	447.89 ± 845.14	227.83 ± 286.49
Absent	23.02 ± 77.03	24.33 ± 92.70	245.69 ± 251.79	353.65 ± 691.80
P	0.095	0.15	0.137	0.527

utilized. To study the correlations using the TNM variable, the Bonferroni multiple comparisons method was utilized.

In the tests utilized, the level of statistical significance for rejection of the nullity hypothesis was set at 0.05% or 5% ($\alpha \leqslant 0.05$), thereby indicating the results that were considered significant.

RESULTS

Analysis of the mesenteric and peripheral levels of the markers

Two statistical analysis methods were performed (one numerical and the other categorical), and each of the markers was analyzed in relation to its peripheral and mesenteric concentrations. With regard to the numerical descriptive measurements of the CEA levels, the mean for CEA (M) was 39.10 mg/L ± 121.19 mg/L and the mean for CEA (P) was 38.5 mg/L ± 122.55 mg/L, with a statistically significant difference ($P < 0.05$). Comparison between the proportions of positive rates of mesenteric and peripheral CEA was done by means of the marginal homogeneity test. No statistical difference was found from this.

With regard to the numerical descriptive measurements of the TPA-M levels, the mean for TPA-M (M) was 325.06 U/L ± 527.29 U/L and the mean for TPA-M (P) was 279.48 U/L ± 455.81 U/L ($P < 0.01$). To compare the evaluations of mesenteric and peripheral TPA-M, the marginal homogeneity test was utilized, from which it was found that rate of positive results was greater for mesenteric TPA-M ($P < 0.05$).

Associations

For both markers and for both mesenteric and peripheral blood, the levels were related to advanced stage of the neoplasia, and especially to stage Ⅳ of TNM. In addition to this association, CEA (M) and CEA (P) presented correlations with venous invasion, and CEA (M) alone correlated with vegetating lesions. Both the mesenteric and peripheral levels of TPA-M were high in non-ulcerated lesions (Table 1).

DISCUSSION

Many studies have been conducted on tumor markers, seeking greater understanding of all the possible ways of using them in diagnoses, staging, prognoses and

detection of neoplastic recurrences[1,5-7,14,17]. Even the location for sample collection has been analyzed, seeking the site that would best translate the serum levels of tumor markers and identify groups of patients with more limited prognoses (with or without liver micrometastases), and also to identify the patients who would most benefit from adjuvant therapy, for example[8,17-20].

Studies have analyzed samples from different markers collected from different points: peripheral veins or the main drainage vein from the neoplasia. The levels of these markers have been found to be higher when sampled closer to the tumors, and thus the peripheral levels do not provide a true reflection of the production of these markers.

The production of markers by diseased cells, the release of these markers and their passage through adjacent tissue, their entry into lymphatic vessels and the bloodstream, the formation of immunocomplexes, metabolism of these markers, their excretion from the liver and absorption by the colorectal wall, are factors that would influence the peripheral levels of these markers[12,13].

The way in which markers arrive in the peripheral blood has still not been clearly established. It could be *via* the portal vein system, the lymphatic system, or both. Previous studies have suggested that CEA arrives in the peripheral blood *via* the portal system[12,13]. These studies have shown that there is a strong association between the mesenteric and peripheral CEA levels and the extent of venous invasion and degree of penetration of this invasion into the colorectal wall. They have also shown that there is a significant increase in the portal levels of CEA soon after the manipulations carried out during the surgical resection of the neoplasia.

In the case of colorectal adenocarcinoma, CEA has become prominent in demonstrating usefulness for following up patients who have undergone surgery with curative intent, with increases in its levels in the event of probable tumor recurrence or development of liver metastases[7,17-19,21].

Cytokeratins have shown greater sensitivity than that of CEA in the initial diagnosis, staging, establishment of prognoses and detection of recurrence in colorectal adenocarcinoma cases[1,2,5].

The studies performed by Tabuchi *et al*[12,13] in Japan have established that there is a statistically significant difference between the mesenteric and peripheral CEA levels, and thus the authors postulate that this marker reaches the peripheral blood *via* the portal system. Positive rates were correlated with certain histopathological variables, such as venous invasion and Dukes classification. These studies have also demonstrated that patients with high mesenteric CEA levels are potentially at risk of developing liver metastases and that such levels have a negative impact on patient survival.

The mesenteric-peripheral CEA gradient has also been utilized, together with the mesenteric levels, for assessing the impact on postoperative survival among patients with colorectal cancer. A study published in Japan in 1990 demonstrated that patients with a mesenteric-peripheral CEA gradient greater than 10 ng/mL would have a worse prognosis[18].

Another study of interest showed that the mesenteric levels and the mesenteric-peripheral gradient were more effective than the utilization of the peripheral levels alone for predicting liver metastases. A study published in Japan in 1998 compared patients with advanced colorectal cancer divided into two groups: with and without liver metastases. The mean mesenteric CEA level and mesenteric-peripheral gradient were greater than the peripheral level in the group with postoperative liver metastases. This suggests that mesenteric assaying of this marker would be more effective for predicting this event[22].

Subsequent studies conducted by other authors have not shown significant differences between the peripheral and mesenteric CEA levels[14-16]. This may be related to the small size of the samples analyzed in these studies.

In the present investigation, the sample was composed of 138 patients who were analyzed retrospectively. All of them underwent peripheral and mesenteric assaying of the CEA and cytokeratin (TPA-M) levels, which were evaluated in relation to seven histopathological variables. Statistically significant differences were found between the peripheral and mesenteric CEA and cytokeratin levels when numerical analysis was performed. When only the positive frequency of the markers was investigated, there was only a difference for cytokeratins. This may signify that the main drainage route for the markers is the portal system.

Both of these markers had high levels in TNM stage IV, both for mesenteric and for peripheral blood. Thus, the markers had significantly higher levels when the neoplastic disease was no longer limited to the colon. This corroborates the findings of Fernandes *et al*[1] (2005), which showed higher marker levels in cases of patients with extra-colonic disease, perhaps signifying the presence of liver or occult lymph node micrometastases[17].

The mesenteric and peripheral CEA levels were higher in the presence of venous invasion, and this reproduces the results from previous studies. This may corroborate the hypothesis that drainage *via* the portal vein system is the fundamental principle for the distribution of this marker[12-16].

In the present study, the cytokeratin levels were also higher in the presence of non-ulcerated lesions. No studies presenting an association between peripheral and mesenteric CEA and cytokeratin levels and the macroscopic characteristics of the lesion were found in a search of the medical literature. In the present investigation, there were associations between mesenteric CEA and vegetating lesions and between mesenteric and peripheral cytokeratins and non-ulcerated lesions. It is believed that subsequent studies will be necessary, in order to analyze and compare ulcerated, vegetating and infiltrative lesions in relation

to survival and the peripheral and mesenteric levels of biological tumor marker, so as to obtain greater depth for the conclusions.

In summary, the present results allow it to be concluded that, for the patients analyzed, there was a significant difference between the CEA and cytokeratin tumor marker levels, with higher levels in the samples collected from the portal vein system than in those obtained from the peripheral blood. The levels increased in accordance with the progression of neoplastic dissemination. High mesenteric and peripheral CEA levels were associated with venous invasion. There were higher assayed cytokeratin levels in patients with non-ulcerated colorectal adenocarcinoma lesions.

REFERENCES

1 **Fernandes LC**, Kim SB, Matos D. Cytokeratins and carcinoembryonic antigen in diagnosis, staging and prognosis of colorectal adenocarcinoma. *World J Gastroenterol* 2005; **11**: 645-648

2 **Fernandes LC**, Kim SB, Saad SS, Matos D. Value of carcinoembryonic antigen and cytokeratins for the detection of recurrent disease following curative resection of colorectal cancer. *World J Gastroenterol* 2006; **12**: 3891-3894

3 **Wilmink AB**. Overview of the epidemiology of colorectal cancer. *Dis Colon Rectum* 1997; **40**: 483-493

4 **Boyle P**, Ferlay J. Cancer incidence and mortality in Europe, 2004. *Ann Oncol* 2005; **16**: 481-488

5 **Correale M**, Arnberg H, Blockx P, Bombardieri E, Castelli M, Encabo G, Gion M, Klapdor R, Martin M, Nilsson S. Clinical profile of a new monoclonal antibody-based immunoassay for tissue polypeptide antigen. *Int J Biol Markers* 1994; **9**: 231-238

6 **Forones NM**, Tanaka M, Machado D, Falcão JB, Giovanoni M. [Carcinoembryonic antigen in diagnosis and monitoring of colorectal cancer] *Arq Gastroenterol* 1997; **34**: 3-6

7 **Kim SB**, Fernandes LC, Saad SS, Matos D. Assessment of the value of preoperative serum levels of CA 242 and CEA in the staging and postoperative survival of colorectal adenocarcinoma patients. *Int J Biol Markers* 2003; **18**: 182-187

8 **Lindmark G**, Gerdin B, Påhlman L, Bergström R, Glimelius B. Prognostic predictors in colorectal cancer. *Dis Colon Rectum* 1994; **37**: 1219-1227

9 **Plebani M**, De Paoli M, Basso D, Roveroni G, Giacomini A, Galeotti F, Corsini A. Serum tumor markers in colorectal cancer staging, grading, and follow-up. *J Surg Oncol* 1996; **62**: 239-244

10 **Gold P**, Freedman SO. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J Exp Med* 1965; **121**: 439-462

11 **Björklund B**. Tissue polypeptide antigen (TPA): Biology, biochemistry, improved assay methodology, clinical significance in cancer and other conditions, and future outlook. *Antibiot Chemother* 1978; **22**: 16-31

12 **Tabuchi Y**, Deguchi H, Imanishi K, Saitoh Y. Comparison of carcinoembryonic antigen levels between portal and peripheral blood in patients with colorectal cancer. Correlation with histopathologic variables. *Cancer* 1987; **59**: 1283-1288

13 **Tabuchi Y**, Deguchi H, Imanishi K, Saitoh Y. Carcinoembryonic antigen levels of peripheral and draining venous blood in patients with colorectal cancer. Correlation with survival. *Cancer* 1992; **69**: 2411-2417

14 **Harłozińska A**, Rachel F, Gawlikowski W, Richter R, Kołodziej J. CEA and NCA levels in peripheral and tumour venous blood of patients with gastric and colonic carcinomas estimated by RIA and EIA methods. *Eur J Surg Oncol* 1991; **17**: 59-64

15 **Moura RM**, Matos D, Galvão Filho MM, D'Ippólito G, Sjzenfeld J, Giuliano LM. Value of CEA level determination in gallbladder bile in the diagnosis of liver metastases secondary to colorectal adenocarcinoma. *Sao Paulo Med J* 2001; **119**: 110-113

16 **Waisberg J**, Contim-Neto L, Oliveira Mda S, Matheus Cde O, Nagashima CA, Goffi FS. Determination of carcinoembryonic antigen levels in peripheral and draining venous blood in patients with colorectal carcinoma. *Arq Gastroenterol* 2004; **41**: 88-92

17 **Finlay IG**, McArdle CS. The identification of patients at high risk following curative resection for colorectal carcinoma. *Br J Surg* 1982; **69**: 583-584

18 **Deguchi H**, Tabuchi Y, Saitoh Y. [CEA levels of draining venous blood and draining-peripheral CEA gradient in colorectal cancer patients: correlation with postoperative survival] *Nippon Geka Gakkai Zasshi* 1990; **91**: 575-580

19 **Finlay IG**, Meek DR, Gray HW, Duncan JG, McArdle CS. Incidence and detection of occult hepatic metastases in colorectal carcinoma. *Br Med J* (Clin Res Ed) 1982; **284**: 803-805

20 **Koch M**, Weitz J, Kienle P, Benner A, Willeke F, Lehnert T, Herfarth C, von Knebel Doeberitz M. Comparative analysis of tumor cell dissemination in mesenteric, central, and peripheral venous blood in patients with colorectal cancer. *Arch Surg* 2001; **136**: 85-89

21 **Hünerbein M**. The value of tumor markers in colorectal cancer. *Recent Results Cancer Res* 1998; **146**: 48-55

22 **Tabuchi Y**, Nakamura T, Kuniyasu T. A predictive value of carcinoembryonic antigen in draining venous blood for colorectal cancer patients with postoperative hematogenous metastases. *Cancer Detect Prev* 1998; **22**: 57-61

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RAPID COMMUNICATION

Endoscopic and histopathological evaluation of acute gastric injury in high-dose acetaminophen and nonsteroidal anti-inflammatory drug ingestion with suicidal intent

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Abstract

AIM: To evaluate endoscopic and histopathologic aspects of acute gastric injury due to ingestion of high-dose acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs) with respect to some risk factors and patient characteristics.

METHODS: The study group consists of 50 patients admitted to emergency department with high dose analgesic ingestion (group Ⅰ) with suicidal intent. Thirty patients with or without mild complaints of dyspepsia (group Ⅱ) were selected as the control group. The study group was stratified according to the use of type and number of analgesics. Endoscopic findings were evaluated according to the Lanza score (LS), expressing the severity of the gastroduodenal damage and biopsies according to a scoring system based on histopathologic findings of acute erosive gastritis.

RESULTS: Gastroduodenal damage was significantly more severe in group Ⅰ compared to group Ⅱ ($P < 0.01$). The LS was similar in both groups Ⅰa and Ⅰb. However LS was significantly higher in patients who had ingested multiple NSAIDs (group Ⅰc) compared to other patients ($P < 0.01$). The LS was correlated to age ($P < 0.01$) and total amount of drug ingested ($P < 0.05$) in group Ⅰ; but it was not correlated with *Helicobacter pylori (H pylori)* infection or duration of exposure ($P > 0.05$). The biopsy score (BS) was higher in group Ⅰ than group Ⅱ ($P < 0.01$), and higher in group Ⅰb than group Ⅰa ($P < 0.05$).

CONCLUSION: The histopathologic damage was more severe among NSAID ingesting patients compared to those ingesting only acetaminophen and there is no significant difference in the endoscopic findings between the groups. There is no significant difference in the LS between the groups. This lack of significance is remarkable in terms of the gastric effects of high-dose acetaminophen.

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Key words: Acute gastric injury; Nonsteroidal anti-inflammatory drug; Acetaminophen; Endoscopic lesion; Gastroscopy

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INTRODUCTION

Analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs) and acetaminophen are extensively used pharmaceutical agents. NSAIDs are well known and widely used for their anti-inflammatory, analgesic and antipyretic properties. Incidence of gastrointestinal side effects associated with NSAIDs is relatively high

and common. Gastrointestinal side effects of NSAIDs are related to various factors such as individual risk factors, the dose and the duration of use. NSAID use presents with a 2.5- to 5-fold increase in the risk of gastrointestinal tract (GI) complications[1]. These complications vary from abdominal discomfort to serious complications like ulceration, bleeding, perforation or obstruction[2]. Risk for adverse gastrointestinal events related to NSAID use are related to the age of the patient (> 60 years), presence of previous complicated/uncomplicated ulcer, use of multiple NSAIDs, dose of NSAIDs, type of drug, the presence of *H pylori* infection, concomitant use of corticosteroids and serious comorbid diseases[1,3]. Besides these, duration of the NSAID use, smoking and alcohol consumption have also been reported to increase the risk of NSAID complications, and the incidence of adverse events decrease with long-term use due to adaptation mechanisms[4,5].

The reports about NSAID use and related adverse events have so far involved therapeutic doses of NSAIDs. Therefore acute effects of short-term, especially high-dose NSAID and acetaminophen use have not been reported. This study was designed to investigate acute gastric injury and histological changes among patients who ingested high dose NSAID with suicidal intent. We evaluated the association of single high dose analgesic use with previous gastric complaints, smoking, food intake, drug dose, duration of exposure, co-existence of *H pylori*, and body position during intake (standing/supine) on the distribution of gastric lesions.

MATERIALS AND METHODS

Study group

This is a prospective, randomized and controlled study. Fifty Patients admitted to the emergency unit after ingestion of high-dose analgesics with suicidal intent (group Ⅰ) were included in the study. The control group consisted of 30 patients with or without mild dyspeptic complaints (group Ⅱ). The patients in the study group were grouped according to the type of analgesic use: Of the 50 patients, 18 ingested only acetaminophen (group Ⅰa), 32 have ingested only NSAIDs (group Ⅰb) and in a subgroup of the NSAID ingesting group 10 ingested multiple types of NSAID simultaneously (group Ⅰc). Exclusion criteria were: antacids, acid suppressants (H_2-blocker, PPI), NSAIDs and steroid use with or without alcohol consumption, established gastric condition and treatment for gastric symptoms, systemic disease and homodynamic disorders and cognitive problems. The control group consisted of patients presenting with or without mild gastric complaints who had not been previously diagnosed with a gastric or any other disease, no history of alcohol use, and who had not used acid suppressants or NSAIDs within the last month. Exclusion of chronic or intermittent NSAIDs users enabled us to compare the endoscopic and histological assessed acute gastric damage with an almost healthy group. The study protocol was approved by the local ethics committee.

Table 1 Maximum recommended daily dose according to drug type[6]

Drug	Dose (mg)
Acetaminophen	4000
Aspirin	4000
Etodolac	1200
Flurbiprofen	300
Ibuprofen	2400
Naproxen sodium	1375

Table 2 Endoscopic grading scale: LS[7,8]

Grade	Scale
0	No visible lesions
1	Redness and hyperemia in the mucosa
2	One or two erosions or hemorrhaging lesions
3	3-10 erosions or hemorrhaging lesions
4	> 10 erosions or hemorrhaging lesions or an ulcer

Note: Erosions are defined as flat, white-based mucosal breaks of any size; Ulcers are defined as mucosal breaks of at least 3 mm or more.

Study design

Demographic characteristics, smoking habits and history of gastric complaints during the last month were compared. The dose and type of drug used with suicidal intent was identified by examination of drug pack physically and information were obtained from the household. Information about time of the ingestion, type and amount of the drug, the occurrence of vomiting after intake, food intake and body position following the ingestion (standing/supine) and gastric lavage after the ingestion were recorded. The amount of ingested drug was standardized based on the pharmacological maximum recommended daily dose and scored in multiples (Table 1)[6].

Within 24 h following drug ingestion, gastroduodenoscopy was performed on the study and control groups. Endoscopic gastric findings were recorded by video endoscopy, and evaluated by a second endoscopy specialist according to the Lanza score (LS) (Table 2)[7,8]. Biopsies were obtained from the antrum for *H pylori* testing from all patients and from lesions if present. Biopsy specimens were stained with hematoxylin-eosin and with modified Giemsa for *H pylori*. In order to evaluate and quantify the histopathologic findings we have made a list of microscopic findings of NSAID-related acute erosive/hemorrhagic gastritis[9,10]. These findings were stratified into a three-scaled scoring algorithm according to the severity of the lesions which we present in Table 3 as biopsy score (BS).

Statistical analysis

Besides descriptive statistical methods (mean, standard deviation, frequency), the Mann-Whitney *U* test, χ^2 test, and Spearman's Rank Correlation Test were used in the

Table 3 Three-scaled histomorphologic scoring algorithm developed by us indicating the severity of the lesions according to microscopic findings of NSAID-related acute erosive/hemorrhagic gastritis

Score	Microscopic findings
1	Superficial epithelium is intact, edema at superficial lamina propria, dilated and congested capillary structures, extravasated erythrocyte and tiny fibrin clots
2	Multiple focal scattered erosions + 1
3	Disappearance of superficial epithelium, presence of proteinose materials on the surface of epithelium, diffuse hemorrhages within lamina propria, transmural ischemic necrosis + 2

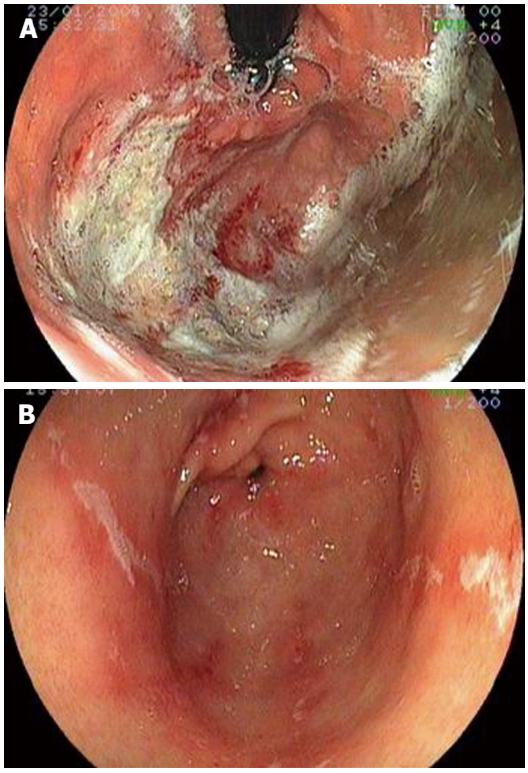

Figure 1 Images of lesions according to the posture after the ingestion. A: Patients in the supine position following ingestion of NSAID; B: Patients in the upright position following ingestion.

assessment of study results. Results were evaluated with a 95% confidence interval (CI) and a significance level of $P < 0.05$.

RESULTS

Patients in group Ⅰ [23.3 ± 7.5 years (range: 14-50)] were younger than those in group Ⅱ [32.3 ± 7.6 years (range: 19-48)] ($P = 0.001$). There was no difference between the two groups in terms of smoking ($P = 0.39$) and *H pylori* positivity ($P = 0.385$) (Table 4). Patients in group Ⅰ a had ingested 1-5 times the maximum daily dose of acetaminophen, while those in group Ⅰ b had ingested 1.2-13.6 times the maximum daily dose of NSAIDs.

Clinical findings

There was no significant difference between group Ⅰ and group Ⅱ with respect to gastric symptoms within the last month. Of the group Ⅰ patients, 12% had nausea, 6% had mild heartburn, 4% had epigastric pain and 4% had hemathinized gastric fluid in nasogastric lavages during the emergency department examination. There was no difference in LS and BS among group Ⅰ patients with or without gastric symptoms during last month.

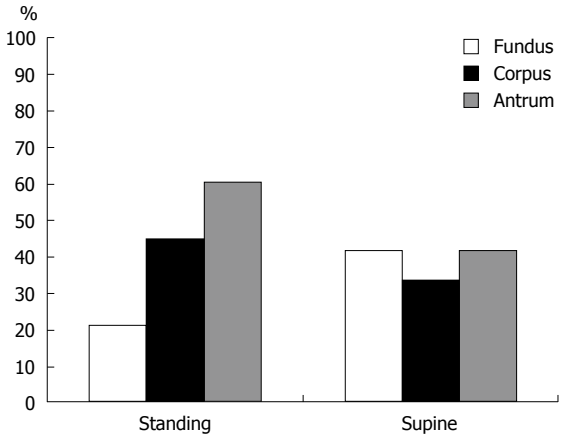

Figure 2 Distribution of the localization of endoscopic lesions in patients in the standing and supine positions following ingestion.

Endoscopic findings

There was no difference between fundus, corpus, and antrum involvement of endoscopic lesions according to body position after drug intake ($P > 0.05$). Although there was no statistically significant difference in the distribution of gastric lesions in the fundus, corpus, or antrum according to body position after ingestion, the allocation of gastric lesions in the fundus were 21.1% in the standing and 41.7% in the supine positions, lesions in the antrum were 60.5% in the upright and 41.7% in the supine positions. Images of lesions according to the posture after the ingestion are demonstrated in Figure 1. Although there was no statistical significance, anatomical distribution of gastric lesions differed according to the body position after the ingestion (Figure 2).

LS

The LS of group Ⅰ (2.3 ± 1.2) was higher than in group Ⅱ (0.77 ± 0.6) ($P < 0.01$). The LS was positively correlated with age in group Ⅰ ($r = 0.39$, $P < 0.01$), with a significant correlation at the 39.1% level. LS increased with age (Figure 3). Conversely, LS was not correlated with smoking habits, food intake, *H pylori* positivity and duration of exposure to the drugs ($P > 0.05$). Interestingly, the difference between the LS of patients who ingested only NSAIDs (group Ⅰ b) was higher but not significant compared to those who ingested only acetaminophen (group Ⅰ a) ($P = 0.08$). The LS of patients who ingested multiple types of NSAIDs was significantly higher than those who took a single type of NSAID ($P < 0.01$). There was a positive correlation between the total amount of ingested drugs and LS ($r = 0.35$, $P < 0.05$) which means that LS increased with increasing drug dose.

Table 4 Comparison of LS and BS in relation to various patient characteristics

		LS (median)	BS (median)
Group	Patients	2.30 ± 1.18 (2)	2.26 ± 0.83 (2.5)
	Control group	0.77 ± 0.57 (1)	1.47 ± 0.73 (1)
	P	0.001	0.001
Age	*r*	0.391	-0.078
	P	0.005	0.592
History of gastric complaints	Positive	2.36 ± 1.43 (3)	2.09 ± 0.83 (2)
	Negative	2.28 ± 1.12 (2)	2.31 ± 0.83 (3)
	P	0.809	0.4
Smoking	Yes	2.08 ± 1.29 (2)	2.24 ± 0.88 (3)
	No	2.52 ± 1.04 (2)	2.28 ± 0.79 (2)
	P	0.234	0.949
Food intake	On full stomach	2.36 ± 1.17 (2)	2.27 ± 0.80 (2)
	On empty stomach	2.17 ± 1.24 (2)	2.23 ± 0.90 (3)
	P	0.576	0.964
H pylori	Positive	2.08 ± 1.19 (2)	2.32 ± 0.75 (2)
	Negative	2.52 ± 1.15 (2)	2 20 ± 0.91 (3)
	P	0.204	0.743
Duration of exposure	*r*	0.14	0.099
	P	0.332	0.492
Total drug dose (as multiples of maximum daily dose)	*r*	0.353	0.219
	P	0.012	0.127
Ingested drug	Acetaminophen only	1.89 ± 1.18 (2)	1.94 ± 0.87 (2)
	NSAID only	2.54 ± 1.21 (3)	2.58 ± 0.70 (3)
	P	0.086	0.013
Multiple NSAIDs	Yes	3.40 ± 0.70 (3.5)	2.50 ± 0.85 (3)
	No	2.02 ± 1.12 (2)	2.20 ± 0.82 (2)
	P	0.001	0.256

Analyses performed with Mann Whitney *U* test. *r*: Spearman's rank correlation test.

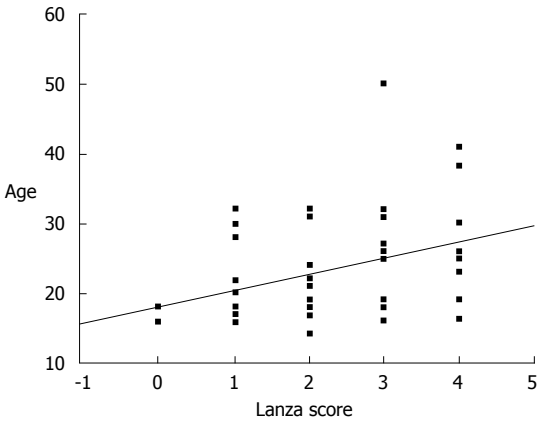

Figure 3 Correlation between age and LS.

BS

The BS of patients in group Ⅰ (2.3 ± 0.8) was significantly higher than in patients of group Ⅱ (1.47 ± 0.73) ($P < 0.01$). There was no correlation between BS and age, smoking habits, food intake, *H pylori* positivity or duration of drug exposure among group Ⅰ ($P > 0.05$). The BS of group Ⅰb patients (2.58 ± 0.70) was higher than in patients in group Ⅰa (1.94 ± 0.87) ($P < 0.05$). The BS of patients who ingested only NSAIDs was higher than in those who ingested only acetaminophen ($P < 0.05$). The BS of patients who took multi-agent NSAIDs and of those who took single-agent NSAIDs were similar ($P > 0.05$). There was no significant correlation between the total amount of ingested drug and BS ($P > 0.05$).

DISCUSSION

Besides personal risk factors, the type and the dose of NSAIDs, duration of exposure to the drug and concomitant use of other drugs play an important role on NSAID-related GI side effects[11]. In our study, we observed a positive correlation between endoscopically observed gastric injury and age, although none of our patients were in the age group (> 60 years) established as an individual risk factor. The gastric effects of NSAIDs vary according to their acute or chronic use. Short-term studies have reported that gastroduodenal mucosal injury is dose-dependent. Acute injury on the gastric superficial epithelium occurs within minutes, whereas subepithelial hemorrhage and erosions occur within hours[12,13]. Asymptomatic endoscopically observed lesions are submucosal hemorrhage, erosions, and ulceration. Intramucosal hemorrhage, petechia, and mucosal erosions have been observed endoscopically with short-term usage. Superficial ulceration typically appears within 1 wk[11]. Following single-dose (650-1300 mg) aspirin intake, gastric lesions occur with a 100% certainty[12]. This gastric mucosal injury has been reported to develop rapidly, within 24 h of ASA intake, erosions have been observed within the first 24 h, and maximal damage appeared within 3 to 7 d[8,14,15]. In our endoscopic evaluation within the first 24 h, hemorrhagic foci, erosions and marked gastric ulceration were observed. There were acute findings in the histomorphological evaluation such as congestion of the superficial lamina propria, edema, extravasated erythrocyte and fibrin aggregates within two patients whose LS was 0.

The gastric effects of long-term NSAID use are diverse. Gastric and duodenal mucosal erosions and gastroduodenal ulcers, hemorrhage, perforation and even fatality due to complications of ulceration can occur[12,16]. In long-term NSAID users, gastric injury is more likely to be more severe and has a higher possibility to be localized in the antrum than the corpus[13,17]. Lesions situated in the antrum might penetrate into the submucosa and tend to be wider in size (> 6 mm)[13,18]. Ulcers are most commonly localized in the stomach and are diffused, multiple and painless[4]. Many patients who show mucosal damage in the form of ulceration have no or mild symptoms, while many cases without visible mucosal damage present with indigestion, abdominal pain, distension and flatulence[8,16,19]. Sudden-onset GI hemorrhage or silent perforation has also been reported following ingestion of NSAIDs[8]. In our study of acute high dose use, physical examination and symptom inquiry did not reveal apparent differences and clinical presentation and endoscopic findings were not correlated as in the chronic NSAID users. None of the patients were admitted for gastric symptoms, they were rather admitted for the long term toxicity concerns. One patient with marked ulcerations had mild epigastric pain. Abdominal palpation was normal for two patients who had extensive superficial ulcerations and hemathinized fluid in nasogastric lavage.

Various studies have reported an increase in the risk of gastrointestinal toxicity with short-term NSAIDs use, and demonstrated that the first month of treatment presents the largest risk, while the risk thereafter decreases with chronic use[4]. A meta-analysis of 16 studies by Gabriel *et al* reported an 8-fold increase in the NSAID-related gastrointestinal risk within the first 1 mo, a 3.3-fold increase within 1-3 mo and 1.9-fold increase for longer than 3 mo of use[5]. LS was 4 in 20% of our cases, which present an example for risk of high-dose NSAIDs in the short-term use. This high percentage may be interpreted as the added ulceration effect of acute local toxic damage without a period of adaptation, thus these patients had multiple and large superficial ulcerations and marked hemorrhagic foci.

Endoscopic evaluations demonstrated that superficial lesions develop acutely, within minutes, and that topical factors prevail in the development of lesions. Acute lesions involve only the mucosa, are small in size, and are more prevalent in the fundus than in the antrum[13,18]. NSAID-related ulceration is most often localized in the greater curvature of the antrum. In adults, in the normal upright position, this region is susceptible to external factors, indicating that this type of ulceration (sump-ulcer) is related to the direct ingestion of corrosive substances[13]. A study on this subject reported more histological damage in the antrum than in the corpus[15]. In an endoscopic evaluation in 6 healthy dogs following administration of carprofen (4 mg/kg) and deracoxib (4 mg/kg), involvement of the fundus, antrum and lesser curvature was reported to deteriorate on the second day of treatment and heal on the fifth day[20]. Since early topical consequences are in the forefront in our cases, gastric lesion involvement in the fundus was observed in 41.7% of the patients who were in the supine position following drug intake, and in the antrum in 60.5% of the patients who remained standing, albeit without statistical significance. Considering that local damage is greater than systemic effects, this may account for the lack of significance of high-dose drug use with more intense topical exposure and localization.

Subsequent doses have been determined to increase the frequency, but not the severity of lesions[14,20]. The damage was greatest on the third day of continuous use, and endoscopic damage was lesser on the seventh day than the first day. Adaptation occurred at around the third day, and was found to be associated with increasing healing processes[14]. Endoscopic gastric damage is primarily dose-related and occurs even in anti-inflammatory doses of NSAIDs. In a study with 1 064 healthy volunteers, 6.7% of the subjects developed ulcerations after a 7-d course of anti-inflammatory dose[19]. Adaptation and resolution occur more slowly with high doses and more rapidly with low doses, and discontinuation accelerates the healing process[15]. We have preferred not to have a chronic NSAID user control group, so that we could demonstrate gastric damage in acute NSAID use.

Bergmann *et al*[21] administered single-dose ketoprofen (25 mg), ibuprofen (200 mg) or aspirin (500 mg) to 12 healthy subjects with empty stomach and found that lesions were similar with ketoprofen and ibuprofen using the LS and they were less local toxic compared to aspirin. Previous publications support the finding of the local damaging effect of short-term, single-dose use of NSAIDs[15]. In our study, the total amount of drug taken was found to be correlated with LS. We found a correlation between the total amount of drug taken and the severity of endoscopic findings. Also, multiple types of NSAID users had more endoscopic gastric damage than acetaminophen only or single NSAID users.

In a study that investigated damage on the gastric mucosa 10 and 60 min after the direct administration of 2 g acetaminophen in 100 mL saline solution, 10 min after administration cellular damage was seen in 3%-5% in the study group and 1%-7% in the control group. Microscopic evaluation showed focal cellular disruption and erythrocyte infiltration. Electron microscopy revealed minimal loss in the cellular apex, but no erosion. The corresponding findings with aspirin are quite different. A 2 g dose of acetaminophen caused minimal mucosal damage[22]. After a 7d continuous treatment on 7 healthy volunteers, aspirin and acetaminophen (1.95 g/d or 2.6 g/d) plus aspirin (1.95 g *vs* 3.9 g) on a full stomach caused escalating mucosal damage with increasing doses in the group that received aspirin. However, the addition of acetaminophen to the aspirin regimen did not cause any deterioration of gastric damage. All doses were received on a full stomach[23]. In the study group, food intake before ingestion of analgesics had no effect on LS or BS. Patients who took acetaminophen had ingested single doses of 4000 to 20 000 mg, and their endoscopic evaluations revealed LS of 0 to 4. The LS of

two patients who took 10 000 mg acetaminophen was 0 and 4, respectively. The unsteadiness of the BS might be due to the non-uniformity of gastric histomorphologic findings. In this study, the determination of endoscopic damage among patients who ingested higher doses of acetaminophen compared to previous studies[22,23] indicate that high-dose acetaminophen use may cause damage, however the damage and dose relationship suggests that factors other than dose also play a role.

Chemical gastropathy may present with separate or concurrent nonspecific histopathologic lesions of various degrees and proportions, and no correlation between histological findings and clinical symptoms, particularly the risk of bleeding, has been documented in gastropathy[24]. Following aspirin ingestion, the inconsistency of biopsy results during histological evaluations may reflect sampling errors, or aspirin-related changes may be unpredictable. None of the biopsies taken from normal-appearing mucosal tissue following aspirin intake yielded normal results. Visible damage may somewhat intensify after the second and third doses, but the extent of damage does not increase after a couple of doses, and may even start to diminish. Although mucosal appearance normalizes, gastric microhemorrhages do not cease to occur during aspirin use[15]. Histomorphological evaluation is significant in such cases[9]. In this study, BS was not correlated with the total amount of drug taken or the use of multiple NSAIDs. However, histological gastric damage was more extensive in patients who ingested NSAIDs compared to those who ingested acetaminophen only. There were no marked histomorphologic differences between biopsy evaluations of acetaminophen and NSAID patients. Nonetheless, the biopsies of all patients who took only acetaminophen revealed acute gastric damage, and six patients with a BS over 3 had ingested 6000 mg (n = 1) or 10 000 mg (n = 5) acetaminophen. The BS and LS of acetaminophen only patients did not correlate to the acetaminophen dose increments (Figure 4). Even the patient with a LS of 0 had findings of erosive gastritis in the histomorphologic evaluation. Also, all patients had more bleeding during endoscopic biopsy procedures compared to routine procedure or chronic NSAID users.

H pylori infection and NSAID-related gastric mucosal damage occur through different pathways and separate mechanisms[16]. Foveolar hyperplasia, edema and vascular ectasia are more extensive in NSAID-related gastritis compared with *H pylori* gastritis[13,17]. Gastric damage related to aspirin use was found to be more limited in patients with *H pylori* eradication, thus indicating that *H pylori* may enhance NSAID-induced gastric damage[25]. It was also reported that ulcer bleeding was more prevalent in patients with *H pylori* infections[26]. Conversely, certain studies have reported that positivity or negativity of *H pylori* in NSAID users made no significant difference[27,28]. In our study, *H pylori* positivity was not related to significant changes in LS or BS in patients who had taken acetaminophen and NSAIDs.

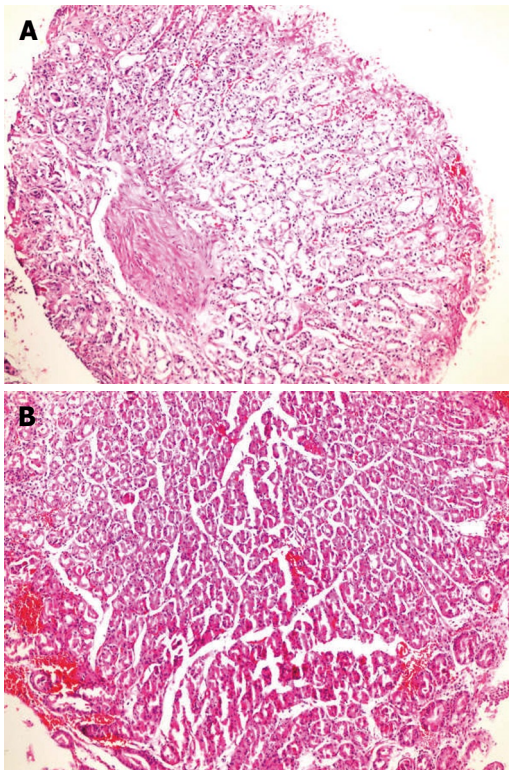

Figure 4 HE stain images, x 100. A: Acetaminophen only (10 000 mg) patient with LS = 4 and BS = 2. Erosion within superficial epithelium, fibrin clots on superficial lamina propria, widespread congested vessel structures and marked edema; B: Acetaminophen only (6000 mg) patient with LS = 3 and BS = 2. Focal erosion within superficial epithelium, extravasated erythrocyte clusters and congested veins.

CONCLUSION

Acute gastric damage of varying degrees occurs in subjects who ingest high doses of NSAIDs and acetaminophen with suicidal intent. The degree of endoscopic acute lesions in high-dose analgesic use also increases with age, total drug dose, concurrent use of multiple types of NSAIDs. We found the BS of patients who ingested only high-dose NSAIDs to be higher than in those who took acetaminophen, while there was no significant difference between gastric lesions. The lack of statistical significance between the LSs of patients who ingested NSAIDs and those who took only acetaminophen is remarkable in terms of the gastric effects of high-dose acetaminophen. It may be concluded that, contrary to current convictions, high-dose acetaminophen may also cause endoscopic acute gastric damage. Body position following intake influences the localization of endoscopic lesions. *H pylori* positivity was not determined as a factor that may affect NSAID- and acetaminophen-related acute gastric damage endoscopically or histopathologically.

In patients in a stable condition, we recommend endoscopy within the first 24 h for the determination of gastric injury and for treatment maintenance in patients who have ingested high-dose analgesics with suicidal intent.

COMMENTS

Background

Analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs) and acetaminophen, are commonly used for the relief of fever, headaches, and other minor aches and pains. The gastrointestinal side effects of NSAIDs are well documented and acetaminophen is accepted to be a safe drug for the gastrointestinal system. Acute effects of short-term, especially high-dose NSAID and acetaminophen use have not been studied adequately.

Research frontiers

Acute gastric injury due to high dose analgesic use. Gastrointestinal safety of acetaminophen. Scoring the histological severity of the gastritis.

Innovations and breakthroughs

This paper is one of the first to document the endoscopic acute gastric damage caused by acute high-dose acetaminophen.

Applications

The results of the present paper may be useful in evaluating the gastrointestinal complications of acute high dose analgesic use. Contrary to current convictions, high-dose acetaminophen, as well as NSAIDs, may also cause endoscopic acute gastric damage. Therefore physicians should be alert at evaluating the gastrointestinal complaints of acetaminophen users as well. The study suggests that clinicians might as well plan endoscopic evaluation in patients who ingested high-dose analgesics with suicidal intent.

Peer review

The importance of the research and the significance of the research contents are significant because as it is mentioned in the manuscript there is actually not much information about acute high-dose acetaminophen causing endoscopic acute gastric damage. The manuscript is very readable. Methods are appropriate and detailed description is provided for any modified or novel methods to allow other investigators to reproduce or validate.

REFERENCES

1 **Lanas A**, Hirschowitz BI. Toxicity of NSAIDs in the stomach and duodenum. *Eur J Gastroenterol Hepatol* 1999; **11**: 375-381
2 **Akarca US**. Gastrointestinal effects of selective and non-selective non-steroidal anti-inflammatory drugs. *Curr Pharm Des* 2005; **11**: 1779-1793
3 **Lanas A**. Prevention and treatment of NSAID-induced gastroduodenal injury. *Curr Treat Options Gastroenterol* 2006; **9**: 147-156
4 **Sung J**, Russell RI, Nyeomans, Chan FK, Chen S, Fock K, Goh KL, Kullavanijaya P, Kimura K, Lau C, Louw J, Sollano J, Triadiafalopulos G, Xiao S, Brooks P. Non-steroidal anti-inflammatory drug toxicity in the upper gastrointestinal tract. *J Gastroenterol Hepatol* 2000; **15** Suppl: G58-G68
5 **Gabriel SE**, Jaakkimainen L, Bombardier C. Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis. *Ann Intern Med* 1991; **115**: 787-796
6 **Burke A**, Smyth E, FitzGerald GA. Analgesic-antipyretic agent; Pharmacotherapy of gout. In: Brunton LL, Lazo JS, Parker KL. Goodman & Gillman's The Pharmacological Basis of Therapeutics. 11th ed. New York: McGraw-Hill, 2006: 671-715
7 **Lanza FL**, Royer GL Jr, Nelson RS, Rack MF, Seckman CC. Ethanol, aspirin, ibuprofen, and the gastroduodenal mucosa: an endoscopic assessment. *Am J Gastroenterol* 1985; **80**: 767-769
8 **Lanza FL**. Endoscopic studies of gastric and duodenal injury after the use of ibuprofen, aspirin, and other nonsteroidal anti-inflammatory agents. *Am J Med* 1984; **77**: 19-24
9 **Noffsinger AE**, Stemmermann GN, Lantz PE, Isaacson PG. The nonneoplastic stomach. In: Fenoglio-Preiser CM. Gastrointestinal Pathology: An Atlas and Text, 3rd edition. Philadelphia: Lippincott Williams & Wilkins, 2008: 135-231
10 **Bhattacharya B**. Non-Neoplastic Disorders of the Stomach. In: Christine A, Iacobuzio-Donahue, Elizabeth Montgomery. Gastrointestinal and Liver Pathology (A Volume in the Series Foundations in Diagnostic Pathology). London: Churchill-Livingstone, 2005: 66-125
11 **Aalykke C**, Lauritsen K. Epidemiology of NSAID-related gastroduodenal mucosal injury. *Best Pract Res Clin Gastroenterol* 2001; **15**: 705-722
12 **Lichtenstein DR**, Syngal S, Wolfe MM. Nonsteroidal antiinflammatory drugs and the gastrointestinal tract. The double-edged sword. *Arthritis Rheum* 1995; **38**: 5-18
13 **Graham DY**. Peptic diseases of the stomach and duodenum. In: Sivak MV. Gastroenterologic Endoscopy. Philadelphia: Saunders, 2000: 615-641
14 **Graham DY**, Smith JL, Dobbs SM. Gastric adaptation occurs with aspirin administration in man. *Dig Dis Sci* 1983; **28**: 1-6
15 **Graham DY**, Smith JL, Spjut HJ, Torres E. Gastric adaptation. Studies in humans during continuous aspirin administration. *Gastroenterology* 1988; **95**: 327-333
16 **Peura DA**. Prevention of nonsteroidal anti-inflammatory drug-associated gastrointestinal symptoms and ulcer complications. *Am J Med* 2004; **117** Suppl 5A: 63S-71S
17 **Haber MM**, Lopez I. Gastric histologic findings in patients with nonsteroidal anti-inflammatory drug-associated gastric ulcer. *Mod Pathol* 1999; **12**: 592-598
18 **Soll AH**, Weinstein WM, Kurata J, McCarthy D. Nonsteroidal anti-inflammatory drugs and peptic ulcer disease. *Ann Intern Med* 1991; **114**: 307-319
19 **Lanza FL**. A review of gastric ulcer and gastroduodenal injury in normal volunteers receiving aspirin and other non-steroidal anti-inflammatory drugs. *Scand J Gastroenterol Suppl* 1989; **163**: 24-31
20 **Dowers KL**, Uhrig SR, Mama KR, Gaynor JS, Hellyer PW. Effect of short-term sequential administration of nonsteroidal anti-inflammatory drugs on the stomach and proximal portion of the duodenum in healthy dogs. *Am J Vet Res* 2006; **67**: 1794-1801
21 **Bergmann JF**, Chassany O, Genève J, Abiteboul M, Caulin C, Segrestaa JM. Endoscopic evaluation of the effect of ketoprofen, ibuprofen and aspirin on the gastroduodenal mucosa. *Eur J Clin Pharmacol* 1992; **42**: 685-687
22 **Ivey RJ**, Silvoso GR, Krause WJ. Effect of paracetamol on gastric mucosa. *Br Med J* 1978; **1**: 1586-1588
23 **Graham DY**, Smith JL. Effects of aspirin and an aspirin-acetaminophen combination on the gastric mucosa in normal subjects. A double-blind endoscopic study. *Gastroenterology* 1985; **88**: 1922-1925
24 **Genta RM**. Differential diagnosis of reactive gastropathy. *Semin Diagn Pathol* 2005; **22**: 273-283
25 **Giral A**, Ozdogan O, Celikel CA, Tozun N, Ulusoy NB, Kalayci C. Effect of Helicobacter pylori eradication on anti-thrombotic dose aspirin-induced gastroduodenal mucosal injury. *J Gastroenterol Hepatol* 2004; **19**: 773-777
26 **Adamopoulos A**, Efstathiou S, Tsioulos D, Tsami A, Mitromaras A, Mountokalakis T. Acute upper gastrointestinal bleeding: comparison between recent users and nonusers of nonsteroidal anti-inflammatory drugs. *Endoscopy* 2003; **35**: 327-332
27 **Pilotto A**, Franceschi M, Leandro G, Di Mario F, Valerio G. The effect of Helicobacter pylori infection on NSAID-related gastroduodenal damage in the elderly. *Eur J Gastroenterol Hepatol* 1997; **9**: 951-956
28 **Niv Y**, Battler A, Abuksis G, Gal E, Sapoznikov B, Vilkin A. Endoscopy in asymptomatic minidose aspirin consumers. *Dig Dis Sci* 2005; **50**: 78-80

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RAPID COMMUNICATION

Non-traumatic splenic rupture: Report of seven cases and review of the literature

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Abstract

AIM: To evaluate seven patients with non-traumatic splenic rupture (NSR). NSR is an uncommon dramatic abdominal emergency that requires immediate diagnosis and prompt surgical treatment to ensure the patient's survival.

METHODS: Within 11 years, seven cases were evaluated for patient characteristics, anamnesis and symptoms, method of diagnosis, findings of laparotomy, and etiology of NSR.

RESULTS: There were six (86%) male and one female (14%) patient, whose mean age was 36 ± 12.8 (17-56) years. We report here four cases of *Plasmodium vivax* malaria (cases Ⅰ-Ⅳ), one case of hemodialysis (case Ⅴ), one case of spontaneous splenic rupture (case Ⅵ), and one case of hairy cell leukemia (case Ⅶ). Splenectomy was performed in all patients. All of them made an uneventful recovery and were discharged in stable condition.

CONCLUSION: NSR is a rare entity that needs a high index of suspicion for diagnosis. Using ultrasonography or computer tomography, and peritoneal aspiration of fresh blood may assist in the diagnosis of NSR. Increased awareness of NSR can enhance early diagnosis and effective treatment.

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Key words: Hairy cell leukemia; Hemodialysis; Malaria; Splenic rupture

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INTRODUCTION

Trauma is the most common cause of splenic rupture, while non-traumatic splenic rupture (NSR) is a rare condition. NSR has been described in the medical literature as a clinical oddity with grave consequences, if unrecognized and untreated[1]. NSR rarely occurs in a histologically proven normal spleen, and in such cases, is called a spontaneous rupture. NSR usually occurs in a diseased spleen and is called a pathologic rupture[2]. Infection, malignancy, metabolic disorders, as well as vascular and hematological diseases, of which only single case reports have been published in the literature, are the usual reasons[3-5]. Recently, some authors have reported that spontaneous splenic rupture has been seen as a factor in malaria[6,7], aortic valve replacement for bacterial endocarditis[8], normal spleen[9], factor Ⅷ deficiency, which is a rare autosomal bleeding disorder with a frequency of 1:2 000 000 in the general population[10], and autologous transplantation for primary systemic amyloidosis[11]. Especially, malaria was discussed retrospectively in all aspects by authors. It has been reported that the disease has started spreading from its hotspots to new areas, including many parts of the United States and southern Europe, including Azerbaijan and Turkey[12]. We report here seven cases with NSR, who were successfully treated by splenectomy.

MATERIALS AND METHODS

The medical records of seven patients with NSR, who

Table 1 Patient characteristics

Cause of NSR	Age/sex	TA (mmHg)	Splenic weight (g)	Diagnosis		Observation time (h)	Amount of free blood (cc)	Grade of splenic injury	Amount of blood transfusion (U)	Duration of hospitalization (d)
				Parasynthesis	Radiological imaging					
Malaria (Case Ⅰ)	56/M	80/60	950	+	USG	4	1500	Ⅲ	3	6
Malaria (Case Ⅱ)	32/M	70/40	1050	+	None	Urgent Laparotomy	1200	Ⅱ	2	11
Malaria (Case Ⅲ)	46/M	90/60	800	+	USG	6	1700	Ⅲ	2	5
Malaria (Case Ⅳ)	17/M	80/50	980	+	USG	5	1500	Ⅲ	5	4
Hemodialysis patient (Case Ⅴ)	29/M	80/40	220	+	USG	3	1500	Ⅲ	3	5
Spontaneous (Case Ⅵ)	30/M	120/70	195	+	USG/CT	18	2000	Ⅱ	6	5
Hairy Cell Leukemia (Case Ⅶ)	42/F	65/40	2540	+	None	Urgent Laparotomy	2500	Ⅲ	5	15

NSR: Nontraumatic splenic rupture; TA: Tension arterial; USG: Ultrasonography; CT: Computerized tomography.

were treated in the Department of General Surgery, Dicle University Hospital, between 1995 and 2006, were reviewed retrospectively. The patients did not have a minor or trivial trauma history before admission. Standard advanced trauma life support resuscitation protocols were used in all patients. All patients received preoperative antibiotics and were maintained on antibiotics for at least 24 h postoperatively. Splenic injury grade was defined in accordance with the Organ Injury Scaling Committee of the American Association of Surgery for Trauma (AAST)[13]. After splenectomy, Pneumovax and *Haemophilus influenzae* vaccines were given. Histopathological analysis of masses was performed.

Patient data were evaluated for their characteristics (Table 1), anamnesis and symptoms, method of diagnosis, findings of laparotomy, and etiology of NSR. The constant variables were expressed as mean ± SD, except where otherwise stated.

RESULTS

Cases I -IV

We determined that NSR was due to malaria in four patients. The mean age of these patients was 37.7 ± 8.48 (17-56) years, and all of them were male. Our area is endemic for malaria, and before patients presented to our hospital, they had been diagnosed as malaria by the Free Malaria Out-Patient Clinic, which treats for free patients who have been taking antimalarial therapy. Trophozoites and schizonts of *Plasmodium vivax* were identified in peripheral blood smears to confirm previous diagnoses in all patients in our institute. No patient had a history of glucose-6-phosphate dehydrogenase deficiency. The abdominal ultrasound scans of three patients showed an enlarged, ruptured spleen and intraperitoneal free blood. Gross examination revealed grayish-brown or dark grey discolored spleen with capsular tears. Microscopy revealed congestion and dilatation of sinusoids, mononuclear infiltration with focal necrosis in capillaries and splenic pulp. Mean hospitalization time of patients who had splenic rupture with malaria etiology was 6.50 ± 1.55 (4-11) d. The patient who was hospitalized for the longest period of 11 d was receiving treatment for duodenal ulcer in the gastroenterology department. He immediately became hypotensive, and had abdominal pain on the day 4 of hospitalization. We performed abdominal parasynthesis, which demonstrated free blood in the peritoneal cavity. Since he was in shock, he underwent explorative laparotomy, and splenectomy was performed. The patient had postoperative wound infection on day 5, and he was discharged on day 11 after therapy.

Case V

A 29-year-old male hemodialysis patient was admitted to our emergency department. Severe left upper abdominal pain developed 2 d prior to admission. He had a history of hypertension and urolithiasis for 6 years, and he was undergoing hemodialysis twice weekly. Abdominal ultrasonography revealed a hyperechogenic mass that was composed of spleen and perisplenic fluid, and another mass, which showed internal echogenicity. A moderate amount of free fluid was present in the pelvic abdomen (Figure 1). Gross examination showed a dark gray spleen, which weighed 220 g, with capsular tears and subcapsular hematoma on the medial side. Microscopy revealed sinus dilatation, and increased fibrous tissue and hemosiderin pigments. The postoperative course was uneventful, and the patient was discharged from hospital on day 5.

Case VI

A healthy 30-year-old man was admitted to the emergency department previously with complaints of abdominal pain. His pain began approximately 3 h before arrival, which he described as being vague periumblical pain. He had no recent illness or trauma and no medical or surgery history. Initially, pain was minimal and he had no peritoneal signs, and his abdomen was non-distended, with normal bowel sounds. He had epigastric and left upper quadrant tenderness with voluntary guarding in this region. He denied taking any drugs, and did not have any symptoms and signs such as nausea, vomiting, diarrhea, melena, hematochezia, cough,

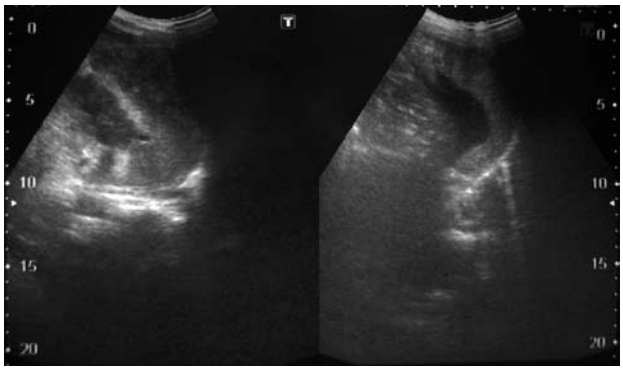

Figure 1 Abdominal ultrasonography of case Ⅴ revealed a hyperechogenic mass composed of spleen and perisplenic fluid.

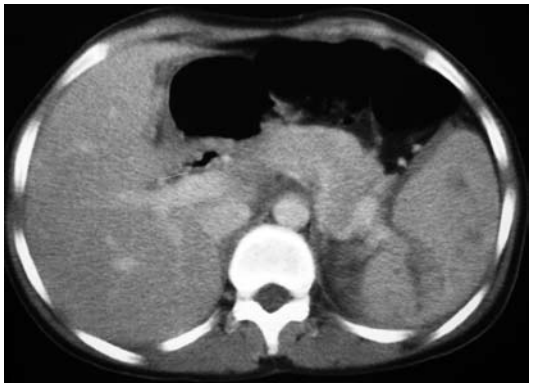

Figure 2 CT of case Ⅵ revealed massive hemoperitoneum and large tears of the splenic hilus.

shortness of breath, weight loss, or fever. Heart and lung examination was normal. The genitourinary examination was normal, without hernia. Abdominal parasynthesis was performed, and free blood in the peritoneal cavity was observed. Abdominal ultrasonography showed perisplenic and left paracolic free fluid. Furthermore, computerized tomography (CT) showed massive hemoperitoneum and large tears of the splenic hilus (Figure 2). In spite of non-operative management at 18 h after arrival at the emergency department, he became hemodynamically unstable and had to be taken to the operating room for emergency surgery. Exploratory laparotomy was performed. After opening of the abdomen, 2000 mL of blood was removed immediately. The spleen was removed without complication and was sent for pathological investigation. The pathology report demonstrated the spleen to be of normal size. The rupture was found in medial side, penetrating into the parenchyma of the spleen. Paired acute-phase and convalescent sera provided no evidence of acute viral infection, and cultures from blood, sputum, stool and urine were negative. Screening for autoantibodies was negative. The patient's hospital course was uneventful, and he was discharged on postoperative day 5.

Case Ⅶ

A 42-year-old woman was admitted to our hospital with a history of weakness, easy fatigability, weight loss, and left upper abdominal pain for one month. After splenomegaly was determined, the patient was hospitalized in our department for etiological investigation. After one day of hospitalization, she developed sudden hypotension, tachycardia, and generalized abdominal pain. There was abdominal distension and peritoneal irritation upon physical examination. We performed abdominal parasynthesis, which demonstrated free blood in the peritoneal cavity. Laparotomy showed extensive hemoperitoneum and markedly enlarged spleen, with areas in which it appeared to be a tumor pushing through the splenic capsule, which was torn and bleeding excessively. Microscopic examination of the spleen revealed that splenic sinuses and cords (Figure 3A) were surrounded by hairy cells in the expanded red pulp (Figure 3B). The patient had a complication of pneumonia on postoperative day 5, which was managed successfully with antibiotic therapy; she made an uneventful recovery and was discharged in stable condition after 15 d.

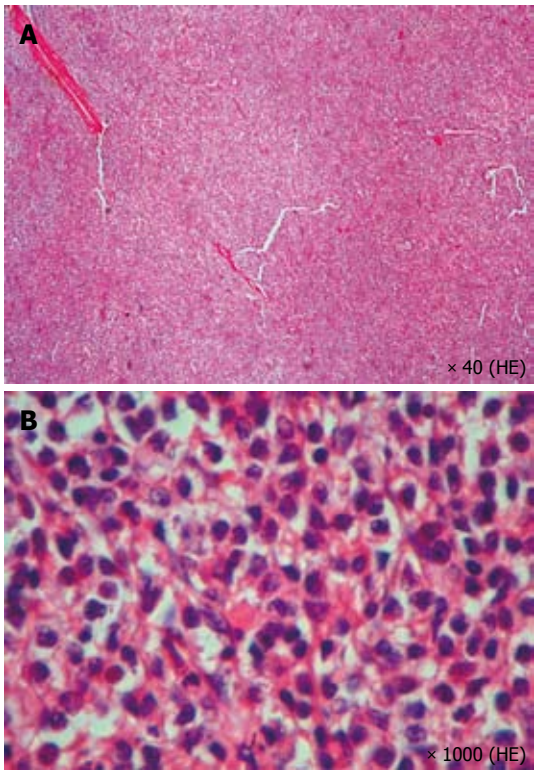

Figure 3 HE staining reveals splenic sinuses and cords of case Ⅶ (A, x 40) surrounded by hairy cells in the expanded red pulp (B, x 1000).

DISCUSSION

NSR may occur in 0.1%-0.5% of patients with no associated trauma[14]. The first cases of spontaneous splenic rupture were reported by Rokitansky[15] in 1861 and Atkinson[16] in 1874.

The actual reason for the rupture is not yet fully understood, although the size of the organ plays a significant role. However, normal-sized organs have been reported with ruptured spleen[17]. Three mechanisms have been implicated in the process. The first of these

mechanisms is increased intrasplenic tension that is caused by cellular hyperplasia and engorgement. The second is that the spleen may be compressed by the abdominal musculature during physiological activities, such as sneezing, coughing and defecation. Finally, vascular occlusion caused by reticular endothelial hyperplasia, which results in thrombosis and infarction, may be involved. This leads to interstitial and subcapsular hemorrhage and stripping of the capsule, which causes further subcapsular hemorrhage. The distended capsule finally gives way[18,19]. A Medline search for cases of NSR revealed that 454 cases were reported between 1966 and February 2007[20,21].

Malaria is the most common cause of pathologic rupture of spleen in the tropics, and life-threatening complications occur in up to an estimated 2% of cases[6,7,12,20,22]. Most cases of pathologic rupture of the spleen in malaria occur during acute infection, and usually during the primary attack[23]. Chronically enlarged spleens are less vulnerable to rupture[20]. When a palpable spleen is present, it is generally recognized within 3-4 d of the onset of symptoms. If the disease goes untreated, the spleen may grow and result in greater average spleen size, as the prevalence of malaria increases[24]. This occurrence is likely caused by rapid hyperplasia and stretching of splenic parenchyma and capsule, a high frequency of small infarctions, hemorrhage and tears, a lack of extensive connective tissue and fibrosis (as found in chronic malarial spleens); an increased risk of minor stress to the spleen (e.g. vomiting, rigors) and a lack of prior immunity[23]. Spontaneous splenic rupture is more common with *P. vivax* than *Plasmodium falciparum* malaria. In Turkey, *P. vivax* is the predominant *Plasmodium* species (99.9%)[18,25]. In all our patients, *P. vivax* was present with pathologic splenic rupture. Patients were experiencing primary attacks of malaria and were in the acute stage of the disease.

Spontaneous rupture of the spleen is extremely rare[2,26-28]. According to Orloff and Peksin[29], a small group of cases exist, in which the only justifiable conclusion is that the spleen ruptures spontaneously without known cause. They identified four criteria for the diagnosis of spontaneous splenic rupture: (1) on thorough questioning, either prior to operation or in retrospect after operation, there should be no history of trauma or unusual activity that could conceivably injure the spleen; (2) there should be no evidence of disease in other organs that are known to affect the spleen adversely and thereby could cause it to rupture; (3) there should be no evidence of perisplenic adhesions or scarring of the spleen that suggests it has been traumatized or ruptured previously; (4) without findings of hemorrhage and rupture, the spleen should be normal on both gross inspection and histological examination. Crate *et al*[30] added a fifth criterion: studies of acute-phase and convalescent sera should not show any significant rise in viral antibody titers that are suggestive of recent infection with viruses associated with splenic involvement. The patient, who was accepted with spontaneous splenic rupture, had these five criteria. The pathophysiology of spontaneous splenic rupture is obscure. It should be considered that undetected structural abnormalities in spleen may cause NSR.

Pathological rupture of the spleen is most commonly seen in the hematological malignancies[31], in which fragmentation and dissolution of the fibrous capsule of the spleen occur by infiltrating atypical lymphocytes, as seen in lymphoma or leukemia[20]. Giagounidis *et al*[32] have reported that male sex, adulthood, severe splenomegaly and cytoreductive chemotherapy are factors that are associated more often with rupture of the spleen in hematological malignancies. The most common finding was splenomegaly, which was present in 96% of patients with hairy cell leukemia[33]. One of the patients with pathological splenic rupture had hairy cell leukemia, with marked splenomegaly. In patients with hematological malignancy, pathological rupture of the spleen often happens unexpectedly, without preceding trauma[34]. Therefore, diagnosis is often difficult, and diagnosis of splenic rupture must be considered in all patients with hematologic malignancies. Acute or subacute new abdominal pain, hypotension and tachycardia, even if there is no previous history of trauma, must be treated aggressively.

Dialysis patients with chronic renal failure (CRF) show a fibrinolysis defect at the level of plasminogen activation. Reduced fibrinolysis may be responsible, along with other factors, for development of thrombosis, atherosclerosis and their complications[35]. Abnormal homeostasis is also common in CRF and is characterized by a tendency to abnormal bleeding and bruising. Prolongation of bleeding time, decreased activity of platelet factor Ⅲ, abnormal platelet aggregation and adhesiveness, and impaired prothrombin consumption contribute to the clotting defects. Besides, edema of the spleen and formation of a subcapsular hematoma (secondary to uremic coagulopathy and use of heparin in those who are on hemodialysis) may occur in uremia[36]. Fluid overload is the major cause of hypertension in uremia; the normotensive state usually can be restored by aggressive ultrafiltration with dialysis. Nevertheless, because of hyper-reninemia, in spite of rigorous salt and water restriction and ultrafiltration, some patients maintain hypertension[37]. We detected hypertension in our patients. The underlying cause for the NSR may be uremic coagulopathy and hypertension.

The most common symptom is left upper quadrant abdominal pain. This pain can become generalized, with distention, tenderness and rigidity in later stages. The abdominal symptoms may be accompanied by pallor, tachycardia, hypotension and oliguria. Eventually, more than half of patients will suffer hemorrhagic shock if the condition is left untreated[27,38]. Diagnosis is based on clinical symptoms and confirmatory diagnostic tests. Some authors have reported that paracentesis is the most effective diagnostic procedure[4,39]. Abdominal ultrasound is an inexpensive and practical way to obtain a quick diagnosis of intraperitoneal fluid accumulation or hematoma, which can be performed at the patient's bedside or in the emergency unit[28]. CT signs of NSR may be useful for predicting rupture, and clearly show

grade of splenic damage severity and intraperitoneal free fluid[2]. We think that abdominal ultrasound can be a good, non-invasive technique, without risk for the patients who are hemodynamically unstable, whereas CT can be useful for patients who are hemodynamically stable. Paracentesis is not only useful in NSR, but also in patients in whom we think there is intraperitoneal hemorrhage and who are unstable. Negative results of paracentesis are not certain to show that there is no hemorrhage, but a positive result indicates the possibility of intraperitoneal hemorrhage. In fact, if paracentesis is positive, it leads us to consider the possibility of intra-abdominal hemorrhage.

The management of spontaneous or pathological splenic hemorrhage has been debated constantly. Among 136 cases of pathological splenic rupture reported in the literature, 88 underwent surgical intervention, 55 (63%) survived and 33 (37%) died. Among 43 patients who did not undergo surgery, 40 died. No information could be obtained for the five remaining cases[32]. Aggressive management with early surgical intervention and appropriate hemoderivative support is important[40]. The survival of patients following splenectomy is probably well correlated with the course of the underlying disease. The trauma literature is replete with data supporting the role of non-operative management of low-grade splenic injuries in hemodynamically stable patients[41,42]. The same principle has been applied for the management of spontaneous splenic rupture[43]. In our series, since six patients were hemodynamically unstable, after rapid fluid and blood infusions, the patients underwent splenectomy. One patient, who had SRS, and was hemodynamically stable at admission, to avoid the risks of splenectomy, was given medical therapy at first, but after 18 h, the patient became unstable; therefore, splenectomy was performed. One patient developed wound infection and another pneumonia, but all patients were discharged uneventfully.

In conclusion, NSR is a rare entity that needs a high index of suspicion for diagnosis. Absence of a history of trauma can make it difficult to reach a diagnosis, which causes delay in treatment. Using ultrasonography or CT, and peritoneal aspiration of fresh blood may assist in the diagnosis of NSR. Rapid diagnosis, aggressive resuscitation, and surgical intervention can lead to a successful outcome in patients with NSR.

COMMENTS

Background

NSR is a rare condition in emergency surgery. NSR may be seen along with different diseases, such as malaria, infections, malignancies, metabolic disorders, as well as vascular and hematological diseases. Also, spontaneous rupture of the spleen may be observed. Absence history of a trauma may not remind a rupture needs a high index of suspicion for diagnosis in spleen.

Research frontiers

The criteria for NSR were first described by Orloff *et al* in 1958 and our patients with NSR were in accordance with these criteria.

Innovations and breakthroughs

Our study emphasized that rapid diagnosis, aggressive resuscitation, and surgical intervention are important for successful outcome in patients with NSR. If the patient with intra-abdominal hemorrhage has no associated trauma, splenic rupture should be considered.

Applications

NSR may be shown in particular in endemic regions of malaria, hematological malignancies, and spontaneous and chronic renal failure.

Peer review

In this study, NSR was presented with different diseases. Diagnosis of NSR, using ultrasonography or CT, and paracentesis, is difficult. Splenectomy may lead to a successful outcome in patients with NSR.

REFERENCES

1 **Hyun BH**, Varga CF, Rubin RJ. Spontaneous and pathologic rupture of the spleen. *Arch Surg* 1972; **104**: 652-657
2 **Torricelli P**, Coriani C, Marchetti M, Rossi A, Manenti A. Spontaneous rupture of the spleen: report of two cases. *Abdom Imaging* 2001; **26**: 290-293
3 **Andrews DF**, Hernandez R, Grafton W, Williams DM. Pathologic rupture of the spleen in non-Hodgkin's lymphoma. *Arch Intern Med* 1980; **140**: 119-120
4 **Bauer TW**, Haskins GE, Armitage JO. Splenic rupture in patients with hematologic malignancies. *Cancer* 1981; **48**: 2729-2733
5 **Gallerani M**, Vanini A, Salmi R, Bertusi M. Spontaneous rupture of the spleen. *Am J Emerg Med* 1996; **14**: 333-334
6 **Jimenez BC**, Navarro M, Huerga H, Lopez-Velez R. Spontaneous splenic rupture due to Plasmodium vivax in a traveler: case report and review. *J Travel Med* 2007; **14**: 188-191
7 **Tauro LF**, Maroli R, D'Souza CR, Hegde BR, Shetty SR, Shenoy D. Spontaneous rupture of the malarial spleen. *Saudi J Gastroenterol* 2007; **13**: 163-167
8 **Dimitrakakis G**, Von Oppell U, Zilidis G, Srivastava A. Splenic rupture complicating aortic valve replacement for bacterial endocarditis. *Interact Cardiovasc Thorac Surg* 2008; **7**: 138-140
9 **Vahid B**, Bosanac A, Marik P. Spontaneous rupture of the normal spleen: a case report. Priory Medical Journals, Surgery On-line, 2005-10-8. Available from: URL: http://www.priory.com/surgery/spleen.pdf
10 **Khalife H**, Muwakkit S, Al-Moussawi H, Dabbous I, Khoury R, Peyvandi F, Abboud MR. Spontaneous splenic rupture in a patient with factor XIII deficiency and a novel mutation. *Pediatr Blood Cancer* 2008; **50**: 113-114
11 **Fernandez de Larrea C**, Cibeira MT, Rovira M, Rosinol L, Esteve J, Blade J. Spontaneous rupture of the spleen as immediate complication in autologous transplantation for primary systemic amyloidosis. *Eur J Haematol* 2008; **80**: 182-184
12 **Ramana B**. Malaria: who is at fault? *World J Surg* 2007; **31**: 2072-2074
13 **Moore EE**, Shackford SR, Pachter HL, McAninch JW, Browner BD, Champion HR, Flint LM, Gennarelli TA, Malangoni MA, Ramenofsky ML. Organ injury scaling: spleen, liver, and kidney. *J Trauma* 1989; **29**: 1664-1666
14 **Lai PK**. Infectious mononucleosis: recognition and management. *Hosp Pract* 1977; **12**: 47-52
15 **Laseter T**, McReynolds T. Spontaneous splenic rupture. *Mil Med* 2004; **169**: 673-674
16 **Badenoch DF**, Maurice HD, Gilmore OJ. Spontaneous rupture of a normal spleen. *J R Coll Surg Edinb* 1985; **30**: 326-327
17 **Zieren J**, Paul M, Scharfenberg M, Muller JM. The spontaneous splenic rupture as first manifestation of mantle cell lymphoma, a dangerous rarity. *Am J Emerg Med* 2004; **22**: 629-631
18 **Yagmur Y**, Kara IH, Aldemir M, Buyukbayram H, Tacyildiz IH, Keles C. Spontaneous rupture of malarial spleen: two case reports and review of literature. *Crit Care* 2000; **4**: 309-313
19 **Patel MI**. Spontaneous rupture of a malarial spleen. *Med J Aust* 1993; **159**: 836-837

20 **Debnath D**, Valerio D. Atraumatic rupture of the spleen in adults. *J R Coll Surg Edinb* 2002; **47**: 437-445
21 **Tataria M**, Dicker RA, Melcher M, Spain DA, Brundage SI. Spontaneous splenic rupture: the masquerade of minor trauma. *J Trauma* 2005; **59**: 1228-1230
22 **Ozsoy MF**, Oncul O, Pekkafali Z, Pahsa A, Yenen OS. Splenic complications in malaria: report of two cases from Turkey. *J Med Microbiol* 2004; **53**: 1255-1258
23 **Zingman BS**, Viner BL. Splenic complications in malaria: case report and review. *Clin Infect Dis* 1993; **16**: 223-232
24 **Russel PF**, West LS, Manwell RD, Macdonald G. Practical Malariology. 2nd ed. London: Oxford University Press, 1963: 371-485
25 **Akdur R**. Epidemiology of malaria [in Turkish]. In: Ozcel MA. Sitma, Malaria. Izmir: Ege Universitesi Basimevi, 1999: 51-118
26 **Tu AS**, Tran MHT, Larsen CR. Spontaneous splenic rupure: report of five cases and a review of the literature. *Emerg Radiol* 1997; **4**: 415-418
27 **Denehy T**, McGrath EW, Breen JL. Splenic torsion and rupture in pregnancy. *Obstet Gynecol Surv* 1988; **43**: 123-131
28 **Klinkert P**, Kluit AB, de Vries AC, Puylaert JB. Spontaneous rupture of the spleen: role of ultrasound in diagnosis, treatment, and monitoring. *Eur J Surg* 1999; **165**: 712-713
29 **Orloff MJ**, Peksin GW. Spontaneous rupture of the normal spleen; a surgical enigma. *Int Abstr Surg* 1958; **106**: 1-11
30 **Crate ID**, Payne MJ. Is the diagnosis of spontaneous rupture of a normal spleen valid? *J R Army Med Corps* 1991; **137**: 50-51
31 **Oinonen R**, Franssila K, Elonen E. Spontaneous splenic rupture in two patients with a blastoid variant of mantle cell lymphoma. *Ann Hematol* 1997; **74**: 33-35
32 **Giagounidis AA**, Burk M, Meckenstock G, Koch AJ, Schneider W. Pathologic rupture of the spleen in hematologic malignancies: two additional cases. *Ann Hematol* 1996; **73**: 297-302
33 **Hoffman MA**. Clinical presentations and complications of hairy cell leukemia. *Hematol Oncol Clin North Am* 2006; **20**: 1065-1073
34 **Biswas S**, Keddington J, McClanathan J. Large B-cell lymphoma presenting as acute abdominal pain and spontaneous splenic rupture; a case report and review of relevant literature. *World J Emerg Surg* 2006; **1**: 35
35 **Opatrny K Jr**. Hemostasis disorders in chronic renal failure. *Kidney Int Suppl* 1997; **62**: S87-S89
36 **Zbrog Z**, Pawlicki L. [Spontaneous rupture of the spleen as a cause of death of a patient with uremia] *Pol Tyg Lek* 1989; **44**: 232-233
37 **Lazarus JM**, Brenner BM. Chronic renal failure. In: Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL, Longo DL. Harrison's Principles of Internal Medicine. 14th ed. USA: The McGraw-Hill, 1998: 1513-1520
38 **Toubia NT**, Tawk MM, Potts RM, Kinasewitz GT. Cough and spontaneous rupture of a normal spleen. *Chest* 2005; **128**: 1884-1886
39 **Altes A**, Brunet S, Martinez C, Soler J, Ayats R, Sureda A, Lopez R, Domingo A. Spontaneous splenic rupture as the initial manifestation of acute lymphoblastic leukaemia: immunophenotype and cytogenetics. *Ann Hematol* 1994; **68**: 143-144
40 **Bernat S**, Garcia Boyero R, Guinot M, Lopez F, Gozalbo T, Canigral G. Pathologic rupture of the spleen as the initial manifestation in acute lymphoblastic leukemia. *Haematologica* 1998; **83**: 760-761
41 **King DR**, Lobe TE, Haase GM, Boles ET Jr. Selective management of injured spleen. *Surgery* 1981; **90**: 677-682
42 **Smith JS Jr**, Wengrovitz MA, DeLong BS. Prospective validation of criteria, including age, for safe, nonsurgical management of the ruptured spleen. *J Trauma* 1992; **33**: 363-368; discussion 368-369
43 **Brichkov I**, Cummings L, Fazylov R, Horovitz JH. Nonoperative management of spontaneous splenic rupture in infectious mononucleosis: the role for emerging diagnostic and treatment modalities. *Am Surg* 2006; **72**: 401-404

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RAPID COMMUNICATION

Helicobacter pylori infection and expression of DNA mismatch repair proteins

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Abstract

AIM: To determine the expression of DNA (MMR) proteins, including hMLH1 and hMSH2, in gastric epithelial cells in the patients with or without *Helicobacter pylori (H pylori)*-infected gastritis.

METHODS: Fifty *H pylori*-positive patients and 50 *H pylori*-negative patients were enrolled in the study. During endoscopy of patients with non-ulcer dyspepsia, two antral and two corpus biopsies were taken for histological examination (Giemsa stain) and for immunohistochemical staining of hMLH1 and hMSH2.

RESULTS: The percentage of epithelial cell nuclei that demonstrated positivity for hMLH1 staining was 84.14 ± 7.32% in *H pylori*-negative patients, while it was 73.34 ± 10.10% in *H pylori*-positive patients ($P < 0.0001$). No significant difference was seen between the two groups regarding the percentage of epithelial cell nuclei that demonstrated positivity for hMSH2 staining (81.16 ± 8.32% in *H pylori*-negative *versus* 78.24 ± 8.71% in *H pylori*-positive patients; $P = 0.09$).

CONCLUSION: This study indicates that *H pylori* might promote development of gastric carcinoma at least in part through its ability to affect the DNA MMR system.

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Key words: *Helicobacter pylori*; DNA mismatch repair; hMLH1, hMSH2

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INTRODUCTION

Helicobacter pylori (H pylori) infection affects about half of the world's population, and gastric carcinoma is one of the most frequent malignancies despite a decrease in incidence and mortality in recent decades[1,2]. The association of *H pylori* with gastric cancer is supported by epidemiological studies that show odds ratios for gastric cancer up to nine-fold greater in *H pylori*-infected individuals[3]. Chronic *H pylori* infection can cause chronic gastritis, which often progresses to gastric atrophy and intestinal metaplasia, which are premalignant lesions of the stomach[4]. Although many epidemiological studies that address the association of *H pylori* infection and gastric cancer have been carried out, fewer advances have been made to understand how long it takes for *H pylori* infection to induce the development of gastric cancer.

The main molecular mechanisms that underlie cancer development include the overexpression of genes, including oncogenes and growth factors or their receptors, and impaired expression of tumor suppressor genes that results from mutation or allelic losses[5,6] and deficiencies of the DNA mismatch repair (MMR) system[7,8].

Impairment of the DNA MMR system is a known mechanism of carcinogenesis and tumor progression

of both sporadic and hereditary human cancers[9,10]. The MMR deficiency leads to the accumulation of base-base mismatches, and short insertion/deletion mispairing during DNA replication, which results in widespread mutation generated as a consequence of DNA replication errors[11]. Most cells deficient in MMR display a high level of genomic instability characterized by changes in simple repetitive sequences, so-called microsatellite instability (MSI). Chronic *H pylori* infection damages the gastric barrier function[12,13] and stimulates gastric cell proliferation[14-19], which leads to mucosal repair[20], but which can also induce cellular DNA damage[18-22].

H pylori gastritis occurs more frequently in individuals with MSI-positive than MSI-negative gastric cancers, which raises the possibility that *H pylori* infection affects the DNA MMR system[23].

MATERIALS AND METHODS

Patients

We examined dyspeptic patients who were referred for endoscopic evaluation to Taleghani hospital, a tertiary hospital in Tehran, Iran. Dyspepsia was defined as persistent or recurrent abdominal pain or discomfort, centered in the upper abdomen, with a duration of at least 3 mo. Abdominal discomfort was characterized by early satiety, fullness, nausea, retching, upper abdominal bloating and anorexia[24,25]. We recruited consecutive patients with non-ulcer dyspepsia upon upper gastrointestinal (GI) endoscopy. Patients were examined using an Olympus GIF-Q30 endoscope (Olympus, Tokyo, Japan). One experienced endoscopist participated in the study, which allowed the inclusion of patients. Patients with duodenal ulcer (circumscribed break of > 5 mm depth in the mucosa, covered with exudate, present in the prepyloric, pyloric, or duodenal bulb region), gastric ulcer (with the above-described mucosal defect located at the angulus or above it), gastric polyps or cancers, bleeding complications, previous gastric resection and those who had been treated with anti-*H pylori* treatment, aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) or antibiotics 2 wk before the study were excluded.

During endoscopy, two antral and two corpus biopsies were taken and fixed in 10% buffered formalin and then embedded in paraffin for histological examination (Giemsa stain) and for immunohisto-chemical staining of hMLH1 and hMSH2. Patients were considered to be *H pylori*-positive when histological demonstration of the bacterium was positive. Fifty *H pylori*-positive and 50 *H pylori*-negative patients were enrolled. The updated Sydney system was used to evaluate pathological findings, such as gastritis severity, gastritis activity, intestinal metaplasia, gastric atrophy and dysplasia[26].

Immunohistochemical staining

Immunohistochemical staining was performed following the Envision method on the gastric biopsy specimens from *H pylori*-positive and -negative patients. Four-micrometer-thick sections were obtained from formalin-fixed paraffin-embedded tissue blocks. The tissue sections were deparaffinized in xylene and rehydrated in graded concentrations of alcohol. Endogenous peroxidase activity was blocked by treating the sections with blocking solution. For antigen retrieval, the sections were treated while boiling in citrate buffer pH 9.0 in a microwave oven. Then sections were incubated with primary antibodies against hMLH1 (BD Biosciences Pharmingen, clone G168-15, 1:100 dilution) and hMSH2 (Calbiochem, Oncogene Sciences, clone FE11, 1:100 dilution). After each step, slides were washed with TBS buffer for 3 min. Then, slides were treated with Envision (DAKO, REAL Envision) for 20 min. To visualize immunoreactivity, 3,3'-diaminobenzidine was used and samples were counterstained with hematoxylin. Intramucosal lymphocytes were used as positive controls. Samples from patients with hereditary non-polyposis colon cancer were used as a negative control. These samples have loss of nuclear staining in tumoral cells[24]. The slides were evaluated by two pathologists who were blinded regarding *H pylori* status.

A case was considered positive for expression of hMLH1 or hMSH2 in the presence of nuclear staining of epithelial cells; however, it was considered negative when there was a complete absence of nuclear staining of the epithelial cells in the presence of an unquestioned internal positive control. The staining intensity was divided into three grades. We counted more than 500 epithelial cells (including glandular neck, foveolar and surface epithelium) in each case at × 200 magnification. Quantitative I analysis was performed by measuring the total number of cells and the positive-staining epithelial cells. The percentage positivity was then calculated[24].

Statistical analysis

The χ^2 test and an unpaired Student's *t* test were used, when appropriate. $P < 0.05$ was considered statistically significant. All data were analyzed by SPSS version 13.0 (SPSS, Chicago, IL, USA).

RESULTS

Fifty *H pylori*-positive patients with a mean age of 41.78 ± 15.21 years and 50 *H pylori*-negative patients with a mean age of 46.58 ± 13.41 years were studied ($P = 0.1$). There was no significant difference in the male to female ratio between the two groups (28/22 in the *H pylori*-positive group *versus* 23/27 in the *H pylori*-negative group; $P = 0.32$). Table 1 outlines the characteristics and pathological data of both groups. As shown in Table 1, pathological findings such as gastritis severity, gastritis activity, intestinal metaplasia, gastric atrophy and dysplasia were not significantly different between the two groups.

The percentage of epithelial cell nuclei that demonstrated positivity for hMLH1 staining was 84.14 ± 7.32% in *H pylori*-negative patients; while it was 73.34 ± 10.10% in *H pylori*-positive patients ($P < 0.0001$). No

Table 1 Demographic and pathological findings of *H pylori*-positive and -negative patients *n* (%)

	Positive group	Negative group	*P* value
Age (mean ± SD, yr)	41.78 ± 15.21	46.58 ± 13.41	0.1
Male:female	28:22	23:27	0.32
Gastritis severity			0.45
1	8 (16)	13 (26)	
2	34 (68)	29 (58)	
3	8 (16)	8 (16)	
Gastritis activity			0.23
0	8 (16)	15 (30)	
1	6 (12)	9 (18)	
2	28 (56)	20 (40)	
3	8 (16)	6 (12)	
Intestinal metaplasia			0.45
Positive	8 (16)	11(22)	
Negative	42 (84)	39(78)	
Atrophy			0.54
Positive	7 (14)	5 (10)	
Negative	43 (86)	45 (90)	
Dysplasia			0.31
Positive	3 (6)	1 (2)	
Negative	47 (94)	49 (98)	

Table 2 Immunohistochemical staining of gastric biopsy specimens from *H pylori*-positive and -negative patients

	Positive group	Negative group	*P* value
hMLH1			
Body			
Area	73.80 ± 11.77	85.28 ± 7.71	0.000
Intensity	2.02 ± 0.65	2.08 ± 0.63	0.64
Antrum			
Area	72.44 ± 11.35	82.36 ± 9.63	0.000
Intensity	1.88 ± 0.59	1.92 ± 0.63	0.75
Overall			
Area	73.34 ± 10.10	84.14 ± 7.32	0.000
Intensity	1.95 ± 0.47	1.99 ± 0.41	0.64
hMSH2			
Body			
Area	77.24 ± 11.36	81.28 ± 10.58	0.07
Intensity	1.96 ± 0.57	1.96 ± 0.64	1.00
Antrum			
Area	78.76 ± 11.24	80.62 ± 10.89	0.40
Intensity	2.04 ± 0.53	1.90 ± 0.65	0.24
Overall			
Area	78.24 ± 8.71	81.16 ± 8.32	0.09
Intensity	1.99 ± 0.42	1.93 ± 0.46	0.50

significant difference was seen between the two groups regarding the percentage of epithelial cell nuclei that were positive for hMSH2 staining (81.16 ± 8.32% in *H pylori*-negative patients *versus* 78.24 ± 8.71% in *H pylori*-positive patients; $P = 0.09$). As shown in Table 2, the immunohistochemical staining in the corpus and antrum was relatively similar for hMLH1 or hMSH2.

Intensity of immmunohistochemical staining for hMLH1 did not differ significantly between the groups (1.99 ± 0.41 in *H pylori*-negative patients *versus* 1.95 ± 0.47 in *H pylori*-positive patients; P = 0.64). For hMSH2, intensity of immmunohistochemical staining was 1.93 ± 0.46 in *H pylori*-negative patients and 1.99 ± 0.42 in *H pylori*-positive patients; however, the difference was not statistically significant ($P = 0.50$).

DISCUSSION

The relationship between *H pylori* infection, gastric mucosal damage, and cell proliferation rate is a matter of debate. One hypothesis regarding how *H pylori* causes gastric carcinoma is through impairment of DNA repair in the gastric epithelium. This results in the accumulation of mutations and a genomic imbalance in the epithelium, which increases the risk of gastric carcinoma[27]. Previous studies have shown that active *H pylori* infection neither was more frequently seen in patients who had MSI-positive gastric carcinomas or intestinal metaplasia nor attach to carcinoma cells *in vivo* It is possible that during chronic gastritis, *H pylori* is physically in direct contact with gastric epithelial cells, and disturbs them at the molecular level. Studies on cytokine induction by *H pylori* support this hypothesis[28,29]. During chronic gastritis, the mucosa undergoes rapid turnover, and increased cell proliferation may permit an increased number of uncorrected mutations that may be induced by inadequate DNA MMR activity. Impairment of the DNA MMR system is a known mechanism of carcinogenesis and tumor progression in sporadic and hereditary human cancers[9,10]. In humans, MMR is mediated by at least six genes, including hMLH1, hMSH2, hMSH3, hMSH6, hPMS2 and hPMS1[30]. Germline mutations in hMSH2 and hMLH1 account for approximate 90% of all reported MMR gene mutations, whereas hPMS2 and hMSH6 account for the remainder[31]. Several studies have shown that hMLH1 and hMSH2 are the two main MMR proteins and the other MMR proteins including hPMS2, hPMS1, and hMSH6 seem to be unstable in the absence of the main MMR proteins[32,33].

Our findings indicate that decreased levels of hMLH1 proteins are seen in gastric epithelial cells in *H pylori*-positive patients. Although the level of hMSH2 proteins was lower in *H pylori*-positive patients, there was no significant difference. Results were the same as the study of Halling *et al*[34], which found that MSI-positive gastric carcinoma is usually associated with a lack of hMLH1 and rarely with a lack of hMSH2. Leung *et al*[28] have demonstrated that active *H pylori* infection is more frequently found in individuals with MSI-positive than in those with MSI-negative gastric cancer, which suggests that *H pylori* infection affects the DNA MMR system during the stepwise progression of gastric carcinogenesis. Park *et al* have studied the expression of hMLH1 and hMSH2 in patients with chronic *H pylori* infection before and after bacterial eradication. They have found that the expression of DNA MMR proteins increases in the gastric mucosa after *H pylori* eradication, which indicates that *H pylori* is associated with impairment of the DNA MMR system. Kim *et al*[27] cocultured gastric cancer cell lines with *H pylori* and then determined MutL and MutS DNA MMR protein and RNA levels. All cell lines showed decreased levels of MutL and MutS DNA MMR proteins in a dose-dependent manner[23]. Lack of an efficient DNA

MMR system can potentially have dramatic effects on the cell genome by allowing the accumulation of mutations in critical regulatory genes.

In this study, immunohistochemical staining of the corpus and antrum was similar for hMLH1 and hMSH2. This indicates that *H pylori* affects DNA MMR stems of gastric epithelium regardless of its location. In conclusion, this study indicates that the oncogenic bacterium *H pylori* might promote development of gastric carcinoma, at least in part through its effect on the DNA MMR system. Impairment of the DNA MMR system represents a novel mechanism of infection-associated cancer promotion.

COMMENTS

Background

Cancer arises from the accumulation of inherited polymorphism (i.e. SNPs) and mutation and/or sporadic somatic polymorphism (i.e. non-germline polymorphism) in cell cycle, DNA repair, and growth signaling genes. Main molecular mechanisms underlying cancer development include the overexpression of genes, including oncogenes and growth factors or their receptors, and impaired expression of tumor suppressor genes resulting from mutation or allelic losses and deficiencies of the DNA mismatch repair (MMR) system.

Research frontiers

During chronic gastritis the mucosa undergoes rapid turnover and increased cell proliferation may permit an increased number of uncorrected mutations that may be induced by inadequate DNA MMR activity. Impairment of the DNA MMR system is a known mechanism of carcinogenesis and tumor progression of both sporadic and hereditary human cancers. fewer advances have been made to understand how long it takes for *Helicobacter pylori (H pylori)* infection to induce the development of gastric cancer. The MMR deficiency leads to the accumulation of base-base mismatches, and the short insertion/deletion mispairs during DNA replication resulting in widespread mutation generated as a consequence of DNA replication errors. Most cells deficient in MMR display a high level of genomic instability characterized by changes in simple repetitive sequences so-called microsatellite instability (MSI).

Innovations and breakthroughs

Chronic *H pylori* infection damages gastric barrier function and stimulates gastric cell proliferation, which leads to mucosal repair, but which can also induce cellular DNA damage. *H pylori* gastritis occurs more frequently in individuals with MSI-positive than those with MSI-negative gastric cancers, raising the possibility that *H pylori* infection affects the DNA MMR system. The present study confirmed *H pylori* might promote development of gastric carcinoma at least in part through the ability to affect the DNA MMR system.

Applications

This study indicates that impairment of the DNA MMR system represents a novel mechanism of infection-associated cancer promotion.

Terminology

The association of *H pylori* with gastric cancer is supported by epidemiological studies showing that the odds ratios for gastric cancer is up to nine-fold greater in *H pylori*-infected individuals. Impairment of the DNA MMR system is a known mechanism of carcinogenesis and tumor progression of both sporadic and hereditary human cancers. Most cells deficient in MMR display a high level of genomic instability characterized by changes in simple repetitive sequences, so-called microsatellite instability (MSI). Chronic *H pylori* infection damages gastric barrier function[12,13] and stimulates gastric cell proliferation[14-19], which leads to mucosal repair[20], but which can also induce cellular DNA damage.

Peer review

Interesting paper but needed some amendment. This paper is adequately presented. The investigation is useful for the progress in the knowledge of this topic.

REFERENCES

1 **Parkin DM**, Pisani P, Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1985. *Int J Cancer* 1993; **54**: 594-606

2 **Landis SH**, Murray T, Bolden S, Wingo PA. Cancer statistics, 1998. *CA Cancer J Clin* 1998; **48**: 6-29

3 **Huang JQ**, Sridhar S, Chen Y, Hunt RH. Meta-analysis of the relationship between Helicobacter pylori seropositivity and gastric cancer. *Gastroenterology* 1998; **114**: 1169-1179

4 **Sipponen P**, Hyvarinen H, Seppala K, Blaser MJ. Review article: Pathogenesis of the transformation from gastritis to malignancy. *Aliment Pharmacol Ther* 1998; **12** Suppl 1: 61-71

5 **Todd R**, Wong DT. Oncogenes. *Anticancer Res* 1999; **19**: 4729-4746

6 **Teh BT**, Larsson C, Nordenskjold M. Tumor suppressor genes (TSG). *Anticancer Res* 1999; **19**: 4715-4728

7 **Liu B**, Nicolaides NC, Markowitz S, Willson JK, Parsons RE, Jen J, Papadopolous N, Peltomaki P, de la Chapelle A, Hamilton SR. Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nat Genet* 1995; **9**: 48-55

8 **Boland CR**. Molecular genetics of hereditary nonpolyposis colorectal cancer. *Ann N Y Acad Sci* 2000; **910**: 50-59; discussion 59-61

9 **Eshleman JR**, Markowitz SD. Mismatch repair defects in human carcinogenesis. *Hum Mol Genet* 1996; **5** Spec No: 1489-1494

10 **Kinzler KW**, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159-170

11 **Kolodner RD**, Marsischky GT. Eukaryotic DNA mismatch repair. *Curr Opin Genet Dev* 1999; **9**: 89-96

12 **Nardone G**, d'Armiento F, Corso G, Coscione P, Esposito M, Budillon G. Lipids of human gastric mucosa: effect of Helicobacter pylori infection and nonalcoholic cirrhosis. *Gastroenterology* 1994; **107**: 362-368

13 **Mauch F**, Bode G, Ditschuneit H, Malfertheiner P. Demonstration of a phospholipid-rich zone in the human gastric epithelium damaged by Helicobacter pylori. *Gastroenterology* 1993; **105**: 1698-1704

14 **Correa P**. Helicobacter pylori and gastric carcinogenesis. *Am J Surg Pathol* 1995; **19** Suppl 1: S37-S43

15 **Lynch DA**, Mapstone NP, Clarke AM, Sobala GM, Jackson P, Morrison L, Dixon MF, Quirke P, Axon AT. Cell proliferation in Helicobacter pylori associated gastritis and the effect of eradication therapy. *Gut* 1995; **36**: 346-350

16 **Lynch DA**, Mapstone NP, Clarke AM, Jackson P, Dixon MF, Quirke P, Axon AT. Cell proliferation in the gastric corpus in Helicobacter pylori associated gastritis and after gastric resection. *Gut* 1995; **36**: 351-353

17 **Brenes F**, Ruiz B, Correa P, Hunter F, Rhamakrishnan T, Fontham E, Shi TY. Helicobacter pylori causes hyperproliferation of the gastric epithelium: pre- and post-eradication indices of proliferating cell nuclear antigen. *Am J Gastroenterol* 1993; **88**: 1870-1875

18 **Fox JG**, Li X, Cahill RJ, Andrutis K, Rustgi AK, Odze R, Wang TC. Hypertrophic gastropathy in Helicobacter felis-infected wild-type C57BL/6 mice and p53 hemizygous transgenic mice. *Gastroenterology* 1996; **110**: 155-166

19 **Correa P**, Ruiz B, Shi TY, Janney A, Sobhan M, Torrado J, Hunter F. Helicobacter pylori and nucleolar organizer regions in the gastric antral mucosa. *Am J Clin Pathol* 1994; **101**: 656-660

20 **Panella C**, Ierardi E, Polimeno L, Balzano T, Ingrosso M, Amoruso A, Traversa A, Francavilla A. Proliferative activity of gastric epithelium in progressive stages of Helicobacter pylori infection. *Dig Dis Sci* 1996; **41**: 1132-1138

21 **Medline A**, Farber E. The multi-step theory of neoplasia. In: Anthony PP, Mc Sween RHM, eds. Recent advances in histopathology. New York: Churchill Livingstone, 1981: 19-34

22 **Yabuki N**, Sasano H, Tobita M, Imatani A, Hoshi T, Kato K, Ohara S, Asaki S, Toyota T, Nagura H. Analysis of cell damage and proliferation in Helicobacter pylori-infected human gastric mucosa from patients with gastric adenocarcinoma. *Am J Pathol* 1997; **151**: 821-829

23 **Kim JJ**, Tao H, Carloni E, Leung WK, Graham DY,

Sepulveda AR. Helicobacter pylori impairs DNA mismatch repair in gastric epithelial cells. *Gastroenterology* 2002; **123**: 542-553
24 **Talley NJ**, Stanghellini V, Heading RC, Koch KL, Malagelada JR, Tytgat GN. Functional gastroduodenal disorders. *Gut* 1999; **45** Suppl 2: II37-II42
25 **Westbrook JI**, McIntosh JH, Talley NJ. The impact of dyspepsia definition on prevalence estimates: considerations for future researchers. *Scand J Gastroenterol* 2000; **35**: 227-233
26 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181
27 **Park DI**, Park SH, Kim SH, Kim JW, Cho YK, Kim HJ, Sohn CI, Jeon WK, Kim BI, Cho EY, Kim EJ, Chae SW, Sohn JH, Sung IK, Sepulveda AR, Kim JJ. Effect of Helicobacter pylori infection on the expression of DNA mismatch repair protein. *Helicobacter* 2005; **10**: 179-184
28 **Leung WK**, Kim JJ, Kim JG, Graham DY, Sepulveda AR. Microsatellite instability in gastric intestinal metaplasia in patients with and without gastric cancer. *Am J Pathol* 2000; **156**: 537-543
29 **Crabtree JE**, Wyatt JI, Trejdosiewicz LK, Peichl P, Nichols PH, Ramsay N, Primrose JN, Lindley IJ. Interleukin-8 expression in Helicobacter pylori infected, normal, and neoplastic gastroduodenal mucosa. *J Clin Pathol* 1994; **47**: 61-66
30 **Jiricny J**. Replication errors: cha(lle)nging the genome. *EMBO J* 1998; **17**: 6427-6436
31 **Lindor NM**, Greene MH. The concise handbook of family cancer syndromes. Mayo Familial Cancer Program. *J Natl Cancer Inst* 1998; **90**: 1039-1071
32 **Leung WK**, Kim JJ, Wu L, Sepulveda JL, Sepulveda AR. Identification of a second MutL DNA mismatch repair complex (hPMS1 and hMLH1) in human epithelial cells. *J Biol Chem* 2000; **275**: 15728-15732
33 **Yao X**, Buermeyer AB, Narayanan L, Tran D, Baker SM, Prolla TA, Glazer PM, Liskay RM, Arnheim N. Different mutator phenotypes in Mlh1- versus Pms2-deficient mice. *Proc Natl Acad Sci USA* 1999; **96**: 6850-6855
34 **Halling KC**, Harper J, Moskaluk CA, Thibodeau SN, Petroni GR, Yustein AS, Tosi P, Minacci C, Roviello F, Piva P, Hamilton SR, Jackson CE, Powell SM. Origin of microsatellite instability in gastric cancer. *Am J Pathol* 1999; **155**: 205-211

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RAPID COMMUNICATION

Colonoscopic perforation: A report from World Gastroenterology Organization endoscopy training center in Thailand

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Abstract

AIM: To determine the incidence of colonoscopic perforation (CP), and evaluate clinical findings, management and outcomes of patients with CP from the World Gastroenterology Organization (WGO) Endoscopy Training Center in Thailand.

METHODS: All colonoscopies and sigmoidoscopies performed between 1999 and 2007 in the Endoscopic unit, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok were reviewed. Incidence of CP, patients' characteristics, endoscopic information, intra-operative findings, management and outcomes were analyzed.

RESULTS: A total of 17357 endoscopic procedures of the colon (13 699 colonoscopies and 3658 flexible sigmoidoscopies) were performed in Siriraj hospital over a 9-year period. Fifteen patients (0.09%) had CP: 14 from colonoscopy and 1 from sigmoidoscopy. The most common site of perforation was in the sigmoid colon (80%), followed by the transverse colon (13%). Perforations were caused by direct trauma from either the shaft or the tip of the endoscope (n = 12, 80%) and endoscopic polypectomy (n = 3, 20%). All patients with CP underwent surgical management: primary repair (27%) and bowel resection (73%). The mortality rate was 13% and postoperative complication rate was 53%.

CONCLUSION: CP is a rare but serious complication following colonoscopy and sigmoidoscopy, with high rates of morbidity and mortality. Incidence of CP was 0.09%. Surgery is still the mainstay of CP management.

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Key words: Colonoscopic perforation; Colonoscopy; Complication; Incidence; Endoscopy training center

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INTRODUCTION

There are an increasing number of patients undergoing endoscopic examination of the colon and rectum for various purposes such as screening and surveillance of colorectal cancer. Endoscopy-related complications could result from preparation for the procedure (such as hypotension and electrolyte imbalance following mechanical bowel preparation), or they could be directly related to the endoscopic procedures (such as post-polypectomy hemorrhage and colonic perforation). Although colonoscopic perforation (CP) is a rare complication, it is associated with a high rate of morbidity and mortality[1-4]. The incidence of CP could be as low as 0.02% in diagnostic colonoscopy and could be as high as 0.6% in therapeutic colonoscopy[5,6]. The

reported morbidity following CP is about 40% and mortality might be up to 14% depending on patients' characteristics and co-morbidities[7]. Most patients with CP require open surgery; however, there is recent evidence that CP can be successfully managed by endoluminal repair[6] and laparoscopic surgery[8-11].

To the best of our knowledge, there is no published literature about CP from any World Gastroenterology Organization (WGO) Endoscopy Training Center. The aims of this study were to determine the incidence of CP following colonoscopy and flexible sigmoidoscopy, and to evaluate clinical findings, management and outcomes of patients with CP from the WGO Endoscopy Training Center in Thailand.

MATERIALS AND METHODS

All colonoscopies and sigmoidoscopies performed between 1999 and 2007 at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, were reviewed. Patients with CP were identified from the prospectively collected database of the Siriraj GI Endoscopy Center or from the hospital information system. Medical records of all CP patients were reviewed. The incidence of CP, patients' characteristics, endoscopic information, intra-operative findings, management and outcomes in CP patients were analyzed. The study was approved by the Institutional Ethics Committee and informed written consent was obtained from each patient.

All data were prepared and compiled using SPSS software (version 10.0 for Windows). Pearson chi-square test or Fisher's exact test was used for comparing categorical data, whereas the Mann-Whitney *U* test was used for comparing non-categorical data. $P < 0.05$ was considered statistically significant. Values were presented as a number (percentage), or mean (range).

RESULTS

Demographic data

A total of 17357 endoscopic procedures of the colon (13699 colonoscopies and 3658 flexible sigmoidoscopies) were performed in Siriraj hospital over a 9 year period. Fifteen patients (0.09%) had CP: 14 from colonoscopy and 1 from sigmoidoscopy. The incidence of CP following colonoscopy was slightly higher than that following sigmoidoscopy (0.1% *vs* 0.03%; $P = 0.22$), with a relative risk ratio of 3.7 (95% confidence intervals = 0.5-28.4).

Patients with CP had an average age of 67 years (range 36-88) and nine patients (60%) were female. Indications for endoscopic examination in these patients were anemia or lower gastrointestinal bleeding ($n = 6$), bowel habit change ($n = 4$), large bowel mass ($n = 2$), non-specific abdominal pain ($n = 1$), refractory inflammatory bowel disease ($n = 1$), and suspected pseudomembranous colitis ($n = 1$).

Table 1 Incidence, findings and management of CP *n* (%)

Characteristics	Data
CP	15 cases
Incidence of CP	
Overall endoscopy ($n = 17357$)	0.09
Colonoscopy ($n = 13699$)	0.10
Flexible sigmoidoscopy ($n = 3658$)	0.03
Site of perforation	
Sigmoid colon	12 (80)
Transverse colon	2 (13)
Ascending colon	1 (7)
Mechanism of perforation	
Direct injury from shaft of scope	7 (47)
Direct injury from tip of scope	5 (33)
Polypectomy	3 (20)
Surgical management[1]	
Primary suture of the perforation	4 (27)
Resection with primary anastomosis	4 (27)
Resection without anastomosis	7 (47)

[1]Four patients (27%) had concomitant colorectal cancer.

Perforations

The most common site of perforation was the sigmoid colon ($n = 12$, 80%), followed by the transverse colon ($n = 2$, 13%) and the ascending colon ($n = 1$, 7%). Based on the endoscopic reports and intra-operative findings, mechanisms of perforation were determined to be direct trauma from the shaft of the endoscope in 7 patients (47%) and from the tip of the endoscope in 5 patients (33%). The others ($n = 3$, 20%) were caused by endoscopic polypectomy. All perforations were immediately recognized during endoscopy except for one electrical injury after polypectomy which caused peritonitis 24 h after the procedure. Details of the characteristics of CP are summarized in Table 1.

Management and outcomes

Non-operative management consisting of bowel rest and intravenous antibiotics was attempted in 2 patients with localized peritonitis; however, their symptoms deteriorated and an operation was eventually required. Therefore, all the patients with CP in this series underwent surgical management. Of these patients, twelve (80%) had medical co-morbidities and four (27%) had concomitant colorectal cancer. Types of operation included primary suture of the perforation in 4 patients (27%), resection and primary anastomosis in 4 patients (27%), and resection without anastomosis in 7 patients (47%).

Postoperative complications were identified in 8 patients (53%): 5 wound infections, 3 pneumonias and 1 antibiotics-associated colitis. There was no difference in age, gender, ASA status, and size of the perforation between the groups who did and did not develop postoperative complications (Table 2). There were 2 deaths (both females aged 76 and 83 years, respectively), accounting for 13% of CP patients and 0.01% of total colonic endoscopy patients. Pneumonia was the primary cause of death in both patients. The average hospital stay of CP patients was 23 d (range 3-92).

Table 2 Comparison of patients' characteristics between the groups who did and did not develop postoperative complications

	Patients with complication	Patients without complication	*P*
	(*n* = 8)	(*n* = 7)	
Age over 60	5 (63)	5 (71)	0.71
Female	6 (75)	3 (43)	0.32
ASA ⩾ 3	5 (63)	4 (57)	0.83
Perforation size ⩾ 5 cm	2 (25)	3 (43)	0.61

Values were given as a number (percentage).

DISCUSSION

The incidence of CP in our study was 0.09%, which was quite similar to that in other larger series (sample size > 30 000 cases)[12-15]. To the best of our knowledge, this is the first report of such an incidence from the WGO Endoscopy training centers. Although it remains inconclusive whether an endoscopy performed by a trainee increases risk of CP, we cannot evaluate such a potential factor because the trainee-to-endoscopist ratio for all procedures in our study was unknown. Anderson and co-workers[16] have reported that there was no significant increased risk of CP performed by training fellows. Training bodies in America, Britain and Australia have recommended a minimum of 50-100 colonoscopies should be performed by a trainee to gain endoscopic competency[17-20].

We found that colonoscopy had an almost fourfold increased incidence of CP compared with sigmoidoscopy, although this did not reach statistical significance. Many investigators have reported that the risk of CP following colonoscopy is 2-4 times higher than that following sigmoidoscopy[13,20]. Other risk factors for CP may include female gender[16], advanced age[12,21], a history of diverticular disease or previous intraabdominal surgery[12], and endoscopic interventions such as polypectomy and endoscopic mucosal resection[6].

In our study, perforation at the sigmoid colon accounted for 80% of all perforation sites. This finding was consistent with that of other studies[9,10,13]. There are three possible mechanisms responsible for CP: mechanical perforation directly from the colonoscope, perforations that occur during therapeutic procedures and, finally, barotrauma from overzealous air insufflation[22,23].

All patients with CP in the present series underwent surgical management. Clearly, the choice between conservative and surgical management depends on clinical factors[24]. Conservative management is reserved for patients in good general condition and without any sign of peritonitis. Surgical management is recommended in patients with diffuse peritonitis, with clinical deterioration under medical treatment, or with a concomitant colonic pathology such as colorectal cancer. In the published literature, less than 20% of patients with CP can be successfully treated by non-surgical approach[14,15,25].

With regard to the choices of operation for colonic perforation, we found that a quarter of CP patients underwent primary suture of the perforation while the others had bowel resection. The rate of primary repair in our study was less than that of other studies, in which the rate of non-resection procedures could be 30%-60%[7,13,15,25,26]. A possible explanation for a relatively low percentage of primary repair in our series might be that half of our patients had a large perforation caused by the shaft of the scope, and many patients were suspected of having underlying colorectal cancer which required bowel resection.

The postoperative morbidity rate was 53% and wound infection was the most common complication. We cannot identify risk factors for developing postoperative complication in CP patients. This may be due to small sample size and limitation in its power. However, some investigators have suggested that such risk factors may include delayed diagnosis, extensive peritoneal contamination, patients using anticoagulants[15], patients having severe co-morbid diseases and a large perforation[7]. The mortality rate of patients with CP in our study was fairly comparable to other reports which ranged from 0% to 14%, depending on patients' coexisting diseases, experience of the care team and hospital setting. Pneumonia was the primary cause of death in our study. Respiratory complications often occur after major abdominal surgery[27-30], particularly in advanced age patients like ours.

In conclusion, colonic perforation is a rare but serious complication following colonoscopy and sigmoidoscopy, with high rates of morbidity and mortality. In this first study into CP carried out in a WGO Endoscopy training center, we found that the incidence of CP was 0.09% and the sigmoid colon was the most common perforation site. Surgery is still the mainstay of CP management.

COMMENTS

Background

Colonoscopic perforation (CP) is a rare, but serious complication of colonoscopy. Rising use of colonoscopy could lead to a high number of endoscopic colonic perforations. Meanwhile, CP could be associated with a significant morbidity and mortality.

Research frontiers

Absent from the published literature is the incidence, management and outcomes of CP reported from World Gastroenterology Organization (WGO) Endoscopy Training Centers.

Innovations and breakthroughs

The Siriraj GI Endoscopy center (Bangkok, Thailand) is one of eight endoscopy training centers accredited by WGO. Incidence of CP in this center was 0.09%. The most common site of the perforation was the sigmoid colon. Direct trauma from either the shaft or tip of the endoscope was the most common cause of perforation. Surgical management remains a mainstay treatment of CP. The mortality rate was 13% and the postoperative complication rate was 53%.

Applications

Although the incidence of CP is very low, it is associated with high rates of morbidity and mortality. Further research might focus on identification of risk factors for CP and improvement of management in these patients.

Terminology

Incidence of CP in the WGO endoscopy training center in Thailand was 0.09%.

Peer review

This is a report of a large experience with endoscopic colonic perforations from a single institution. It is well organized. The complications and their management are clearly documented. It is a valuable contribution and should be accepted for publication.

REFERENCES

1 **Avgerinos DV**, Llaguna OH, Lo AY, Leitman IM. Evolving management of colonoscopic perforations. *J Gastrointest Surg* 2008; **12**: 1783-1789
2 **Iqbal CW**, Cullinane DC, Schiller HJ, Sawyer MD, Zietlow SP, Farley DR. Surgical management and outcomes of 165 colonoscopic perforations from a single institution. *Arch Surg* 2008; **143**: 701-706; discussion 706-707
3 **Farley DR**, Bannon MP, Zietlow SP, Pemberton JH, Ilstrup DM, Larson DR. Management of colonoscopic perforations. *Mayo Clin Proc* 1997; **72**: 729-733
4 **Orsoni P**, Berdah S, Verrier C, Caamano A, Sastre B, Boutboul R, Grimaud JC, Picaud R. Colonic perforation due to colonoscopy: a retrospective study of 48 cases. *Endoscopy* 1997; **29**: 160-164
5 **Rathgaber SW**, Wick TM. Colonoscopy completion and complication rates in a community gastroenterology practice. *Gastrointest Endosc* 2006; **64**: 556-562
6 **Taku K**, Sano Y, Fu KI, Saito Y, Matsuda T, Uraoka T, Yoshino T, Yamaguchi Y, Fujita M, Hattori S, Ishikawa T, Saito D, Fujii T, Kaneko E, Yoshida S. Iatrogenic perforation associated with therapeutic colonoscopy: a multicenter study in Japan. *J Gastroenterol Hepatol* 2007; **22**: 1409-1414
7 **Garbay JR**, Suc B, Rotman N, Fourtanier G, Escat J. Multicentre study of surgical complications of colonoscopy. *Br J Surg* 1996; **83**: 42-44
8 **Kilic A**, Kavic SM. Laparoscopic colotomy repair following colonoscopic polypectomy. *JSLS* 2008; **12**: 93-96
9 **Mattei P**, Alonso M, Justinich C. Laparoscopic repair of colon perforation after colonoscopy in children: report of 2 cases and review of the literature. *J Pediatr Surg* 2005; **40**: 1651-1653
10 **Hansen AJ**, Tessier DJ, Anderson ML, Schlinkert RT. Laparoscopic repair of colonoscopic perforations: indications and guidelines. *J Gastrointest Surg* 2007; **11**: 655-659
11 **Bleier JI**, Moon V, Feingold D, Whelan RL, Arnell T, Sonoda T, Milsom JW, Lee SW. Initial repair of iatrogenic colon perforation using laparoscopic methods. *Surg Endosc* 2008; **22**: 646-649
12 **Korman LY**, Overholt BF, Box T, Winker CK. Perforation during colonoscopy in endoscopic ambulatory surgical centers. *Gastrointest Endosc* 2003; **58**: 554-557
13 **Lüning TH**, Keemers-Gels ME, Barendregt WB, Tan AC, Rosman C. Colonoscopic perforations: a review of 30,366 patients. *Surg Endosc* 2007; **21**: 994-997
14 **Cobb WS**, Heniford BT, Sigmon LB, Hasan R, Simms C, Kercher KW, Matthews BD. Colonoscopic perforations: incidence, management, and outcomes. *Am Surg* 2004; **70**: 750-757; discussion 757-758
15 **Iqbal CW**, Chun YS, Farley DR. Colonoscopic perforations: a retrospective review. *J Gastrointest Surg* 2005; **9**: 1229-1235: discussion 1236
16 **Anderson ML**, Pasha TM, Leighton JA. Endoscopic perforation of the colon: lessons from a 10-year study. *Am J Gastroenterol* 2000; **95**: 3418-3422
17 **Thomas-Gibson S**, Williams CB. Colonoscopy training--new approaches, old problems. *Gastrointest Endosc Clin N Am* 2005; **15**: 813-827
18 **Wexner SD**, Garbus JE, Singh JJ. A prospective analysis of 13,580 colonoscopies. Reevaluation of credentialing guidelines. *Surg Endosc* 2001; **15**: 251-261
19 **Balfour TW**. Training for colonoscopy. *J R Soc Med* 2001; **94**: 160-161
20 **Jones IT**. Training in colonoscopy: a personal view. *Aust N Z J Surg* 1998; **68**: 316-317
21 **Gatto NM**, Frucht H, Sundararajan V, Jacobson JS, Grann VR, Neugut AI. Risk of perforation after colonoscopy and sigmoidoscopy: a population-based study. *J Natl Cancer Inst* 2003; **95**: 230-236
22 **Damore LJ 2nd**, Rantis PC, Vernava AM 3rd, Longo WE. Colonoscopic perforations. Etiology, diagnosis, and management. *Dis Colon Rectum* 1996; **39**: 1308-1314
23 **Luchette FA**, Doerr RJ, Kelly K, Kulaylat M, Stephan RM, Hassett JM. Colonoscopic impaction in left colon strictures resulting in right colon pneumatic perforation. *Surg Endosc* 1992; **6**: 273-276
24 **Donckier V**, André R. Treatment of colon endoscopic perforations. *Acta Chir Belg* 1993; **93**: 60-62
25 **Araghizadeh FY**, Timmcke AE, Opelka FG, Hicks TC, Beck DE. Colonoscopic perforations. *Dis Colon Rectum* 2001; **44**: 713-716
26 **Tulchinsky H**, Madhala-Givon O, Wasserberg N, Lelcuk S, Niv Y. Incidence and management of colonoscopic perforations: 8 years' experience. *World J Gastroenterol* 2006; **12**: 4211-4213
27 **Lohsiriwat V**, Chinswangwatanakul V, Lohsiriwat S, Akaraviputh T, Boonnuch W, Methasade A, Lohsiriwat D. Hypoalbuminemia is a predictor of delayed postoperative bowel function and poor surgical outcomes in right-sided colon cancer patients. *Asia Pac J Clin Nutr* 2007; **16**: 213-217
28 **Serejo LG**, da Silva-Júnior FP, Bastos JP, de Bruin GS, Mota RM, de Bruin PF. Risk factors for pulmonary complications after emergency abdominal surgery. *Respir Med* 2007; **101**: 808-813
29 **Brooks-Brunn JA**. Predictors of postoperative pulmonary complications following abdominal surgery. *Chest* 1997; **111**: 564-571
30 **Lohsiriwat V**, Lohsiriwat D, Boonnuch W, Chinswang-watanakul V, Akaraviputh T, Lert-Akayamanee N. Pre-operative hypoalbuminemia is a major risk factor for postoperative complications following rectal cancer surgery. *World J Gastroenterol* 2008; **14**: 1248-1251

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RAPID COMMUNICATION

A novel device for endoscopic submucosal dissection, the Fork knife

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Abstract

AIM: To introduce and evaluate the efficacy and technical aspects of endoscopic submucosal dissection (ESD) using a novel device, the Fork knife.

METHODS: From March 2004 to April 2008, ESD was performed on 265 gastric lesions using a Fork knife (Endo FS®) (group A) and on 72 gastric lesions using a Flexknife (group B) at a single tertiary referral center. We retrospectively compared the endoscopic characteristics of the tumors, pathological findings, and sizes of the resected specimens. We also compared the *en bloc* resection rate, complete resection rate, complications, and procedure time between the two groups.

RESULTS: The mean size of the resected specimens was 4.27 ± 1.26 cm in group A and 4.29 ± 1.48 cm in group B. The *en bloc* resection rate was 95.8% (254/265 lesions) in group A and 93.1% (67/72) in group B. Complete ESD without tumor cell invasion of the resected margin was obtained in 81.1% (215/265) of group A and in 73.6% (53/72) of group B. The perforation rate was 0.8% (2/265) in group A and 1.4% (1/72) in group B. The mean procedure time was 59.63 ± 56.12 min in group A and 76.65 ± 70.75 min in group B ($P < 0.05$).

CONCLUSION: The Fork knife (Endo FS®) is useful for clinical practice and has the advantage of reducing the procedure time.

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Key words: Fork knife; Novel device; Endoscopic submucosal dissection; Flexknife; Procedure time

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INTRODUCTION

The emergence of endoscopic submucosal dissection (ESD) has allowed the achievement of histologically complete *en bloc* resection of gastric tumors regardless of size, which permits the resection of previously non-resectable tumors[1-4]. Although the fundamental incision and dissection method are the same for all ESD procedures, ESD can be classified by the type of knife that is used; for example, an IT knife (Olympus Co., Tokyo, Japan)[5-8], a Flexknife (Olympus Co.)[9], or a Hook knife (Olympus Co.)[10]. Each step of ESD, such as marking of a lesion, injection, incision and dissection, may require a different accessory or a knife, depending on the location and shape of the tumor[11]. Therefore, when performing ESD, the accessories may be frequently changed through the working channel of the endoscope[12,13], which results in a longer procedure time and a delay in controlling gastrointestinal (GI) bleeding. A two-channel endoscope can use one accessory or two accessories at the same time during a procedure[14]. However, two-channel endoscopes have a thicker diameter, which may cause additional discomfort to the

patient and impede the field of view in some situations. Consequently, the development of an instrument capable of multiple functions, such as injection, incision, dissection, coagulation, and irrigation, without the need for changing accessories is an attractive idea.

The Fork knife (Endo FS®; Kachu Technology Co., Seoul, Korea) is a device that was developed to enable an endoscopist to perform the multiple steps of ESD without the need to change accessories during the procedure. The Fork knife is capable of performing every step of ESD, including marking, injection, saline irrigation, and coagulation, and thus may enable the endoscopist to reduce the procedure time.

The aim of the present study was to introduce and evaluate the efficacy and technical aspects of ESD performed with a Fork knife, which we made ourselves in cooperation with the Kachu Technology Company.

MATERIALS AND METHODS

Fork knife

The Fork knife (Endo FS®) has two interchangeable knives, a fixed flexible snare and a forked knife, which form a single working unit, and has an inlet for material injection or saline irrigation during the procedure (Figures 1 and 2). The knives can be changed during a procedure by using two switches, the fork knob and core knob, located on the center of the body (Figure 2).

Fixed flexible snare: The first of the two knives that constitute the Fork knife is the fixed flexible snare (Figure 3A), which is operated by sliding the core knob switch forward. The blade is shaped into an elongated loop much like the Flexknife. We mainly use the fixed flexible snare for marking and making incisions around lesions. Marking around a lesion can be done using the tip of the fixed flexible snare. Additionally, the tip can be used for the incision and dissection of a lesion. Although the incision and dissection techniques are similar to those used with a Flexknife, the fixed flexible snare has the advantage of a fixed exposed body length with the snare located inside the tube, which allows the snare to be used more firmly than a Flexknife during dissection. An endoscopist does not need to be concerned about controlling the length of the body to prevent perforation in the event of a sudden movement by the patient, such as belching or coughing. The length of the blade, which can be adjusted using the switch on the center of the body, is usually set to 2.5 ± 1.0 mm in order to minimize the risk of perforation.

Forked knife: The second knife is the forked knife, which has a double-tipped blade (longer tip length, 2 mm; shorter tip length, 1.5 mm) with a forked shape (Figure 3B). The forked knife is located on the opposite side from the fixed snare knife and is operated by sliding the fork knob switch forward. It is a form of a needle knife with an M-shape that maximizes the power applied to contacted surfaces, which is advantageous for dissection and coagulation. The longer tip of the forked knife can be used as an injection needle for making a submucosal cushion, or for injecting agents such as epinephrine. The opening of the needle is located in the center of the knife, between both tips, so that the mucosa must be injected deeply and at an oblique angle for maximum injection into the submucosa. We use the forked knife mainly for submucosal dissection performed in the proximal to distal direction of the endoscope, under direct visualization of the dissection area.

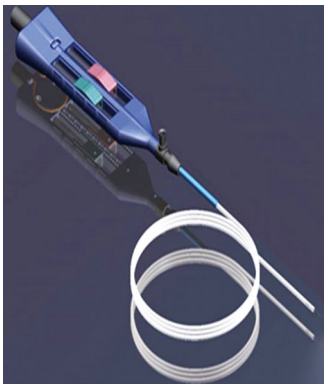

Figure 1 The Fork knife (Endo FS®).

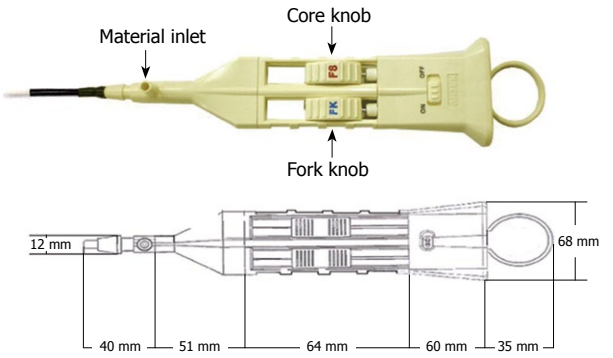

Figure 2 Working body of the Fork knife. Two switches, the fork knob and core knob, are located on the center of the body and enable the knives to be changed during the procedure. A material inlet for injection and irrigation is located forward of the body.

Multi-functions: The Fork knife has a built-in irrigation system *via* the material inlet, which allows saline irrigation while using either knife (Figure 3C). This allows the endoscopist to perform the dissection more comfortably and with prompt control of bleeding to maintain a clear endoscopic view. Additionally, by softly touching a vessel with the tip of knife and using the forced coagulation mode (40 W), an endoscopist can coagulate small vessels that may be exposed during a procedure. Thus, the use of the Forked knife permits better and faster control of any bleeding during a procedure, which reduces the time during which the endoscopic view is obscured.

Patients

From March 2004 to April 2008, we performed 715 ESDs on gastric lesions. We enrolled 337 patients with gastric lesions who underwent ESD from January 2006 to April 2008. One endoscopist, who performed ESD in

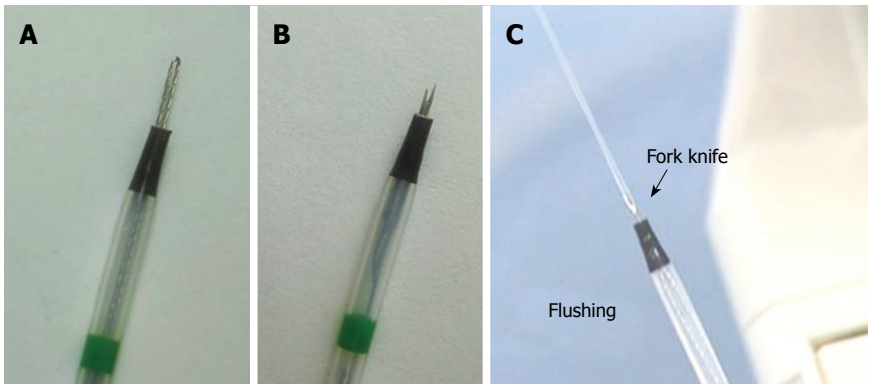

Figure 3 Close-up views of the two interchangeable knives of the Fork knife. A: Fixed flexible snare knife; B: Forked knife with a double-bladed tip; C: Saline irrigation can be performed using either knife.

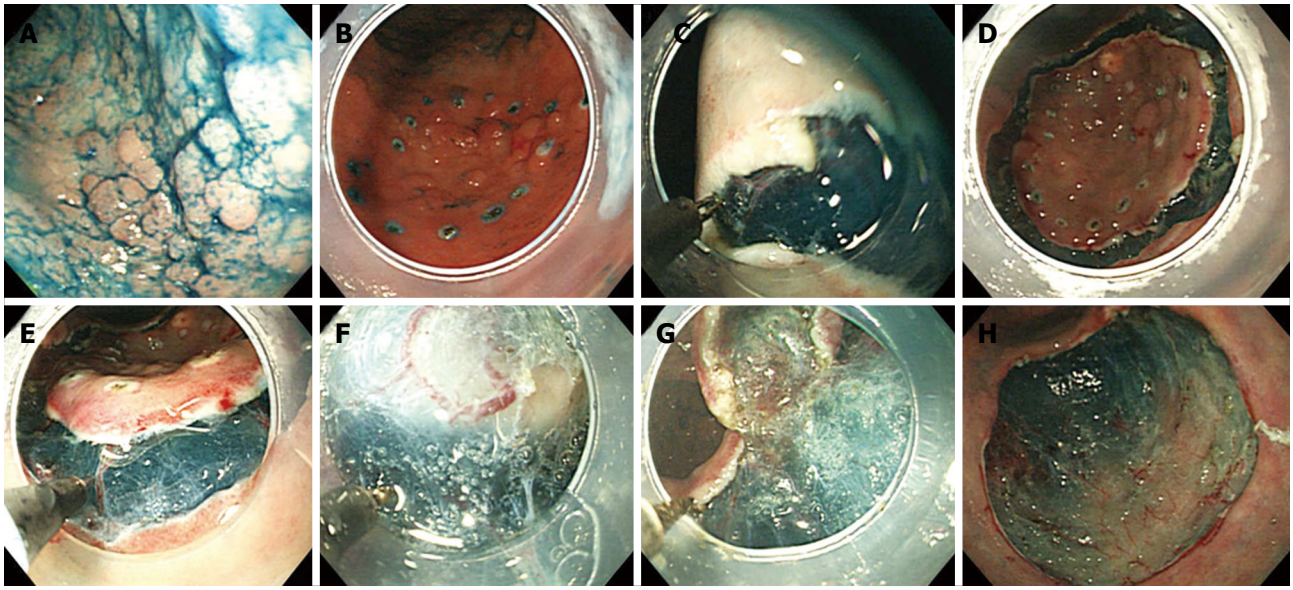

Figure 4 ESD using the Fork knife. A: Indigo carmine spray for deciding the tumor border; B: Circumferential marking by the fixed flexible snare tip with soft coagulation current; C: Making an incision with a fixed flexible snare in ENDOCUT mode; D: Circumferential mucosal incision at the periphery of the marking dots; E: Dissecting the submucosal layer with a forked knife; F: Additional submucosal injection using the long tip of the forked knife without changing accessories; G: Dissecting the submucosal layer with a fixed flexible snare; H: Large ESD defect after complete en bloc resection.

over 400 cases, performed ESD on gastric lesions during the investigation period. ESD was performed using the Fork knife from January 2006 to October 2007, and the Flexknife was used from November 2007 to April 2008. We designed this study prospectively and reviewed retrospectively all of the enrolled ESD data. A total of 265 lesions were dissected with the Fork knife (group A), and 72 lesions were dissected with the Flexknife (group B). This study was approved by the institutional review boards of our hospital.

Methods

Patients were sedated with intravenous diazepam (20-40 mg) while in the endoscopic suite, and conscious sedation was maintained with additional injections during the procedure. All patients had a performance status of < 2 on the Eastern Cooperative Oncology Group scale and fulfilled the following expanded ESD indication established by the Japanese Gastroenterologic Endoscopic Society: (1) non-ulcerated, differentiated-type mucosal carcinoma, regardless of tumor size; and (2) differentiated-type mucosal carcinoma with an ulcer scar < 30 mm[15]. The Fork knife was used for all ESD steps in group A, as shown in Figure 4.

Marking: After spraying of indigo carmine dye (Figure 4A), using the fixed flexible snare tip of the Fork knife, circumferential markings were made at 5 mm around the outline of the lesion, with 2-mm intervals between each marking dot, using a soft coagulation current of 40-50 W (ICC 200EA, ERBE Elektromedizin GmbH, Tübingen, Germany) (Figure 4B). For marking the lesion, the length of the fixed snare tip of the knife was set to approximate 1 mm, and then using the tip, the mucosa was touched lightly with electricity for 1 s.

Injection: Using the long tip of the forked knife, 2-5 mL of a saline/epinephrine solution was injected into the submucosa until the mucosa was raised. Additional injections were repeated as necessary during the procedure, without changing accessories.

Mucosal incision: After the lesion was lifted, the knife was subsequently changed to the fixed flexible snare for

incision around the lesion. The fixed flexible snare was used to make the incision along the markings, using an electrosurgical generator in ENDOCUT mode, effect 3 (output, 80 W) (Figure 4C and D). The tip of the knife was set to approximate 2 mm. Precutting was not needed.

Dissection: Followed by incision around the lesion with the fixed flexible snare, dissection was done with either the forked knife or the fixed-flexible snare, with an electrical current of 40-50 W for swift coagulation (Figure 4F-H).

Bleeding: If bleeding occurs during dissection, saline can be irrigated without delay using either blades of the knife. Usually for small vessels that are smaller than or similar to the tip of the knives, the fixed flexible snare or the forked knife is sufficient to coagulate the vessels, with an electrical current of 40 W for forced coagulation. This allows the endoscopist to prompt control of bleeding (Figure 4E). Instead of a Fork knife, a Flexknife was used for ESD in group B, according to the same steps as described for group A.

Measurements

We compared the endoscopic appearance of tumors, location of tumors, *en bloc* resection rate, complete resection rate, size of resected specimens, complications, pathological findings, and procedure time between the two groups. The resected specimens were divided into two categories, *en bloc* and piecemeal. Complete resection was identified when the lateral and vertical margins of the specimen were free from tumor involvement. The resected specimens were classified into three size categories based on the longest diameter (< 3 cm, 3 to < 5 cm, and ≥ 5 cm), and the proportion of each category was compared between the two groups. To observe the complications of bleeding and perforation, blood samples, radiological examinations, and follow-up endoscopy were performed 1 and 2 d after ESD. Control of bleeding, difficult dissection caused by fibrosis, and lesion size were the major factors that affected the procedure time. Bleeding was identified when a hemostatic treatment such as endoscopic clipping and/or electrocoagulation was required during or after the procedure. Perforation was identified by endoscopy just after the resection and/or by the presence of free air on plain abdominal radiography. Fibrosis was identified as fibrotic tissue observable during dissection; the fibrosis rate was compared with the procedure time. The procedure time was measured as the time between the marking of the first dot and the withdrawal of the endoscope. The mean procedure time was compared between the two groups.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (version 12.0; SPSS Inc., Chicago, IL, USA). The data are presented as the mean ± SD or the median and range. Categorical parameters were compared using the χ^2 or Fisher's exact test, and continuous variables were compared with Student's *t*-test. $P < 0.05$ was considered significant.

Table 1 Baseline and tumor characteristics of the patients in each group

Characteristic	Group A (*n* = 265)	Group B (*n* = 72)
Gender (M/F) (%)	189 (71.3)/76 (28.7)	51 (70.8)/21 (29.2)
Mean age (yr)	62.4 ± 9.5	63.3 ± 9.3
Size of specimen (cm)	4.27 ± 1.26	4.29 ± 1.48
Pathologic report, *n* (%)		
TALG	51 (19.2)	10 (13.9)
TAHG/CIS	30 (11.3)	9 (12.5)
Adenocarcinoma WD	96 (36.2)	24 (33.3)
Adenocarcinoma MD	64 (24.2)	19 (26.4)
Adenocarcinoma PD	15 (5.7)	7 (9.7)
Signet ring cell type	8 (3.0)	3 (4.2)
Other tumor[1]	1 (0.4)	0

TALG: Tubular adenoma lower-grade dysplasia; TAHG: Tubular adenoma high-grade dysplasia; CIS: Carcinoma *in situ*; WD: Well-differentiated; MD: Moderately differentiated; PD: Poorly differentiated. [1]Carcinoid tumor.

Table 2 Clinical aspects of gastric cancer in the two groups *n* (%)

Clinical aspect	Group A (*n* = 210)	Group B (*n* = 53)
Tumor depth		
Mucosal layer	175 (83.3)	43 (81.1)
Submucosal layer	35 (16.7)	10 (18.9)
Endoscopic appearance		
Protruded/Elevated	77 (36.7)	21 (39.6)
Flat	17 (8.1)	4 (7.5)
Depressed	59 (28.1)	19 (35.8)
Mixed	57 (27.1)	9 (17.1)
Tumor location		
Cardia, Fundus	11 (5.2)	4 (7.5)
Body	54 (25.7)	15 (28.3)
Angle	29 (13.8)	12 (22.6)
Antrum, Pylorus	115 (54.8)	21 (39.6)
Subtotal gastrectomy state	1 (0.5)	1 (1.9)

RESULTS

The mean age of group A was 62.4 ± 9.5 years and 63.3 ± 9.3 years in group B (Table 1). Gender ratios were similar between groups A and B. The number of differentiated adenocarcinomas was similarly high in both groups (36.2% in group A, 33.3% in group B), and the pathological distribution and mean size of the resected specimens were similar between the groups. Undifferentiated, signet ring cell cancer was confirmed based on resected specimens after ESD, instead of by initial biopsy before ESD. The mean longest diameter of the resected specimens was 4.27 ± 1.26 cm in group A and 4.29 ± 1.48 cm in group B.

Table 2 summarizes the depth, location, and endoscopic appearance of the tumors in the two groups. The table excludes adenomatous lesions and is limited to only those lesions confirmed to be cancerous by

Table 3 Comparison of procedure time and lesions in the two groups *n* (%)

	Group A (*n* = 265)	Group B (*n* = 72)	*P*
Procedure time (min)	59.63 ± 56.12	76.65 ± 70.75	0.043
Fibrosis			NS
Yes	42 (15.8)	11 (15.3)	
No	223 (84.2)	61 (84.7)	
Specimen size			NS
< 3 cm	26 (9.8)	7 (9.7)	
3 to < 5 cm	177 (66.8)	42 (65.3)	
⩾ 5 cm	62 (23.4)	18 (25)	
Ulcer lesion			0.041
Yes	36 (13.6)	5 (6.9)	
No	229 (86.4)	67 (93.1)	

NS: Not significant.

Table 4 Resection type and complication rates in the two groups *n* (%)

	Group A (*n* = 265)	Group B (*n* = 72)
Resection		
En bloc	254 (95.8)	67 (93.1)
Piecemeal	11 (4.2)	5 (6.9)
Complication		
None	250 (94.3)	70 (97.2)
Bleeding	13 (4.9)	1 (1.4)
Perforation	2 (0.8)	1 (1.4)

Table 5 Comparison of ESD in the two groups *n* (%)

ESD	Group A (*n* = 265)	Group B (*n* = 72)
Complete	215 (81.1)	53 (73.6)
Incomplete	47 (17.7)	18 (25)
Could not be evaluated	3 (1.1)	1 (1.4)

pathological reports. The tumor depth distribution was similar between the two groups (83.3% of mucosal layer cancer in group A, 81.1% in group B), although group B showed a higher portion of submucosal cancers. The endoscopic appearance showed a similar pattern of distribution between the two groups. The occurrence of depressed lesions was higher in group B than in group A (35.8% *vs* 28.1%), and the occurrence of mixed lesions was lower in group B than in group A (17.1% *vs* 27.1%). With regard to tumor location, group A showed a higher proportion of antral area cancers (54.8%) than group B (39.6%), but the locations were not significantly different between the two groups.

The mean procedure time was significantly shorter in group A (59.63 ± 56.12 min), compared with group B (76.65 ± 70.75 min, $P < 0.05$) (Table 3). Other features such as fibrosis and resected specimen size, which play important roles in determining the procedure time, were similar between the groups. However, ulcerous lesions were significantly more common in group A than in group B (13.6% *vs* 6.9%, $P < 0.05$). Size of specimens was similarly distributed between the two groups.

The *en bloc* resection rate tended to be higher in group A (254, or 95.8% of cases) than in group B (67, or 93.1% of cases) (Table 4). The overall complication rate was 6.4% in group A and 2.8% in group B. Bleeding was more common in group A, but not significantly so. Perforation was observed in three cases in both group, and all cases were treated by conservative management with endoscopic clipping and fasting.

The *en bloc* resection rates were high for specimens of all size categories and were not statistically different between the two groups. *En bloc* resection was performed for 92.3% of lesions < 3 cm, 97.7% of lesions 3 to < 5 cm, and 91.9% of lesions ⩾ 5 cm in group A. In group B, *en bloc* resection was performed for 85.7% of lesions < 3 cm, 95.7% of lesions 3 to < 5 cm, and 88.9% of lesions ⩾ 5 cm. The rate for complete ESD was higher in group A than in group B (Table 5), but the difference was not significant. Overall, four cases could not be evaluated regarding the invasion of cancer in the resected margins because of cellular autolysis and coagulation artifacts.

DISCUSSION

The aim of this study was to introduce and evaluate the efficacy and technical aspects of ESD using a novel device, the multi-functional, convenient Fork knife.

ESD is an innovative technique that improves the rate of successful *en bloc* resection at an early stage of GI neoplasia[1-4]. Since Hirao *et al*[16] first introduced the ESD technique in 1988, many investigators have improved the technique and designed several devices, such as the needle-knife[16], insulated-tip (IT) knife[5-8], and Flexknife[9], that allow *en bloc* resection of widespread tumors. ESD and EMR techniques were developed mainly in Japan[16-18]. However, these techniques have now become major therapeutic modalities in Korea[19], which has a high incidence of early gastric cancer similar to that in Japan, for the treatment of early gastric cancer associated with minimal risk for lymph node metastasis[20,21]. Performing ESD is technically difficult and carries a high risk for complications such as perforation and bleeding[22-25]. The IT knife, which is commonly used for ESD, has been reported to have complication rates of 5%-22%[7,8,26]. There are no standard methods for preventing complications, and no rules exist for selecting proper devices during ESD. The proper devices for safe ESD are determined by the preference of the endoscopist and the circumstances of the dissection. Currently, ESD requires that the accessories be frequently changed through the working channel of the endoscope, according to the procedure being performed[12,13]. Using a two-channel endoscope is more convenient because two accessories can be loaded into the endoscope at the same time[14]. However, the thicker endoscopic diameter causes additional discomfort to the patient and increases the bend angle of the endoscope. The obtuse bend angle of the scope increases the difficulty of each step of ESD and complicates the response to specific conditions such as bleeding.

The Fork knife is a new device developed for ESD. It consists of two knives, a fixed flexible snare knife and a forked knife, in a single working unit. These two

knives can be interchanged easily and with minimal time during the procedure. The Fork knife has the advantage of being multi-functional, such as marking, injection, incision, irrigation, dissection, and bleeding control. The fixed flexible snare tip can be used for marking and performing incisions in the early steps of ESD, and the forked knife can be used for injections and for dissection of the submucosal layer. Both knives can also perform simple irrigation and coagulation of point bleeding from exposed vessels, without changing accessories during the procedure. This enables the endoscopist to perform ESD more conveniently and easily and thus potentially shortens the total procedure time.

The devices needed during ESD vary according to the technique used, such as a one-knife or multi-knife method. There is no principle that ESD should be performed with only one device (one-knife method). It depends on the preference of the endoscopist who performs ESD or on the technical difficulty of the target lesion. We prefer a multi-knife method and believe that it allows safer and easier ESD procedures. Also, the Fork knife enables endoscopists to perform ESD without changing accessories during the whole procedure time.

The knife tip of the Fork knife and the Flexknife are similar to that of a needle knife, and afford easier horizontal than vertical dissection. Nevertheless, a skilled endoscopist can dissect a lesion without considering the direction of the knife relative to the plane of the wall.

In the present study, most of the conditions such as tumor location, endoscopic appearance, resected specimen size, and fibrosis that can affect procedure time were similar between the two groups. However, ulcerations were significantly more common in group A than in group B. The rates of complete ESD, *en bloc* resection, and bleeding were higher in group A, but the difference was not significant. The bleeding rate in group A was 4.9%, whereas bleeding rates of 0.7% and 22% have been reported for the Flexknife[9] and IT knife[8]. The definition of bleeding in our study, i.e., all hemostatic processes that occurred during the procedure, was broader than that in the Flexknife study[9], which may account, at least in part, for the difference in the reported rates. Despite the tendency toward more frequent bleeding complications in group A, the procedure time was significantly shorter in the Fork knife than in the Flexknife group. The shorter procedure time cannot be attributed to differences in the distributions of resected specimen size, tumor location, or endoscopic appearance, as all of these factors were similar between the two groups. It is likely that the shorter procedure time in group A resulted from the faster control of bleeding with the Fork knife compared with the Flexknife.

This study was limited by the different numbers of enrolled patients in each group; group A had four times the number of subjects in group B. This may present concerns regarding statistical comparisons between the groups. Another matter to consider is that ESD using the Fork knife was performed by a single endoscopist, who developed the design. Therefore, the endoscopist may have taken exceptional care to avoid complications or to shorten the procedure time. Also, we did not take into consideration the technical improvement of the endoscopist that resulted from his accumulated experience. Nevertheless, it is our belief that the multi-functional Fork knife can potentially reduce the time required for ESD and make ESD a more acceptable procedure for experienced endoscopists. The aim of this study was not to compare resection techniques such as ESD with the Flexknife *vs* ESD with the Fork knife, but to report on our preliminary experience of using a multi-functional and convenient tool that may save time.

In conclusion, the Fork knife is a multi-functional instrument that can perform various therapeutic endoscopic procedures such as marking, injection, dissection, coagulation, and simple saline irrigation, without the need to change accessories during procedures. The major advantage of the Fork knife is that it allows endoscopists to perform ESD more easily. The Fork knife may be a very useful device for performing ESD.

COMMENTS

Background

Each step of ESD, such as lesion marking, injection, incision, and dissection, may require a different accessory or a knife, depending on the location and shape of the tumor. Therefore, when performing ESD, the accessories may need to be changed frequently through the working channel of the endoscope, prolonging the procedure and delaying the control of GI bleeding.

Research frontiers

Many investigators have improved the technique of ESD and have designed various knives that enable the endoscopist to perform ESD more easily, such as the IT knife, Hookknife, Flexknife, and triangle-tipped knife. These knives have contributed to the development of ESD techniques, and each has merits and demerits.

Innovations and breakthroughs

The authors introduced and evaluated the efficacy and technical aspects of ESD using a novel device, a convenient multi-functional Fork knife. The Fork knife consists of two knives in a single working unit: a fixed flexible snare knife and a forked knife. These two knives can be interchanged easily and have the advantage of being multifunctional; they can be used for marking, injecting, incising, irrigating, dissecting, and controlling bleeding. This enables the endoscopist to perform ESD more conveniently, thus shortening the procedure.

Applications

The Fork knife may be useful for ESD of gastric lesions, such as early gastric cancer and gastric adenoma. The feasibility and safety of the novel device is similar to that of other knives but it may actually be more convenient because of its design.

Terminology

The ESD technique was first introduced in 1988 in Japan. ESD is an innovative technique that improves the rate of successful *en bloc* resection of early-stage GI neoplasms.

Peer review

An interesting article on a timely topic, endoscopic submucosal resection of early gastric cancer. The authors compared two newer instruments and found no real difference between them. The paper is well written, the study well conceived, and I learned a lot about the new treatments of early gastric cancer.

REFERENCES

1 **Watanabe K**, Ogata S, Kawazoe S, Watanabe K, Koyama T, Kajiwara T, Shimoda Y, Takase Y, Irie K, Mizuguchi M, Tsunada S, Iwakiri R, Fujimoto K. Clinical outcomes of EMR for gastric tumors: historical pilot evaluation between endoscopic submucosal dissection and conventional

mucosal resection. *Gastrointest Endosc* 2006; **63**: 776-782
2 **Yokoi C**, Gotoda T, Hamanaka H, Oda I. Endoscopic submucosal dissection allows curative resection of locally recurrent early gastric cancer after prior endoscopic mucosal resection. *Gastrointest Endosc* 2006; **64**: 212-218
3 **Kakushima N**, Yahagi N, Fujishiro M, Kodashima S, Nakamura M, Omata M. Efficacy and safety of endoscopic submucosal dissection for tumors of the esophagogastric junction. *Endoscopy* 2006; **38**: 170-174
4 **Fujishiro M**, Yahagi N, Nakamura M, Kakushima N, Kodashima S, Ono S, Kobayashi K, Hashimoto T, Yamamichi N, Tateishi A, Shimizu Y, Oka M, Ogura K, Kawabe T, Ichinose M, Omata M. Successful outcomes of a novel endoscopic treatment for GI tumors: endoscopic submucosal dissection with a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar. *Gastrointest Endosc* 2006; **63**: 243-249
5 **Miyamoto S**, Muto M, Hamamoto Y, Boku N, Ohtsu A, Baba S, Yoshida M, Ohkuwa M, Hosokawa K, Tajiri H, Yoshida S. A new technique for endoscopic mucosal resection with an insulated-tip electrosurgical knife improves the completeness of resection of intramucosal gastric neoplasms. *Gastrointest Endosc* 2002; **55**: 576-581
6 **Miyazaki S**, Gunji Y, Aoki T, Nakajima K, Nabeya Y, Hayashi H, Shimada H, Uesato M, Hirayama N, Karube T, Akai T, Nikaidou T, Kouzu T, Ochiai T. High en bloc resection rate achieved by endoscopic mucosal resection with IT knife for early gastric cancer. *Hepatogastroenterology* 2005; **52**: 954-958
7 **Ono H**, Kondo H, Gotoda T, Shirao K, Yamaguchi H, Saito D, Hosokawa K, Shimoda T, Yoshida S. Endoscopic mucosal resection for treatment of early gastric cancer. *Gut* 2001; **48**: 225-229
8 **Ohkuwa M**, Hosokawa K, Boku N, Ohtu A, Tajiri H, Yoshida S. New endoscopic treatment for intramucosal gastric tumors using an insulated-tip diathermic knife. *Endoscopy* 2001; **33**: 221-226
9 **Kodashima S**, Fujishiro M, Yahagi N, Kakushima N, Ichinose M, Omata M. Endoscopic submucosal dissection for gastric neoplasia: experience with the flex-knife. *Acta Gastroenterol Belg* 2006; **69**: 224-229
10 **Oyama T**, Tomori A, Hotta K, Morita S, Kominato K, Tanaka M, Miyata Y. Endoscopic submucosal dissection of early esophageal cancer. *Clin Gastroenterol Hepatol* 2005; **3**: S67-S70
11 **Gotoda T**. A large endoscopic resection by endoscopic submucosal dissection procedure for early gastric cancer. *Clin Gastroenterol Hepatol* 2005; **3**: S71-S73
12 **Shim CS**. Endoscopic mucosal resection: an overview of the value of different techniques. *Endoscopy* 2001; **33**: 271-275
13 **Monkewich GJ**, Haber GB. Novel endoscopic therapies for gastrointestinal malignancies: endoscopic mucosal resection and endoscopic ablation. *Med Clin North Am* 2005; **89**: 159-186, ix
14 **Neuhaus H**, Costamagna G, Deviere J, Fockens P, Ponchon T, Rosch T. Endoscopic submucosal dissection (ESD) of early neoplastic gastric lesions using a new double-channel endoscope (the "R-scope"). *Endoscopy* 2006; **38**: 1016-1023
15 **Gotoda T**, Yamamoto H, Soetikno RM. Endoscopic submucosal dissection of early gastric cancer. *J Gastroenterol* 2006; **41**: 929-942
16 **Hirao M**, Masuda K, Asanuma T, Naka H, Noda K, Matsuura K, Yamaguchi O, Ueda N. Endoscopic resection of early gastric cancer and other tumors with local injection of hypertonic saline-epinephrine. *Gastrointest Endosc* 1988; **34**: 264-269
17 **Nakajima T**. Gastric cancer treatment guidelines in Japan. *Gastric Cancer* 2002; **5**: 1-5
18 **Uedo N**, Iishi H, Tatsuta M, Ishihara R, Higashino K, Takeuchi Y, Imanaka K, Yamada T, Yamamoto S, Yamamoto S, Tsukuma H, Ishiguro S. Longterm outcomes after endoscopic mucosal resection for early gastric cancer. *Gastric Cancer* 2006; **9**: 88-92
19 **Kim JJ**, Lee JH, Jung HY, Lee GH, Cho JY, Ryu CB, Chun HJ, Park JJ, Lee WS, Kim HS, Chung MG, Moon JS, Choi SR, Song GA, Jeong HY, Jee SR, Seol SY, Yoon YB. EMR for early gastric cancer in Korea: a multicenter retrospective study. *Gastrointest Endosc* 2007; **66**: 693-700
20 **Tajima Y**, Nakanishi Y, Ochiai A, Tachimori Y, Kato H, Watanabe H, Yamaguchi H, Yoshimura K, Kusano M, Shimoda T. Histopathologic findings predicting lymph node metastasis and prognosis of patients with superficial esophageal carcinoma: analysis of 240 surgically resected tumors. *Cancer* 2000; **88**: 1285-1293
21 **Gotoda T**, Yanagisawa A, Sasako M, Ono H, Nakanishi Y, Shimoda T, Kato Y. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. *Gastric Cancer* 2000; **3**: 219-225
22 **Fujishiro M**. Endoscopic submucosal dissection for stomach neoplasms. *World J Gastroenterol* 2006; **12**: 5108-5112
23 **Onozato Y**, Ishihara H, Iizuka H, Sohara N, Kakizaki S, Okamura S, Mori M. Endoscopic submucosal dissection for early gastric cancers and large flat adenomas. *Endoscopy* 2006; **38**: 980-986
24 **Imagawa A**, Okada H, Kawahara Y, Takenaka R, Kato J, Kawamoto H, Fujiki S, Takata R, Yoshino T, Shiratori Y. Endoscopic submucosal dissection for early gastric cancer: results and degrees of technical difficulty as well as success. *Endoscopy* 2006; **38**: 987-990
25 **Gotoda T**, Friedland S, Hamanaka H, Soetikno R. A learning curve for advanced endoscopic resection. *Gastrointest Endosc* 2005; **62**: 866-867
26 **Takeuchi Y**, Uedo N, Iishi H, Yamamoto S, Yamamoto S, Yamada T, Higashino K, Ishihara R, Tatsuta M, Ishiguro S. Endoscopic submucosal dissection with insulated-tip knife for large mucosal early gastric cancer: a feasibility study (with videos). *Gastrointest Endosc* 2007; **66**: 186-193

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RAPID COMMUNICATION

XRCC1 genetic polymorphism Arg399Gln and gastric cancer risk: A meta-analysis

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Abstract

AIM: To evaluate the association between X-ray cross-complementing gene 1 (XRCC1) genetic polymorphism Arg399Gln and gastric cancer risk by means of meta-analysis.

METHODS: We searched PubMed and NCBI up to June 1, 2008. A total of 16 clinical trials and reports were identified, but only 8 trials qualified under our selection criteria. Statistical analysis was performed with the software program Review Manage, version 4.2.8.

RESULTS: Of the 8 case-control studies selected for this meta-analysis, a total of 1334 gastric cancer cases and 2194 controls were included. For Arg399Gln, the Gln/Gln genotype carriers did not have a decreased cancer risk compared with those individuals with the Arg/Arg genotype (OR = 0.92, 95% CI, 0.71-1.19; P = 0.51). Similarly, no associations were found in the recessive and dominant modeling (Gln/Gln *vs* Arg/Gln + Arg/Arg: OR = 0.96; 95% CI, 0.77-1.19; P = 0.70 and Gln/Gln + Arg/Gln *vs* Arg/Arg: OR = 0.90, 95% CI, 0.77-1.05; P = 0.18).

CONCLUSION: No association is found between the XRCC1 polymorphism Arg399Gln and gastric cancer risk.

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Key words: Gastric cancer; Gene polymorphism; X-ray cross-complementing gene 1; Meta-analysis

Peer reviewers: Dr. Mark S Pearce, Pediatric and Lifecourse

INTRODUCTION

Gastric cancer is a leading cause of death worldwide, with nearly a million new cases diagnosed each year. It is the fourth most common cancer and the second leading cause of cancer death worldwide[1]. Multiple environmental and lifestyle factors may increase the risk of gastric cancer (GC), including tobacco use[2], a diet poor in fresh fruits and vegetables or rich in salt[3], and *Helicobacter pylori* (*H pylori*) infection[4]. However, not all of those who have been exposed to the risk factors will develop gastric cancer, suggesting the inter-individual differences in susceptibility. These differences may in part be caused by genetic variation, such as single nucleotide polymorphism (SNPs)[5] in DNA repair gene that increase susceptibility to the DNA damage resulting from carcinogens, particularly when these SNPs are located within the coding or regulating regions causing altered protein expression.

One of the important DNA repair protein is X-ray cross-complementing gene 1 (XRCC1)[6], acting as a scaffolding protein for the base excision repair (BER) and single-strand break repair (SSBR). These overlapping pathways participate in the constitutive response to endogenous mutagens and exogenous exposures, including tobacco smoke. Specifically, XRCC1-mediated pathways repair damage to DNA bases, from oxidation or covalent binding of nonbulky electrophiles, and to the deoxyribose phosphate backbone. Quick resolution of this genetic damage is imperative because repair intermediates, such as abasic sites and SSB, are generally more genotoxic and cytotoxic than the initial lesion.

Three common polymorphisms within the XRCC1 gene have been identified at codon 194, 280 and 399 (Arg194Trp, Arg280His, and Arg399Gln)[7]. These nonconservative amino acid changes may alter XRCC1 function. This change in protein biochemistry leads to the supposition that variant alleles may diminish repair kinetics, thereby influencing susceptibility to adverse health effects, including cancer.

Shen *et al*[8] first reported an association between XRCC1 codon 399 polymorphisms and gastric cancer. Since the publication of this report, many studies have appeared in the literature either supporting or negating the association. To clarify the effect of XRCC1 codon 399 polymorphisms on the risk of gastric cancer, we have undertaken a systematic review and meta-analysis.

MATERIALS AND METHODS

Literature search strategy

We searched in the Medline and Chinese National Knowledge Infrastructure (CNKI), covering all papers published up to June 1, 2008, with a combination of the following keywords: gastric cancer and XRCC1. We evaluated potentially relevant publications by examining their titles and abstracts and procured the most relevant publications for a closer examination. Besides the database search, the reference lists of the selected papers were also screened for other potential articles that may have been missed in the initial search. The search and evaluation were conducted from January to June 2008.

The following criteria were used for the literature selection for the meta-analysis: (1) The articles should be published in either English or Chinese between January 1989 and March 2008; (2) Only the case-control studies were considered; (3) The paper should clearly describe gastric cancer diagnoses and the sources of cases and controls; (4) The authors must offer the size of the sample, odds ratios (ORs) and their 95% confidence intervals (CIs) or the information that can help infer the results in the papers; (5) The definition of the exposure/risk genotypes was similar in all papers; (6) The methods of data collection and analysis should be statistically acceptable; (7) Those publications that presented data allowing such outcomes to be derived were also included.

Accordingly, the following exclusion criteria were also used: (1) The design and the definition of the exposure were obviously different from those of the selected papers; (2) Not offering the source of cases and controls and other essential information; (3) Reviews and repeated literatures were also excluded.

Data extraction

To minimize the bias and to improve the reliability, two reviewers checked all potentially relevant studies independently. Data on the following characteristics were also extracted: the first author, year of publication, journal, study population, number of genotyped cases and controls, and odds ratios and their confidence intervals.

Meta analysis

The strength of the associations between gastric cancer and the XRCC1 polymorphism was estimated by ORs and 95% CI. For the Arg399Gln polymorphism, we first estimated the risk of the variant genotype Gln/Gln and compared with the wild-type Arg/Arg homozygote, and then evaluated the risks of Gln/Gln *vs* (Arg/Gln + Arg /Arg) and (Arg/Gln + Gln/Gln) *vs* Arg/Arg, which assumed the recessive and dominant effect of the variant Gln399 allele.

We assessed the departure from the Hardy-Weinberg equilibrium for the control group in each study using the HWE program (http://linkage.rockefeller.edu/ott/linkutil.htm) for goodness of fit. The Peto Mantel-Haenszel fixed effect model or DerSimonian Laird random effect model was selected to summarize the combined *OR* dependent on the results of heterogeneity test among individual studies. And the heterogeneity was considered significant if $P < 0.05$. If there was no heterogeneity, fixed effects model was used; otherwise, a random effect model based on the DerSimonian and Laird estimator was used[9]. Inverted funnel plots were used to provide diagnosis of publication biases.

All of the statistical analyses were performed with Review Manager (version 4.2.8, The Cochrane Collaboration).

RESULTS

Literature search and meta-analysis databases

We selected 16 published papers dealing with case-control studies of the polymorphisms .We reviewed all papers in accordance with the criteria defined above, and excluded 8 papers because their study designs were different from others or they did not list data clearly enough for further analysis or repeated literatures. Hence, data were available from 8 case-control studies, including 1334 gastric cancer cases and 2194 controls (Table 1)[8,10-16]. We established a database according to the extracted information from each article. Given in Table 1 are the lists of the publication year, first author, and the number of cases and controls for each XRCC1 codon 399 genotype. Other necessary information is also listed in the forest plots in our meta-analysis. No qualified researches were acquired before 2000, which suggested that the researches between XRCC1 polymorphism and gastric cancer were started very late.

Meta analysis

The Gln/Gln genotype carriers did not have an increased cancer risk compared with those individuals with the Arg/Arg genotype (OR, 0.92; 95% CI, 0.71-1.19; $P = 0.51$). Similarly, no associations were found in the recessive and dominant modeling (Gln/Gln *vs* Arg/Gln + Arg/Arg: OR, 0.96; 95% CI, 0.77-1.19; $P = 0.70$ and Gln/Gln + Arg/Gln *vs* Arg/Arg: OR, 0.90; 95% CI, 0.77-1.05; $P = 0.18$). We did not find any associations between Arg399Gln polymorphism and gastric cancer risk in our study (Figure 1).

In all of the studies, the genotype frequencies were

Table 1 Distribution of XRCC1 genotype among gastric cancer cases and controls included in the meta-analysis

Distribution			XRCC1 condon 399						HWE
			Prostate cancer			Control			
First author	Area	Yr	Arg/Gln	Arg/Gln	Gln/Gln	Arg/Gln	Arg/Gln	Gln/Gln	
Shen *et al*[8]	China	2000	13	83	92	13	59	64	Yes
Lee *et al*[10]	South Korea	2002	110	71	9	94	69	9	Yes
Ratnasinghe *et al*[11]	China	2004	49	34	3	192	193	33	Yes
Duarte *et al*[12]	Brazil	2005	73	67	20	70	57	23	Yes
Huang *et al*[13]	Poland	2005	124	121	36	166	179	45	Yes
Song *et al*[14]	China	2006	46	48	8	54	44	3	Yes
Zhang *et al*[15]	China	2006	136	82	18	369	275	64	Yes
Ruzzo *et al*[16]	Italy	2007	10	45	36	10	59	50	Yes

HWE: Hardy-Weinberg equilibrium.

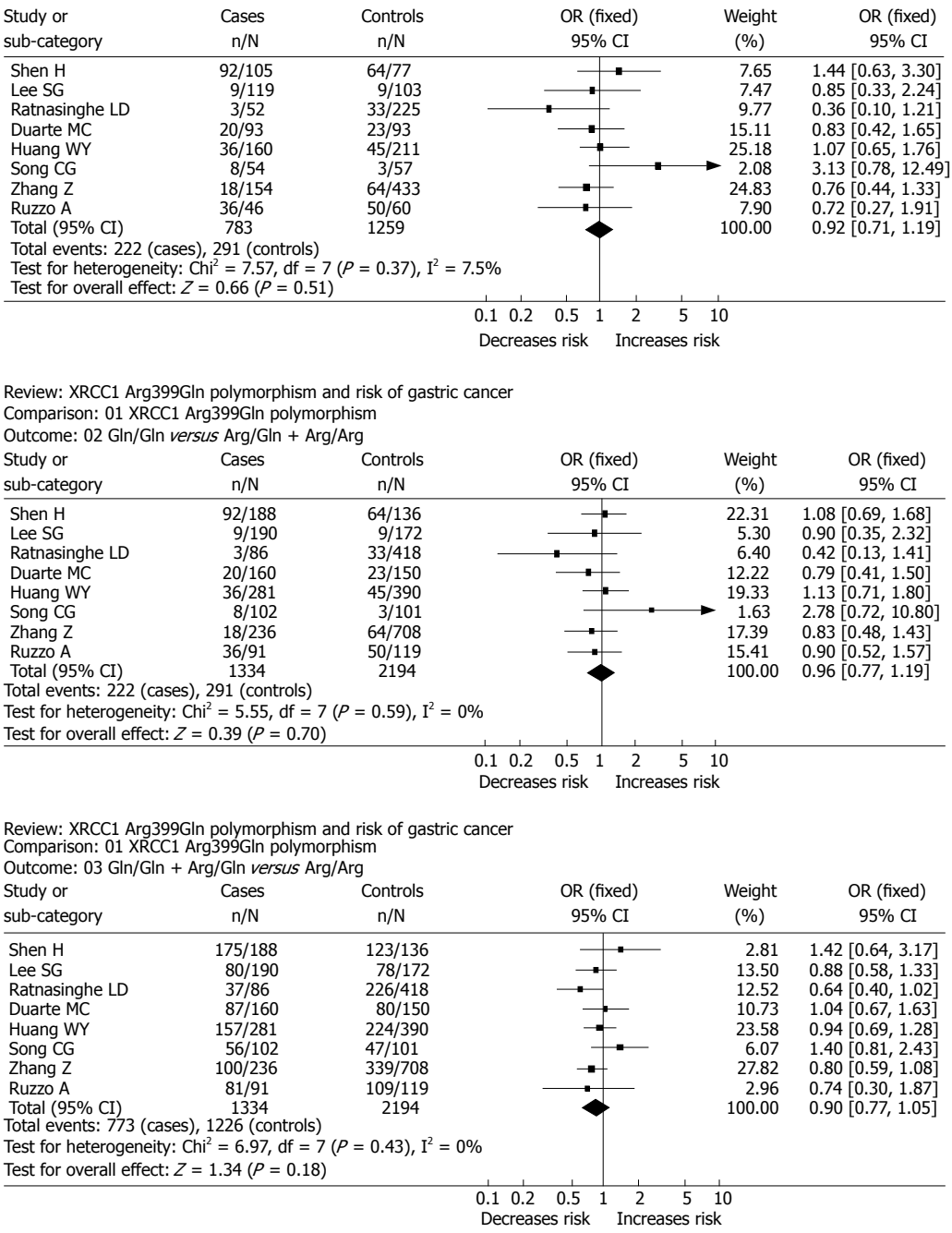

Figure 1 Forest plot of a meta-analysis of the association between XRCC1 polymorphism Arg399Gln and gastric cancer risk (fixed-effects mode).

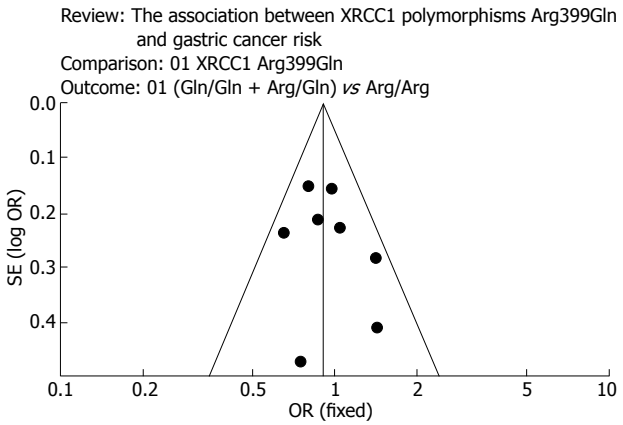

Figure 2 Funnel plot to explore publication bias. The graphical funnel plot of the 8 published studies appears to be asymmetrical.

consistent with Hardy-Weinberg equilibrium (Table 1). No significant between-study heterogeneities were present in the heterogeneity tests. In the funnel plot analysis of publication biases, the shape of the funnel plot (Figure 2) appeared to be approximately symmetrical, and the magnitude of the main ORs was in dispersion on the left side of 1. Therefore, the funnel plot analysis suggested that publication biases might not have a significant influence on the results of the XRCC1 gene.

DISCUSSION

XRCC1 protein is an important component of the BER pathway, which fixes base damage and DNA single strand breaks caused by ionizing radiation and alkylating agents. The XRCC1 protein has no known catalytic activity but serves to orchestrate BER through its role as a central scaffolding protein for DNA ligase Ⅲ, DNA polymerase β, and PARP, and also through its function in recognizing and binding to single-strand breaks[17]. The Arg399Gln polymorphism[18] is located in the region of the BRCT- I interaction domain of XRCC1 with ADPribosepolymerase, the presence of the variant 399Gln allele has been shown to be associated with measurable reduced DRC as assessed by the persistence of DNA adducts, tumor-suppressor gene P53 mutations, increased red blood cell glycophorin A, elevated levels of sister chromatid exchanges and prolonged cell-cycle delay.

To the best of our knowledge, it is the first systematic review that has investigated the association of XRCC1 codon 399 polymorphisms and gastric cancer, and no evidence has shown any associations between Arg399Gln polymorphism and gastric cancer susceptibility. One possible explanation is that human cells have five different DNA repair systems, characterized by the involvement of more than 150 proteins coded by genes, the majority of which are polymorphic. Thus, given that inter-individual variation in DNA repair capacity is in part explained by SNPs in repair genes, it is possible that the analyzed variants do not influence the basal level of damage. Nevertheless, based on our study, it is worth noting that, even if a common variant in the functional region of a definitively meaningful gene had an effect on human disease, such as cancer, it may play only a minor role in the disease causation.

Although the relationship is inconsistent in bladder cancer[19], other previous studies have found that the XRCC1 399Gln/Gln genotype is associated with an increased risk of lung cancer among Asians[20] and breast cancer in African Americans[21]. Our present and previous studies were similar in most aspects, and the reasons for this apparent difference in risk with different tumors are as yet unknown, possibly due to the overall heterogeneity in the studies that find strong positive or inverse associations. Many meta-analyses of several gene-disease associations have shown that initially promising associations often gravitate toward null over time[22].

In our study, no significant between-study heterogeneities were present in the heterogeneity tests, indicating that our present combined analyses were unbiased, and no obvious publication bias existed in our meta-analysis, since the "funnel plot" was symmetrically plotted. However, serious limitations were still inherited from the published studies. First, our assessment was based on relatively few studies, and because the papers included in our meta-analysis were limited to those published in either English or Chinese only in the periods between 1989 and 2008, it is possible that some relevant published studies and unpublished studies that are likely to have null results were not included, which may have biased the results. Therefore, although the test for publication bias was not statistically significant, possible bias, especially the outcome-reporting bias, still could not be ruled out. Second, most studies selected case subjects from Asian populations; therefore, we did not conduct a subgroup analysis according to different ethnic groups. The other studies were from Brazil, Poland and Italy, which have different patterns of lifestyle risk factors, genetic backgrounds, and gastric cancer risks. Third, we did not test for gene x environment interactions because of the issue of multiple testing and the lack of sufficient studies. Nevertheless, it is possible that these polymorphisms alter risk in subgroups of the population that have been exposed to specific environmental and lifestyle factors. Therefore, larger and well-designed studies are needed to further evaluate the association between XRCC1 polymorphism and gastric cancer risk.

In summary, our meta-analysis evaluated the relationship between genetic polymorphisms and gastric cancer risk and revealed that XRCC1 polymorphism Arg399Gln could not alter susceptibility to gastric cancer.

COMMENTS

Background

A G to A transition of the X-ray cross-complementing gene 1 (XRCC1) gene at codon 399 has been implicated as a risk factor for gastric cancer, but individual studies have been inconclusive or controversial. The aim of this meta-analysis

was to clarify the effect of XRCC1 Arg399Gln polymorphism on the risk of gastric cancer.

Research frontiers

To date, there have been many studies on the association between XRCC1 genetic polymorphism Arg399Gln and gastric cancer risk, but no meta-analyses.

Innovations and breakthroughs

XRCC1 polymorphism Arg399Gln could not alter susceptibility to gastric cancer. Further studies are needed to prove it.

Applications

It can be seen from this paper that XRCC1 polymorphism Arg399Gln could not alter susceptibility to gastric cancer. It suggests that, even if a common variant in the functional region of a definitively meaningful gene had an effect on human disease, such as cancer, it may play only a minor role in the disease causation.

Peer review

The study is nicely designed and the analytical data appear to be scientifically sound. The outcome clearly shows that XRCC1 polymorphism Arg399Gln could not alter susceptibility to gastric cancer.

REFERENCES

1 **Crew KD**, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**: 354-362

2 **Yang L**. Incidence and mortality of gastric cancer in China. *World J Gastroenterol* 2006; **12**: 17-20

3 **Kushi LH**, Byers T, Doyle C, Bandera EV, McCullough M, McTiernan A, Gansler T, Andrews KS, Thun MJ. American Cancer Society Guidelines on Nutrition and Physical Activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity. *CA Cancer J Clin* 2006; **56**: 254-281; quiz 313-314

4 **Masuda G**, Tokunaga A, Shirakawa T, Togashi A, Kiyama T, Kato S, Matsukura N, Bou H, Watanabe M, Tajiri T. Helicobacter pylori infection, but not genetic polymorphism of CYP2E1, is highly prevalent in gastric cancer patients younger than 40 years. *Gastric Cancer* 2007; **10**: 98-103

5 **Zienolddiny S**, Campa D, Lind H, Ryberg D, Skaug V, Stangeland L, Phillips DH, Canzian F, Haugen A. Polymorphisms of DNA repair genes and risk of non-small cell lung cancer. *Carcinogenesis* 2006; **27**: 560-567

6 **Caldecott KW**, Aoufouchi S, Johnson P, Shall S. XRCC1 polypeptide interacts with DNA polymerase beta and possibly poly (ADP-ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' in vitro. *Nucleic Acids Res* 1996; **24**: 4387-4394

7 **Whitehouse CJ**, Taylor RM, Thistlethwaite A, Zhang H, Karimi-Busheri F, Lasko DD, Weinfeld M, Caldecott KW. XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. *Cell* 2001; **104**: 107-117

8 **Shen H**, Xu Y, Qian Y, Yu R, Qin Y, Zhou L, Wang X, Spitz MR, Wei Q. Polymorphisms of the DNA repair gene XRCC1 and risk of gastric cancer in a Chinese population. *Int J Cancer* 2000; **88**: 601-606

9 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188

10 **Lee SG**, Kim B, Choi J, Kim C, Lee I, Song K. Genetic polymorphisms of XRCC1 and risk of gastric cancer. *Cancer Lett* 2002; **187**: 53-60

11 **Ratnasinghe LD**, Abnet C, Qiao YL, Modali R, Stolzenberg-Solomon R, Dong ZW, Dawsey SM, Mark SD, Taylor PR. Polymorphisms of XRCC1 and risk of esophageal and gastric cardia cancer. *Cancer Lett* 2004; **216**: 157-164

12 **Duarte MC**, Colombo J, Rossit AR, Caetano A, Borim AA, Wornrath D, Silva AE. Polymorphisms of DNA repair genes XRCC1 and XRCC3, interaction with environmental exposure and risk of chronic gastritis and gastric cancer. *World J Gastroenterol* 2005; **11**: 6593-6600

13 **Huang WY**, Chow WH, Rothman N, Lissowska J, Llaca V, Yeager M, Zatonski W, Hayes RB. Selected DNA repair polymorphisms and gastric cancer in Poland. *Carcinogenesis* 2005; **26**: 1354-1359

14 **Song CG**, Lu HS, Huang CM, Liu X, Hou PF, Zhang XF. Relationship between gene polymorphism of XRCC1 Arg399Gln and the risk of gastric cancer patients in Fujian. *Zhonghua Shiyan Waike Zazhi* 2006; **23**: 1021

15 **Zhang Z**, Miao XP, Tan W, Guo YL, Zhang XM, Lin DX. [Correlation of genetic polymorphisms in DNA repair genes ADPRT and XRCC1 to risk of gastric cancer] *Ai Zheng* 2006; **25**: 7-10

16 **Ruzzo A**, Canestrari E, Maltese P, Pizzagalli F, Graziano F, Santini D, Catalano V, Ficarelli R, Mari D, Bisonni R, Giordani P, Giustini L, Lippe P, Silva R, Mattioli R, Torresi U, Latini L, Magnani M. Polymorphisms in genes involved in DNA repair and metabolism of xenobiotics in individual susceptibility to sporadic diffuse gastric cancer. *Clin Chem Lab Med* 2007; **45**: 822-828

17 **Marintchev A**, Mullen MA, Maciejewski MW, Pan B, Gryk MR, Mullen GP. Solution structure of the single-strand break repair protein XRCC1 N-terminal domain. *Nat Struct Biol* 1999; **6**: 884-893

18 **Lunn RM**, Langlois RG, Hsieh LL, Thompson CL, Bell DA. XRCC1 polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. *Cancer Res* 1999; **59**: 2557-2561

19 **Wang C**, Sun Y, Han R. XRCC1 genetic polymorphisms and bladder cancer susceptibility: a meta-analysis. *Urology* 2008; **72**: 869-872

20 **Kiyohara C**, Takayama K, Nakanishi Y. Association of genetic polymorphisms in the base excision repair pathway with lung cancer risk: a meta-analysis. *Lung Cancer* 2006; **54**: 267-283

21 **Duell EJ**, Millikan RC, Pittman GS, Winkel S, Lunn RM, Tse CK, Eaton A, Mohrenweiser HW, Newman B, Bell DA. Polymorphisms in the DNA repair gene XRCC1 and breast cancer. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 217-222

22 **Ioannidis JP**, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG. Replication validity of genetic association studies. *Nat Genet* 2001; **29**: 306-309

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RAPID COMMUNICATION

Value of ultrasound examination in differential diagnosis of pancreatic lymphoma and pancreatic cancer

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Abstract

AIM: To investigate the value of clinical manifestations and ultrasound examination in the differential diagnosis of pancreatic lymphoma and pancreatic cancer.

METHODS: The clinical and ultrasonic characteristics of 12 cases of pancreatic lymphoma and 30 cases of pancreatic cancer were retrospectively analyzed.

RESULTS: Statistically significant differences were found in the course of disease, back pain, jaundice, carcino-embryonic antigen (CEA) and CA19-9 increase, palpable abdominal lump, superficial lymph node enlargement, fever and night sweats, lesion size, bile duct expansion, pancreatic duct expansion, vascular involvement, retroperitoneal (below the renal vein level) lymph node enlargement, and intrahepatic metastasis between pancreatic lymphoma and pancreatic cancer. There were no significant differences in age of onset, gender ratio, weight loss, nausea and vomiting, lesion position, the echo of the lesion, and the blood flow of the lesion.

CONCLUSION: Pancreatic lymphoma should be considered for patients with long lasting symptoms, superficial lymph node enlargement, palpable abdominal lump, fever and night sweats, relatively large lesions, and retroperitoneal (below the level of the renal vein) lymph node enlargement. A diagnosis of pancreatic cancer should be considered more likely in the patients with relatively short disease course, jaundice, back pain, CEA and CA19-9 increase, relatively small lesions, bile duct expansion, obvious pancreatic duct expansion, peripheral vascular wrapping and involvement, or intrahepatic metastases.

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INTRODUCTION

Among the pancreatic tumors, there are much similarities in clinical manifestations and imaging characteristics of pancreatic cancer and pancreatic lymphoma and thus they are easy to be misdiagnosed[1-4]. However, the treatment and prognosis of pancreatic cancer and pancreatic lymphoma are quite different, and the differential diagnosis of these two diseases is therefore very important[5-7]. We retrospectively analyzed clinical and ultrasonic data of 12 cases of pancreatic lymphoma and 30 cases of pancreatic cancer to study the differentiation between the two diseases.

MATERIALS AND METHODS

Subjects

Forty-two pancreatic lesions were retrospectively analyzed. Patients with lymphoma (n = 12) and patients with pancreatic cancer (n = 30) whose diagnoses were confirmed by surgical pathology at our hospital from April 2001 to December 2007 were studied. All patients received ultrasound examinations before treatment.

Instruments and methods

Instruments: GE Logiq 7, Philips HDI-5000, HD-11 color Doppler ultrasound diagnostic instrument were used. The probe frequency was 8-12 MHz; speed range, blood flow filtering and color gain were adjusted to achieve blood flow maps with the best display status.

Ultrasound examination: Patients were placed in the supine position, the gray-scale ultrasound was used

Table 1 Clinical manifestation of pancreatic lymphoma and pancreatic cancer

	Pancreatic lymphoma	Pancreatic cancer	*P*
Age of onset (yr)	52.8 ± 3.4	53.8 ± 4.2	NS
Gender ratio (male/female)	10/2	24/6	NS
Course of disease	3 mo to 1 yr	4 wk to 3 mo	< 0.05
Weight loss	10	27	NS
Nausea and vomiting	4	12	NS
Fever and night sweats	5	1	< 0.05
Back pain	1	18	< 0.05
Jaundice	3	20	< 0.05
Palpable abdominal lump	6	0	< 0.05
Superficial lymph node enlargement	5	1	< 0.05
CEA, CA19-9 increase	0	18	< 0.05

P < 0.05, the two groups were significantly different.

to observe the size of the pancreatic lesion, lesion position, lesion echo, the main pancreatic duct, bile duct, peripancreatic vessels, and retroperitoneal lymph nodes. Then the color hemodromogram of the lesion area was displayed and divided into 3 grades: no blood flow signal, some blood flow signals, and abundant blood flow signals.

Statistical analysis

SAS V8.1 statistical software was used for data analysis. Statistical analyses were done for patient age, course of disease and lesion size using Wilcoxon's rank sum test. Fisher's exact probability and the χ^2 test were carried out for analyses of sex, back pain, jaundice, palpable abdominal lump, weight loss, nausea and vomiting, fever and night sweats, carcino-embryonic antigen (CEA) or CA19-9 increases, superficial lymph node enlargement, lesion position, lesion echo, bile duct expansion, pancreatic duct expansion, vascular invasion, retroperitoneal (below the renal vein level) lymph node enlargement, intrahepatic metastasis, and blood flow. $P < 0.05$ was taken as an indicator of a significant difference between the two groups.

RESULTS

Clinical manifestations

The clinical characteristics of pancreatic lymphoma and pancreatic cancer were compared. There was no significant difference in age of onset, gender ratio, weight loss, or nausea and vomiting between the two groups. The course of disease, back pain, jaundice, CEA and CA19-9 increase, palpable abdominal lump, superficial lymph node enlargement, fever and night sweats were significantly different between the two groups. This means that the disease course of the pancreatic lymphoma is longer, and superficial lymph node enlargement, palpable lump, and fever and night sweats occur more frequently in pancreatic lymphoma. In pancreatic cancer, back pain, jaundice, and CEA and CA19-9 increases occur more frequently (Table 1).

Table 2 Ultrasonic manifestations of pancreatic lymphoma and pancreatic cancer

	Pancreatic lymphoma	Pancreatic cancer	*P*
Lesion position			
Pancreatic head	7	19	NS
Pancreatic body & tail	3	7	NS
Diffuse	2	4	NS
Lesion echo	All 12 cases had low echo	All 30 cases had low echo	NS
Lesion size	79.6 ± 10.7 mm	34.7 ± 5.5 mm	< 0.05
Blood flow of the lesion	11 cases did not show any blood flow signals, 1 case showed some blood flow signals	None of the 30 cases showed blood flow signals	NS
Bile duct expansion	3	20	< 0.05
Pancreatic duct expansion	2	18	< 0.05
Blood vessel involvement	2	15	< 0.05
Retroperitoneal (below the renal vein) lymph node enlargement	5	1	< 0.05
Intrahepatic metastasis	0	6	< 0.05

P < 0.05, the two groups were significantly different.

Ultrasonic manifestations

The ultrasound characteristics of pancreatic lymphoma and of pancreatic cancer were compared. There were no significant differences in lesion position, lesion echo, or blood flow of the lesion between the two groups. The lesions size, bile duct expansion, pancreatic duct expansion, vascular involvement, retroperitoneal (below the renal vein) lymph node enlargement, and intrahepatic metastasis were significantly different (Table 2). In other words, the lesion size of pancreatic lymphoma is relatively larger, which is prone to causing enlargement of the lymph nodes below the renal vein level. Pancreatic cancer tends to cause bile duct and pancreatic duct expansion more frequently and is more likely to affect the blood vessels and lead to intrahepatic metastasis.

DISCUSSION

The incidence of pancreatic cancer has grown substantially in developed countries and in some developing countries. Currently, it is one of the top ten malignant tumors, and its prognosis is very poor. The 1-year survival rate is below 20% and the 5-year survival rate is only 3%[1,5,8]. Pancreatic lymphoma is a rare pancreatic tumor that accounts for only 1% of all pancreatic tumors. Its main pathological type is B-cell non-Hodgkin's lymphoma and the overwhelming majority of cases involve pancreatic infiltration by systemic lymphoma. It can also originate from the pancreas and show primary pancreatic manifestations, which are called primary pancreatic lymphoma (PPL). The average survival time of pancreatic lymphoma is 2-6.5 years, which is better than the prognosis for pancreatic cancer[9-11]. There are much similarities in the clinical and imaging examination of the two diseases so that they are easily misdiagnosed. However, the

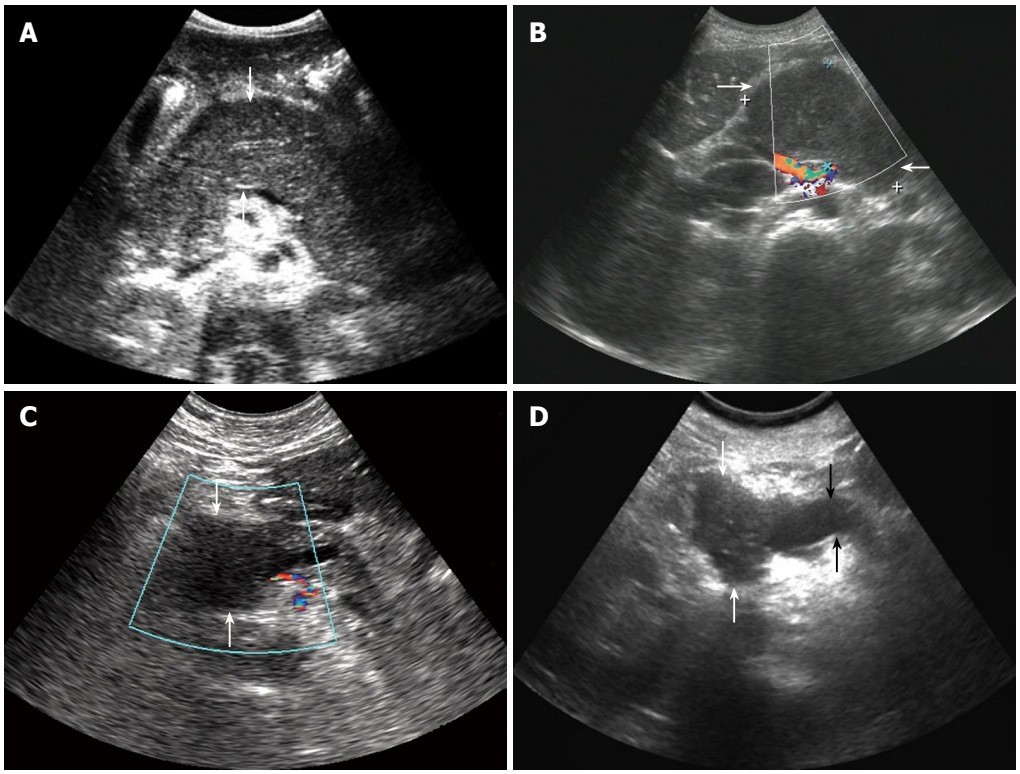

Figure 1 ultrasonic manifestation. A: Diffuse pancreatic lymphoma (arrows): the pancreas showed diffuse enlargement and the echo decreased; B: Pancreatic lymphoma (arrows): a low echo lump was seen in the pancreatic head. There were no blood flow signals; C: Pancreatic cancer (arrows): a low echo lump was seen in the pancreatic head. There were no blood flow signals; D: Pancreatic cancer: a low echo lump appeared in the pancreatic head (white arrows) and the main pancreatic duct (black arrows) was obviously expanded.

treatment and the prognosis of the two diseases are quite different. Therefore, the differential diagnosis of these two diseases is important.

Comparison of clinical characteristics of pancreatic lymphoma and pancreatic cancer

There was no significant difference in the age of onset, gender ratio, weight loss, nausea and vomiting between the two diseases. Therefore, these characteristics cannot be used for the differential diagnosis.

The course of disease of the patients with pancreatic lymphoma is usually longer than that of the patients with pancreatic cancer. As the lesion is relatively large in pancreatic lymphoma, an abdominal lump is easily palpable. In our series, an abdominal lump was palpable in 6 (50%) patients in the pancreatic lymphoma group. In addition, as the majority of the pancreatic lymphoma cases originate from systemic lymphoma, characteristics of the lymphoma such as superficial lymph node enlargement and fever and night sweats are likely to occur. Those characteristics are relatively rare in patients with pancreatic cancer. Compared with pancreatic lymphoma, jaundice and back pain are more likely to occur in patients with pancreatic cancer. This is because pancreatic cancer has perivascular and perineural growth[12-15]. It is easy for pancreatic cancer to cause jaundice by bile duct infiltration or oppression or to cause back pain by erosion of the retroperitoneal nerve, which is more obvious at night. Regarding laboratory tests, since the pancreatic cancer is a tumor with duct infiltration, most patients show increases in two tumor markers, CEA and CA19-9.Those markers will usually not increase in patients with pancreatic lymphoma[16-18].

Comparison of ultrasonic characteristics of pancreatic lymphoma and pancreatic cancer

There were no significant differences in the lesion position, lesion echo, or the blood flow of the lesion between the two diseases. Pancreatic head lesions are commonly seen in both diseases. In this study, pancreatic head lesions accounted for 58.3% of cases in the pancreatic lymphoma group and 63.3% of cases in the pancreatic cancer group. In addition, some lesions located in the body or the tails of the pancreas were diffuse (Figure 1A). All the lesions had low echo, and most lesions did not have any blood flow signals (Figure 1B and C).

The lesion sizes were significantly different between the two diseases. The lesion of the pancreatic lymphoma is usually greater than that of the pancreatic cancer. In this study, the lesion size of the pancreatic lymphoma was approximately 79.6 ± 10.7 mm, and the lesion size of the pancreatic cancer was approximately 34.7 ± 5.5 mm. Other literature reports showed similar results that the diameter of the pancreatic lymphoma lump is usually greater than 70 mm, and the diameter of the pancreatic cancer is usually less than 50 mm[19-22]. A possible reason is that the early symptoms of pancreatic lymphoma are not obvious and diagnosis is made relatively late; therefore the lesions are relatively big when they are found.

Bile duct expansion was significantly different between the two diseases. Pancreatic cancer readily infiltrates and oppresses the bile duct so that bile duct expansion occurs more frequently in pancreatic cancer than in pancreatic lymphoma. In this study, intrahepatic and extrahepatic bile duct expansion occurred in 20 (66.7%) cases of pancreatic cancer, but in only 3 (25%)

cases of pancreatic lymphoma.

Pancreatic duct expansion was statistically significant between the two diseases. In this study, 18 (60%) cases of pancreatic cancer showed pancreatic duct expansion (Figure 1D), while only 2 (16.7%) cases of pancreatic lymphoma did so. Another study showed that the pancreatic duct in pancreatic lymphoma can be discontinuous or narrow, expansion is rare, and the ratio of the expanded pancreatic duct to the anteroposterior diameter of the gland is less than 0.5. However, this ratio in pancreatic cancer (in more than 89% of cases) is often greater than 0.5[23-26].

The two diseases were significantly different regarding blood vessel involvement. The pancreatic cancer frequently wraps and erodes the blood vessels. There were 15 (50%) cases of pancreatic cancer with vascular involvement in this study; there were only 2 (16.7%) cases of pancreatic lymphoma with vascular involvement. The pancreatic lymphoma mainly pushes the surrounding blood vessels.

The two diseases were significantly different in retroperitoneal (below the renal vein level) lymph node enlargement. Peri-pancreatic and retro-peritoneal lymph node enlargement can occur in both pancreatic cancer and pancreatic lymphoma, but pancreatic lymphoma is often part of a systemic lymphoma. Therefore, there are more sites of lymph node enlargement in pancreatic lymphoma than in pancreatic cancer. It has been reported in the literature that retroperitoneal lymph node enlargement below the level of the renal vein seldom appears in pancreatic cancer[27-29]. In this study, 5 (41.7%) patients with pancreatic lymphoma showed lymph node enlargement below the level of the renal vein, but only 1 (3.3%) case of pancreatic cancer showed similar lymph node enlargement.

The two diseases were significantly different in intrahepatic metastasis. Intrahepatic metastasis occurred more easily in patients with pancreatic cancer compared with pancreatic lymphoma[30]. There were 6 (20%) cases of pancreatic cancer with intrahepatic metastasis in this study while no intrahepatic metastasis occurred in pancreatic lymphoma.

In summary, clinical and ultrasonic manifestations can aid in the differentiation between pancreatic lymphoma and pancreatic cancer. For the differential diagnosis of the two diseases, pancreatic lymphoma should be considered for patients with long lasting symptoms, superficial lymph node enlargement, palpable abdominal lump, fever and night sweats, relatively large lesions, and retroperitoneal (below the level of the renal vein) lymph node enlargement. A diagnosis of pancreatic cancer should be considered more likely for patients with relatively short disease course, jaundice, back pain, CEA and CA19-9 increase, relatively small lesions, bile duct expansion, obvious pancreatic duct expansion, peripheral vascular wrapping and involvement, or intrahepatic metastases. The diagnosis can be confirmed by biopsy or postoperative pathological results.

COMMENTS

Background

Differential diagnosis of pancreatic masses is a frequent clinical challenge. Differential diagnosis is difficult between pancreatic cancer and pancreatic lymphoma because there are a lot of similarities in clinical manifestations and imaging characteristics of pancreatic cancer and pancreatic lymphoma among pancreatic tumors. However, the treatment and prognosis of pancreatic cancer and pancreatic lymphoma are quite different, and the differential diagnosis of these two diseases is therefore, very important.

Research frontiers

Further researches are needed in order to improve the diagnosis of pancreatic cancer and pancreatic lymphoma. Availability of ultrasound examination and clinical manifestations may provide more information for the diagnosis of pancreatic cancer and pancreatic lymphoma.

Innovations and breakthroughs

Because the morbidity of pancreatic lymphoma is low, in this study, 12 cases of pancreatic lymphoma were important which could provide much favorable information for diagnosis.

Applications

Clinical and ultrasonic manifestations can aid in the differentiation between pancreatic lymphoma and pancreatic cancer.

Peer review

This is an interesting paper. The contents are important and significant because there is no much information about pancreatic lymphoma in the existing literature.

REFERENCES

1 **Miura F**, Takada T, Amano H, Yoshida M, Furui S, Takeshita K. Diagnosis of pancreatic cancer. *HPB* (Oxford) 2006; **8**: 337-342

2 **Mulkeen AL**, Yoo PS, Cha C. Less common neoplasms of the pancreas. *World J Gastroenterol* 2006; **12**: 3180-3185

3 **Leite NP**, Kased N, Hanna RF, Brown MA, Pereira JM, Cunha R, Sirlin CB. Cross-sectional imaging of extranodal involvement in abdominopelvic lymphoproliferative malignancies. *Radiographics* 2007; **27**: 1613-1634

4 **Sheth S**, Fishman EK. Imaging of uncommon tumors of the pancreas. *Radiol Clin North Am* 2002; **40**: 1273-1287, vi

5 **Mortenson MM**, Katz MH, Tamm EP, Bhutani MS, Wang H, Evans DB, Fleming JB. Current diagnosis and management of unusual pancreatic tumors. *Am J Surg* 2008; **196**: 100-113

6 **Paissan A**, Wachs A, Arias M, Abeldaño A, Frider B. [Obstructive jaundice associated Burkitt's lymphoma mimicking pancreatic carcinoma] *Acta Gastroenterol Latinoam* 2007; **37**: 246-249

7 **Canto MI**. Screening and surveillance approaches in familial pancreatic cancer. *Gastrointest Endosc Clin N Am* 2008; **18**: 535-553, x

8 **Helmstaedter L**, Riemann JF. Pancreatic cancer-EUS and early diagnosis. *Langenbecks Arch Surg* 2008; **393**: 923-927

9 **Ros PR**, Mortelé KJ.Imaging features of pancreatic neoplasms. *JBR-BTR* 2001; **84**: 239-249

10 **Basu A**, Patil N, Mohindra P, Zade B, Gujral S, Muckaden MA, Laskar S. Isolated non-Hodgkin's lymphoma of the pancreas: case report and review of literature. *J Cancer Res Ther* 2007; **3**: 236-239

11 **Aloui-Kasbi N**, Mbarek S, Bellagha I, Hammou A. [Primary T-cell lymphoma of the pancreas in children] *Tunis Med* 2005; **83**: 114-116

12 **Gardner TB**, Chari ST. Endoscopic ultrasonography and pancreatic cancer. *Minerva Gastroenterol Dietol* 2008; **54**: 161-176

13 **Rabinowitz CB**, Prabhakar HB, Sahani DV. Recent advances in imaging of pancreatic neoplasms. *Cancer Treat Res* 2008; **143**: 229-254

14 **Battula N**, Srinivasan P, Prachalias A, Rela M, Heaton N. Primary pancreatic lymphoma: diagnostic and therapeutic

dilemma. *Pancreas* 2006; **33**: 192-194
15 **Dietrich CF**, Braden B, Hocke M, Ott M, Ignee A. Improved characterisation of solitary solid pancreatic tumours using contrast enhanced transabdominal ultrasound. *J Cancer Res Clin Oncol* 2008; **134**: 635-643
16 **Choi EK**, Byun JH, Lee SJ, Jung SE, Park MS, Park SH, Lee MG. Imaging findings of leukemic involvement of the pancreaticobiliary system in adults. *AJR Am J Roentgenol* 2007; **188**: 1589-1595
17 **McCauley AM**, Gottlieb KT. Primary pancreatic lymphoma coexisting with chronic lymphocytic leukemia: EUS findings. *Gastrointest Endosc* 2008; **68**: 188-189
18 **Jayanthi V**, Randhir J, Rajesh N. Problems in diagnosing lymphoma of the pancreas with computed tomography. A case report. *J Gastrointestin Liver Dis* 2007; **16**: 101-103
19 **Sata N**, Kurogochi A, Endo K, Shimura K, Koizumi M, Nagai H. Follicular lymphoma of the pancreas: a case report and proposed new strategies for diagnosis and surgery of benign or low-grade malignant lesions of the head of the pancreas. *JOP* 2007; **8**: 44-49
20 **Leite NP**, Kased N, Hanna RF, Brown MA, Pereira JM, Cunha R, Sirlin CB. Cross-sectional imaging of extranodal involvement in abdominopelvic lymphoproliferative malignancies. *Radiographics* 2007; **27**: 1613-1634
21 **Shah S**, Mortele KJ. Uncommon solid pancreatic neoplasms: ultrasound, computed tomography, and magnetic resonance imaging features. *Semin Ultrasound CT MR* 2007; **28**: 357-370
22 **Ji Y**, Kuang TT, Tan YS, Chen Y, Zeng HY, Jin DY. Pancreatic primary lymphoma: a case report and review of the literature. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 622-626
23 **Rickes S**, Malfertheiner P. Echo-enhanced ultrasound--a new imaging modality for the differentiation of pancreatic lesions. *Int J Colorectal Dis* 2006; **21**: 269-275
24 **Kalra MK**, Maher MM, Mueller PR, Saini S. State-of-the-art imaging of pancreatic neoplasms. *Br J Radiol* 2003; **76**: 857-865
25 **Ishida H**, Konno K, Ishida J, Naganuma H, Komatsuda T, Sato M, Watanabe S. Abdominal lymphoma: differentiation from pancreatic carcinoma with Doppler US. *Abdom Imaging* 2002; **27**: 461-464
26 **Lee JH**, Yu JS, Kim H, Kim JK, Kim TH, Kim KW, Park MS, Kim JH, Kim YB, Park C. Solid pseudopapillary carcinoma of the pancreas: differentiation from benign solid pseudopapillary tumour using CT and MRI. *Clin Radiol* 2008; **63**: 1006-1014
27 **Keter D**, Melzer E. Endoscopic ultrasound in clinical practice. *Acta Gastroenterol Latinoam* 2008; **38**: 146-151
28 **Malbora B**, Avci Z, Alioglu B, Tutar NU, Ozbek N. A case with mature B-cell acute lymphoblastic leukemia and pancreatic involvement at the time of diagnosis. *J Pediatr Hematol Oncol* 2008; **30**: 87-89
29 **Stoopen ME**. [Imaging of cancer of the pancreas] *Rev Gastroenterol Mex* 2007; **72** Suppl 2: 160-164
30 **Peeters E**, Op de Beeck B, Osteaux M. Primary pancreatic and renal non-Hodgkin's lymphoma: CT and MR findings. *JBR-BTR* 2001; **84**: 108-110

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RAPID COMMUNICATION

Non-thermal ablation of rabbit liver VX2 tumor by pulsed high intensity focused ultrasound with ultrasound contrast agent: Pathological characteristics

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Abstract

AIM: To investigate the pathological characteristics of non-thermal damage induced by pulsed high intensity focused ultrasound (PHIFU) combined with ultrasound contrast agent (UCA), SonoVue (Bracco SpA, Milan, Italy) in rabbit liver VX2 tumor.

METHODS: Liver VX2 tumor models were established in 20 rabbits, which were divided randomly into PHIFU combined with ultrasound contrast agent group (PHIFU + UCA group) and sham group. In the PHIFU + UCA group, 0.2 mL of SonoVue was injected intravenously into the tumor, followed by ultrasound exposure of I_{SP} 5900 W/cm^2. The rabbits were sacrificed one day after ultrasound exposure. Specimens of the exposed tumor tissues were obtained and observed pathologically under light microscope and transmission electron microscope. The remaining tumor tissues were sent for 2,3,5-Triphenyltetrazolium chloride (TTC) staining.

RESULTS: Before TTC staining, tumor tissues in both the sham and the PHIFU + UCA groups resembled gray fish meat. After TTC staining, the tumor tissues were uniformly stained red, with a clear boundary between tumor tissue and normal tissue. Histological examination showed signs of tumor cell injury in PHIFU + UCA group, with cytoplasmic vacuoles of various sizes, chromatin margination and karyopyknosis. Electron microscopic examination revealed tumor cell volume reduction, karyopyknosis, chromatin margination, intercellular space widening, the presence of high electron-density apoptotic bodies and vacuoles in cytoplasm.

CONCLUSION: The non-thermal effects of PHIFU combined with UCA can be used to ablate rabbit liver VX2 tumors.

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Key words: Pulsed high intensity focused ultrasound; Ultrasound contrast agent; Non-thermal effects; Rabbit liver VX2 tumors; Histopathology

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INTRODUCTION

High-intensity focused ultrasound (HIFU) is a rapidly developing non-invasive technology for tumor treatment. In recent years it has been widely used for treating a variety of solid tumors, such as liver tumor, bone tumor, and breast cancer. Mechanism for therapeutic actions of HIFU includes thermal effects and non-thermal effects with the latter dominated by cavitational effects. Thermal effects and cavitational effects work together to produce coagulative necrosis in targeted tumor and thus therapeutic purpose is achieved[1]. Glynn *et al*[2] have reported that cavitational effects of HIFU can be completed in one acoustic cycle, indicating that in the process of coagulative necrosis, cavitational effects precede thermal effects. Furthermore, cavitational effects will inhibit cell growth, increase membrane permeability, damage cellular DNA,

promote cell apoptosis and inhibit cell proliferation[3]. Therefore, efforts to reach treatment goals solely by non-thermal effects dominated by cavitational effects have become very meaningful.

Adjusting acoustic parameters of pulsed high intensity focused ultrasound (PHIFU) can control thermal effects and non-thermal effects; short duty cycle and high intensity favor the occurrence of cavitation[4]. Roberts *et al*[5] used mechanical and cavitational effects generated by pulse focused ultrasound to ablate rabbit kidney and they found that stable and predictable morphological lesions can be achieved by controlling certain parameters. Hall *et al*[6] used cavitational effects of PHIFU to ablate tissues (histotripsy), and concluded that cavitation causes orderly and predictable histologic changes. Our preliminary study results show that bovine liver can be ablated non-thermally by PHIFU using the following parameters: frequency 0.87 MHz, spatial peak intensity I_{SP} 5900 W/cm^2, pulse repetition frequency 100 Hz, and duty cycle 15%[7]. Considering blood circulation, *in vivo* tissues should be less susceptible to heating under same experimental conditions and acoustic parameters. It has been reported that ultrasound contrast agent (UCA) can enhance cavitational effects[8]. Therefore, we combined PHIFU and UCA to produce non-thermal damage in rabbit liver VX2 tumor *in vivo*, and investigated the pathological characteristics of the non-thermal damage.

MATERIALS AND METHODS

Animal model

Twenty New Zealand white rabbits of either gender, 3-4 mo old and weighing 2.0 ± 0.16 kg were used in this study. Animals were fasted 24 h before experiments; abdomen was shaved using 10% Na_2S; all rabbits were anesthetized by intramuscular injection of 0.1 mL/kg Sumianxin (Institute of Veterinary Medicine, Agriculture and Ranching University of Changchun, China). One block (bout 1 cm^3) of active tumor tissues was taken from the rabbit inoculated with VX2 tumor (VX2 squamous carcinoma cell line was offered by Funabashi Farm Company in Kyoto, Japan), washed with normal saline, and then subdivided into small tissue pieces about 1 mm^3. The 20 rabbits were fixed in prone position and routinely disinfected. A median incision was made below the Xiphoid process to expose the right lobe of liver, where a hole about 1-2 cm deep was made using ophthalmological forceps; then 2 ready tissue pieces were implanted into each hole. Bleeding points were stopped with gelatin sponge and then the abdominal wall was sutured. Skin incision was disinfected; 1 mL Suxingling was injected intramuscularly. After tumor inoculation, the rabbits were fed for 2-3 wk and then used in this study.

The experiment was approved ethically and scientifically by our university and complied with Practice for Laboratory Animals in China.

Preparation of UCA suspension

SonoVue® (Bracco Company, Italy) was used. After the plastic cap of the vial was removed, 5 mL sterile normal saline was added into the vial. The vial was shaken vigorously for about 20 s before the milky white suspension was ready.

HIFU equipment and experimental procedures

Model JC High Intensity Focused Ultrasound Tumor Therapeutic System [Chongqing Haifu (HIFU) Technology Co. Ltd., China] was used in this study and has been described in detail previously[9]. HIFU parameters used were as follows: ultrasound frequency 0.87 MHz, focal length 150 mm, I_{SP} 5900 W/cm^2, pulse repetition frequency 100 Hz, and duty cycle 15%.

Experiment started on the 15th day after VX2 tumor inoculation. The rabbit abdomen was shaved with 10% Na_2S one day before experiment. They were anaesthetized by intraperitoneal injection of 3% pentobarbital sodium solution (30 mg/kg body weight) 10 min prior to the experiment and then fixed in prone position on the treatment bed. The 20 rabbits were randomly divided into sham and PHIFU + UCA groups, with 10 rabbits in each group. In the sham group, the target tumor was localized under the assistance of B-mode ultrasonography; after the power generator was turned off, the whole target tumor was irradiated for 90 s. In the PHIFU + UCA group, after the tumor was localized using B-mode ultrasonography, 0.2 mL SonoVue was injected rapidly via ear marginal veins, followed by rapid injection of 1 mL normal saline. Fifteen seconds after the injection, the tumors were exposed to HIFU for 90 s.

Specimen

Twenty-four hours after the HIFU exposure, rabbits in both groups were killed using excessive anesthetics. The abdomen was opened to excise the liver. Multiple-spot sampling was done from the tumor center to the tumor periphery. One mm^3 tumor piece was sampled, double-fixed by glutaraldehyde and osmium acid, embedded in epoxy resin, sectioned ultra-thinly and ultra-structural changes of the targeted tissues were observed under transmission electron microscope. Another 1 mm^3 tumor piece was HE stained and observed under light microscope. The remaining tumor tissues were stained at 37℃ TTC solution for 15 min and observed grossly.

RESULTS

Gross observation

VX2 tumor model was successfully established in all the rabbits. Gross observation found that the implanted tumor was located within hepatic parenchyma, and they were spherical, elliptical or nodular shaped. The tumors resembled the appearance of gray fish meat and the cells were non-encapsulated. There was a clear demarcation between the tumor and the normal surrounding tissues. Before TTC staining, tumor tissues in PHIFU + UCA group resembled gray fish meat and they were demarcated clearly from surrounding normal liver tissues with a sharp boundary (Figure 1A). After the TTC staining the tumor

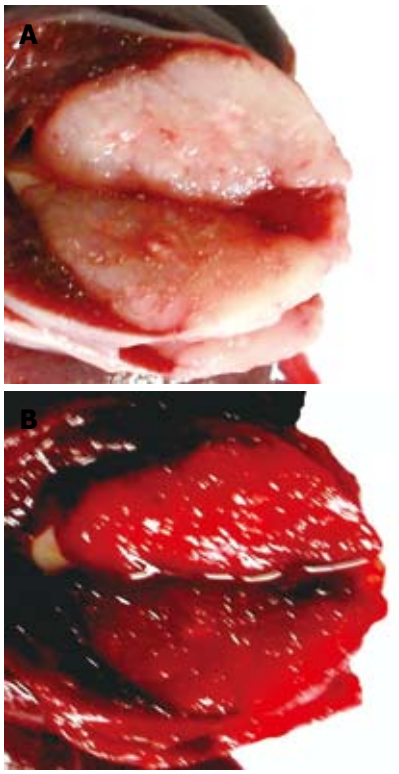

Figure 1 Gross specimen from PHIFU + UCA group before and after TTC staining. A: Before TTC staining, tumor tissues resembled gray fish meat, and the boundary between tumor and normal tissues was very clear; B: After TTC staining, tumor tissues were uniformly stained red, without unstained regions.

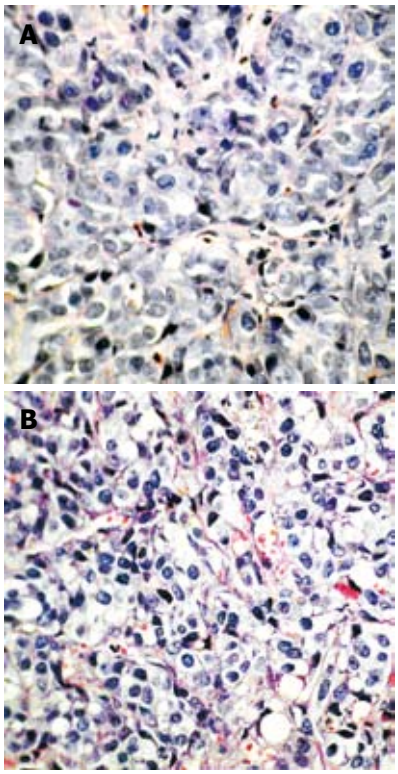

Figure 2 Light microscopic pathology of the targeted VX2 tumor rabbit liver tissues in sham group (A) and PHIFU + UCA group (B) (HE, x 400). A: In sham group, tumor cells were large, irregularly arranged and with irregular morphology. The nuclei were large and deeply H&E stained, with great karyoplasmic ratio and increased mitosis. B: In PHIFU + UCA group, tumor cytoplasm in all 10 rabbits was lightly stained, with cytoplasmic vacuoles of various sizes, chromatin margination and karyopyknosis.

tissues were uniformly stained red without any unstained regions. There was a clear demarcation between the tumor and the normal tissues (Figure 1B).

Light microscopy

Sham group: Under low power lens, VX2 tumor cells in the sham group appeared mass-flake like or an invasive cancer nest, with reduced connective tissues and an unclear demarcation between the tumor and mesenchymal cells. Under high power lens, tumor cells were large, irregularly arranged with an irregular morphology. The nuclei were large and deeply stained, with great karyoplasmic ratio and increased mitosis (Figure 2A).

PHIFU + UCA group: Tumor cytoplasm in all 10 rabbits was lightly stained, with cytoplasmic vacuoles of various sizes, chromatin margination and karyopyknosis (Figure 2B).

Electron microscopy

Sham group: Tumor cells in the sham group appeared polymorphic or spindle-shaped of various sizes and with signs of mitosis. The nuclei were large, deformed, with clear nuclear membranes and rich euchromatin. The chromatin particles were large with intranuclear pseudoinclusions and there were multiple visible nucleoli (Figure 3A).

PHIFU + UCA group: Tumor cells were reduced in size and in some tumor cells, karyopyknosis was revealed. These tumors showed chromatin margination, intercellular space widening (Figure 3B), the presence of high electron-density apoptotic bodies (Figure 3C) and various vacuoles of different sizes in the cytoplasm (Figure 3D).

DISCUSSION

In recent years, the use of microwave, radio frequency, laser and ultrasound as thermal source to ablate solid tumors has been applied in clinical use[10]. Among these treatments, image-guided high-intensity focused ultrasound (HIFU) *in situ* ablation of solid tumors is believed to be the most promising non-invasive modality. Ultrasound beams from an extracorporeal source can propagate through overlying tissues and be brought to a tight focus in the target tissue. The temperature at focus will elevate to 65℃ instantly (0.5-5 s), causing irreversible coagulative necrosis which is grossly visible and histopathologically confirmed. Coagulative necrosis is induced through a combination of thermal and cavitational effects[1]. Some researchers believe that the thermal ablation mechanism has shortcomings. Due to the individual diversity of tissue characteristics and blood perfusion, the size and morphology of thermal lesion cannot be accurately predicted. The local thermal effects may also damage the adjacent tissues and it is possible that uneven heating in the target region will cause leaping injury[11]. In order to avoid the defects of thermal effects, non-thermal effects of ultrasound may offer an alternative.

When acoustic intensity in the target tissue is higher than 1500 W/cm^2, non-thermal effects (mechanical and cavitational effects) will occur. These include forming non-thermal lesion[12], damaging DNA, promoting cell apoptosis, inhibiting cell proliferation[13] and even causing histotripsy[14]. Thermal and non-thermal effects can be effectively controlled by adjusting exposure parameters of PHIFU; short duty cycle and high intensity favor the occurrence of cavitation[4]. PHIFU parameters used in this study, ultrasound frequency 0.87 MHz, I_{SP} 5900 W/cm^2,

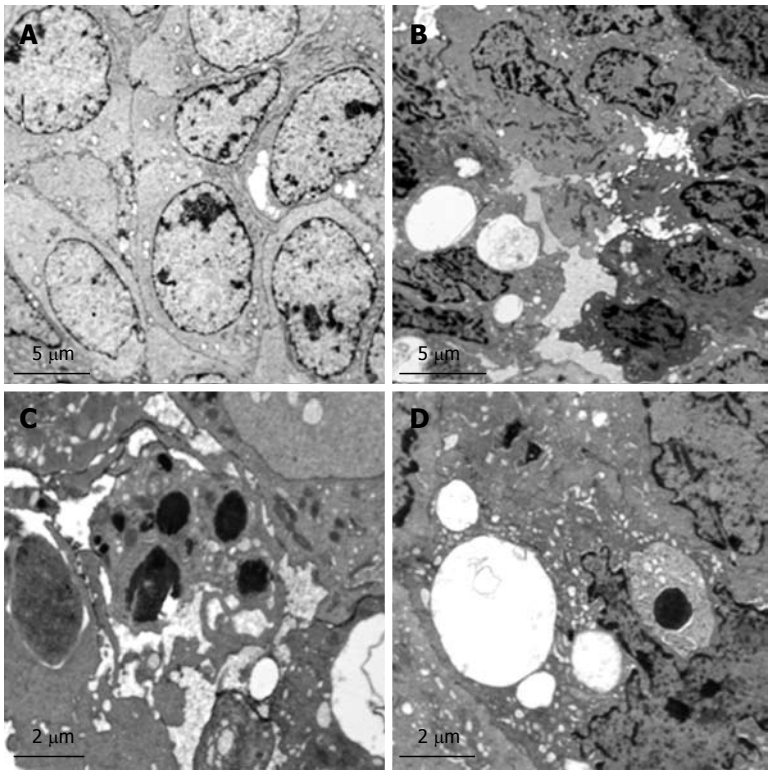

Figure 3 Ultrastructure of the targeted VX2 tumor tissues in sham group and PHIFU + UCA group under transmission electron microscope. A: Tumor cells in sham group were pleomorphic or spindle-shaped of various sizes and with mitosis; nuclei were large and deformed, with clear nuclear membranes and rich euchromatin; chromatin particles were large with intranuclear pseudo-inclusions; there were multiple visible nucleoli (sham group, lead dyeing, x 3500); B: Tumor cells reduced in size; in some tumor cells, karyopyknosis was revealed, with chromatin margination and intercellular space widening (PHIFU + UCA group, lead dyeing, x 4000); C: High electron-density apoptotic bodies were present (PHIFU + UCA group, lead dyeing, x 8000); D: Vacuoles were present in cytoplasm of tumor cells (PHIFU + UCA group, lead dyeing, x 8000).

pulse repetition frequency 100 Hz, and duty cycle 15%, have been demonstrated previously to be able to induce non-thermal damage in bovine liver[7]. These parameters are also consistent with non-thermal parameters reported elsewhere[12].

TTC staining assesses dehydrogenase activity and is a staining method for gross specimens. The main principle is that in the presence of NADH, active dehydrogenase in viable tissues will reduce colorless oxidative TTC to red reductive TTC; hence viable tissue can be stained red while necrotic tissue can not. HIFU causes instant temperature rise to above 65℃ at focus; at such a high temperature, dehydrogenase is deactivated, hence tissues within the target region cannot be stained. Based on this principle, Chang Shufang *et al*[15] applied TTC staining to observe HIFU-induced coagulative necrosis grossly. In one of our preliminary studies, the same parameters were used to form non-thermal damage *in vitro* bovine liver tissues[7]. Considering the cooling effect of blood circulation *in vivo* animals, it is inferred that temperature elevation under same exposure parameters should be lower in this study. In this study, we found that tissues in PHIFU + UCA group were stained red by TTC, indicating that focal temperature was not high enough to deactivate dehydrogenase, which further proved that temperature in the targeted tissues did not elevate to the level required for formation of thermal coagulation.

Lesions caused by non-thermal effects have characteristic pathological changes quite different from those of thermal lesions. Ashush *et al*[16] observed the following morphological changes after HIFU cavitation: cell shrinkage, vacuole formation, chromatin condensation, karyorrhexis and the formation of apoptotic bodies. Kieran *et al*[11] studied non-thermal lesion by changing ultrasound intensity and duty cycle. Their histological observation showed that within certain intensity and duty cycle, vacuoles were formed in the cells, with blanched and dense liquid inside the vacuoles. This study found that PHIFU in combination with UCA can intensify non-thermal effects of HIFU and thus achieve thermal ablation. Light microscopy displayed abundant vacuoles of various sizes in the cytoplasm and chromatin margination and karyopyknosis in some cells. Electron microscopic examination revealed presence of karyopyknosis and chromatin margination in some cells, intercellular space widening, the presence of high electron-density apoptotic bodies and many vacuoles of various sizes in the cytoplasm. These results are consistent with previous reports, indicating that by combining PHIFU with UCA, non-thermal effects can be intensified to achieve non-thermal tumor ablation. Immunohistochemical detection found that PHIFU combined with UCA can promote tumor cell apoptosis and inhibit tumor cell proliferation, which will be reported in another article.

Future studies are needed in many aspects, such as cavitation detection, temperature monitoring and other means to detect non-thermal effects; how to optimize combination between UCA and HIFU exposure parameters; means to control and monitor cavitational lesions; long-term outcomes of non-thermal tumor ablation.

COMMENTS

Background

Image-guided high-intensity focused ultrasound (HIFU) *in situ* ablation of solid tumors is believed to be a promising non-invasive modality. Ultrasound beams

from an extracorporeal source can propagate through overlying tissues and be brought to a tight focus in the target tissue. The temperature at focus will elevate to 65℃ instantly (0.5-5 s), causing irreversible coagulative necrosis. Mechanism for therapeutic actions of HIFU includes thermal effects and non-thermal effects with the latter dominated by cavitational effects. Due to the individual diversity of tissue characteristics and blood perfusion, the size and morphology of thermal lesion cannot be accurately predicted. The local thermal effects may also damage the adjacent tissues and it is possible that uneven heating in the target region will cause leaping injury. In order to avoid the defects of thermal effects, non-thermal effects of ultrasound may offer an alternative.

Research frontiers

Adjusting acoustic parameters of pulsed high intensity focused ultrasound (PHIFU) can control thermal effects and non-thermal effects; short duty cycle and high intensity favor the occurrence of cavitation. Ultrasound contrast agent (UCA) can enhance cavitation effects. Lesions caused by non-thermal effects have characteristic pathological changes quite different from those of thermal lesions. Therefore, the authors combined PHIFU and UCA to produce non-thermal damage in rabbit liver VX2 tumor *in vivo*, and investigated the pathological characteristics of the non-thermal damage.

Innovations and breakthroughs

Non-thermal damage for tumor is the modality different from thermal damage. Previous studies showed that HIFU could ablate tumors by thermal effects. Short duty cycle and high intensity of PHIFU favor the occurrence of cavitation, and ultrasound contrast agent can enhance cavitation effects. The present study used PHIFU with short duty cycle and high intensity combined with UCA to damage rabbit liver VX2 tumor by non-thermal effect. Histopathology was used to evaluate the result.

Applications

The present study showed that the non-thermal effects of PHIFU combined with UCA could be used to ablate rabbit liver VX2 tumors. And this study would help promote a new scheme of therapy for tumors.

Peer review

This is an interesting investigation, demonstrating the effect of intensity focused ultrasound combined with ultrasound contrast agent on liver metastases.

REFERENCES

1 **Kennedy JE**. High-intensity focused ultrasound in the treatment of solid tumours. *Nat Rev Cancer* 2005; **5**: 321-327
2 **Glynn HR**, Ronald AR, Patrick AE, Yang XM. Bubbles and HIFU: the good, the bad, and the ugly. 2nd International Symposium on Therapeutic Ultrasound; 2002 July 29-August 1, Seattle, USA. Washington, 2002: 120-131
3 **Feril LB Jr**, Kondo T, Zhao QL, Ogawa R, Tachibana K, Kudo N, Fujimoto S, Nakamura S. Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents. *Ultrasound Med Biol* 2003; **29**: 331-337
4 **Kieran K**, Hall TL, Parsons JE, Wolf JS, Fowlkes JB, Cain CA, Roberts WW. Exploring the acoustic parameter space in ultrasound therapy: defining the threshold for cavitational effects. 6th International Symposium on Therapeutic Ultrasound; 2006 Aug 30-Sep 2; Oxford, UK, 2006: 185-190
5 **Roberts WW**, Hall TL, Ives K, Wolf JS Jr, Fowlkes JB, Cain CA. Pulsed cavitational ultrasound: a noninvasive technology for controlled tissue ablation (histotripsy) in the rabbit kidney. *J Urol* 2006; **175**: 734-738
6 **Hall TL**, Kieran K, Fowlkes JB, Cain CA, Roberts WW. Temporal trends in the histology of the rabbit kidney after cavitational tissue ablation. 6th International Symposium on Therapeutic Ultrasound; 2006 Aug 30-Sep 2; Oxford, UK, 2006: 191-197
7 **Liu CM**, Chen JY, Ke D, Xu J, Lei L, Li FQ, Wang ZB. Duty cycle affect on in vitro bovine liver treated with pulsed high intensity focused ultrasound. *Zhongguo Chaosheng Yixue Zazhi* 2007; **23**: 567-570
8 **Luo W**, Zhou X, Tian X, Ren X, Zheng M, Gu K, He G. Enhancement of ultrasound contrast agent in high-intensity focused ultrasound ablation. *Adv Ther* 2006; **23**: 861-868
9 **Wu F**, Wang ZB, Zhu H, Chen WZ, Zou JZ, Bai J, Li KQ, Jin CB, Xie FL, Su HB. Feasibility of US-guided high-intensity focused ultrasound treatment in patients with advanced pancreatic cancer: initial experience. *Radiology* 2005; **236**: 1034-1040
10 **Goldberg SN**, Gazelle GS, Mueller PR. Thermal ablation therapy for focal malignancy: a unified approach to underlying principles, techniques, and diagnostic imaging guidance. *AJR Am J Roentgenol* 2000; **174**: 323-331
11 **Kieran K**, Hall TL, Parsons JE, Wolf JS Jr, Fowlkes JB, Cain CA, Roberts WW. Refining histotripsy: defining the parameter space for the creation of nonthermal lesions with high intensity, pulsed focused ultrasound of the in vitro kidney. *J Urol* 2007; **178**: 672-676
12 **Parsons JE**, Cain CA, Abrams GD, Fowlkes JB. Pulsed cavitational ultrasound therapy for controlled tissue homogenization. *Ultrasound Med Biol* 2006; **32**: 115-129
13 **Tran BC**, Seo J, Hall TL, Fowlkes JB, Cain CA. Microbubble-enhanced cavitation for noninvasive ultrasound surgery. *IEEE Trans Ultrason Ferroelectr Freq Control* 2003; **50**: 1296-1304
14 **Xu Z**, Hall TL, Fowlkes JB, Cain CA. Effects of acoustic parameters on bubble cloud dynamics in ultrasound tissue erosion (histotripsy). *J Acoust Soc Am* 2007; **122**: 229-236
15 **Chang SF**, Gu ML, Wu F, Bai J, Zou JZ, Zhu SY, Li CY, Hu K, Du YH, Li Y, Xiang LK, Wang ZB. The Application of Triphenyl Tetrazolium Choloride Staining in the Observing of Biological Focal Field of High Intensity Ultrasound. *Zhongguo Chaosheng Yixue Zazhi* 2000; **16**: 641-644
16 **Ashush H**, Rozenszajn LA, Blass M, Barda-Saad M, Azimov D, Radnay J, Zipori D, Rosenschein U. Apoptosis induction of human myeloid leukemic cells by ultrasound exposure. *Cancer Res* 2000; **60**: 1014-1020

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CASE REPORT

An unusual presentation of primary sclerosing cholangitis

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Abstract

This case report describes the unusual presentation of a patient who had findings which were initially suggestive of a type Ⅳ choledochal cyst. Her liver biopsy demonstrated biliary cirrhosis. She was treated with endoscopic retrograde cholangiopancreatography and biliary stent exchanges over one year. Her cholangiogram one year later demonstrated resolution of the biliary cystic dilation which led to her initial diagnosis, with beading and stricturing of the hepatic ducts consistent with primary sclerosing cholangitis. Liver-associated enzymes and physical findings also improved. A liver biopsy one year later demonstrated a marked improvement in hepatic fibrosis with no evidence of cirrhosis.

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Key words: Biliary cysts; Cholangiogram; Endoscopic retrograde cholangiopancreatography; Liver fibrosis; Primary sclerosing cholangitis

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic progressive disorder characterized by diffuse intrahepatic and extrahepatic bile duct inflammation, stricturing, and fibrosis; progressing to cirrhosis and hepatic failure. PSC occurs more commonly in men, is frequently associated with ulcerative colitis (73%) and is a risk factor for cholangiocarcinoma[1]. We report an unusual case of PSC, which was initially diagnosed as a choledochal cyst.

CASE REPORT

A 30-year-old female presented with a 6-mo history of yellow eyes, dark urine and clay-colored stools. Two years earlier, evaluation at another facility revealed elevated liver-associated enzymes (LAEs). She was diagnosed with Caroli's disease on the basis of non-invasive imaging.

On our evaluation, scleral icterus and hepatosplenomegaly were found on physical examination. Her total bilirubin was 20.9 mg/dL (normal 0.2-1.3 mg/dL), direct bilirubin 18.0 mg/dL (0.0-0.4 mg/dL), alkaline phosphatase (ALK) 957 U/L (38-126 U/L), aspartate aminotransferase (AST) 104 U/L (14-50 U/L) and alanine aminotransferase (ALT) 56 U/L (9-52 U/L). Abdominal computed tomography (CT) scanning showed dilation of the biliary ducts involving the right and left lobes (Figure 1). The common bile duct appeared normal. Magnetic resonance cholangiopancreatography (MRCP) showed intrahepatic biliary ductal dilation including the right and left hepatic ducts consistent with choledochal cysts (Figure 2). Endoscopic retrograde cholangiopancreatography (ERCP) revealed cystic dilation of the left and right hepatic ducts and a common duct stricture (Figure 3A). The intrahepatic ducts appeared irregular and had focal areas of dilation. A 10 FR 7 cm biliary stent was placed with good drainage of bile and contrast. Cytologic specimens of the stricture were benign.

Liver biopsy demonstrated biliary-type cirrhosis with cholestasis and bile ductular proliferation. Based upon cholangiography and histological findings, we diagnosed type ⅣB choledochal cyst and biliary cirrhosis. Biliary stent exchanges were performed over the following year with gradual improvement in LAEs. ERCP performed one year after our evaluation revealed diffusely irregular intrahepatic and extrahepatic bile ducts and total resolution of the cystic dilation and common duct stricture (Figure 3B). Her bilirubin was 0.6 mg/dL, ALK 220 U/L, ALT 36 U/L, and AST 38 U/L. Liver biopsy showed only focal cholestasis, mild portal inflammation,

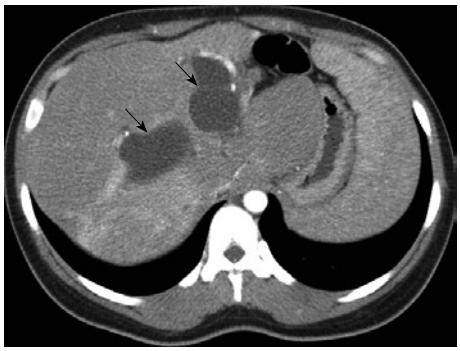

Figure 1 CT scan illustrating saccular dilations (arrows) of right and left hepatic ducts.

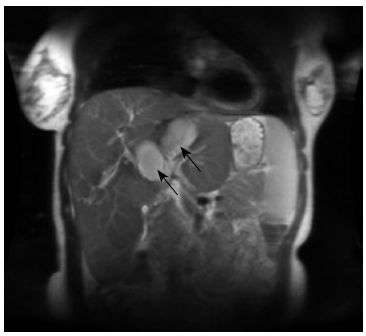

Figure 2 MRCP demonstrating saccular dilation (arrows) of the right and left hepatic ducts.

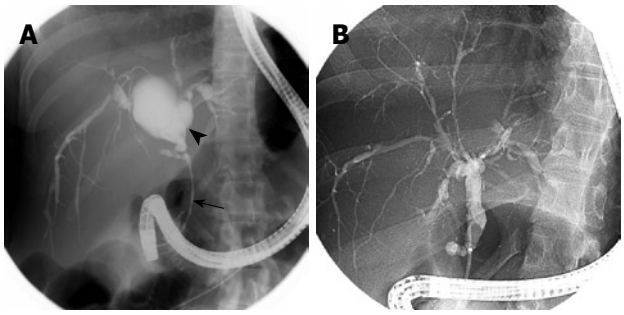

Figure 3 ERCP. A: Initial cholangiogram showing dilated extrahepatic ducts consistent with diagnosis of type IV choledochal cysts (arrowhead), and 7 cm common duct stricture (arrow); B: Final balloon occlusion cholangiogram performed one year after stenting showing resolution of the common duct stricture and cystic biliary dilation. The intrahepatic ducts are diffusely irregular.

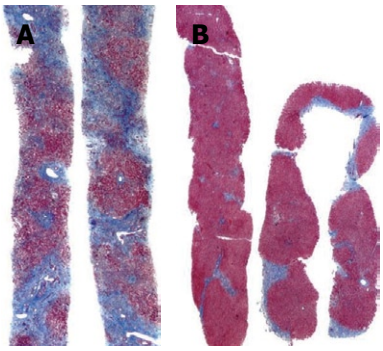

Figure 4 Liver biopsy. A: Pretreatment Trichome stain showed cirrhosis; B: Trichrome stain one year later following biliary stenting showed marked improvement in degree of fibrosis.

and irregular portal and bridging fibrosis. There was a marked improvement in the degree of fibrosis when compared to her initial liver biopsy (Figure 4), and there was no longer evidence of cirrhosis.

DISCUSSION

We present a patient with an unusual presentation of PSC, with imaging and histological findings which initially led to a diagnosis of a type ⅣB choledochal cyst and biliary cirrhosis. The diagnosis of PSC was made after her subsequent cholangiogram demonstrated pruning and stenosis of the intrahepatic ducts, with resolution of the cystic biliary dilation. PSC is a slow progressive disease, which ultimately leads to hepatic failure. Cholangiography is the gold standard for the diagnosis of PSC[2]. Orthotopic liver transplantation may be used for patients with PSC with end stage liver disease[3].

Hepatic fibrosis is triggered by recurrent liver injury, which results in the activation of perivascular stellate cells and other myofibroblasts[4]. This process may be reversible in its early stages, and there are reports of regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis[5]. However, it is unusual to have significant improvement in hepatic fibrosis after years of liver injury. In our patient, pre- and post-treatment liver biopsies documented a regression in hepatic fibrosis. It is our belief that the histologic improvement in our patient can be attributed to relief of mechanical obstruction by stenting and drainage of the common bile duct. We acknowledge the possibility of liver sampling error; however, the overall improvement in her clinical picture supports a cause and effect relationship between biliary stricture therapy and regression of fibrosis.

REFERENCES

1 **Matsuda T**, Matsutani T, Tsuchiya Y, Okihama Y, Egami K, Yoshioka M, Maeda S, Onda M. A clinical evaluation of lymphangioma of the large intestine: a case presentation of lymphangioma of the descending colon and a review of 279 Japanese cases. *J Nippon Med Sch* 2001; **68**: 262-265
2 **Kim J**, Han D, Hong CH, Lee HL, Kim JP, Sohn JH, Hahm JS. Colonic lymphangiomatosis associated with protein-losing enteropathy. *Dig Dis Sci* 2005; **50**: 1747-1753
3 **Watanabe T**, Kato K, Sugitani M, Hasunuma O, Sawada T, Hoshino N, Kaneda N, Kawamura F, Arakawa Y, Hirota T. A case of multiple lymphangiomas of the colon suggesting colonic lymphangiomatosis. *Gastrointest Endosc* 2000; **52**: 781-784
4 **Wildhaber BE**, Chardot C, Coultre CL, Genin B. Total Laparoscopic Excision of Retroperitoneal Cystic Lymphangioma. *J Laparoendosc Adv Surg Tech A* 2006; **16**: 530-533
5 **Sato K**, Maekawa T, Yabuki K, Tomita N, Eguchi M, Matsumoto M, Sugiyama N. Cystic lymphangiomas of the colon. *J Gastroenterol* 1999; **34**: 520-524

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CASE REPORT

Concomitant gastric adenocarcinoma and stromal tumor in a woman with polymyalgia rheumatica

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Abstract

Gastrointestinal stromal tumors (GISTs) are rare neoplasms (1%) of the gastrointestinal tract and to our knowledge only rare cases of synchronous presentation of gastric carcinomas and GISTs are reported in the literature. A 72-year-old female with a simultaneous presentation of gastric adenocarcinoma and GIST is presented. Moreover, due to polymyalgia rheumatica the patient received corticosteroids as treatment for the last 3 years. The concomitant occurrence of these neoplasms may involve common carcinogenic factors and there could be an association with polymyalgia rheumatica either as a paraneoplastic presentation or due to its treatment with corticosteroids.

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Key words: Gastric adenocarcinoma; Gastrointestinal stromal tumor; Paraneoplastic presentation; Polymyalgia rheumatica; Corticosteroids

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare neoplasms (1%) of the gastrointestinal tract[1]. It is believed that they originate from interstitial cells of Cajal (ICCs) or their precursors, which regulate gut motility. This hypothesis is based on observations that both GISTs and ICCs express the receptor tyrosine kinase KIT (c-KIT)[2]. Polymyalgia rheumatica is a clinical syndrome characterized by pain and stiffness in the neck, shoulders and hips, fatigue, weight loss and low-grade fever and rarely is presented as a paraneoplastic manifestation[3]. We report a case of synchronous occurrence of gastric adenocarcinoma and GIST in a 72-year-old female with polymyalgia rheumatica and provide a review of the literature. To the best of our knowledge this is the first case of a synchronous presentation of a gastric carcinoma and a GIST in a patient with polymyalgia rheumatica.

CASE REPORT

A 72-year-old woman was admitted to our department in September of 2007 because of epigastric pain lasting at least three months. Laboratory tests revealed elevation of the erythrocyte sedimentation rate (ESR) to 45 mm per hour and the presence of a microcytic, hypochromic anemia. All other studies were normal, except esophagogastroscopy which revealed a tumor in her stomach and a subtotal gastrectomy was performed. In the pathologic specimen of stomach, two different neoplasms were revealed: an adenocarcinoma and a GIST. The adenocarcinoma was a diffuse type according to Lauren classification (Figure 1). The gastrointestinal tumor of median diameter 1.8 cm was composed of spindle and epithelioid cells without atypia. Low mitotic activity (1/50 high powered field), low membranous CD117(KIT) positivity (Figure 2), median CD34 positivity, low positivity for smooth muscle actin (SMA) and scatter desmin positive cells were observed. Consequently, she received six cycles with the combination of cisplatin and fluorouracil (5-FU) and since April of 2008 has been disease free. From her past medical history it should

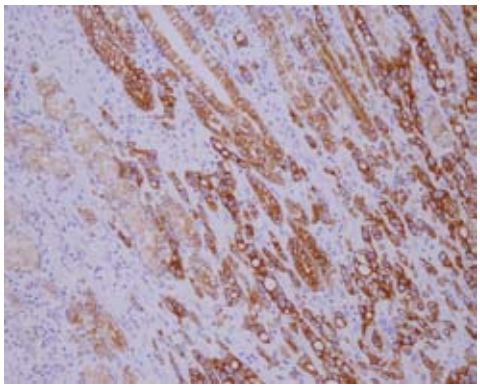

Figure 1 A diffuse type adenocarcinoma according to Lauren classification (x 20).

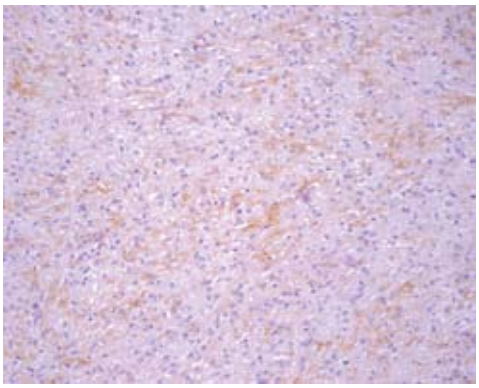

Figure 2 Spindle cells with low membranous positive CD117 immunostaining (x 20).

be noted that she has been suffering from polymyalgia rheumatica since October of 2004, and she has been receiving corticosteroids as treatment.

DISCUSSION

GISTs are rare neoplasms and the stomach (39%-60%), small bowel (30%-42%), colon-rectum (5%-10%) and esophagus (5%) are the most common locations[4]. The cell morphology is usually spindle shaped (70%), epithelioid (20%) or mixed. GISTs are immunohistochemically positive for KIT expression (90-95%) and often express BCL-2 (80%), CD34 (70%), SMA (35%), S-100 (10%) and desmin (5%)[5].

The malignancy potential of these neoplasms is highly variable and ranges from benign to malignant-aggressive tumors. Malignant proportion is about 20%-45% of all GISTs and they often lead to intra-abdominal metastases. On the contrary extra-abdominal metastases are very rare[6]. GIST size and cell proliferation rate are the most important prognostic factors. Differential diagnosis mainly includes leiomyomas and leiomyosarcomas.

They usually present as sporadic cancers but have also been reported as a familial occurrence. GISTs with extraadrenal paraganglioma and pulmonary chondroma occurred in patients with Carney's triad[7]. They are resistant to chemotherapeutic treatment, and radiotherapy and surgery is the initial treatment, while the administration of imatinib has become standard first-line therapy for patients with unresectable or metastatic GIST. Imatinib is an inhibitor of receptor tyrosine kinases including KIT, platelet-derived growth factor receptors (PDGFRs), colony stimulating factor 1 receptor (c-FMS), breakpoint cluster region and abl gene fusion protein (BCR-ABL) and specifically blocks the adenosine-5'-triphosphate (ATP) binding site[8].

Rare cases of concurrent presentation of gastric adenocarcinoma and GIST have been reported in the literature[9,10]. Some researchers claim that their presentation is based on coincidence; meanwhile, others support the hypothesis that some unknown carcinogens provoke the simultaneous proliferation and oncogenesis of epithelial and stromal cells. Cohen *et al*[11] reported that exposure to both acetylsalicylic acid and nitrosoguanidine causes synchronous development of both gastric cancer and leiomyosarcoma.

The relation between polymyalgia rheumatica and giant cell arteritis is well known[3]. There is debate as to whether there is an association with the occurrence of malignancy. Case reports present a possible association of polymyalgia rheumatica with breast cancer, colon cancer and non-Hodgkin lymphoma[12-14]. On the contrary, other researchers failed to reveal a paraneoplastic mechanism[15].

A hypothesis of a relationship between glucocorticoid and gastric cancer has also been reported[16]. Kodama *et al*[17] reported a possible linkage between glucocorticoid excess and a malignant transformation in the epithelial cells of the esophagus. It should also be noted that systemic glucocorticoid administration is associated with skin cancers and non-Hodgkin lymphoma[18].

To the best of our knowledge this is the first case of a synchronous presentation of a gastric carcinoma and a GIST in a patient with polymyalgia rheumatica. The concomitant occurrence of these neoplasms may involve common carcinogenic factors and could be associated with polymyalgia rheumatica either through a paraneoplastic mechanism or due to its treatment with corticosteroids.

REFERENCES

1 **Nilsson B**, Bumming P, Meis-Kindblom JM, Oden A, Dortok A, Gustavsson B, Sablinska K, Kindblom LG. Gastrointestinal stromal tumors: the incidence, prevalence, clinical course, and prognostication in the preimatinib mesylate era--a population-based study in western Sweden. *Cancer* 2005; **103**: 821-829

2 **Kindblom LG**, Remotti HE, Aldenborg F, Meis-Kindblom JM. Gastrointestinal pacemaker cell tumor (GIPACT): gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal. *Am J Pathol* 1998; **152**: 1259-1269

3 **Bird HA**. Criteria for polymyalgia rheumatica. Tale without end. *J Rheumatol* 2008; **35**: 188-189

4 **Hersh MR**, Choi J, Garrett C, Clark R. Imaging gastrointestinal stromal tumors. *Cancer Control* 2005; **12**: 111-115

5 **Fletcher CD**, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: A consensus approach.

Hum Pathol 2002; **33**: 459-465
6 **Miettinen M**, Lasota J. Gastrointestinal stromal tumors--definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. *Virchows Arch* 2001; **438**: 1-12
7 **Carney JA**. Gastric stromal sarcoma, pulmonary chondroma, and extra-adrenal paraganglioma (Carney Triad): natural history, adrenocortical component, and possible familial occurrence. *Mayo Clin Proc* 1999; **74**: 543-552
8 **Buchdunger E**, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, Lydon NB. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* 2000; **295**: 139-145
9 **Lin YL**, Tzeng JE, Wei CK, Lin CW. Small gastrointestinal stromal tumor concomitant with early gastric cancer: a case report. *World J Gastroenterol* 2006; **12**: 815-817
10 **Wronski M**, Ziarkiewicz-Wroblewska B, Gornicka B, Cebulski W, Slodkowski M, Wasiutynski A, Krasnodebski IW. Synchronous occurrence of gastrointestinal stromal tumors and other primary gastrointestinal neoplasms. *World J Gastroenterol* 2006; **12**: 5360-5362
11 **Cohen A**, Geller SA, Horowitz I, Toth LS, Werther JL. Experimental models for gastric leiomyosarcoma. The effects of N-methyl-N'-nitro-N-nitrosoguanidine in combination with stress, aspirin, or sodium taurocholate. *Cancer* 1984; **53**: 1088-1092
12 **Kehler T**, Curkovic B. Polymyalgia rheumatica and colon malignacy: case report. *Clin Rheumatol* 2006; **25**: 764-765
13 **Keith MP**, Gilliland WR. Polymyalgia rheumatica and breast cancer. *J Clin Rheumatol* 2006; **12**: 199-200
14 **Kuttikat A**, Keat A, Hughes R, Hakim A, Chakravarty K. A case of polymyalgia rheumatica, microscopic polyangiitis, and B-cell lymphoma. *Nat Clin Pract Rheumatol* 2006; **2**: 686-690; quiz 1p following 691
15 **Myklebust G**, Wilsgaard T, Jacobsen BK, Gran JT. No increased frequency of malignant neoplasms in polymyalgia rheumatica and temporal arteritis. A prospective longitudinal study of 398 cases and matched population controls. *J Rheumatol* 2002; **29**: 2143-2147
16 **Kodama M**, Kodama T, Takagi I, Kodama M. Relation between the hormonal and epidemiological aspects of esophageal cancer in Japan. *Anticancer Res* 1992; **12**: 1671-1681
17 **Kodama M**, Kodama T. In search of the cause of gastric cancer. *In Vivo* 2000; **14**: 125-138
18 **Sorensen HT**, Mellemkjaer L, Nielsen GL, Baron JA, Olsen JH, Karagas MR. Skin cancers and non-hodgkin lymphoma among users of systemic glucocorticoids: a population-based cohort study. *J Natl Cancer Inst* 2004; **96**: 709-711

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CASE REPORT

Pneumatosis cystoides intestinalis associated with massive free air mimicking perforated diffuse peritonitis

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Abstract

While pneumatosis cystoides intestinalis (PCI) is a rare disease entity associated with a wide variety of gastrointestinal and non-gastrointestinal disorders, PCI associated with massive intra- and retroperitoneal free air is extremely uncommon, and is difficult to diagnose differentially from perforated peritonitis. We present two cases of PCI associated with massive peritoneal free air and/or retroperitoneal air that mimicked perforated peritonitis. These cases highlight the clinical importance of PCI that mimics perforated peritonitis, which requires emergency surgery. Preoperative imaging modalities and diagnostic laparoscopy are useful to make an accurate diagnosis.

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Key words: Pneumatosis cystoides intestinalis; Perforated peritonitis; Corticosteroid therapy; Peritoneal free air

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INTRODUCTION

Pneumatosis cystoides intestinalis (PCI) is a rare disease characterized by the presence of multiple gas-filled cysts in the subserosal or submucosal wall of the large and/or small intestine. Patients either stay asymptomatic or present with gastrointestinal symptoms, including nausea, vomiting, diarrhea and abdominal pain. While the exact pathogenesis of PCI is not well understood, two theories, mechanical and bacterial, have been proposed based on the known clinical associations[1]. PCI is commonly associated with chronic obstructive pulmonary disease, intestinal obstruction[2], ischemic bowel disease[3], necrotizing enterocolitis in premature infants[4], immunodeficiency such as AIDS[5], bacterial and/or viral infection[6,7] and drug therapy[8-13]. PCI may usually be found in a subserosal or submucosal location, and bowel distention and/or intraperitoneal free air can appear when the PCI is sufficiently extensive. However, PCI showing massive intraperitoneal free air and/or retroperitoneal air is extremely uncommon and only a limited number of cases have been documented[5,14]. We present two cases of PCI that mimicked perforated peritonitis, which required emergency surgery. Previously reported cases with PCI are reviewed and the significance of preoperative imaging is discussed.

CASE REPORT

Case 1 was a 58-year-old Japanese woman, who received corticosteroid therapy for rheumatoid arthritis in the department of internal medicine at our hospital. She complained of abdominal pain and distention and was referred to our department. The abdominal roentgenogram showed massive intraperitoneal free air (Figure 1A), dilatation of the intestine and circular collection of intestinal gas (Figure 1B). The abdominal computed tomography (CT) scan revealed ascites and

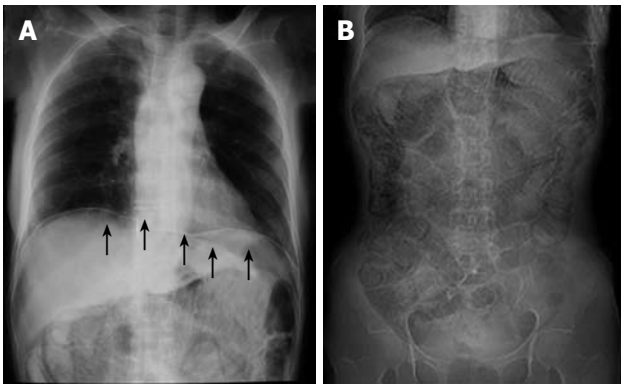

Figure 1 Abdominal roentgenogram. A: Massive intraperitoneal free air (arrows); B: dilatation of the intestine and circular collection of intestinal gas.

air collection within the bowel wall, which extended from the jejunum to the splenic flexure of the colon, which indicated that the air was present within the bowel wall (Figure 2). The laboratory data just before the operation indicated that white blood cell count was 14900/mm^3 and the serum C-reactive protein (CRP) level was 4.0 mg/dL. Although PCI was strongly suspected, the patient received corticosteroid therapy for the last 10 years, which may have masked the severe abdominal symptoms. The possibility of perforated peritonitis associated with unexplained ascites could not be eliminated, therefore emergency laparotomy was performed. At surgery, a large amount of free air exhausted when the abdomen was opened. Multiple gas-filled subserosal vesicles scattered throughout the surface of bowel wall and the mesentery were found, which were almost distributed entirely in the small intestine and the colon (data not shown). While there was a small amount of serous ascitic fluid in the abdominal cavity, the fluid was clear. Despite meticulous exploration of the abdomen, no evidence suggestive of hollow viscus perforation was found. The abdomen was irrigated with 1000 mL of saline solution. Culture of the ascitic fluid obtained at surgery was negative for any microorganism. Postoperatively, the patient received oxygen therapy for 4 d and the signs of PCI completely disappeared 5 d after surgery. The patient was discharged from the hospital 19 d after surgery. No recurrence of PCI has been found to date.

Case 2 was a 25-year-old man who had been receiving treatment for the colonic type of Crohn's disease for the last 12 years. While he had received repeated doses of infliximab 31 times from 2001 to 2005, administration was discontinued because of the appearance of colonic stenosis. He was then followed up by treatment with azathioprine and 5-aminosalicylic acid. After the patient underwent total colonofiberoscopy and barium enema, abdominal distention continued for 2 wk. The patient then complained of abdominal pain and was referred to our department in June 2006. The abdominal plain roentgenogram revealed massive intra-abdominal free air and retroperitoneal air (Figure 3). The abdominal CT scan revealed massive peritoneal free air throughout the abdomen and a small amount of ascites (Figure 4). The laboratory data just before the operation indicated that white blood cell count was 4600/mm^3 and serum CRP level was 1.8 mg/dL. To eliminate the possibility of perforated peritonitis and to confirm the diagnosis of PCI, diagnostic laparoscopy was performed. The laparoscopic findings revealed multiple glistering, translucent, gas-filled subserosal vesicles scattered throughout the surface of the bowel wall, the mesentery and omentum, which was compatible with PCI (data not shown). Although a small amount of ascitic fluid was present in the abdominal cavity, there was no finding suggestive of perforated peritonitis. Culture of the ascitic fluid obtained at surgery was negative for any microorganism. The postoperative course was uneventful and the elemental diet was started 8 d after surgery to facilitate the recovery. The patient was discharged from the hospital 19 d after diagnostic laparoscopy. The patient has remained healthy and no recurrence of PCI has been found to date.

DISCUSSION

PCI affects men more commonly than women, with a peak incidence at 30-50 years of age[15]. The sigmoid colon and its proximal side of the gastrointestinal tract has been shown to be the area that is predisposed to PCI, which is similar to diverticular disease[16]. The majority of patients are asymptomatic, therefore, the incidence of PCI may be underestimated. As a result of the high incidence of associated conditions, mortality rate of PCI in a collected series has been reported to be as high as 33%[17]. Furthermore, mortality rate of patients of PCI observed without surgery has been reported to be 18%, which suggests that emergency laparotomy is occasionally required.

The striking finding of preoperative imaging analysis in the present cases was the presence of massive intraperitoneal free air and/or retroperitoneal air that mimicked perforated diffuse peritonitis. Despite the fact that rupture of subserosal cysts of PCI results in pneumoperitoneum without peritonitis, accumulation of a large amount of intraperitoneal free air throughout the abdomen is extremely uncommon and only a limited number of cases have been documented previously[14,18,19]. Particularly, the presence of free retroperitoneal air as well as intraperitoneal air was detected in the plain abdominal roentgenogram and abdominal CT scan in case 2, which could also be signs strongly suggestive of gastrointestinal perforation. Although it is extremely rare, the presence of free retroperitoneal air caused by PCI has been reported previously[5,14]. Plain abdominal roentgenography is the most useful and easy way to ensure the diagnosis of PCI. Circular collection of gas in the bowel and mesentery is characteristic of PCI, and a review of 919 cases of PCI has shown positive abdominal roentgenography in two-thirds of patients[20]. Furthermore, recent reports have shown that abdominal CT and ultrasonography are useful methods for the

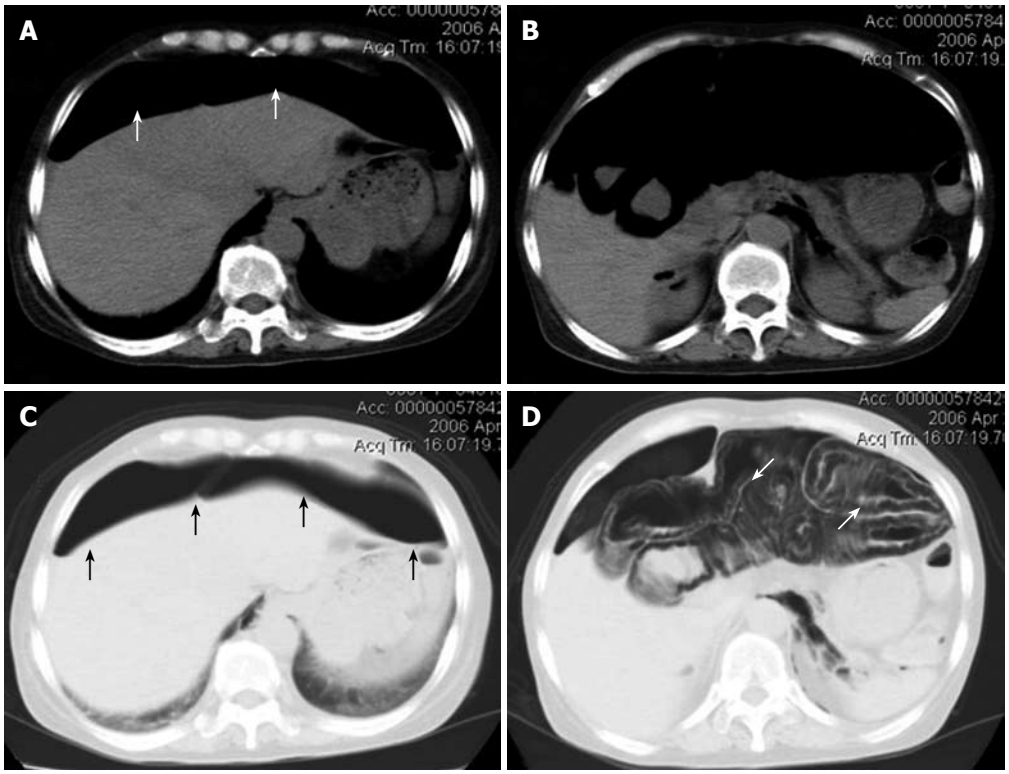

Figure 2 Abdominal CT scan images. Massive intraperitoneal free air extending throughout the abdominal cavity (white arrows, A and B), and a stranded appearance of air collection within the bowel wall detected in the lung window setting, which extended from the jejunum to the splenic flexure of the colon. This indicated that the air was present within the bowel wall (white arrows, C and D). Black arrows indicate massive intraperitoneal air (C).

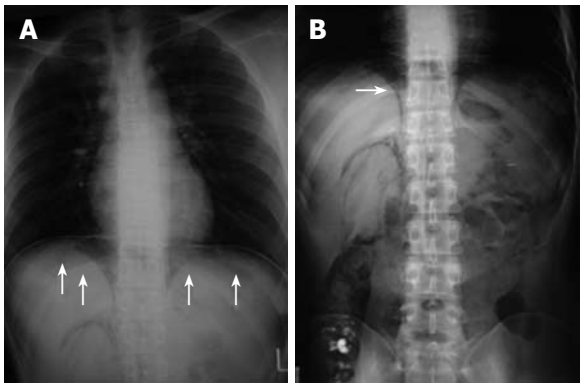

Figure 3 Roentgenogram of chest and abdomen. It revealed massive intra-abdominal free air (arrows, A) and pneunoretroperitoneum (arrow, B).

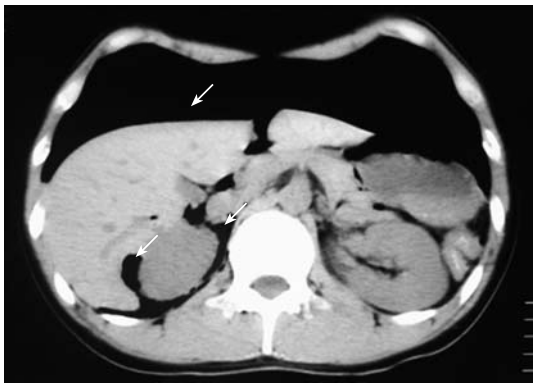

Figure 4 Abdominal CT scan, revealing massive peritoneal free air (arrows) that extended throughout the entire abdomen.

diagnosis of PCI[19,21,22]. A stranded appearance within the air collection of the bowel wall in the CT images is characteristic of PCI, which indicates that the air is present within the bowel wall[1]. In particular, lung window settings have been reported to be important in the detection of intramural air, and obviate the need for intraluminal contrast to outline the circumferential pattern of PCI[22]. This technical observation could have potential clinical significance in the evaluation of patients with suspected intestinal ischemia or gangrene, in whom the appearance of intramural air is an ominous prognostic sign. Although these CT images were useful for the diagnosis of PCI in the present cases, the possibility of gastrointestinal perforation could not be completely eliminated because of the massive intraperitoneal free air and ascites.

In the present cases, because no gastrointestinal perforation occurred, no particular surgical intervention was performed on the bowel associated with PCI. Oxygen therapy was performed in case 1 and the signs of PCI substantially disappeared 5 d after surgery. Although oxygen was first used by Forgacs *et al*[23] in 1973 for the treatment of PCI, the minimal concentration and the optimal duration of oxygen have not been established. It was initially suggested that arterial oxygen concentrations in excess of 300 mmHg were required, which could be accomplished by delivering 70%-75% humidified oxygen at 8 L/min *via* a mask. Wyatt[24] has suggested that aggressive oxygen therapy should be continued for at least 48 h after complete radiological disappearance of all cysts, and that recurrent cysts are to the result of inadequate initial treatment rather than actual recurrence. No recurrence of PCI was detected during 2 years follow-up in the present cases.

Laparoscopic examination was performed on case 2 to precisely determine the cause of massive

intraperitoneal free air, retroperitoneal air and non-infectious ascites. Although there was no gastrointestinal perforation in either of our cases, the cause of the ascites remains uncertain. Unexplained ascites associated with PCI has been reported previously[25]. The ascites disappeared as the PCI improved postoperatively in our cases, which suggests a possible link between PCI and ascites.

In summary, these cases highlight the clinical importance of PCI associated with massive intraperitoneal free air and/or retroperitoneal air that mimic perforated peritonitis, which required emergency surgery. Preoperative imaging modalities are useful to make precise diagnosis and further diagnostic laparoscopic examination is a useful adjunct to ensure the diagnosis of PCI.

REFERENCES

1 **Heng Y**, Schuffler MD, Haggitt RC, Rohrmann CA. Pneumatosis intestinalis: a review. *Am J Gastroenterol* 1995; **90**: 1747-1758
2 **Luks FI**, Chung MA, Brandt ML, Hertecant J, Roy CC, Blanchard H, Bensoussan AL. Pneumatosis and pneumoperitoneum in chronic idiopathic intestinal pseudoobstruction. *J Pediatr Surg* 1991; **26**: 1384-1386
3 **Schulze CG**, Blum U, Haag K. Hepatic portal venous gas. Imaging modalities and clinical significance. *Acta Radiol* 1995; **36**: 377-380
4 **Rabinowitz JG**, Siegle RL. Changing clinical and roentgenographic patterns of necrotizing enterocolitis. *AJR Am J Roentgenol* 1976; **126**: 560-566
5 **Wood BJ**, Kumar PN, Cooper C, Silverman PM, Zeman RK. Pneumatosis intestinalis in adults with AIDS: clinical significance and imaging findings. *AJR Am J Roentgenol* 1995; **165**: 1387-1390
6 **Yale CE**, Balish E. The natural course of Clostridium perfringens--induced pneumatosis cystoides intestinalis. *J Med* 1992; **23**: 279-288
7 **Mannes GP**, de Boer WJ, van der Jagt EJ, Meinesz AF, Meuzelaar JJ, van der Bij W. Pneumatosis intestinalis and active cytomegaloviral infection after lung transplantation. Groningen Lung Transplant Group. *Chest* 1994; **105**: 929-930
8 **Shindelman LE**, Geller SA, Wisch N, Bauer JJ. Pneumatosis cystoides intestinalis: a complication of systemic chemotherapy. *Am J Gastroenterol* 1981; **75**: 270-274
9 **Hashimoto S**, Saitoh H, Wada K, Kobayashi T, Furushima H, Kawai H, Shinbo T, Funakoshi K, Takahashi H, Shibata A. Pneumatosis cystoides intestinalis after chemotherapy for hematological malignancies: report of 4 cases. *Intern Med* 1995; **34**: 212-215
10 **Galm O**, Fabry U, Adam G, Osieka R. Pneumatosis intestinalis following cytotoxic or immunosuppressive treatment. *Digestion* 2001; **64**: 128-132
11 **Candelaria M**, Bourlon-Cuellar R, Zubieta JL, Noel-Ettiene LM, Sánchez-Sánchez JM. Gastrointestinal pneumatosis after docetaxel chemotherapy. *J Clin Gastroenterol* 2002; **34**: 444-445
12 **Hisamoto A**, Mizushima T, Sato K, Haruta Y, Tanimoto Y, Tanimoto M, Matsuo K. Pneumatosis cystoides intestinalis after alpha-glucosidase inhibitor treatment in a patient with interstitial pneumonitis. *Intern Med* 2006; **45**: 73-76
13 **Shih IL**, Lu YS, Wang HP, Liu KL. Pneumatosis coli after etoposide chemotherapy for breast cancer. *J Clin Oncol* 2007; **25**: 1623-1625
14 **Schulenburg A**, Herold C, Eisenhuber E, Oberhuber G, Volc-Platzer B, Greinix HT, Reiter E, Keil F, Kalhs P. Pneumatosis [correction of Pneumocystis] cystoides intestinalis with pneumoperitoneum and pneumoretroperitoneum in a patient with extensive chronic graft-versus-host disease. *Bone Marrow Transplant* 1999; **24**: 331-333
15 **KOSS LG**. Abdominal gas cysts (pneumatosis cystoides intestinorum hominis); an analysis with a report of a case and a critical review of the literature. *AMA Arch Pathol* 1952; **53**: 523-549
16 **Gagliardi G**, Thompson IW, Hershman MJ, Forbes A, Hawley PR, Talbot IC. Pneumatosis coli: a proposed pathogenesis based on study of 25 cases and review of the literature. *Int J Colorectal Dis* 1996; **11**: 111-118
17 **Knechtle SJ**, Davidoff AM, Rice RP. Pneumatosis intestinalis. Surgical management and clinical outcome. *Ann Surg* 1990; **212**: 160-165
18 **Hwang J**, Reddy VS, Sharp KW. Pneumatosis cystoides intestinalis with free intraperitoneal air: a case report. *Am Surg* 2003; **69**: 346-349
19 **Ryback LD**, Shapiro RS, Carano K, Halton KP. Massive pneumatosis intestinalis: CT diagnosis. *Comput Med Imaging Graph* 1999; **23**: 165-168
20 **Jamart J**. Pneumatosis cystoides intestinalis. A statistical study of 919 cases. *Acta Hepatogastroenterol* (Stuttg) 1979; **26**: 419-422
21 **Vernacchia FS**, Jeffrey RB, Laing FC, Wing VW. Sonographic recognition of pneumatosis intestinalis. *AJR Am J Roentgenol* 1985; **145**: 51-52
22 **Connor R**, Jones B, Fishman EK, Siegelman SS. Pneumatosis intestinalis: role of computed tomography in diagnosis and management. *J Comput Assist Tomogr* 1984; **8**: 269-275
23 **Forgacs P**, Wright PH, Wyatt AP. Treatment of intestinal gas cysts by oxygen breathing. *Lancet* 1973; **1**: 579-582
24 **Wyatt AP**. Prolonged symptomatic and radiological remission of colonic gas cysts after oxygen therapy. *Br J Surg* 1975; **62**: 837-839
25 **Tsega E**. Pneumatosis cystoides intestinalis with unexplained ascites in Ethiopian patients. *Br J Surg* 1975; **62**: 379-382

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CASE REPORT

Gastric outlet obstruction caused by heterotopic pancreas: A case report and a quick review

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Abstract

A 46-year-old Chinese woman presented with nausea, recurrent vomiting, and abdominal pain. Gastroduodenal endoscopic examination revealed an oval-shaped submucosal tumor at the prepyloric area on the posterior wall of the stomach. A degenerated gastrointestinal stromal tumor was suspected. Distal gastrectomy was performed and a histological diagnosis of heterotopic pancreas (HPs) was confirmed. The patient had an uneventful postoperative course and was discharged 7 d after operation. The patient remains healthy and symptom-free in the follow-up of 6 mo. This is a report of a case of gastric outlet obstruction resulting from pancreatic heterotopia in the gastric antrum in an adult woman.

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Key words: Gastric; Outlet obstruction; Heterotopic pancreas

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INTRODUCTION

Heterotopic pancreas (HPs), a rare entity, is defined as the presence of pancreatic tissue outside its normal localization and without anatomic and vascular continuity with the pancreas itself. Other terms such as pancreatic rest, ectopic, aberrant, or accessory pancreas are also used [1]. It can occur anywhere in the gastrointestinal (GI) tract. The etiology of HP is unknown. In most cases, HP does not cause symptoms, but it can occasionally present as nausea, vomiting and abdominal pain. Peptic ulceration and upper GI bleeding are rare presentations, as are malignant degeneration, pancreatitis and pseudocysts[2]. HP tissue is found in persons of all ages and slightly more often in man[3]. HP has been reported as the cause of gastric outlet obstruction in infant[4] or child[5]. This is a report of a case of gastric outlet obstruction resulting from pancreatic heterotopias in the gastric antrum in an adult woman.

CASE REPORT

A 46-year-old woman was admitted to our hospital with a 5-year history of chronic epigastric pain and recurrent vomiting after meal. The patient had an unremarkable medical history. The physical examination was normal. She was afebrile with stable vital signs. The abdomen was soft and nontender with no palpable mass and normal bowel sounds. Stools tested negative for occult blood. Hematologic examination and blood chemical findings were normal as well.

Gastroscopy showed antritis and a submucosal lesion in the prepyloric posterior gastric wall (Figure 1). Proton pump inhibitors were started but did not relieve the symptoms. A computer tomography scan showed

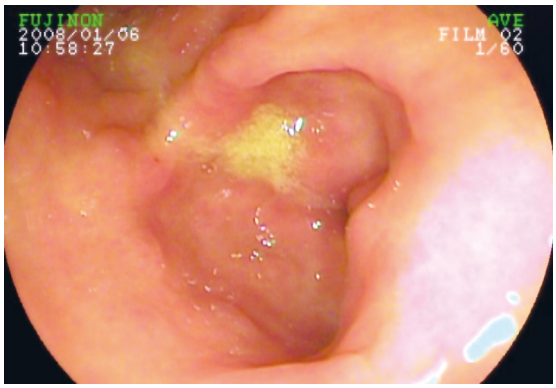

Figure 1 Submucosal lesion in the prepyloric posterior gastric wall.

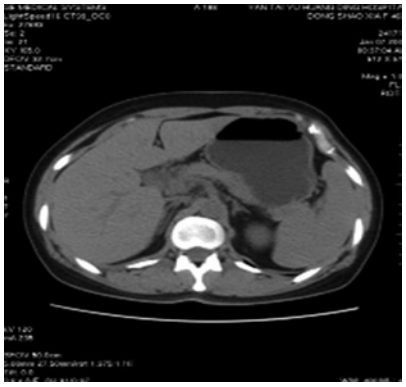

Figure 2 CT scan shows thickness of posterior gastric wall, measuring 1 cm x 1.5 cm in the lower body of the stomach.

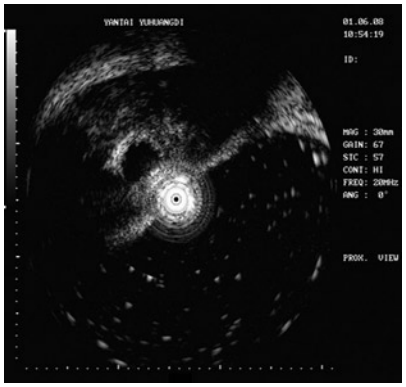

Figure 3 EUS reveals a lesion of 1.5 cm in diameter, with low and high complex echo-genicity, located within either the third or the fourth echo-layers.

thickness of posterior gastric wall, measuring 1 cm × 1.5 cm in the lower body of the stomach (Figure 2). Endoscopic ultrasonography revealed a lesion of 1.5 cm in diameter, with low and high complex echogenicity around this tissue, located within either the third or fourth echo-layers (Figure 3).

The results of biopsy were nondiagnostic, and disclosed only mild gastritis. Attempts of ploric dilation once proved unsuccessful, because the patient remained symptomatic: recurrent vomiting and epigastric pain despite helicobacter eradication and ploric dilation. A surgical exploration was proposed.

The lesion was removed with gastrectomy and gastrojejunostomy due to a presumed diagnosis of gastric outlet obstruction and submucosal tumor and because endoscopic biopsy was not diagnostic. The resected mass measured 1.5 cm × 2.0 cm × 1.0 cm with no ulceration or excessive induration. Some mucosal areas were remarkable for the presence of heterotopic pancreatic tissue with fully developed acini and ducts (Figure 4). The margins of the resection were negative, and the lymph nodes were benign.

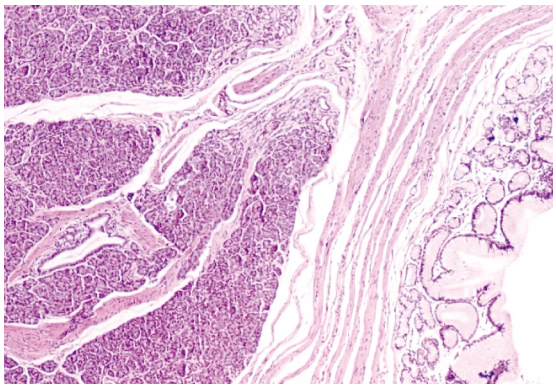

Figure 4 Heterotopic pancreatic tissue with fully developed acini and ducts. Islet is not found.

The postoperative course was uneventful. After the operation, vomiting episodes ceased, the patient gained 3 kg of body weight, and returned to full physical activity. The patient remained healthy and symptom-free in the follow-up of 6 mo.

DISCUSSION

The first case report of HP was made by Schultz in 1729. It is possible that early in fetal life, during rotation of the foregut and fusion of the dorsal and ventral parts of the pancreas, small parts are separated from it, and continue to develop in the wrong location[3]. Most often, HP is found in the stomach, duodenum and jejunum, but it may also be found anywhere in the digestive tract, intra-abdominally, in the mediastinum, and in the lung[1].

HP tissue is often found incidentally in patients operated on for other reasons or during autopsies. The condition is relatively uncommon, it has been found in 1%-2% of patients in autopsy studies and encountered in about 1 of 500 operations in the upper abdomen[6]. Macroscopically, HP consists of lobular white or yellow nodular tumors about 1-4 cm in diameter[1]. The mean size of HP was 1.52 cm in diameter (range 0.2-4 cm)[7]. A central mucosal depression, also described as volcanic of crater-like, is often recognized at endoscopy. The endoscopic appearance of gastric depends on the localization, HP may present with various symptoms. Nausea (27%) and vomiting, epigastric pain (27%) and ulceration (27%) were the most frequent clinical signs[7]. Heartburn and dyspepsia are also frequent for gastric HP, but often there are symptoms due to mechanical obstruction[1]. In the series of 34 patients with pancreatic heterotopia of Armstrong *et al*, 8 lesions were located in the stomach and 6 of those had produced symptoms[8]. There was a significant correlation between the size of the lesion and the presence of symptoms. Armstrong *et al* found that symptoms are related to the size of HP lesion. They postulated that size greater than 1.5 cm is more likely to be of clinical significance[8]. Other less

common symptoms were bleeding mimicking jejunal tumor[9], pancreatitis, and malignant transformation[10-12].

Macroscopically, the tissue often localized in the submucosa, but may also be found in the muscularis mucosa, subserously or in the serosa. In some cases, it streches through several or all of these layers[3]. Although imaging studies such as computer tomography (CT), radiographic contrast studies, and upper endoscopy are of assistance in the initial assessment of patients, the preoperative diagnosis of pancreatic heterotopia is often difficult.

Ormarsson *et al* reported that all patients examined with CT were inconclusive[1]. Cho *et al* also reported CT findings interpreted as HP in only two cases (17%)[13]. A diagnosis can occasionally be made on the basis of endoscopic biopsies. Histological examinations are inconclusive in about 50% of the cases because normal gastric mucosa covers the lesions[14]. Only in 4 out 10 patients did a biopsy lead to the correct diagnosis[1]. In most cases, however, the diagnosis is confirmed only by surgical resection.

Endoscopic ultrasonography (EUS) is widely used to evaluate submucosal lesions in the upper GI tract[15]. In a retrospective study of postresection histologic features compared with preoperative EUS findings in 10 patients with gastric HP, there was a close correlation between histology and EUS findings. EUS-guided aspiration has been reported to be helpful in diagnosis of HP[16]. If endoscopic resection is considered, EUS is also extremely useful for pre-excision assessment[17].

Most patients with HP are asymptomatic and require no treatment. The lesion is usually discovered incidentally. There was no correlation between the histological type of HP and the presence of symptoms[1,18]. Surgery is frequently needed to make a definitive diagnosis and to plan further treatment because the differential diagnosis of pancreatic rests includes leiomyoma, lymphoma, carcinoid tumors, and other malignancies[19].

If HP is discovered as an incidental finding, local excision is recommended. When HP results in symptoms, the lesion should be resected[20,21]. Increasingly, some are removed endoscopically with satisfactory postoperative results. Endoscopic excision can be considered in select cases depending on the size and location of the mass, especially for treating the benign lesions of HP.

REFERENCES

1 **Ormarsson OT**, Gudmundsdottir I, Marvik R. Diagnosis and treatment of gastric heterotopic pancreas. *World J Surg* 2006; **30**: 1682-1689

2 **Chou SJ**, Chou YW, Jan HC, Chen VT, Chen TH. Ectopic pancreas in the ampulla of vater with obstructive jaundice. A case report and review of literature. *Dig Surg* 2006; **23**: 262-264

3 **Pang LC**. Pancreatic heterotopia: a reappraisal and clinicopathologic analysis of 32 cases. *South Med J* 1988; **81**: 1264-1275

4 **Ozcan C**, Celik A, Guclu C, Balik E. A rare cause of gastric outlet obstruction in the newborn: Pyloric ectopic pancreas. *J Pediatr Surg* 2002; **37**: 119-120

5 **Ertem D**, Tutar E, Cam S, Ugras M, Pehlivanoglu E. Severe gastric outlet obstruction in a child with ectopic pancreas: is there a role for Helicobacter pylori? *J Pediatr Gastroenterol Nutr* 2006; **43**: 385-387

6 **Matsushita M**, Hajiro K, Okazaki K, Takakuwa H. Gastric aberrant pancreas: EUS analysis in comparison with the histology. *Gastrointest Endosc* 1999; **49**: 493-497

7 **Eisenberger CF**, Gocht A, Knoefel WT, Busch CB, Peiper M, Kutup A, Yekebas EF, Hosch SB, Lambrecht W, Izbicki JR. Heterotopic pancreas--clinical presentation and pathology with review of the literature. *Hepatogastroenterology* 2004; **51**: 854-858

8 **Armstrong CP**, King PM, Dixon JM, Macleod IB. The clinical significance of heterotopic pancreas in the gastrointestinal tract. *Br J Surg* 1981; **68**: 384-387

9 **Joo YE**, Kim HS, Choi SK, Rew JS, Park CS, Kim YJ, Kim SJ. Massive gastrointestinal bleeding caused by ectopic pancreas mimicking jejunal tumor. *Digestion* 2001; **64**: 133-136

10 **Ura H**, Denno R, Hirata K, Saeki A, Hirata K, Natori H. Carcinoma arising from ectopic pancreas in the stomach: endosonographic detection of malignant change. *J Clin Ultrasound* 1998; **26**: 265-268

11 **Jeong HY**, Yang HW, Seo SW, Seong JK, Na BK, Lee BS, Song GS, Park HS, Lee HY. Adenocarcinoma arising from an ectopic pancreas in the stomach. *Endoscopy* 2002; **34**: 1014-1017

12 **Song DE**, Kwon Y, Kim KR, Oh ST, Kim JS. Adenocarcinoma arising in gastric heterotopic pancreas: a case report. *J Korean Med Sci* 2004; **19**: 145-148

13 **Cho JS**, Shin KS, Kwon ST, Kim JW, Song CJ, Noh SM, Kang DY, Kim HY, Kang HK. Heterotopic pancreas in the stomach: CT findings. *Radiology* 2000; **217**: 139-144

14 **Laurent T**, Fournier D, Doenz F, Karaaslan T, Wassmer FA. Complex lesion of the gastric wall: an unusual presentation of ectopic pancreas. *J Clin Ultrasound* 1995; **23**: 438-441

15 **Yasuda K**, Cho E, Nakajima M, Kawai K. Diagnosis of submucosal lesions of the upper gastrointestinal tract by endoscopic ultrasonography. *Gastrointest Endosc* 1990; **36**: S17-S20

16 **Rodriguez FJ**, Abraham SC, Allen MS, Sebo TJ. Fine-needle aspiration cytology findings from a case of pancreatic heterotopia at the gastroesophageal junction. *Diagn Cytopathol* 2004; **31**: 175-179

17 **Dolan RV**, ReMine WH, Dockerty MB. The fate of heterotopic pancreatic tissue. A study of 212 cases. *Arch Surg* 1974; **109**: 762-765

18 **Lai EC**, Tompkins RK. Heterotopic pancreas. Review of a 26 year experience. *Am J Surg* 1986; **151**: 697-700

19 **McLean A**, Fairclough P. Endoscopic ultrasound--current applications. *Clin Radiol* 1996; **51**: 83-98

20 **Matsushita M**, Hajiro K, Okazaki K, Takakuwa H. Preoperative histological diagnosis of heterotopic pancreas. *Dig Dis Sci* 1999; **44**: 552

21 **Tanaka K**, Tsunoda T, Eto T, Yamada M, Tajima Y, Shimogama H, Yamaguchi T, Matsuo S, Izawa K. Diagnosis and management of heterotopic pancreas. *Int Surg* 1993; **78**: 32-35

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
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June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
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June 10-13, Istanbul, Turkey
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June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
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July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

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September 17, Mainz, Germany
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September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
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Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

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www.negf.org
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59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

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Neurogastroenterology & Motility Joint International Meeting 2008
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November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

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May 17-20, Denver, Colorado, USA
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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Books

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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

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[1]Passed away on October 20, 2007
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Contents

Contents

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EDITORIAL

Surgical outcome of adenosquamous carcinoma of the pancreas

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Abstract

Adenosquamous carcinoma is rare, accounting for 3%-4% of all pancreatic carcinoma cases. These tumors are characterized by the presence of variable proportions of mucin-producing glandular elements and squamous components, the latter of which should account for at least 30% of the tumor tissue. Recently, several reports have described cases of adenosquamous carcinoma of the pancreas. However, as the number of patients who undergo resection at a single institute is limited, large studies describing the clinicopathological features, therapeutic management, and surgical outcome for adenosquamous carcinoma of the pancreas are lacking. We performed a literature review of English articles retrieved from Medline using the keywords 'pancreas' and 'adenosquamous carcinoma'. Additional articles were obtained from references within the papers identified by the Medline search. Our subsequent review of the literature revealed that optimal adjuvant chemotherapy and/or radiotherapy regimens for adenosquamous carcinoma of the pancreas have not been established, and that curative surgical resection offers the only chance for long-term survival. Unfortunately, the prognosis of the 39 patients who underwent pancreatic resection for adenosquamous carcinoma was very poor, with a 3-year overall survival rate of 14.0% and a median survival time of 6.8 mo. Since the postoperative prognosis of adenosquamous carcinoma of the pancreas is currently worse than that of pancreatic adenocarcinoma, new adjuvant chemotherapies and/or radiation techniques should be investigated as they may prove indispensible to the improvement of surgical outcomes.

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Key words: Adenosquamous carcinoma of the pancreas; Pancreatectomy; Surgical outcome; Survival after pancreatic resection

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INTRODUCTION

The majority of malignant tumors in the pancreas are adenocarcinomas. Adenosquamous carcinoma of the pancreas occurs less frequently with an incidence of 3%-4%[1]. These tumors are a malignant epithelial carcinoma of the pancreas and are characterized by the presence of variable proportions of both glandular and squamous components. At least 30% of the neoplasm should be comprised of the squamous component[1,2]. Recently, several reports have described cases of adenosquamous carcinoma of the pancreas[3-6]. However, as the number of patients who undergo resection at a single institute is limited, large studies describing the clinicopathological features, therapeutic management and surgical outcome for adenosquamous carcinoma of the pancreas are lacking.

To the best of our knowledge, our survey of the English literature reporting on adenosquamous carcinoma of the pancreas, which was found on Medline, revealed that only 39 intent-to-cure surgical resections had been performed and had clearly presented data (Table 1)[7-28]. The purpose of this study was to clarify the surgical outcome including survival rates after surgery, and to determine the prognostic factors of adenosquamous carcinoma of the pancreas by conducting a retrospective analysis of the 39 patients.

PATIENTS

Our survey of the literature from 1980 to the end of 2007 revealed that 45 patients underwent surgical resection for adenosquamous carcinoma of the pancreas[7-28].

Table 1 Clinical and pathological data for the 39 cases that underwent surgical resection for adenosquamous carcinoma of the pancreas

Author	Ref	Yr	Age	Sex	Location	Surgery	Size (cm)	Cx	RT	Rec site	Survival
Ishikawa	7	1980	67	M	Body	DP	10.0	-	-	Widespread metastasis	4 mo
			53	F	Head	PD	4.2	-	-		2 d
			61	M	Head	PD	4.5	-	-		12 mo
Wilczynski	8	1984	68	M	Head, body	PD	4.5	-	-		20 d
Yamaguchi	9	1991	60	M	Head	PD	ND	-	-	ND	3 mo
			52	F	Head	PD	ND	-	-	ND	7 mo
			44	F	Head	PD	ND	-	-	ND	5 mo
			56	F	Head	PD	ND	-	-	ND	4 mo
			56	M	Head	PD	ND	-	-	ND	5 mo[1]
			68	F	Head	PD	ND	-	-	ND	5 mo[1]
			49	F	Body	DP	ND	-	-	ND	5 mo
			61	M	Tail	DP	ND	-	-	ND	14 mo[1]
Motojima	10	1992	52	M	Body, tail	DP	7.0	ND	ND	Systemic metastasis	3 mo
			75	M	Head	PD	3.0	ND	ND	Liver	10 mo
			75	F	Head	PD	6.0	ND	ND	Liver	8 mo
Tanaka	11	1994	48	F	Head	PD	4.2	+	-	ND	7 mo
Makiyama	12	1995	58	M	Head	PD	5.0	-	-	Peritoneum	18 mo
Onoda	13	1995	64	M	Body, tail	DP	7.0	+	-	Liver, peritoneum	3 mo
Campman	14	1997	65	F	Body, tail	DP	7.5	ND	ND	ND	ND
Kuji	15	1997	73	M	Body, tail	TP	6.0	-	-	ND	2 mo
Nabae	16	1998	60	M	Body	DP	6.0	-	IOR	ND	4 mo
			73	M	Head	PD	ND	-	-	Liver	10 mo
Myung	17	1998	64	M	Head	PD	3.5	-	-	ND	4 mo[1]
Lozano	18	1998	75	M	Head, body	PD	4.5	+	+	ND	ND[1]
			42	M	Head	PD	3.5	+	+	ND	ND[1]
Aranha	19	1999	52	M	Head	PD	3.2	+	+	Systemic metastasis	13 mo
			62	M	Head	PD	3.0	+	+	Systemic metastasis	14 mo
Komatsuda	20	2000	67	M	Body	DP	5.0	-	-	Peritoneum	6 mo
Yavus	21	2000	51	M	Head	PD	4.0	ND	ND	-	36 mo[1]
			48	M	Head	PD	2.0	ND	ND	ND	ND
Yamaue	22	2001	63	F	Head	PD	4.5	+	+	-	40 mo[1]
Kardon	23	2001	ND	ND	Head	PD	ND	-	-	ND	33 mo[1]
Murakami	24	2003	41	M	Head	PD	3.0	-	+	Peritoneum	5 mo
Rahemtullah	25	2003	ND	ND	Head	PD	ND	ND	ND	ND	13 mo[1]
			ND	ND	Head	PD	ND	ND	ND	ND	ND
Alwaheeb	26	2005	45	M	Head	PD	6.0	-	-	ND	ND
Hsu	27	2005	66	M	Head	PD	3.5	-	-	ND	2.5 mo
			38	F	Head	PD	3.8	-	+	ND	6.8 mo
Jamali	28	2007	75	M	Head	PD	3.0	+	-	Liver	6 mo

Cx: Chemotherapy; RT: Radiotherapy; Rec site: Recurrence site; ND: Not described; PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; TP: Total pancreatectomy. [1]Surviving patients.

Of these, six patients were excluded due to a lack of clear data. The remaining 39 patients were analyzed in this study (Table 1) and included 25 men, 11 women, and three patients of unknown sex with a mean age of 59.0 years (range, 38-75 years). The prognosis outcome of each case was obtained from the published data. The clinicopathological data associated with the pancreatic adenosquamous carcinomas described in these case reports were evaluated, and included tumor location, type of operation, tumor size, whether chemotherapy and radiotherapy had been administered, recurrence sites, and survival times. All of the patients had undergone surgery involving an attempted curative resection. Survival rates were generated using the Kaplan-Meier method and compared using the log-rank test[29]. Values were expressed appropriately as the mean ± SD. Differences in proportions were evaluated by the Pearson chi-square test. A value of $P < 0.05$ was considered to be statistically significant.

DIAGNOSIS OF ADENOSQUAMOUS CARCINOMA OF THE PANCREAS

Table 1 lists 39 patients who had undergone surgical resection for adenosquamous carcinoma of the pancreas. Adenosquamous carcinomas have not been associated with any specific clinical syndromes[2,30]. Each of the 39 patients presented clinical symptoms such as abdominal pain, back pain, painless jaundice, anorexia, and/or body weight loss (data not shown). Accurate preoperative diagnosis of adenosquamous carcinoma of the pancreas is very difficult, because imaging studies have revealed no characteristic features that can facilitate the differentiation of this tumor type from ordinary invasive ductal carcinoma. One study reported that intense Gallium-67 citrate uptake was observed in adenosquamous carcinoma of the pancreas, indicating that Gallium-67 citrate scintigraphy might be useful in detecting these carcinomas[15]. However, more detailed

imaging data are required to improve the ability to diagnose this rare disease.

Adenosquamous carcinoma of the pancreas appears to be larger than ordinary pancreatic adenocarcinoma. The tumors in the 27 cases for which the relevant data was available had a mean size of 4.8 ± 1.8 cm (range, 2-10 cm; Table 1). Preoperative cytological or pathological diagnosis of adenosquamous carcinoma of the pancreas is reportedly rare[12,16-18,21,24,26,30]. However, the two malignant cellular components of adenosquamous carcinoma can be recognized in aspirated smears[17,18,24]. A careful search for glandular differentiation is warranted when the squamous component predominates, particularly if squamous carcinoma specimens only are obtained by biopsy or fine needle aspiration biopsy[12,16]. Adenosquamous carcinoma of the pancreas has no specific radiological findings or serum data, including tumor markers such as carcinoembryonic antigen, carbohydrate antigen 19-9, or squamous cell carcinoma antigen[12,22]. Physicians should try to remember to consider adenosquamous carcinoma of the pancreas in the differential diagnosis of ordinary pancreatic adenocarcinoma, especially if the patient has severe abdominal symptoms and/or a large tumor size[2,30]. Recently, preoperative and intraoperative cytological examinations have been diagnostically correct, however these findings did not alter treatment decisions or survival[30].

MANAGEMENT FOR RESECTABLE ADENOSQUAMOUS CARCINOMA OF THE PANCREAS

Since adenosquamous carcinomas are uncommon tumors with a poor prognosis, the outcomes associated with various therapeutic interventions are not well defined.

Table 1 lists the tumor location and operative method used in the 39 cases analyzed here. Three main operative methods were performed: pancreaticoduodenectomy (PD) including pylorus-preserving PD (PPPD) in 30 cases (76.9%); distal pancreatectomy (DP) in eight cases (20.5%); and total pancreatectomy (TP) in one case (2.6%). Tumors were located in the head alone in 28 cases (76.9%), in the head and body in two cases, and in the body and/or tail of the pancreas in nine cases (23.1%). Although adenosquamous carcinoma of the pancreas has different clinicopathological features to pancreatic adenocarcinoma, the treatment strategy of patients with adenosquamous carcinoma is dealt with in the same manner as patients with adenocarcinoma. Surgical treatment remains the only curative management option that is seriously considered for adenosquamous carcinoma of the pancreas.

To date, only eight patients have received adjuvant chemotherapy, indicating that postoperative adjuvant chemotherapy is not usually administered to patients with adenosquamous carcinoma of the pancreas (Table 1). Tanaka et al reported that the size of an unresectable adenosquamous carcinoma of the pancreas was reduced by neo-adjuvant chemotherapy consisting of a combination of interferon-α, tumor necrosis factor-α, and 5-fluorouracil[11]. However, the patient only survived 7 mo after surgery[11]. In this case, although neo-adjuvant chemotherapy might not have contributed to prolonging the patient's survival, the ability of the chemotherapy to reduce the size of the tumor from one that was unresectable to one that could be resected was confirmed. In the current study, the adjuvant chemotherapy group had a 2- or 3-year cumulative survival rate of 16.7% and a median survival period of 7 mo (Table 2). In comparison, the group who did not receive adjuvant chemotherapy had a 2-year cumulative survival rate of 9.2% and a median survival period of 5 mo ($P = 0.364$). Almost all of the patients in the adjuvant chemotherapy group were treated with a 5-fluorouracil-based regimen. Recently, adjuvant chemotherapy using new drug agents has been considered as the standard therapeutic option following resection for pancreatic adenocarcinoma, and several reports suggest that adjuvant chemotherapy with gemcitabine is responsible for a significant increase in patient survival[31-33]. Postoperative administration of gemcitabine also significantly delayed the development of recurrent disease after complete resection of pancreatic cancer compared with observation alone[34]. However, information regarding gemcitabine use in cases with adenosquamous carcinoma of the pancreas is not available as previous reports lack such data. Further investigations examining whether adjuvant chemotherapy using gemcitabine will improve surgical outcome in patients with adenosquamous carcinoma of the pancreas are therefore warranted.

There are no published prospective randomized controlled trials investigating radiotherapy treatment of pancreatic adenosquamous carcinoma following curative resection, only retrospective studies. Limitations of the present study include the errors and biases inherent in a small retrospective study design. Two retrospective studies investigating the benefit of radiotherapy following curative resection for pancreatic carcinoma showed no significant difference in the overall survival between patients who were or were not treated with radiotherapy[35,36]. In the current study, patients who had received intra- and/or postoperative radiotherapy had a 2- or 3-year cumulative survival rate of 20.0% and a median survival period of 13 mo (Table 2). By comparison, the non radiotherapy group had a 2-year cumulative survival rate of 9.0% and a median survival period of 6 mo ($P = 0.284$). There was no significant difference in survival between patients who did and did not receive radiotherapy.

PROGNOSIS AFTER PANCREATIC RESECTION

The overall 1-, 2-, and 3-year survival rates after pancreatic resection were 25.5%, 14.0%, and 14.0%, respectively (Figure 1). Table 1 shows operative mortality occurred in two patients during the early 1980s[7,8]. One patient died of myocardial infarction 2 d after undergoing PD and another died of numerous postoperative complications including electrolyte disturbance from massive abdominal fluid

Table 2 Clinical characteristics after surgical resection for adenosquamous carcinoma of the pancreas

Characteristics	No. of patients	Survival rate (%)			Median survival in months (range)	*P* value
		1 yr	2 yr	3 yr		
Overall	39	25.5	14.0	14.0	6.8 (4.6-9.0)	
Age (yr)						
< 60	16	26.9	9.0	9.0	6.8 (4.4-9.2)	0.975
> 60	20	20.4	13.6	13.6	6.0 (1.3-10.7)	
Gender						
Male	25	28.4	8.5	8.5	6.0 (1.1-10.9)	0.842
Female	11	12.0	12.0	12.0	6.8 (5.0-8.6)	
Tumor location						
Head	30	34.8	17.9	17.9	8.0 (5.3-10.7)	0.017
Body or tail	9	11.1	-	-	4.0 (2.6-5.4)	
Operation type						
PD	30	33.4	17.2	17.2	8.0 (5.2-10.8)	0.063
DP or TP	9	12.5	-	-	4.0 (2.7-5.3)	
LN metastasis						
Present	14	20.0	-	-	5.0 (2.0-8.0)	0.134
Absent	8	50.0	50.0	50.0	5	
Chemotherapy						
Yes	8	50.0	16.7	16.7	7.0 (0.0-15.4)	0.364
No	23	18.4	9.2	-	5.0 (3.0-7.0)	
Radiotherapy						
Yes	7	60.0	20.0	20.0	13.0 (0.0-26.3)	0.284
No	23	18.0	9.0	-	6.0 (4.0-8.0)	

PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; TP: Total pancreatectomy; LN metastasis: Lymph node metastasis.

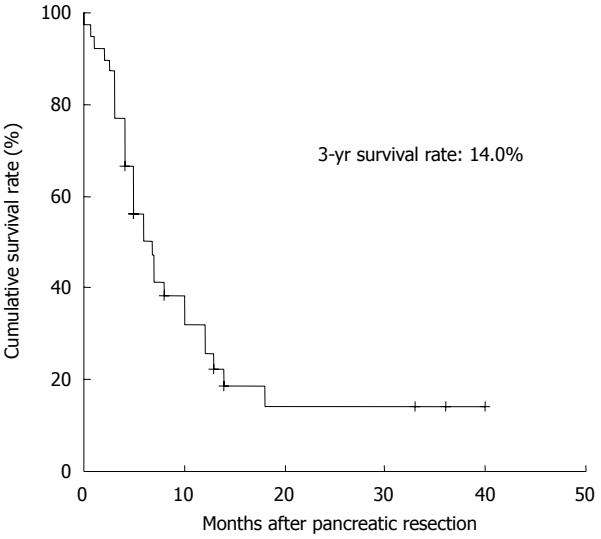

Figure 1 Survival after surgical resection for adenosquamous carcinoma of the pancreas (*n* = 39).

losses, acute renal failure and eventually congestive heart failure 20 d after undergoing PD[7,8]. Univariate analysis of the different prognostic factors predicted to contribute to patient prognosis showed that tumor location was the only unfavorable prognostic factor. Median survival of patients with a tumor located in the body and/or tail (4 mo) was significantly worse than those with tumors located in the head (8 mo) (Table 2). Prognostic differences based on tumor location may relate to tumor size, as the size of a distal pancreatic tumor (7.3 ± 1.8 cm) was significantly larger than that of a proximal pancreatic tumor (4.7 ± 1.9 cm, $P = 0.002$). Age, gender, type of operative procedure, and lymph node metastasis were not significant prognostic factors.

Recently, long-term survival after PD for pancreatic adenocarcinoma has improved, and the number of patients surviving for five or 10 years has increased[37-39]. On the other hand, the prognosis for the 39 patients with adenosquamous carcinoma in this study was poor, with a 3-year overall survival rate of only 14.0%, and includes two patients with hospital mortality. A patient surviving for five years post-resection has not been reported yet (Table 1). This suggests that adenosquamous carcinoma of the pancreas has greater malignant potential than adenocarcinoma of the pancreas. A previous report also found that squamous cell carcinomas grow at twice the speed of adenocarcinomas[31]. Therefore, once an adenocarcinoma has transformed into an adenosquamous carcinoma, the carcinoma may exhibit a higher degree of malignancy[40].

CONCLUSION

Even though curative resection for adenosquamous carcinoma of the pancreas was performed in the 39 patients, prognosis remained poor because systemic metastases in the liver and peritoneal dissemination were the major sites of recurrence (Table 1). In addition, tumor recurrence occurred during the early stages of the postoperative period in a large number of patients. Yamaue et al. reported that it might be preferable not to perform a pancreatic resection if a pancreatic tumor is diagnosed as an adenosquamous carcinoma[22]. Consensus of opinion regarding the surgical indication required for this type of tumor has not been reached yet. Elucidating

a surgical treatment strategy based on the appropriate surgical indication is essential for improving the surgical outcome of adenosquamous carcinoma of the pancreas.

The results of this current study indicate that tumor location may be an important factor in determining the appropriate surgical indication. Namely, surgical resection may be better suited for proximal pancreatic tumors than for distal tumors because the proximal location of tumors was the only significant favorable prognostic factor found in this study. Furthermore, exploration of new radiation techniques and chemotherapeutic regimens using new drug agents such as gemcitabine may be required because conventional chemotherapy and radiotherapy treatments did not contribute to survival benefit. The incorporation of novel 'molecularly targeted' agents into therapy will also be required to improve surgical outcome.

Although adenosquamous carcinoma of the pancreas has a poor prognosis even after curative resection, we must continue to endeavor to improve the surgical outcome of this tumor, because despite its rarity, it occurs worldwide. More data, including epidemiological and pathological findings, will be required to determine the appropriate surgical indication for this tumor.

REFERENCES

1 **Klöppel G**, Hruban RH, Longnecker DS, Adler G, Kern SE, Partanen TJ. Tumours of the exocrine pancreas. In: Hamilton SR, Aaltonen LA, eds. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Digestive System. Lyon: IARC Press, 2000: 219-251

2 **Hruban RH**, Pitman MB, Klimsta DS. Tumors of the pancreas. In: AFIP Atlas of Tumor Pathology. Series 4, Fascicle 6, 2007: 165-168

3 **Nakao A**, Fujii T, Sugimoto H, Kanazumi N, Nomoto S, Kodera Y, Inoue S, Takeda S. Oncological problems in pancreatic cancer surgery. *World J Gastroenterol* 2006; **12**: 4466-4472

4 **Oettle H**, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, Schramm H, Fahlke J, Zuelke C, Burkart C, Gutberlet K, Kettner E, Schmalenberg H, Weigang-Koehler K, Bechstein WO, Niedergethmann M, Schmidt-Wolf I, Roll L, Doerken B, Riess H. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* 2007; **297**: 267-277

5 **Neoptolemos JP**, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, Beger H, Fernandez-Cruz L, Dervenis C, Lacaine F, Falconi M, Pederzoli P, Pap A, Spooner D, Kerr DJ, Buchler MW. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med* 2004; **350**: 1200-1210

6 **Shaib Y**, Davila J, Naumann C, El-Serag H. The impact of curative intent surgery on the survival of pancreatic cancer patients: a U.S. Population-based study. *Am J Gastroenterol* 2007; **102**: 1377-1382

7 **Ishikawa O**, Matsui Y, Aoki I, Iwanaga T, Terasawa T, Wada A. Adenosquamous carcinoma of the pancreas: a clinicopathologic study and report of three cases. *Cancer* 1980; **46**: 1192-1196

8 **Wilczynski SP**, Valente PT, Atkinson BF. Cytodiagnosis of adenosquamous carcinoma of the pancreas. Use of intraoperative fine needle aspiration. *Acta Cytol* 1984; **28**: 733-736

9 **Yamaguchi K**, Enjoji M. Adenosquamous carcinoma of the pancreas: a clinicopathologic study. *J Surg Oncol* 1991; **47**: 109-116

10 **Motojima K**, Tomioka T, Kohara N, Tsunoda T, Kanematsu T. Immunohistochemical characteristics of adenosquamous carcinoma of the pancreas. *J Surg Oncol* 1992; **49**: 58-62

11 **Tanaka N**, Ohoida J, Matuno T, Gouchim A, Iwagaki H, Moreira LF, Orita K. Response of adenosquamous carcinoma of the pancreas to interferon-alpha, tumor necrosis factor-alpha and 5-fluorouracil combined treatment. *Anticancer Res* 1994; **14**: 2739-2742

12 **Makiyama K**, Takuma K, Zea-Iriarte WL, Ikuno N, Kawatomi M, Mori N, Ishino T, Yonemitsu N. Adenosquamous carcinoma of the pancreas. *J Gastroenterol* 1995; **30**: 798-802

13 **Onoda N**, Kang SM, Sugano S, Yamashita Y, Chung YS, Sowa M. Mucoepidermoid carcinoma of the pancreas: report of a case. *Surg Today* 1995; **25**: 843-847

14 **Campman SC**, Fajardo MA, Rippon MB, Kraegel SA, Ruebner BH. Adenosquamous carcinoma arising in a mucinous cystadenoma of the pancreas. *J Surg Oncol* 1997; **64**: 159-162

15 **Kuji I**, Sumiya H, Taki J, Nakajima K, Yokoyama K, Kinuya S, Kinuya K, Ichikawa A, Konishi S, Michigishi T, Tonami N. Intense Ga-67 uptake in adenosquamous carcinoma of the pancreas. *Ann Nucl Med* 1997; **11**: 41-43

16 **Nabae T**, Yamaguchi K, Takahata S, Utsunomiya N, Matsunaga H, Sumiyoshi K, Chijiiwa K, Tanaka M. Adenosquamous carcinoma of the pancreas: report of two cases. *Am J Gastroenterol* 1998; **93**: 1167-1170

17 **Myung SJ**, Kim MH, Lee SK, Seo DW, Kim YS, Min YI. Adenosquamous carcinoma of the pancreas: differentiation from pancreatic pseudocyst. *Gastrointest Endosc* 1998; **47**: 410-413

18 **Lozano MD**, Panizo A, Sola IJ, Pardo-Mindan FJ. FNAC guided by computed tomography in the diagnosis of primary pancreatic adenosquamous carcinoma. A report of three cases. *Acta Cytol* 1998; **42**: 1451-1454

19 **Aranha GV**, Yong S, Olson M. Adenosquamous carcinoma of the pancreas. *Int J Pancreatol* 1999; **26**: 85-91

20 **Komatsuda T**, Ishida H, Konno K, Sato M, Watanabe S, Furuya T, Ishida J. Adenosquamous carcinoma of the pancreas: report of two cases. *Abdom Imaging* 2000; **25**: 420-423

21 **Yavuz E**, Kapran Y, Ozden I, Bulut T, Dizdaroglu F. Pancreatobiliary adenosquamous carcinoma (report of two cases). *Pathologica* 2000; **92**: 323-326

22 **Yamaue H**, Tanimura H, Onishi H, Tani M, Kinoshita H, Kawai M, Yokoyama S, Uchiyama K. Adenosquamous carcinoma of the pancreas: successful treatment with extended radical surgery, intraoperative radiation therapy, and locoregional chemotherapy. *Int J Pancreatol* 2001; **29**: 53-58

23 **Kardon DE**, Thompson LD, Przygodzki RM, Heffess CS. Adenosquamous carcinoma of the pancreas: a clinicopathologic series of 25 cases. *Mod Pathol* 2001; **14**: 443-451

24 **Murakami Y**, Yokoyama T, Yokoyama Y, Kanehiro T, Uemura K, Sasaki M, Morifuji M, Sueda T. Adenosquamous carcinoma of the pancreas: preoperative diagnosis and molecular alterations. *J Gastroenterol* 2003; **38**: 1171-1175

25 **Rahemtullah A**, Misdraji J, Pitman MB. Adenosquamous carcinoma of the pancreas: cytologic features in 14 cases. *Cancer* 2003; **99**: 372-378

26 **Alwaheeb S**, Chetty R. Adenosquamous carcinoma of the pancreas with an acantholytic pattern together with osteoclast-like and pleomorphic giant cells. *J Clin Pathol* 2005; **58**: 987-990

27 **Hsu JT**, Yeh CN, Chen YR, Chen HM, Hwang TL, Jan YY, Chen MF. Adenosquamous carcinoma of the pancreas. *Digestion* 2005; **72**: 104-108

28 **Jamali M**, Serra S, Chetty R. Adenosquamous carcinoma of the pancreas with clear cell and rhabdoid components. A case report. *JOP* 2007; **8**: 330-334

29 **Kaplan EL**, Meier P. Nonparametric estimation from

incomplete observations. *J Am Stat Assoc* 1958; **53**: 457-481
30 **Madura JA**, Jarman BT, Doherty MG, Yum MN, Howard TJ. Adenosquamous carcinoma of the pancreas. *Arch Surg* 1999; **134**: 599-603
31 **O'Connor JK**, Sause WT, Hazard LJ, Belnap LP, Noyes RD. Survival after attempted surgical resection and intraoperative radiation therapy for pancreatic and periampullary adenocarcinoma. *Int J Radiat Oncol Biol Phys* 2005; **63**: 1060-1066
32 **Neoptolemos JP**, Dunn JA, Stocken DD, Almond J, Link K, Beger H, Bassi C, Falconi M, Pederzoli P, Dervenis C, Fernandez-Cruz L, Lacaine F, Pap A, Spooner D, Kerr DJ, Friess H, Buchler MW. Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. *Lancet* 2001; **358**: 1576-1585
33 **Neuhaus P**, Oettle H, Post H, Gellert K, Ridwelski K, Schramm H, Zurke C, Fahlke G, Langrehr J, Reiss H. A randomized, prospective, multicenter, phase III trial of adjuvant chemotherapy with gemcitabine vs. observation in patients with resected pancreatic cancer. *Proc Am Soc Clin Oncol* 2005; **23**: 311s
34 **Oettle H**, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, Schramm H, Fahlke J, Zuelke C, Burkart C, Gutberlet K, Kettner E, Schmalenberg H, Weigang-Koehler K, Bechstein WO, Niedergethmann M, Schmidt-Wolf I, Roll L, Doerken B, Riess H. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* 2007; **297**: 267-277
35 **Ihse I**, Andersson R, Ask A, Ewers SB, Lindell G, Tranberg KG. Intraoperative radiotherapy for patients with carcinoma of the pancreas. *Pancreatology* 2005; **5**: 438-442
36 **Nakagohri T**, Kinoshita T, Konishi M, Takahashi S, Tanizawa Y. Clinical results of extended lymphadenectomy and intraoperative radiotherapy for pancreatic adenocarcinoma. *Hepatogastroenterology* 2007; **54**: 564-569
37 **Nitecki SS**, Sarr MG, Colby TV, van Heerden JA. Long-term survival after resection for ductal adenocarcinoma of the pancreas. Is it really improving? *Ann Surg* 1995; **221**: 59-66
38 **Matsuno S**, Egawa S, Fukuyama S, Motoi F, Sunamura M, Isaji S, Imaizumi T, Okada S, Kato H, Suda K, Nakao A, Hiraoka T, Hosotani R, Takeda K. Pancreatic Cancer Registry in Japan: 20 years of experience. *Pancreas* 2004; **28**: 219-230
39 **Schnelldorfer T**, Ware AL, Sarr MG, Smyrk TC, Zhang L, Qin R, Gullerud RE, Donohue JH, Nagorney DM, Farnell MB. Long-term survival after pancreatoduodenectomy for pancreatic adenocarcinoma: is cure possible? *Ann Surg* 2008; **247**: 456-462
40 **Iemura A**, Yano H, Mizoguchi A, Kojiro M. A cholangiocellular carcinoma nude mouse strain showing histologic alteration from adenocarcinoma to squamous cell carcinoma. *Cancer* 1992; **70**: 415-422

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Adult eosinophilic gastroenteritis and hypereosinophilic syndromes

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Abstract

Eosinophilic gastroenteritis (EGE) in the adult is a distinctive pathologically-based disorder characterized by an eosinophil-predominant mucosal inflammatory process. Most often, the disorder is detected during endoscopic investigation for abdominal pain or diarrhea. Other causes of gastric and intestinal mucosal eosinophilia require exclusion, including parasitic infections and drug-induced causes. Occasionally, the muscle wall or serosal surface may be involved. EGE appears to be more readily recognized, in large part, due to an evolution in the imaging methods used to evaluate abdominal pain and diarrhea, in particular, endoscopic imaging and mucosal biopsies. Definition of EGE, however, may be difficult, as the normal ranges of eosinophil numbers in normal and abnormal gastric and intestinal mucosa are not well standardized. Also, the eosinophilic inflammatory process may be either patchy or diffuse and the detection of the eosinophilic infiltrates may vary depending on the method of biopsy fixation. Treatment has traditionally focused on resolution of symptoms, and, in some instances, eosinophil quantification in pre-treatment and post-treatment biopsies. Future evaluation and treatment of EGE may depend on precise serological biomarkers to aid in definition of the long-term natural history of the disorder and its response to pharmacological or biological forms of therapy.

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Key words: Eosinophils; Eosinophilic gastroenteritis; Eosinophilic gastritis; Eosinophilic enteritis

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INTRODUCTION

Eosinophilic gastroenteritis (EGE) is a distinct eosinophil-predominant inflammatory process in gastric or small intestinal mucosal biopsies. Although most published material has focused largely on the pediatric population, this manuscript focuses on the adult with EGE. Most often in adults, EGE is detected during endoscopic investigation for abdominal pain or diarrhea[1-4]. Although the endoscopic changes are non-specific (Figure 1), chronic or recurring symptoms are present and other causes of intestinal eosinophilia require exclusion (e.g. parasitic infections, medications)[1-4]. The mucosa of the stomach, intestine, or both may be involved most frequently. In one specific classification schema that included different forms of intestinal eosinophilic infiltration, involvement of the muscular layer or serosa was also described, but even in these, concomitant mucosal involvement was often present[5].

EGE has been considered an uncommon, even rare disorder but this may well depend on its definition as well as the method of detection. In a single clinical practice, only less than 1% of all upper endoscopic studies during an 8-year period showed changes that led to a diagnosis of EGE[6]. Biopsies are now commonly done during routine endoscopic evaluation, even if the mucosa is visually normal or if only non-specific changes are present. Finally, it is speculated that there may be some environmental factors (or allergen) in the diet or air that plays a critical role in the emergence of an eosinophil-predominant mucosal inflammatory process.

HISTOPATHOLOGICAL CRITERIA

Most often, the diagnosis of EGE is defined by histological evaluation of endoscopic biopsies. There are some potential diagnostic issues and pitfalls. First,

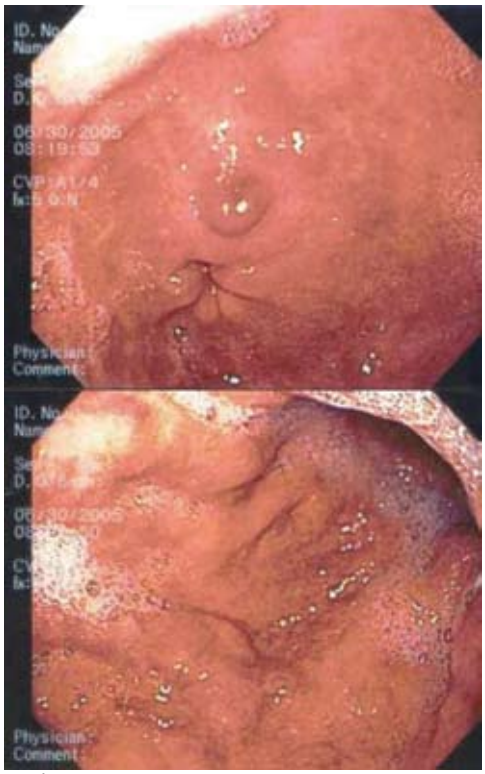
GE clinic

Figure 1 Endoscopic views of gastric antrum and gastric body showing non-specific erythematous and thickened mucosal folds with pre-pyloric pseudopolypoid change.

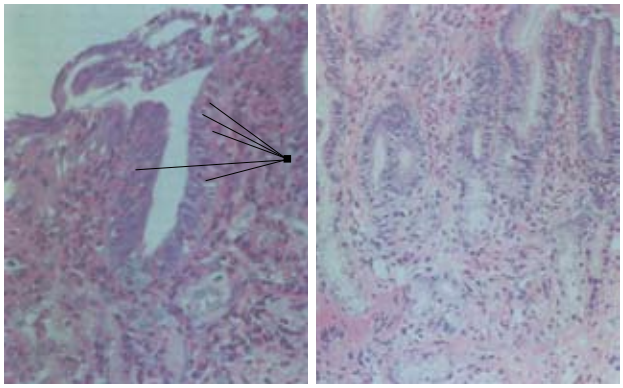

Figure 2 Photomicrographs show a formalin-fixed biopsy on the left with arrows delineating intraepithelial eosinophils along with numerous eosinophils in the lamina propria, while a Bouin's fixed biopsy on the right from an adjacent endoscopic biopsy shows a paucity of eosinophils..

pathologists, like endoscopists, have varying levels of expertise in mucosal biopsy interpretation. It is appreciated that there may be many "shades of grey" in the definition of EGE and the experience of the pathologist may determine disease recognition. To date, however, precise information on intra- and inter-observer error in the interpretation of biopsies in EGE is still needed. Second, eosinophils may normally be detected in the gastric and intestinal mucosa (as opposed to the normal esophagus), and only limited numbers of studies (mainly in children) have tried to quantify normal compared to abnormal numbers in health and different inflammatory disease states, e.g. ulcerative colitis[7,8]. Third, these studies are also limited, to some degree, by the inherent "patchy" nature of the eosinophilic inflammatory process in EGE since the numbers of eosinophils may differ in biopsies obtained from different sites. Finally, fixation methods may be critical in the definition of eosinophils in gastric and intestinal biopsies. For example, Bouin's solution (often used for gastric or intestinal biopsies), can result in "bleaching" of eosinophil granules making detection much more difficult. If EGE is suspected, routine formalin fixation has been shown to provide more optimal material for routine staining (Figure 2)[9].

CLINICAL FEATURES

Prior studies have suggested that EGE is a male-predominant clinical disorder[1]. Some believe that clinical features may reflect extent, location and depth of infiltration of this eosinophilic inflammatory process within the gastrointestinal tract[1,5]. Abdominal pain and diarrhea are common. Weight loss may occur, in part related to malabsorption. Iron deficiency associated with blood loss as well as protein-losing enteropathy may also be seen. If muscular layers are involved, obstruction or, even an acute abdomen has been recorded[1-4], while serosal involvement may be associated with evidence of ascites[5]. Peripheral blood eosinophilia has been recorded in up to 70%, but this is not specific for EGE and should lead to exclusion of other disorders, specifically parasitic infections[3]. In some with EGE, increased serum IgE levels may be seen, but this is also not specific. Endoscopic evaluation might permit definition of the extent of the inflammatory process in the upper gastrointestinal tract. In rare reports[10,11], celiac disease has been linked to EGE but as completely independent disorders.

Treatment is largely aimed at resolving symptoms. Medications used in EGE are largely based on empiric observation and experience. Because of the rarity of EGE, there are no controlled treatment trials available. Steroids have been used as a traditional form of therapy to reduce the inflammatory process[1-4], however, these may cause steroid-related effects, especially because of their recurring need over prolonged periods. Other remedies have been used but their effectiveness still requires definition. These include: proton pump inhibitors[5], mast cell stabilizers[12,13], ketotifen[9,14], leukotriene antagonists[15], octreotide[16] and surgical resection of involved intestinal segments[17]. This lengthening therapeutic list might be construed as a clear reflection of the limited forms of effective therapy that are currently available.

HYPEREOSINOPHILIC SYNDROMES

The hypereosinophilic syndromes (HES) represent a heterogeneous group of rare disorders that have been defined in the past by persistent blood eosinophilia for more than 6 mo with evidence of organ involvement. The cause is unknown. HES may include a broad

spectrum of disorders, including familial or genetically-based eosinophilia and more sinister neoplastic disorders, including eosinophilic leukemia[1,19]. About 25% of cases with HES, however, have eosinophilic infiltration in the gastrointestinal tract, and in some, this inflammatory process is reportedly localized only in gastric or intestinal mucosa.

The onset of HES is generally described between ages of 20 and 50 years with a male predominance[1]. Abdominal pain and diarrhea with malabsorption have been described. In comparison with those with disease localized in some other non-intestinal sites, involvement of the intestinal tract has been associated with a limited prognosis, lympho-proliferative disorders[20], and, in some, a fatal outcome[1]. Unfortunately, there may be little to distinguish EGE and HES, especially if the latter is early in the clinical course and localized to the gastric and intestinal mucosa alone. In some, steroids, immune-suppressants and even biological agents have been used[21]. Clearly, long-term clinical studies are needed to define and elucidate the natural history of EGE, a relatively unique inflammatory process, and to determine if there is a potential HES risk.

REFERENCES

1 **Straumann A**. Idiopathic eosinophilic gastrointestinal diseases in adults. *Best Pract Res Clin Gastroenterol* 2008; **22**: 481-496
2 **Cello JP**. Eosinophilic gastroenteritis--a complex disease entity. *Am J Med* 1979; **67**: 1097-1104
3 **Talley NJ**, Shorter RG, Phillips SF, Zinsmeister AR. Eosinophilic gastroenteritis: a clinicopathological study of patients with disease of the mucosa, muscle layer, and subserosal tissues. *Gut* 1990; **31**: 54-58
4 **Khan S**, Orenstein SR. Eosinophilic gastroenteritis. *Gastroenterol Clin North Am* 2008; **37**: 333-348
5 **Klein NC**, Hargrove RL, Sleisenger MH, Jeffries GH. Eosinophilic gastroenteritis. *Medicine* (Baltimore) 1970; **49**: 299-319
6 **Gorrepati N**, Desai H, Fox S, Amin M, Stecevic V, Desai T. An eight-year study of 30 consecutive patients with eosinophilic gastroenteritis in one gastroenterology practice: eosinophilic gastroenteritis is more common than previously appreciated and may not necessarily require corticosteroid therapy. *Gastroenterology* 2007; **132**: A610 (Abstract)
7 **Lowichik A**, Weinberg AG. A quantitative evaluation of mucosal eosinophils in the pediatric gastrointestinal tract. *Mod Pathol* 1996; **9**: 110-114
8 **DeBrosse CW**, Case JW, Putnam PE, Collins MH, Rothenberg ME. Quantity and distribution of eosinophils in the gastrointestinal tract of children. *Pediatr Dev Pathol* 2006; **9**: 210-218
9 **Freeman HJ**. Histologic evaluation of gastric biopsies for diagnosis and treatment in eosinophilic gastroenteritis. *Can J Gastroenterol* 1993; **7**: 343-348
10 **Bennett RA**, Whitelock T 3rd, Kelley JL Jr. Eosinophilic gastroenteritis, gluten enteropathy, and dermatitis herpetiformis. *Am J Dig Dis* 1974; **19**: 1154-1161
11 **Butterfield JH**, Murray JA. Eosinophilic gastroenteritis and gluten-sensitive enteropathy in the same patient. *J Clin Gastroenterol* 2002; **34**: 552-553
12 **Moots RJ**, Prouse P, Gumpel JM. Near fatal eosinophilic gastroenteritis responding to oral sodium chromoglycate. *Gut* 1988; **29**: 1282-1285
13 **Di Gioacchino M**, Pizzicannella G, Fini N, Falasca F, Antinucci R, Masci S, Mezzetti A, Marzio L, Cuccurullo F. Sodium cromoglycate in the treatment of eosinophilic gastroenteritis. *Allergy* 1990; **45**: 161-166
14 **Melamed I**, Feanny SJ, Sherman PM, Roifman CM. Benefit of ketotifen in patients with eosinophilic gastroenteritis. *Am J Med* 1991; **90**: 310-314
15 **Neustrom MR**, Friesen C. Treatment of eosinophilic gastroenteritis with montelukast. *J Allergy Clin Immunol* 1999; **104**: 506
16 **Rausch T**, Gyr K, Wegmann W, Germer M, Meier R. [Symptomatic therapy of severe diarrhea in eosinophilic gastroenteritis with the somatostatin analog octreotide (Sandostatin)] *Schweiz Med Wochenschr Suppl* 1997; **89**: 9S-13S
17 **Katz AJ**, Goldman H, Grand RJ. Gastric mucosal biopsy in eosinophilic (allergic) gastroenteritis. *Gastroenterology* 1977; **73**: 705-709
18 **Sacher P**. [Surgical aspects of eosinophilic gastroenteritis (author's transl)] *Z Kinderchir* 1981; **34**: 25-30
19 **Weller PF**, Bubley GJ. The idiopathic hypereosinophilic syndrome. *Blood* 1994; **83**: 2759-2779
20 **Simon HU**, Plotz SG, Dummer R, Blaser K. Abnormal clones of T cells producing interleukin-5 in idiopathic eosinophilia. *N Engl J Med* 1999; **341**: 1112-1120
21 **Plotz SG**, Simon HU, Darsow U, Simon D, Vassina E, Yousefi S, Hein R, Smith T, Behrendt H, Ring J. Use of an anti-interleukin-5 antibody in the hypereosinophilic syndrome with eosinophilic dermatitis. *N Engl J Med* 2003; **349**: 2334-2339

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REVIEW

Practical guidelines for diagnosis and early management of drug-induced liver injury

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Abstract

The spectrum of drug-induced liver injury (DILI) is both diverse and complex. The first step in diagnosis is a suspicion of DILI based on careful consideration of recent comprehensive reports on the disease. There are some situations in which the suspicion of DILI is particularly strong. Exclusion of other possible etiologies according to the pattern of liver injury is essential for the diagnosis. In patients with suspected DILI, diagnostic scales, such as the Councils for International Organizations of Medical Sciences/ Roussel Uclaf Causality Assessment Method (CIOMS/ RUCAM) scale, may be helpful for the final diagnosis. Early management of DILI involves prompt withdrawal of the drug suspected of being responsible, according to serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin (T-Bil). However, as DILI patients may show resolution of liver injury without discontinuation of the drug, it should be carefully evaluated whether the suspected drug should be discontinued immediately with adequate consideration of the importance of the medication.

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Key words: Diagnosis; Drug-induced liver injury; Guidelines; Management

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INTRODUCTION

Drug-induced liver injury (DILI) is a common liver disease which generally occurs between 5 and 90 d after drug ingestion. The clinical picture of the disease is variable, ranging from transient mild elevation of liver enzymes to fulminant liver failure leading to death. DILI has been reported to be a cause of fulminant liver failure in 13%-30% of cases[1-3]. DILI is divided into three types: hepatocellular, cholestatic, and mixed according to the Councils for International Organizations of Medical Sciences (CIOMS)[4,5]. Hepatocellular type is defined by alanine aminotransferase (ALT) > 2 ULN (upper limits of normal) or R $\geqslant$ 5, where R is the ratio of serum activity of ALT/serum activity of alkaline phosphatase (ALP), both of which are expressed as multiples of the ULN. Liver injury is likely to be more severe in hepatocellular type than in cholestatic/mixed type, and patients with elevated bilirubin levels in hepatocellular liver injury indicating serious liver injury with fatalities, are found at a rate of 0.7 to 1.3/100 000 individuals receiving a given drug[2]. Cholestatic type is defined by ALP > 2 ULN or R $\leqslant$ 2 and mixed type is defined by ALT > 2 ULN and 2 < R < 5. Patients with cholestatic/ mixed type are likely to develop chronic disease more frequently than those with hepatocellular type[6]. For most drugs, the risk of liver injury is estimated to be 1-10/100 000 persons exposed. A recent report indicated that DILI occurs in 1/100 patients hospitalized in internal medicine departments[7]. Thus, DILI is not a rare condition and sometimes leads to serious disease. Rapid and accurate diagnosis of DILI is important in daily practice. However, diagnosis of DILI is not easy and is mainly based on circumstantial evidence. As there is no gold standard for diagnosis, it is essential to exclude other possible etiologies for accurate diagnosis. A number of scoring systems have been proposed,

but even experts may make different judgments using these systems[8]. This review summarizes recent trends regarding DILI and proposes practical guidelines for its diagnosis and early management.

RECENT REPORTS ON DILI

A recent report on DILI based on the database of the World Health Organization (WHO) indicated that the number of cases of DILI has been increasing since the 1990s[9]. The WHO began monitoring adverse drug reactions in 1968, and there are more than 3 million reports on their database (http://www.who-umc.org). This large database is useful for obtaining information on previous reports regarding adverse reactions to drugs. Acetaminophen, drugs against human immunodeficiency virus (HIV), troglitazone, anti-convulsants (such as valproate), analgesics, antibiotics, and anti-cancer drugs are common causative agents of DILI with fatalities (Table 1)[9]. Therefore, particular attention should be paid to patients taking one or more of these drugs who show liver injury. Analysis of 461 cases in Spain over a 10-year period indicated that amoxicillin/clavulanate was the most common drug involved in DILI (59/461 cases, 12.8%)[10]. Moreover, in addition to amoxicillin/clavulanate, they reported that bentazepam, atorvastatin, and captopril were frequent causative drugs leading to chronic liver damage[6]. In a retrospective study in Italy, hydroxymethylglutaryl-CoA reductase inhibitors were the most frequent causative drugs among 1069 cases of DILI (4.5% of cases of adverse drug reactions)[11]. Other studies also showed acetaminophen, anti-retroviral therapy, antibiotics, lipid-lowering drugs, and anti-convulsants to be responsible for DILI[12-18]. In recent analyses in Asia, traditional alternative medicines were reported to be the most common causes of DILI, in contrast to those in Western countries[19]. Table 2 summarizes the drugs suspected to be responsible for DILI and the types of liver injury reported in the literature from various regions[3,6,7,10,12,17-19]. In general, antibiotics, non-steroidal anti-inflammatory drugs, and anti-convulsants are frequent causative drugs of DILI. Importantly, although not shown in Table 2, two or more drugs were suspected to be responsible for DILI in about 10% of cases[10,13]. Furthermore, it is notable that the incidences of DILI caused by herbal remedies or traditional medicines have been increasing over the last decade. The causative drugs for DILI are therefore becoming more diverse and complex. The first and most important step in managing cases of suspected DILI is to gain a detailed understanding of the causative drugs. In the United States of America, the Food and Drug Administration (FDA) records drug toxicity (http://www.fda.gov/medwatch), and the Drug Induced Liver Injury Network was established in 2003 to collect data on DILI in a prospective manner[1]. A similar network is also in place in Spain[6]. A worldwide network which collects all the reports on adverse drug reactions is needed to provide comprehensive information on DILI, which would facilitate accurate diagnosis and early management.

Table 1 Common causative agents of drug-induced liver injury with fatalities fatalities

Drug	*n* (%)
Acetaminophen	305 (16.9)
Anti-HIV[1]	
Stavudine, didanosine, nevirapine, zidovudine	303 (16.8)
Troglitazone	211 (11.7)
Anticonvulsants (valproate, phenytoin)	187 (10.3)
Anti-cancer	223 (12.3)
Flutamide	59 (3.3)
Cyclophosphamide	56 (3.1)
Methotrexate	55 (3.0)
Cytarabine	53 (2.9)
Antibiotics	158 (8.7)
Trovafloxacin	57 (3.2)
Sulfa/trimethoprim	52 (2.9)
Clarithromycin	51 (2.8)
Anesthetic	
Halothane	85 (4.8)
Anti-tuberculosis	
Isoniazid	57 (3.2)
Diclofenac	56 (3.1)
Oxycodone	56 (3.1)

[1]human immunodeficiency virus.

PRACTICAL DIAGNOSIS OF DILI

Situations in which DILI should be suspected

In daily clinical practice, DILI can always be a cause of liver injury in patients taking medications. However, there are some situations in which DILI should be particularly suspected and are as follows[20]: (1) Start of a new drug in the past 3 mo, (2) Presence of rash or eosinophilia, (3) Mixed type (hepatocellular and cholestatic) liver injury, (4) Cholestasis with normal hepatobiliary imaging and (5) Acute or chronic hepatitis without autoantibodies or hypergammaglobulinemia. Although DILI cannot be excluded if patients with any type of liver injury do not meet these criteria, their consideration may lead to early diagnosis of DILI.

Risk factors for DILI

Recognition of risk factors provides clues for the diagnosis of DILI, and some scoring systems include these elements. Host factors which may be associated with DILI are listed in Table 3. Age, gender, pregnancy, and alcohol intake are estimated as risk factors for patients, and liver injury with these risk factors is thought to be related to acute cytolytic hepatitis[21]. In a recent analysis, age was reported to be the most important determinant in biochemical expression of amoxicillin/clavulanate hepatotoxicity, probably because of the slower drug elimination related to advanced age[22]. In contrast, adverse events associated with valproate or erythromycin are more common in childhood[23]. On the other hand, a retrospective study indicated that most patients with drug-induced acute liver failure undergoing

Table 2 Drugs suspected of being responsible for at least two cases of drug-induced liver injury and the types of liver injury reported in recent literature

Use	Drugs	Hepatocellular	Cholestatic	Mixed
Anti-microbial	Amoxicillin-clavulanate	28	26	23
	Azithromycin	0	8	0
	Trovafloxacin	5	0	1
	Erythromycin	2	4	3
	Clindamycin	2	0	0
	Nitrofurantoin	1	1	0
	Levofloxacin	0	0	1
	Ciprofloxacin	2	1	1
	Flucloxacillin	0	7	1
	Sulfasalazine	1	0	1
	INH + RIP + PIZ	24	6	32
	HAART	4	1	1
	Dapsone	2	0	0
Anti-inflammatory	Acetaminophen	40	0	0
	Diclofenac	18	8	3
	Nimesulide	7	2	0
	Ibuprofen	8	3	9
Anti-convulsant	Carbamazepine	6	1	3
	Valproic acid	4	1	3
	Bentazepam	5	0	2
Psychiatric	Paroxetine	4	1	2
	Disulfiram	2	0	0
	Tetrabamate	6	1	0
Anti-cancer	Flutamide	12	1	5
	Methotrexate	3	0	0
Lipid-lowering	Atorvastatin	6	2	2
	Fenofibrate	1	0	2
Gastrointestinal	Ebrotidine	23	0	2
For circulation	Captopril	1	0	1
Anti-coagulant	Ticlopidine	8	5	1
For endocrine	Thiamazole	1	4	0
Immunosuppressant	Azathioprine	5	4	2
Others	Medical herbs	26	3	2
	OTC health supplements	3	0	0

INH: Isoniazid; RIP: Rifampicin; PIZ: Pirazinamide. HAART: Highly active antiretroviral therapy. 40 cases from United States of America between 1998 and 2006; 28 cases from Spain between 1995-2005; 88 cases from Switzerland between 1996 and 2000; 461 cases from Spain between 1994 and 2004; 29 cases from United States of America between 1999 and 2003; 34 cases from France between 1997 and 2000; 77 cases from Sweden between 1995 and 2005; 31 cases from Asia between 2004 and 2006.

liver transplantation were female[24]. Thus, age and female gender may affect the clinical course of DILI. As immune responses to drugs and altered drug metabolism are possible mechanisms in DILI pathogenesis, different immune status or drug metabolism according to age or gender may lead to differences in the occurrence of DILI[25,26]. However, Shapiro and Lewis reported that factors such as age (over 55 years old), gender (female dominant), or the history of alcohol intake were not specific for DILI based on the evaluation of recent DILI cases using the CIOMS/RUCAM scale[27]. Therefore, risk factors for DILI must be analyzed carefully in future. Moreover, genetic factors for drug metabolism, such as polymorphisms of cytochrome P (CYP) 450 or deficiency of N-acetyltransferase, have been reported to contribute to DILI[28,29]. Interestingly, a recent report suggested an association between the daily dose of drug ingested and idiosyncratic DILI, and the number of cases and poor outcome of DILI were reported to increase in a dose-dependent manner[30]. Furthermore, underlying liver disease or systemic viral infection may increase susceptibility to DILI. In particular, DILI caused by anti-tuberculous therapy is known to be increased in patients with hepatitis B or C virus infection[31]. Anti-retroviral therapy in HIV infection was reported to induce severe hepatitis and lead to acute liver failure[32]. The mechanisms by which HIV infection predisposes patients to severe DILI are unknown, but activation or sensitization of the innate immune system by HIV infection may be involved. Moreover, hepatic steatosis in nonalcoholic fatty liver disease (NAFLD) increases the risk of DILI[33]. Mitochondrial dysfunction or the existence of oxidative stress seen in NAFLD may affect the occurrence and severity of DILI.

Clinical diagnosis of DILI

There are few clinical features associated specifically with DILI. Although fever, rash, arthralgia, and eosinophilia are symptoms and signs of an immunoallergic reaction to a drug, they can also be seen without taking any drugs and the frequencies in patients with DILI are not high. General fatigue, appetite loss, and splenomegaly, often seen in patients with viral hepatitis that may be helpful for differential diagnosis at initial presentation, are also

Table 3 Axes and scores of four representative scales utilized for diagnosis of drug-induced liver injury

NADRPS		CIOMS/RUCAM		M&V		DDW-J	
Axis	**Score**	**Axis**	**Score**	**Axis**	**Score**	**Axis**	**Score**
Chronological criteria		Chronological criteria		Chronological criteria		Chronological criteria	
Illegibility in onset	-1 to +2	From drug intake until onset	+1 to +2	From drug intake until onset	+1 to +3	From drug intake until onset	+1 to +2
		From drug withdrawal until onset	0 to +1	From drug withdrawal until onset	-3 to +3	From drug withdrawal until onset	0 to +1
Course of the reaction							
	0 to +1	Course of the reaction	-2 to +3	Course of the reaction	-3 to +3	Course of the reaction	-2 to +3
		Risk factors Age	0 to +1			Risk factors	
		Alcohol (or Pregnancy)[1]	0 to +1			Alcohol (or Pregnancy)[1]	0 to +1
		Concomitant therapy	-3 to 0				
Exclusion of other causes	-1 to +2	Exclusion of other causes	-3 to +2	Exclusion of other causes	-3 to +3	Exclusion of other causes	-3 to +2
		Previous information	0 to +2	Previous information	0 to +2	Previous information	0 to +1
Rechallenge	-1 to +2	Rechallenge	-2 to +3	Rechallenge	0 to +3	Rechallenge	0 to +3
Placebo response	0 to +1						
Drug concentration and monitoring	0 to +1			Extrahepatic manifestations rash, fever, arthralgia, eosinophilia, cytopenia	0 to +3	Extrahepatic manifestations eosinophilia	0 to +1
Dose relationship	0 to +1						
Previous exposure and cross-reactivity	0 to +1						
Any objective evidence	0 to +1					DLST	0 to +2
⩾ 9	Definitive	> 8	Definitive	⩾ 18	Definitive	⩾ 5	Definitive
5 to 8	Probable	6 to 8	Probable	14 to 17	Probable	3 to 4	Probable
1 to 4	Possible	3 to 5	Possible	10 to 13	Possible	⩽ 2	Unlikely
⩽ 0	Unlikely	1 to 2	Unlikely	6 to 9	Unlikely		
		⩽ 0	Excluded	⩽ 5	Excluded		

[1]Cholestatic/Mixed cases; DLST: Drug lymphocyte stimulation test.

not common in non-fulminant DILI. As there are many causes of liver injury, it is essential to exclude other etiologies in the diagnosis of DILI. Other etiologies include viral hepatitis (hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis E virus, EB virus, cytomegalovirus, human herpes virus-6, parvovirus B19, *etc*.), biliary diseases such as cholelithiasis, alcohol abuse, NAFLD, autoimmune liver diseases, and hereditary diseases, such as hemochromatosis, α_1-antitrypsin deficiency, and Wilson's disease. Among these possible causes of liver injury, diagnosis of acute onset autoimmune hepatitis (AIH) is sometimes difficult, because scores using the International Autoimmune Hepatitis Group scoring system for the diagnosis of AIH, serum IgG levels or antinuclear antibody titers are often low in acute AIH. Histological examination of the liver may be useful for the differential diagnosis. Taken together, a low threshold of suspicion, thorough history including recent and past drug exposure, exclusion of other possible etiologies, or occupational hazards with exposure to potential toxins, are important in making an accurate diagnosis of DILI[20,34]. Some clinical scales are available for the diagnosis of DILI. However, it is impractical to apply these diagnostic scales for each patient with liver injury taking medications. In addition, most patients take more than one drug, and identification of a single drug as a causative agent is difficult, even in cases where DILI is strongly suspected, using these scales. Moreover, patients with underlying liver or systemic diseases which also affect liver biochemical tests, complicate the diagnosis of DILI.

Clinical scales available for diagnosis of DILI (Table 3)

As there are no standard criteria for diagnosis of DILI, various clinical scales have been developed. The Naranjo Adverse Drug Reactions Probability Scale (NADRPS) was proposed in 1981 for assessment of adverse drug reactions[35]. NADRPS has been widely used for DILI due to its simplicity and wide applicability, despite not being developed specifically for diagnosis of DILI. Although simplicity is important for practical use, NADRPS has been reported to have low sensitivity and negative predictive values, and to exhibit a limited capability to distinguish among adjacent categories of probability such as "possible" and "probable"[36]. In the early 1990s, the diagnostic scale called the Council for International Organizations of Medical Sciences (CIOMS) or Roussel Uclaf Causality Assessment Method (RUCAM), was proposed at the International Consensus Meeting by Danan and Benichou[4]. It was also called the French method, because Danan had previously reported the diagnostic criteria for acute cytolytic hepatitis in France[21]. CIOMS/RUCAM is applied for classification of the pattern of liver injury, hepatocellular type, cholestatic type, or mixed type, as described above. This scale is determined by a score based on 7 criteria, including temporal relationship, clinical course (response after withdrawal of drug), risk factors, concomitant drugs, exclusion of other non-drug

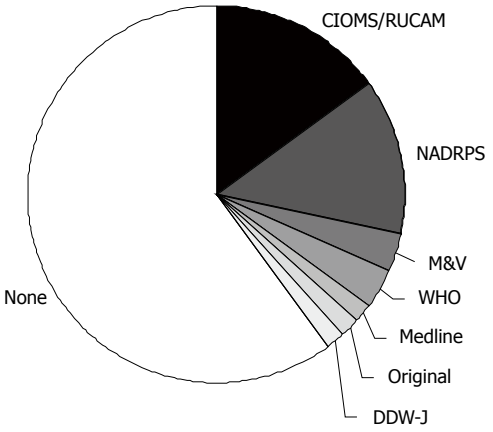

Figure 1 Percentages of methods for causality assessment utilized for diagnosis of DILI, reported during the last decade.

etiologies, likelihood of a reaction based on package labeling, and rechallenge. It has been widely used as a standardized scale with high reliability, reproducibility, and specificity. More recently, Maria and Victorino (M&V) reported a scale called the clinical diagnostic scale (CDS), which simplified the CIOMS/RUCAM using only 5 criteria[37]. It has often been noted that false negative judgments are often made in cholestatic DILI cases because the pattern of liver injury is not taken into consideration in the M&V scale[38]. Moreover, DILI cases with long latency periods and evolution to chronic disease after withdrawal (especially cholestatic type) were poorly diagnosed, and there was no agreement in cases of fulminant hepatitis[39]. The M&V scale emphasizes the immunoallergic reactions, such as extrahepatic manifestations[40]. In Japan, a diagnostic scale was proposed by reference to the CIOMS/RUCAM scale in Digestive Disease Week Japan (DDW-J) 2004, and includes a drug-lymphocyte stimulation test (DLST) as a diagnostic factor[41]. The DDW-J scale was reported to have higher sensitivity than the CIOMS/RUCAM (93.8% *vs* 77.8%, respectively) in the analysis of 127 Japanese patients. However, this scale must be evaluated in non-Japanese patients to verify its universal usefulness.

A review of 61 case reports of DILI in the PubMed database over the last decade regarding diagnostic methods used[42-102] (Figure 1, Table 4) revealed that the CIOMS/RUCAM was the most widely utilized for diagnosis of DILI (10/61 case reports, 16.4%), followed by NADRPS (8/61, 13.1%), M&V (CDS) (2/61, 3.3%), WHO database (2/61, 3.3%), Medline (1/61, 1.6%), Original (1/61, 1.6%), DDW-J (1/61, 1.6%), and none (38/61, 62.3%). The case reports using the WHO database[88,98] or Medline[77] based DILI diagnosis on reports of the suspected drug as a causative drug in the database in addition to circumstantial evidence (history of drug intake, onset of liver injury, and exclusion of other causes). In the case of original criteria[73], DILI diagnosis was made using the following criteria: occurrence of hepatic damage directly related to drug administration, exclusion of other causes of hepatitis, recovery of hepatic function tests after cessation of drug therapy, and liver histology. Although the CIOMS/RUCAM scale is the most widely used and thus currently seems to be the standard method for diagnosis of DILI, it should be emphasized that many physicians still make a diagnosis of DILI based on their own judgment probably because of the complexity of the scoring systems available.

Additional tests to confirm the diagnosis of DILI and identify a single causative drug

As mentioned above, patients are often taking several drugs only one of which is responsible for liver injury in most cases. However, even when clinical scales for DILI strongly suggest a given drug as a cause of liver injury, identification of the single causative drug cannot be established with these scales. Rechallenge with a potential causative drug to establish a diagnosis is one of the diagnostic methods in the CIOMS/RUCAM criteria[4,21]; however, it is not advised and may be contraindicated from an ethical viewpoint. As an alternative way to establish the diagnosis of DILI and the identification of a single causative drug, some additional tests using samples from the patient, such as peripheral blood, could be helpful. One of the most commonly used methods is DLST, which is performed as follows[103]: Lymphocytes collected from the heparinized peripheral blood of patients are incubated with various dilutions of the suspected causative drug. Lymphocyte proliferative response is evaluated by monitoring ^{3}H-thymidine uptake. DLST is widely used in Japan and is incorporated into the diagnostic criteria in Japan (DDW-J scale). However, sensitivity is around 50% and the lymphocyte response to the suspected causative drug may not necessarily be related to liver injury. Another test using peripheral blood of patients is the leukocyte migration test (LMT), which has been reported to be more useful than DLST[104]. This test involves assaying the chemotaxis of granulocytes from one chamber to another chamber containing mononuclear cells, due to the chemotactic factor produced by the mononuclear cells after incubation with the suspected drug solution. Furthermore, Murata *et al*[105] reported a cytokine production test as a method to analyze the immunological pathogenesis of DILI, which also showed high sensitivity for diagnosis. In this analysis, HepG2 cells, which reserve the activities of metabolic enzyme such as CYT450, are first incubated with the suspected drug diluents, and the mixtures of the extract and culture medium of HepG2 are then incubated with peripheral blood lymphocytes isolated from the patients. Intracytoplasmic cytokine profiles of the lymphocytes, such as interferon-γ, tumor necrosis factor-α, or interleukin-2, are finally evaluated by flow cytometry. Although these tests are useful methods for the diagnosis or identification of a single causative drug, they are not simple to perform, and may not be feasible for routine examination. However, if a single causative drug cannot be determined, patients may have to avoid several drugs, mostly non-hepatotoxic drugs, for the

Table 4 Diagnostic methods used for diagnosis of drug-induced liver injury during the last decade

Drug	Type[2]	Criteria	Country	Yr
Acetoaminophen[1]	H	None	Italy	2008
Dexketoprofen trometamol	H	None	Spain	2008
Anabolic-androgenic steroids	C	None	Mexico	2008
Quizalofop-p-ethyl	M	CIOMS/RUCAM	Greece	2007
Amoxicillin/clavulanate	M	None	USA	2007
Fenofibrate	H	None	Poland	2007
INH/RMP/PZA	M	None	USA	2007
Risperidone, Quetiapine	C	NADRPS	USA	2007
Clindamycin	C	NADRPS	Turkey	2007
Bupropion	M	CIOMS/RUCAM, M&V	USA	2007
Flutamide, Cyproterone	H	CIOMS/RUCAM	Spain	2007
Levothyroxine	H	DDW-J	Japan	2007
5-Fluorouracil[1]	H, M	NADRPS	New Zealand	2007
Sairei-to	H	LMT[3]	Japan	2007
Terbinafine	H	NADRPS, CIOMS/RUCAM	USA	2007
Ezetimide	H	None	USA	2007
Terbinafine	M	None	USA	2007
Infliximab[1]	H, C	None	Colombia	2007
Methylenedioxymethamphetamine	M	None	Canada	2006
Methylprednisolone	H	NADRPS	Turkey	2006
Shen-min	H	CIOMS/RUCAM	China	2006
Nimesulide	H	None	Italy	2006
Nevirapine	H	None	France	2006
Sirolimus	H	None	Poland	2005
Amiodarone	H	None	Japan	2005
Proguanil, Chloroquine	M	CIOMS/RUCAM	France	2005
Sulpyrine, Clarithromycin	H	None	Japan	2005
Glimepiride	C	None	Greece	2005
Flucloxacillin	M	None	Australia	2005
Sulbactam/ampicillin	C	NADRPS	Turkey	2004
Hydrochlorothiazide	M	NADRPS	Israel	2004
Ketoconazole	M	Original criteria	Korea	2003
Nimesulide	M	None	Turkey	2003
Ramipril	C	None	Canada	2003
Gemcitabine	M	None	USA	2003
Amoxicillin/clavulanate, Ciprofloxacin	H	Medline	USA	2003
Bupropion, Carbimazole	H	NADRPS	Singapole	2003
Ciprofloxacin	H	CIOMS/RUCAM	Germany	2003
6-Thioguanine	H	None	USA	2003
Terfenadine, Oxatomide	M	None	Japan	2002
Pioglitazone	M	None	USA	2002
Danazol	H	None	Japan	2001
Levofloxacin	H	None	USA	2001
Captopril[1]	M	None	Israel	2001
Pioglitazone	H	None	Japan	2001
Celecoxib	M	None	USA	2001
Nimesulide	M	WHO database	Switzerlamd	2001
Flutamide[1]	H	CIOMS/RUCAM, M&V	Spain	2001
Risperidone	C	None	Germany	2001
Zafirlukast	H	None	USA	2000
Troglitazone	H	None	USA	2000
Stavudine[1]	H	None	USA	2000
Bentazepam[1]	M	None	Spain	2000
Rosiglitazone	H	None	USA	2000
Nitrofurantoin	M	None	Israel	1999
Nimesulide[1]	H, M	CIOMS/RUCAM	Belgium	1998
Omeprazole	H	WHO database	Switzerlamd	1998
Troglitazone	M	None	USA	1998
Acarbose[1]	H	None	Japan	1998
Benzylpenicillin	H	CIOMS/RUCAM	Switzerland	1997
Terbinafine	M	None	France	1997

[1]Cases reported in multiple numbers, not in a single case, are summarized. [2]Type of liver injury. H: hepatocellular; C: cholestatic; M: mixed. [3]LMT, Lymphocyte migration test.

rest of their lives, seriously limiting treatment of other diseases. Therefore, these tests should be considered in selected cases.

Role of histological examination of the liver for the diagnosis of DILI

The features of liver histology in drug-induced hepatitis

are as follows: (1) demarcated perivenular (acinar zone 3) necrosis; (2) minimal hepatitis with canalicular cholestasis; (3) poorly developed portal inflammatory reaction; (4) abundant neutrophils; (5) abundant eosinophils; and (6) epithelioid-cell granulomas[106]. However, liver histology in DILI may not be diagnostic in most cases. Moreover, centrilobular necrosis with minimal portal inflammation is relatively characteristic of DILI, but similar histological features can be seen in acute-onset autoimmune hepatitis. Plasma cell infiltration in portal tracts, which is often prominent in autoimmune hepatitis, may be helpful for differential diagnosis in such cases. The major role of histological examination is therefore to exclude other possible causes of liver injury rather than to make a final diagnosis of DILI. Therefore, it is not recommended as a routine or early examination for the diagnosis of DILI.

EARLY MANAGEMENT FOR DILI

As described above, DILI has a wide spectrum of manifestations, ranging from asymptomatic mild biochemical abnormalities to severe hepatitis with jaundice. In most cases of DILI, liver injury would be expected to improve following discontinuation of the drug suspected to be responsible. On the other hand, some DILI patients may even show resolution of liver injury without discontinuation of the drug. Therefore, it should be carefully evaluated whether the suspected drug should be discontinued with adequate consideration of the importance of the medication. However, once liver injury progresses to acute liver failure, this has a high fatality rate without liver transplantation[107]. Although there are no definitive criteria for cessation of the suspected causative drug, some textbooks suggest that ALT less than 5 × ULN and no symptoms allow continuation of the suspected drug with close observation, whereas ALT of more than 8 × ULN indicates the need to discontinue the suspected drug[108,109]. Another textbook suggests that the suspected drug should be stopped only when abnormalities in serum bilirubin, albumin, or prothrombin time-international normalized ratio (PT-INR) are found in addition to elevated serum ALT[20]. Zimmerman reported that elevation of transaminase activities in combination with jaundice suggests serious liver injury with fatalities. These findings were discussed at the National Institutes of Health in Bethesda, and are recognized as Hy's rule for monitoring DILI, which states that elevation of liver enzymes (AST or ALT more than 3 × ULN or ALP more than 1.5 × ULN) in combination with elevated bilirubin (more than 3 × ULN) at any time after starting a new drug may imply serious liver injury and the suspected drug should be stopped[110]. Two recent studies have shown that hepatocellular liver injury with jaundice is sometimes fatal even if the suspected drug is stopped[9,10]. On the other hand, a recent study showed that cases fulfilling Hy's rule did not always lead to death from DILI[18]. As many drugs can induce asymptomatic elevation of liver enzyme levels without severe hepatotoxicity, mild elevations in transaminases do not always require withdrawal of the causative drug. Based on these observations, the FDA recently proposed draft guidelines (http://www.fda.gov/cder/guidance/7507dft.htm) in which ALT greater than 8 × ULN, ALT greater than 5 × ULN for two weeks, ALT greater than 3 × ULN in association with serum bilirubin greater than 2 × ULN, more than 1.5 × PT-INR, or symptoms of liver injury should be used to predict severe hepatotoxicity and recommend discontinuing the drug[2]. Hepatocellular liver injury with severe jaundice should be treated carefully, and requires prompt referral to a center with hepatologists. As mentioned above, severe liver injury and fatality occur in cases of hepatocellular injury with jaundice. On the other hand, cholestatic DILI cases could be observed with continuation of the suspected causative drug, except if symptoms related to liver injury occur, such as jaundice, elevation of serum bilirubin (more than 3 × ULN), or prolongation of PT-INR (more than 1.5 × ULN). There have been no reports of beneficial therapies except the use of N-acetylcysteine for acetaminophen hepatotoxicity. Corticosteroid therapy may be used in DILI cases with evident hypersensitivity, but it does not have proven benefits[107]. Management of DILI involves prompt withdrawal of the drug suspected to be responsible. A positive de-challenge is a 50% decrease in serum ALT within 8 d of discontinuation of the suspected drug in the hepatocellular type, which is also included in the CIOMS/RUCAM criteria[5,21]. On the other hand, improvement of biliary enzymes after cessation of the suspected drug usually requires a longer period in cholestatic type. However, the time course after cessation of the suspected drug does not always help in early diagnosis and management of DILI, because some patients should be evaluated promptly and managed as suspected DILI on first presentation.

PROPOSAL OF PRACTICAL GUIDELINES FOR DIAGNOSIS AND EARLY TREATMENT OF DILI

Many drugs can cause abnormalities in liver function tests without any symptoms suggestive of liver disease. Preplanned liver function tests should be performed whenever treatment with a new drug is started. In patients with abnormalities in liver function tests without an obvious cause, a careful history, including not only hospital medications but also herbal remedies or supplements, should first be obtained. History taking should also include drug dosage, administration route, previous administration, any concomitant drugs, alcohol consumption, and underlying chronic liver disease and symptoms such as arthralgia. Moreover, family history of adverse drug reactions may be useful for the diagnosis of DILI. On physical examination, patients should be checked for fever, rash, or jaundice. In particular, jaundice should be evaluated carefully,

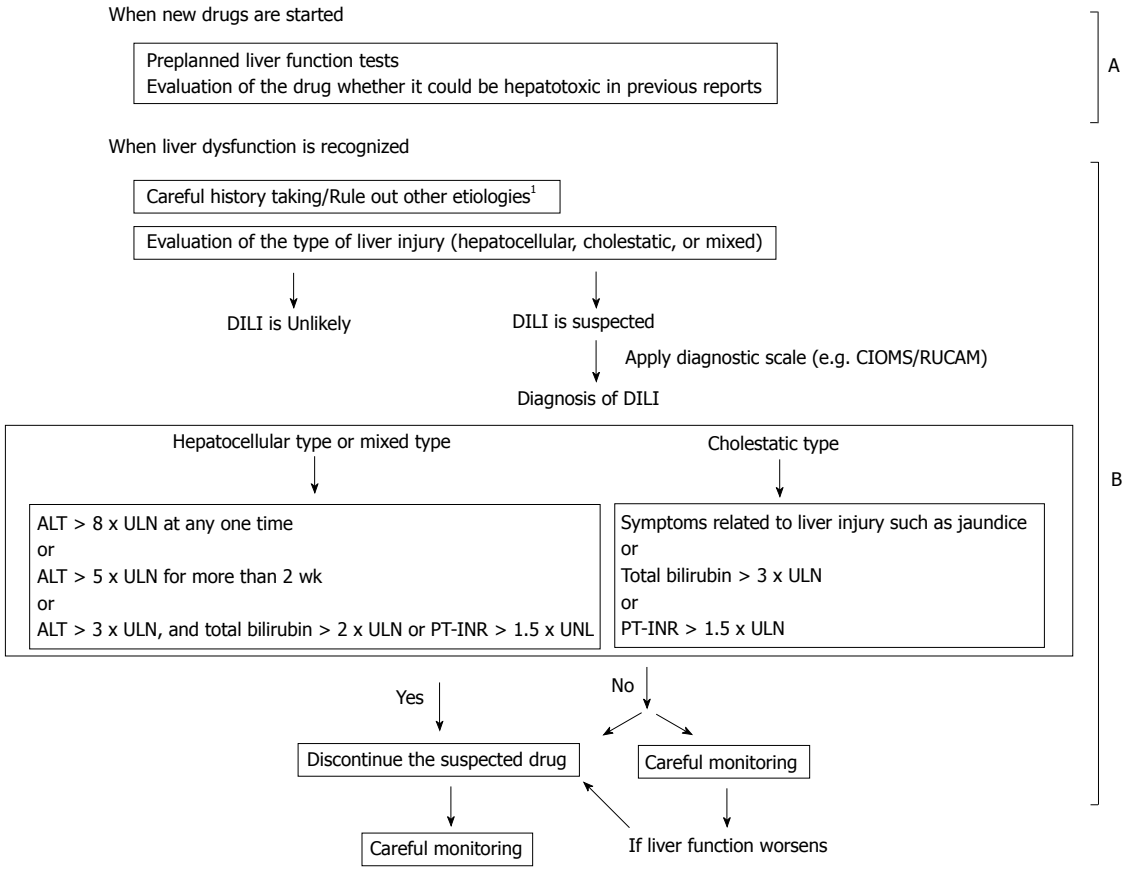

Figure 2 Algorithm for management of DILI. A: When new drugs are started; B: When liver dysfunction is recognized. The type, severity, and causes of liver injury should be assessed promptly. [1]Imaging studies such as ultrasonography should be performed in cases with suspected bile duct disorders.

Table 5 Examinations that should be performed in a patient with suspected DILI

Test	Subjects that can be evaluated
Hematological test[1]	Determination of the type of liver injury (the ratio of ALT and ALP)
Blood count (including eosinophils)	
Biochemical test[1]	
Aspartate aminotransferase (AST)	Possibility (e.g. Increase in eosinophil count, the existence of mixed type liver injury without any biliary disorders on imaging studies, High IgG level (> 2 g/dL) is suspicious of autoimmune hepatitis. Antibodies against hepatitis virus may be false-negative especially in the early phase of infection. Instead, measurement of viral RNA or DNA may be useful for the diagnosis. HDV (requires concomitant HBV infection) and HEV are relatively rare in advanced countries. Although, liver injury caused by EBV or CMV is also relatively rare, young patients with possible DILI should be checked for EBV or CMV).
Alanine aminotransferase (ALT)	
Lactate dehydrogenase	
γ-glutamyl transpeptidase (γ-GTP)	
Alkaline phosphatase (ALP)	
Total bilirubin (including direct and indirect bilirubin)	
Albumin	
Choline esterase (ChE)	
Total cholesterol (Cho)	
Coagulation test[1]	
Prothrombin time international ratio (PT-INR)	
Serological test[1]	
IgG, IgA, IgM	Severity (Marked increase or decrease in white blood cell count, decrease in platelet count. Increase in bilirubin level, decrease in albumin, ChE or Cho levels. Decrease in the ratio of direct/total bilirubin (< 0.67). Prolongation of PT-INR).
Anti-nuclear antibody (ANA)	
Anti-mitochondrial antibody (AMA or M2)	
Viral serology	
IgM anti-HA[1]	
HBsAg[1], IgM-HBc[1], anti-HBc, HBV-DNA	
HCV-Ab[1], HCV-RNA	
HDV-Ab, HDV-DNA	
HEV-Ab, HEV-RNA	
IgM-EBV	
IgM-CMV	
Imaging study	
Ultrasonography (US)[1]	

[1]Tests which should be carried out first. Ig: Immunoglobulin; HA: Hepatitis A; HBsAg: Hepatitis B surface antigen; HBc: Hepatitis B core; HBV: Hepatitis B virus; HCV: Hepatitis C virus; Ab: Antibody; HDV: Hepatitis D virus; HEV: Hepatitis E virus; EBV: Epstein-Barr virus; CMV: Cytomegalovirus.

because it is a sign of severe liver injury indicating the necessity for prompt cessation of the suspected drug. Liver function tests including serum transaminase, ALP, γ-glutamyl transpeptidase, and bilirubin, as well as hematological tests including eosinophil count and coagulation tests should be performed. Classification of the pattern of liver injury should be done as early as possible because clinical course, possible etiologies, and causative drugs are different for each pattern[111]. Other etiologies, such as viral infection, autoimmune liver disease, or biliary disease, should be excluded by serological tests or imaging studies if necessary. DILI cases with severe hepatitis showing elevation of serum bilirubin to more than 3 × ULN may lead to liver failure, and should be treated carefully with referral to the hepatologist after discontinuing all suspected drugs. The list of recommended tests which should be performed in the diagnosis of DILI in patients with liver injury are shown in Table 5. Although accidental readministration of the causative drug may be beneficial for diagnosis of DILI, it may lead to severe liver injury and may even be fatal, and so is not recommended. Moreover, the probability of DILI should also be evaluated using a diagnostic scoring system, such as the CIOMS/RUCAM criteria. However, there is as yet no gold standard set of diagnostic criteria. The initial treatment usually involves withdrawal of the suspected drug. If the causative drug cannot be discontinued because the patient is receiving many drugs or the underlying disease is serious, medications may be continued with careful monitoring. Additional tests, such as the DLST, LMT, or cytokine production test, may be beneficial to identify the causative drug (Figure 2).

CONCLUSION

The spectrum of DILI is both diverse and complex. Although liver injury is often mild and does not require treatment in these patients, DILI may lead to severe hepatitis with a risk of death. Therefore, adequate initial management after achieving an accurate diagnosis is important for physicians. Although the incidence of DILI is reported to be increasing, the precise frequency is difficult to estimate because of the lack of a worldwide monitoring system and the lack of a gold standard for diagnosis. Establishment of a worldwide network for monitoring the adverse events of drugs and a universal diagnostic system for DILI are important for accurate diagnosis, and may lead to better management of DILI.

REFERENCES

1 **Hussaini SH**, Farrington EA. Idiosyncratic drug-induced liver injury: an overview. *Expert Opin Drug Saf* 2007; **6**: 673-684

2 **Norris W**, Paredes AH, Lewis JH. Drug-induced liver injury in 2007. *Curr Opin Gastroenterol* 2008; **24**: 287-297

3 **Carey EJ**, Vargas HE, Douglas DD, Balan V, Byrne TJ, Harrison ME, Rakela J. Inpatient admissions for drug-induced liver injury: results from a single center. *Dig Dis Sci* 2008; **53**: 1977-1982

4 **Danan G**, Benichou C. Causality assessment of adverse reactions to drugs--I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries. *J Clin Epidemiol* 1993; **46**: 1323-1330

5 **Bénichou C**. Criteria of drug-induced liver disorders. Report of an international consensus meeting. *J Hepatol* 1990; **11**: 272-276

6 **Andrade RJ**, Lucena MI, Kaplowitz N, García-Muñoz B, Borraz Y, Pachkoria K, García-Cortés M, Fernández MC, Pelaez G, Rodrigo L, Durán JA, Costa J, Planas R, Barriocanal A, Guarner C, Romero-Gomez M, Muñoz-Yagüe T, Salmerón J, Hidalgo R. Outcome of acute idiosyncratic drug-induced liver injury: Long-term follow-up in a hepatotoxicity registry. *Hepatology* 2006; **44**: 1581-1588

7 **Meier Y**, Cavallaro M, Roos M, Pauli-Magnus C, Folkers G, Meier PJ, Fattinger K. Incidence of drug-induced liver injury in medical inpatients. *Eur J Clin Pharmacol* 2005; **61**: 135-143

8 **Arimone Y**, Miremont-Salamé G, Haramburu F, Molimard M, Moore N, Fourrier-Réglat A, Bégaud B. Inter-expert agreement of seven criteria in causality assessment of adverse drug reactions. *Br J Clin Pharmacol* 2007; **64**: 482-288

9 **Björnsson E**, Olsson R. Suspected drug-induced liver fatalities reported to the WHO database. *Dig Liver Dis* 2006; **38**: 33-38

10 **Andrade RJ**, Lucena MI, Fernández MC, Pelaez G, Pachkoria K, García-Ruiz E, García-Muñoz B, González-Grande R, Pizarro A, Durán JA, Jiménez M, Rodrigo L, Romero-Gomez M, Navarro JM, Planas R, Costa J, Borras A, Soler A, Salmerón J, Martin-Vivaldi R. Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* 2005; **129**: 512-521

11 **Motola D**, Vargiu A, Leone R, Cocci A, Salvo F, Ros B, Meneghelli I, Venegoni M, Cutroneo PM, Vaccheri A, Velo G, Montanaro N. Hepatic adverse drug reactions: a case/non-case study in Italy. *Eur J Clin Pharmacol* 2007; **63**: 73-79

12 **Vuppalanchi R**, Liangpunsakul S, Chalasani N. Etiology of new-onset jaundice: how often is it caused by idiosyncratic drug-induced liver injury in the United States? *Am J Gastroenterol* 2007; **102**: 558-562; quiz 693

13 **Jinjuvadia K**, Kwan W, Fontana RJ. Searching for a needle in a haystack: use of ICD-9-CM codes in drug-induced liver injury. *Am J Gastroenterol* 2007; **102**: 2437-2443

14 **Hussaini SH**, O'Brien CS, Despott EJ, Dalton HR. Antibiotic therapy: a major cause of drug-induced jaundice in southwest England. *Eur J Gastroenterol Hepatol* 2007; **19**: 15-20

15 **Bower WA**, Johns M, Margolis HS, Williams IT, Bell BP. Population-based surveillance for acute liver failure. *Am J Gastroenterol* 2007; **102**: 2459-2463

16 **Akhtar AJ**, Shaheen M. Jaundice in African-American and Hispanic patients with AIDS. *J Natl Med Assoc* 2007; **99**: 1381-1385

17 **Sgro C**, Clinard F, Ouazir K, Chanay H, Allard C, Guilleminet C, Lenoir C, Lemoine A, Hillon P. Incidence of drug-induced hepatic injuries: a French population-based study. *Hepatology* 2002; **36**: 451-455

18 **De Valle MB**, Av Klinteberg V, Alem N, Olsson R, Björnsson E. Drug-induced liver injury in a Swedish University hospital out-patient hepatology clinic. *Aliment Pharmacol Ther* 2006; **24**: 1187-1195

19 **Wai CT**, Tan BH, Chan CL, Sutedja DS, Lee YM, Khor C, Lim SG. Drug-induced liver injury at an Asian center: a prospective study. *Liver Int* 2007; **27**: 465-474

20 **Chitturi S**, Farrell GC. Drug-induced liver disease. In: Schiff ER, Sorrell MF, Maddrey WC. Schiff's diseases of the liver. 9th ed. Tokyo: Lippincott Williams & Wilkins, 2003: 1059–1127

21 **Danan G**. Causality assessment of drug-induced liver injury.

Hepatology Working Group. *J Hepatol* 1988; **7**: 132-136
22 **Lucena MI**, Andrade RJ, Fernández MC, Pachkoria K, Pelaez G, Durán JA, Villar M, Rodrigo L, Romero-Gomez M, Planas R, Barriocanal A, Costa J, Guarner C, Blanco S, Navarro JM, Pons F, Castiella A, Avila S. Determinants of the clinical expression of amoxicillin-clavulanate hepatotoxicity: a prospective series from Spain. *Hepatology* 2006; **44**: 850-856
23 **Maddrey WC**. Drug-induced hepatotoxicity: 2005. *J Clin Gastroenterol* 2005; **39**: S83-S89
24 **Russo MW**, Galanko JA, Shrestha R, Fried MW, Watkins P. Liver transplantation for acute liver failure from drug induced liver injury in the United States. *Liver Transpl* 2004; **10**: 1018-1023
25 **Andrade RJ**, Lucena MI, Alonso A, García-Cortes M, García-Ruiz E, Benitez R, Fernández MC, Pelaez G, Romero M, Corpas R, Durán JA, Jiménez M, Rodrigo L, Nogueras F, Martín-Vivaldi R, Navarro JM, Salmerón J, de la Cuesta FS, Hidalgo R. HLA class II genotype influences the type of liver injury in drug-induced idiosyncratic liver disease. *Hepatology* 2004; **39**: 1603-1612
26 **Chessman D**, Kostenko L, Lethborg T, Purcell AW, Williamson NA, Chen Z, Kjer-Nielsen L, Mifsud NA, Tait BD, Holdsworth R, Almeida CA, Nolan D, Macdonald WA, Archbold JK, Kellerher AD, Marriott D, Mallal S, Bharadwaj M, Rossjohn J, McCluskey J. Human leukocyte antigen class I-restricted activation of CD8+ T cells provides the immunogenetic basis of a systemic drug hypersensitivity. *Immunity* 2008; **28**: 822-832
27 **Shapiro MA**, Lewis JH. Causality assessment of drug-induced hepatotoxicity: promises and pitfalls. *Clin Liver Dis* 2007; **11**: 477-505
28 **Huang YS**, Su WJ, Huang YH, Chen CY, Chang FY, Lin HC, Lee SD. Genetic polymorphisms of manganese superoxide dismutase, NAD(P)H:quinone oxidoreductase, glutathione S-transferase M1 and T1, and the susceptibility to drug-induced liver injury. *J Hepatol* 2007; **47**: 128-134
29 **Huang YS**, Chern HD, Su WJ, Wu JC, Chang SC, Chiang CH, Chang FY, Lee SD. Cytochrome P450 2E1 genotype and the susceptibility to antituberculosis drug-induced hepatitis. *Hepatology* 2003; **37**: 924-930
30 **Lammert C**, Einarsson S, Saha C, Niklasson A, Bjornsson E, Chalasani N. Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: search for signals. *Hepatology* 2008; **47**: 2003-2009
31 **Lee BH**, Koh WJ, Choi MS, Suh GY, Chung MP, Kim H, Kwon OJ. Inactive hepatitis B surface antigen carrier state and hepatotoxicity during antituberculosis chemotherapy. *Chest* 2005; **127**: 1304-1311
32 **Núñez M**. Hepatotoxicity of antiretrovirals: incidence, mechanisms and management. *J Hepatol* 2006; **44**: S132-S139
33 **Tarantino G**, Conca P, Basile V, Gentile A, Capone D, Polichetti G, Leo E. A prospective study of acute drug-induced liver injury in patients suffering from non-alcoholic fatty liver disease. *Hepatol Res* 2007; **37**: 410-415
34 **Norris S**. Drug- and toxin-induced liver disease. In: Bacon BR, O'Grady JG, Kinkhabwala M, Schilsky ML. Comprehensive clinical hepatology. 2nd ed. St. Louis: Mosby, 2006: 497–516
35 **Naranjo CA**, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts EA, Janecek E, Domecq C, Greenblatt DJ. A method for estimating the probability of adverse drug reactions. *Clin Pharmacol Ther* 1981; **30**: 239-245
36 **García-Cortés M**, Lucena MI, Pachkoria K, Borraz Y, Hidalgo R, Andrade RJ. Evaluation of naranjo adverse drug reactions probability scale in causality assessment of drug-induced liver injury. *Aliment Pharmacol Ther* 2008; **27**: 780-789
37 **Maria VA**, Victorino RM. Development and validation of a clinical scale for the diagnosis of drug-induced hepatitis. *Hepatology* 1997; **26**: 664-669
38 **Aithal GP**, Rawlins MD, Day CP. Clinical diagnostic scale: a useful tool in the evaluation of suspected hepatotoxic adverse drug reactions. *J Hepatol* 2000; **33**: 949-952
39 **Lucena MI**, Camargo R, Andrade RJ, Perez-Sanchez CJ, Sanchez De La Cuesta F. Comparison of two clinical scales for causality assessment in hepatotoxicity. *Hepatology* 2001; **33**: 123-130
40 **Kaplowitz N**. Causality assessment versus guilt-by-association in drug hepatotoxicity. *Hepatology* 2001; **33**: 308-310
41 **Watanabe M**, Shibuya A. Validity study of a new diagnostic scale for drug-induced liver injury in Japan-comparison with two previous scales. *Hepatol Res* 2004; **30**: 148-154
42 **Grieco A**, Miele L, Forgione A, Ragazzoni E, Vecchio FM, Gasbarrini G. Mild hepatitis at recommended doses of acetaminophen in patients with evidence of constitutionally enhanced cytochrome P450 system activity. *J Clin Pharm Ther* 2008; **33**: 315-320
43 **Zabala S**, Calpe MJ, Pérez G, Lerín FJ, Mouronval L. Neutropenia, thrombocytopenia and hepatic injury associated with dexketoprofen trometamol therapy in a previously healthy 35-year-old woman. *J Clin Pharm Ther* 2008; **33**: 79-81
44 **Sánchez-Osorio M**, Duarte-Rojo A, Martínez-Benítez B, Torre A, Uribe M. Anabolic-androgenic steroids and liver injury. *Liver Int* 2008; **28**: 278-282
45 **Elefsiniotis IS**, Liatsos GD, Stamelakis D, Moulakakis A. Case report: mixed cholestatic/hepatocellular liver injury induced by the herbicide quizalofop-p-ethyl. *Environ Health Perspect* 2007; **115**: 1479-1481
46 **Jakab SS**, West AB, Meighan DM, Brown RS Jr, Hale WB. Mycophenolate mofetil for drug-induced vanishing bile duct syndrome. *World J Gastroenterol* 2007; **13**: 6087-6089
47 **Kedzia A**, Krysiak R, Madej A, Okopień B. [Is every case of muscle damage during hypolipemic therapy the side effect of this therapy? A case report] *Pol Arch Med Wewn* 2007; **117**: 473-476
48 **Markov M**, Patel K, Raeesy A, Bant A, Van Thiel DH, Nadir A. Liver and pancreatic injury induced by antituberculous therapy. *Dig Dis Sci* 2007; **52**: 3275-3281
49 **Wright TM**, Vandenberg AM. Risperidone- and quetiapine-induced cholestasis. *Ann Pharmacother* 2007; **41**: 1518-1523
50 **Aygun C**, Kocaman O, Gurbuz Y, Senturk O, Hulagu S. Clindamycin-induced acute cholestatic hepatitis. *World J Gastroenterol* 2007; **13**: 5408-5410
51 **Humayun F**, Shehab TM, Tworek JA, Fontana RJ. A fatal case of bupropion (Zyban) hepatotoxicity with autoimmune features: Case report. *J Med Case Reports* 2007; **1**: 88
52 **Miquel M**, Soler A, Vaqué A, Ojanguren I, Costa J, Planas R. Suspected cross-hepatotoxicity of flutamide and cyproterone acetate. *Liver Int* 2007; **27**: 1144-1147
53 **Kawakami T**, Tanaka A, Negoro S, Morisawa Y, Mikami M, Hojo M, Yamamoto T, Uegaki S, Aiso M, Kawasaki T, Ishii T, Kuyama Y, Fukusato T, Takikawa H. Liver injury induced by levothyroxine in a patient with primary hypothyroidism. *Intern Med* 2007; **46**: 1105-1108
54 **Brooks AJ**, Begg EJ, Chapman BA, Fitzharris BM. Two cases of severe liver injury possibly related to 5-fluorouracil and calcium folinate. *Intern Med J* 2007; **37**: 344-345
55 **Aiba T**, Takahashi T, Suzuki K, Okoshi S, Nomoto M, Uno K, Aoyagi Y. Liver injury induced by a Japanese herbal medicine, sairei-to (TJ-114, Bupleurum and Hoelen Combination, Chai-Ling-Tang) R1. *J Gastroenterol Hepatol* 2007; **22**: 762-763
56 **Paredes AH**, Lewis JH. Terbinafine-induced acute autoimmune hepatitis in the setting of hepatitis B virus infection. *Ann Pharmacother* 2007; **41**: 880-884
57 **Liu Q**, Tobias H, Petrovic LM. Drug-induced liver injury associated with ezetimibe therapy. *Dig Dis Sci* 2007; **52**: 602-605
58 **Perveze Z**, Johnson MW, Rubin RA, Sellers M, Zayas C, Jones JL, Cross R, Thomas K, Butler B, Shrestha R. Terbinafine-induced hepatic failure requiring liver

transplantation. *Liver Transpl* 2007; **13**: 162-164
59 **Buyse S**, Vibert E, Sebagh M, Antonini T, Ichai P, Castaing D, Samuel D, Duclos-Vallée JC. Liver transplantation for fulminant hepatitis related to nevirapine therapy. *Liver Transpl* 2006; **12**: 1880-1882
60 **Brncić N**, Kraus I, Visković I, Mijandrusić-Sincić B, Vlahović-Palcevski V. 3,4-methylenedioxymethamphetamine (MDMA): an important cause of acute hepatitis. *Med Sci Monit* 2006; **12**: CS107-CS109
61 **Topal F**, Ozaslan E, Akbulut S, Küçükazman M, Yüksel O, Altiparmak E. Methylprednisolone-induced toxic hepatitis. *Ann Pharmacother* 2006; **40**: 1868-1871
62 **Cárdenas A**, Restrepo JC, Sierra F, Correa G. Acute hepatitis due to shen-min: a herbal product derived from Polygonum multiflorum. *J Clin Gastroenterol* 2006; **40**: 629-632
63 **Gasbarrini A**, Rapaccini GL, Rutella S, Zocco MA, Tittoto P, Leone G, Pola P, Gasbarrini G, Di Campli C. Rescue therapy by portal infusion of autologous stem cells in a case of drug-induced hepatitis. *Dig Liver Dis* 2007; **39**: 878-882
64 **Tobon GJ**, Cañas C, Jaller JJ, Restrepo JC, Anaya JM. Serious liver disease induced by infliximab. *Clin Rheumatol* 2007; **26**: 578-581
65 **Niemczyk M**, Wyzgał J, Perkowska A, Porowski D, Paczek L. Sirolimus-associated hepatotoxicity in the kidney graft recipient. *Transpl Int* 2005; **18**: 1302-1303
66 **Oikawa H**, Maesawa C, Sato R, Oikawa K, Yamada H, Oriso S, Ono S, Yashima-Abo A, Kotani K, Suzuki K, Masuda T. Liver cirrhosis induced by long-term administration of a daily low dose of amiodarone: a case report. *World J Gastroenterol* 2005; **11**: 5394-5397
67 **Wielgo-Polanin R**, Lagarce L, Gautron E, Diquet B, Lainé-Cessac P. Hepatotoxicity associated with the use of a fixed combination of chloroquine and proguanil. *Int J Antimicrob Agents* 2005; **26**: 176-178
68 **Miyakawa R**, Ichida T, Yamagiwa S, Miyaji C, Watanabe H, Sato Y, Yokoyama H, Tsukada C, Ishimoto Y, Sugahara S, Yang XH, Abo T, Asakura H. Hepatic natural killer and natural killer T cells markedly decreased in two cases of drug-induced fulminant hepatic failure rescued by living donor liver transplantation. *J Gastroenterol Hepatol* 2005; **20**: 1126-1130
69 **Chounta A**, Zouridakis S, Ellinas C, Tsiodras S, Zoumpouli C, Kopanakis S, Giamarellou H. Cholestatic liver injury after glimepiride therapy. *J Hepatol* 2005; **42**: 944-946
70 **Dobson JL**, Angus PW, Jones R, Crowley P, Gow PJ. Flucloxacillin-induced aplastic anaemia and liver failure. *Transpl Int* 2005; **18**: 487-489
71 **Köklü S**, Köksal AS, Asil M, Kiyici H, Coban S, Arhan M. Probable sulbactam/ampicillin-associated prolonged cholestasis. *Ann Pharmacother* 2004; **38**: 2055-2058
72 **Arinzon Z**, Alexander P, Berner Y. Hydrochlorothiazide induced hepato-cholestatic liver injury. *Age Ageing* 2004; **33**: 509-510
73 **Kim TH**, Kim BH, Kim YW, Yang DM, Han YS, Dong SH, Kim HJ, Chang YW, Lee JI, Chang R. Liver cirrhosis developed after ketoconazole-induced acute hepatic injury. *J Gastroenterol Hepatol* 2003; **18**: 1426-1429
74 **Ozgür O**, Hacihasanoğlu A, Karti SS, Ovali E. Nimesulide-induced fulminant hepatitis. *Turk J Gastroenterol* 2003; **14**: 208-210
75 **Yeung E**, Wong FS, Wanless IR, Shiota K, Guindi M, Joshi S, Gardiner G. Ramipril-associated hepatotoxicity. *Arch Pathol Lab Med* 2003; **127**: 1493-1497
76 **Robinson K**, Lambiase L, Li J, Monteiro C, Schiff M. Fatal cholestatic liver failure associated with gemcitabine therapy. *Dig Dis Sci* 2003; **48**: 1804-1808
77 **Zaidi SA**. Hepatitis associated with amoxicillin/clavulanic acid and/or ciprofloxacin. *Am J Med Sci* 2003; **325**: 31-33
78 **Khoo AL**, Tham LS, Lee KH, Lim GK. Acute liver failure with concurrent bupropion and carbimazole therapy. *Ann Pharmacother* 2003; **37**: 220-223
79 **Goetz M**, Galle PR, Schwarting A. Non-fatal acute liver injury possibly related to high-dose ciprofloxacin. *Eur J Clin Microbiol Infect Dis* 2003; **22**: 294-296
80 **Rulyak SJ**, Saunders MD, Lee SD. Hepatotoxicity associated with 6-thioguanine therapy for Crohn's disease. *J Clin Gastroenterol* 2003; **36**: 234-237
81 **Suyama T**, Fujiwara H, Takenouchi K, Ito M. Drug eruption and liver injury caused by terfenadine and oxatomide. *Eur J Dermatol* 2002; **12**: 385-386
82 **Pinto AG**, Cummings OW, Chalasani N. Severe but reversible cholestatic liver injury after pioglitazone therapy. *Ann Intern Med* 2002; **137**: 857
83 **Hayashi T**, Takahashi T, Minami T, Akaike J, Kasahara K, Adachi M, Hinoda Y, Takahashi S, Hirayama T, Imai K. Fatal acute hepatic failure induced by danazol in a patient with endometriosis and aplastic anemia. *J Gastroenterol* 2001; **36**: 783-786
84 **Karim A**, Ahmed S, Rossoff LJ, Siddiqui RK, Steinberg HN. Possible levofloxacin-induced acute hepatocellular injury in a patient with chronic obstructive lung disease. *Clin Infect Dis* 2001; **33**: 2088-2090
85 **Schattner A**, Kozak N, Friedman J. Captopril-induced jaundice: report of 2 cases and a review of 13 additional reports in the literature. *Am J Med Sci* 2001; **322**: 236-240
86 **Maeda K**. Hepatocellular injury in a patient receiving pioglitazone. *Ann Intern Med* 2001; **135**: 306
87 **Nachimuthu S**, Volfinzon L, Gopal L. Acute hepatocellular and cholestatic injury in a patient taking celecoxib. *Postgrad Med J* 2001; **77**: 548-550
88 **Merlani G**, Fox M, Oehen HP, Cathomas G, Renner EL, Fattinger K, Schneemann M, Kullak-Ublick GA. Fatal hepatoxicity secondary to nimesulide. *Eur J Clin Pharmacol* 2001; **57**: 321-326
89 **García Cortés M**, Andrade RJ, Lucena MI, Sánchez Martínez H, Fernández MC, Ferrer T, Martín-Vivaldi R, Peláez G, Suárez F, Romero-Gómez M, Montero JL, Fraga E, Camargo R, Alcántara R, Pizarro MA, García-Ruiz E, Rosemary-Gómez M. Flutamide-induced hepatotoxicity: report of a case series. *Rev Esp Enferm Dig* 2001; **93**: 423-432
90 **Krebs S**, Dormann H, Muth-Selbach U, Hahn EG, Brune K, Schneider HT. Risperidone-induced cholestatic hepatitis. *Eur J Gastroenterol Hepatol* 2001; **13**: 67-69
91 **Reinus JF**, Persky S, Burkiewicz JS, Quan D, Bass NM, Davern TJ. Severe liver injury after treatment with the leukotriene receptor antagonist zafirlukast. *Ann Intern Med* 2000; **133**: 964-968
92 **Li H**, Heller DS, Leevy CB, Zierer KG, Klein KM. Troglitazone-induced fulminant hepatitis: report of a case with autopsy findings. *J Diabetes Complications* 2000; **14**: 175-177
93 **Miller KD**, Cameron M, Wood LV, Dalakas MC, Kovacs JA. Lactic acidosis and hepatic steatosis associated with use of stavudine: report of four cases. *Ann Intern Med* 2000; **133**: 192-196
94 **Andrade RJ**, Lucena MI, Aguilar J, Lazo MD, Camargo R, Moreno P, García-Escaño MD, Marquez A, Alcántara R, Alcáin G. Chronic liver injury related to use of bentazepam: an unusual instance of benzodiazepine hepatotoxicity. *Dig Dis Sci* 2000; **45**: 1400-1404
95 **Al-Salman J**, Arjomand H, Kemp DG, Mittal M. Hepatocellular injury in a patient receiving rosiglitazone. A case report. *Ann Intern Med* 2000; **132**: 121-124
96 **Schattner A**, Von der Walde J, Kozak N, Sokolovskaya N, Knobler H. Nitrofurantoin-induced immune-mediated lung and liver disease. *Am J Med Sci* 1999; **317**: 336-340
97 **Van Steenbergen W**, Peeters P, De Bondt J, Staessen D, Büscher H, Laporta T, Roskams T, Desmet V. Nimesulide-induced acute hepatitis: evidence from six cases. *J Hepatol* 1998; **29**: 135-141
98 **Christe C**, Stoller R, Vogt N. Omeprazole-induced hepatotoxicity? A case report. *Pharmacoepidemiol Drug Saf* 1998; **7** Suppl 1: S41-S44
99 **Neuschwander-Tetri BA**, Isley WL, Oki JC, Ramrakhiani S,

Quiason SG, Phillips NJ, Brunt EM. Troglitazone-induced hepatic failure leading to liver transplantation. A case report. *Ann Intern Med* 1998; **129**: 38-41
100 **Fujimoto Y**, Ohhira M, Miyokawa N, Kitamori S, Kohgo Y. Acarbose-induced hepatic injury. *Lancet* 1998; **351**: 340
101 **Bauer TM**, Bircher AJ. Drug-induced hepatocellular liver injury due to benzylpenicillin with evidence of lymphocyte sensitization. *J Hepatol* 1997; **26**: 429-432
102 **Mallat A**, Zafrani ES, Metreau JM, Dhumeaux D. Terbinafine-induced prolonged cholestasis with reduction of interlobular bile ducts. *Dig Dis Sci* 1997; **42**: 1486-1488
103 **Takikawa H**, Takamori Y, Kumagi T, Onji M, Watanabe M, Shibuya A, Hisamochi A, Kumashiro R, Ito T, Mitsumoto Y, Nakamura A, Sakaguchi T. Assessment of 287 Japanese cases of drug induced liver injury by the diagnostic scale of the International Consensus Meeting. *Hepatol Res* 2003; **27**: 192-195
104 **Usui K**, Oda Y, Kubota R, Negishi K, Uno K, Tsunematsu S, Kumagai N, Komiyama T. Clinical application of the leukocyte migration test and new diagnostic criteria for identifying causative agents in patients with drug-induced liver injury. *Hepatogastroenterology* 2007; **54**: 1752-1757
105 **Murata H**, Shimizu Y, Okada K, Higuchi K, Watanabe A. Detection and analysis of intracytoplasmic cytokines in peripheral blood mononuclear cells in patients with drug-induced liver injury. *J Hepatol* 2003; **38**: 573-582
106 **Scheuer PJ**, Lefkowitch JH. Drug and toxins. In: Scheuer PJ, Lefkowitch JH. Liver biopsy interpretation. 7th ed. Philadelphia: Elsevier Saunders, 2006: 125-144
107 **Lee WM**. Drug-induced hepatotoxicity. *N Engl J Med* 1995; **333**: 1118-1127
108 **Kaplowitz N**, DeLeve LD. Drug-induced liver disease, 1st ed. New York: Informa Healthcare, 2003: 227-242
109 **Zimmerman HJ**. Hepatotoxicity: The adverse effects of drugs and other chemicals on the liver. 2nd ed. Philadelphia: Lippincott Williams & Wilkins, 1999: 428-433
110 **Vuppalanchi R**, Teal E, Chalasani N. Patients with elevated baseline liver enzymes do not have higher frequency of hepatotoxicity from lovastatin than those with normal baseline liver enzymes. *Am J Med Sci* 2005; **329**: 62-65
111 **Bleibel W**, Kim S, D'Silva K, Lemmer ER. Drug-induced liver injury: review article. *Dig Dis Sci* 2007; **52**: 2463-2471

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TOPIC HIGHLIGHT

Carlos J Pirola, PhD, FAHA, *Series Editor*

Dynamic localization of hepatocellular transporters in health and disease

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Abstract

Vesicle-based trafficking of hepatocellular transporters involves delivery of the newly-synthesized carriers from the rough endoplasmic reticulum to either the plasma membrane domain or to an endosomal, submembrane compartment, followed by exocytic targeting to the plasma membrane. Once delivered to the plasma membrane, the transporters usually undergo recycling between the plasma membrane and the endosomal compartment, which usually serves as a reservoir of pre-existing transporters available on demand. The balance between exocytic targeting and endocytic internalization from/to this recycling compartment is therefore a chief determinant of the overall capability of the liver epithelium to secrete bile and to detoxify endo and xenobiotics. Hence, it is a highly regulated process. Impaired regulation of this balance may lead to abnormal localization of these transporters, which results in bile secretory failure due to endocytic internalization of key transporters involved in bile formation. This occurs in several experimental models of hepatocellular cholestasis, and in most human cholestatic liver diseases. This review describes the molecular bases involved in the biology of the dynamic localization of hepatocellular transporters and its regulation, with a focus on the involvement of signaling pathways in this process. Their alterations in different experimental models of cholestasis and in human cholestatic liver disease are reviewed. In addition, the causes explaining the pathological condition (e.g. disorganization of actin or actin-transporter linkers) and the mediators involved (e.g. activation of cholestatic signaling transduction pathways) are also discussed. Finally, several experimental therapeutic approaches based upon the administration of compounds known to stimulate exocytic insertion of canalicular transporters (e.g. cAMP, tauroursodeoxycholate) are described.

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Key words: Hepatocellular transporters; Cholestasis; cAMP; Bile salts; Vesicular trafficking; Endocytosis; Signaling pathways

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INTRODUCTION

Bile secretion is a highly-regulated process. Such regulation is aimed at coping with the physiological demand for hepatocellular transport of endo- and xenobiotics. This is achieved by modulation of the constitutive expression, dynamic localization or intrinsic activity of relevant transport systems located at the sinusoidal (basolateral) and canalicular (apical) membranes of the hepatocyte.

Modulation of carrier transport activity may occur at different time scales. Long-term regulations occur by changes in carrier turnover, which leads to modification of the synthesis-degradation balance. Altered synthesis rate involves transcriptional or translational changes in carrier expression. On the other hand, modification of the carrier degradation rate is a post-translational

process. This latter event may involve, as an initiating step, sustained internalization of the carrier protein from its plasma membrane domain, followed by lysosomal breakdown.

In contrast to this irreversible fate, transitory, reversible changes in transporter localization by vesicle-mediated insertion/internalization from/to an endosomal recycling compartment may occur as part of a short-term, physiological mechanism aimed at quickly modulating carrier density at the plasma membrane. This is a tightly regulated process, and the signaling mediators involved are being actively characterized.

Apart from its role in biliary physiology, changes in the proper localization of hepatocellular carriers also occur in a number of pathological conditions, and they may partly explain the cholestatic manifestations in these liver diseases. This has encouraged investigators to better understand the mechanisms involved in this particular pathomechanism at a molecular level, and to envisage and test in experimental models of cholestasis new therapeutic approaches based upon its prevention.

This article aims to give an overview of this subject, by summarizing the current information available in the literature on physiological regulation and cholestatic changes in hepatocellular carrier dynamic localization, as well as its beneficial modulation by therapeutic agents.

HEPATOCELLULAR TRANSPORT SYSTEMS

The hepatocyte is a polarized cell that expresses differential transport systems in its plasma membrane domains. These transporters play a key role in the vectorial transfer of solutes and water from sinusoidal blood into bile, thus contributing to bile formation and the biliary excretion of many xenobiotics. Most of these transport proteins have been identified by molecular cloning, and their transport properties characterized by functional studies. Their localization and transport function are shown in Figure 1.

Sinusoidal solute uptake transporters

Liver sinusoids possess a specific architecture that allows passage of organic compounds bound to albumin through endothelial fenestrae into the space of Disse, from where they can be taken up by the sinusoidal transport systems of the hepatocytes[1].

Basolateral uptake transporters can be divided into Na^+-dependent and Na^+-independent systems. Na^+-dependent uptake involves co-transport of solutes with Na^+, and is driven by the electrochemical Na^+ gradient generated and maintained by the Na^+/K^+-ATPase, which is strategically localized at the sinusoidal membrane. The Na^+-independent transport of organic anions is driven primarily by anion exchange.

Bile salts are the predominant organic solutes in bile, and the main determinants of bile flow[2]. Bile salts are mainly taken up by the Na^+/taurocholate co-transporting polypeptide (NTCP/Ntcp for humans and rodents, respectively; also known as SLC10A1/Slc10a1)[3]. A remaining fraction is taken up by a Na^+-independent transport system mediated by the organic anion-transporting polypeptide (OATP/Oatp) family of transporters[4,5]. In addition to conjugated and unconjugated bile salts, Oatps/OATPs accept other cholephilic compounds, including glucuronidated (and maybe unconjugated) bilirubin, exogenous organic anions (e.g. sulphobromophthalein), leukotrienes, estrogen-conjugates (e.g. estrone-3-sulfate or estradiol-17-β-d-glucuronide), thyroid hormones, mycotoxins, and numerous xenobiotics[3,6-8]. Four OATPs have been cloned and characterized from human liver, namely: OATP1A2 (SLCO1A2/SLC21A3; formerly, OATP-A), OATP1B1 (SLC21A6; formerly, OATP-C or LST-1), OATP1B3 (SLC21A8; formerly, OATP-8) and OATP2B1 (SLC21A9; formerly, OATP-B). There are three Oatps identified in rats, namely: Oatp1a1 (*Slc21a1*; formerly, Oatp1), Oatp1a4 (*Slc21a5*; formerly, Oatp2) and Oatp1b2 (*Slc21a10*; formerly, Oatp4 or Lst-1). Oatp1b2 is the rodent ortholog of both OATP1B1 and OATP1B3[9].

Hepatocellular uptake of organic cations is mediated by two separate transport systems, which depends on the substrate molecular size[10]. Thus, small (type Ⅰ) organic cations are taken up by the organic cation transporter, OCT1/Oct1 (SLC22A1/Slc22a1), which is electrogenic in nature. On the other hand, human OATP-A (but not the remaining members of the OATP family) and rat Oatp2 mediate the uptake of bulky (type Ⅱ) organic cations.

Canalicular solute export transporters

After traversing the cell by Fick's diffusion, mostly bound to high-affinity cytosolic proteins, cholephilic compounds are excreted into bile mainly by ATP-dependent pumps of the superfamily of ATP-binding cassette (ABC) transporters, in particular those belonging to the family of multidrug-resistance proteins, MDR/Mdr, or to the family of multidrug-resistance-associated proteins, MRP/Mrp.

MDRs/Mdrs are members of the ABC superfamily that were originally described in cancer cell lines, where they confer resistance to therapeutic agents. Three gene products were identified in rodents, Mdr1a (Abcb1a), Mdr1b (Abcb1b) and Mdr2 (Abcb4), and two in humans, MDR1 (ABCB1) and MDR 3 (ABCB4). MDR1/Mdr1 functions as an efflux pump for a wide range of amphiphilic, bulky type Ⅱ cationic drugs, together with other hydrophobic compounds, such as endogenous and exogenous metabolites or toxins, steroid hormones, hydrophobic peptides and even glycolipids[8]. Two closely related but functionally distinct Mdr1 isoforms, mdr1a and mdr1b are present in the murine but not in the human phenotype[11]. MDR3/Mdr2 functions as a flippase, which translocates phosphatidylcholine (PC) from the inner to the outer leaflet of the canalicular membrane, followed by release of PC-containing vesicles from the outer leaflet into bile, a process facilitated by the detergent properties of luminal bile salts[12].

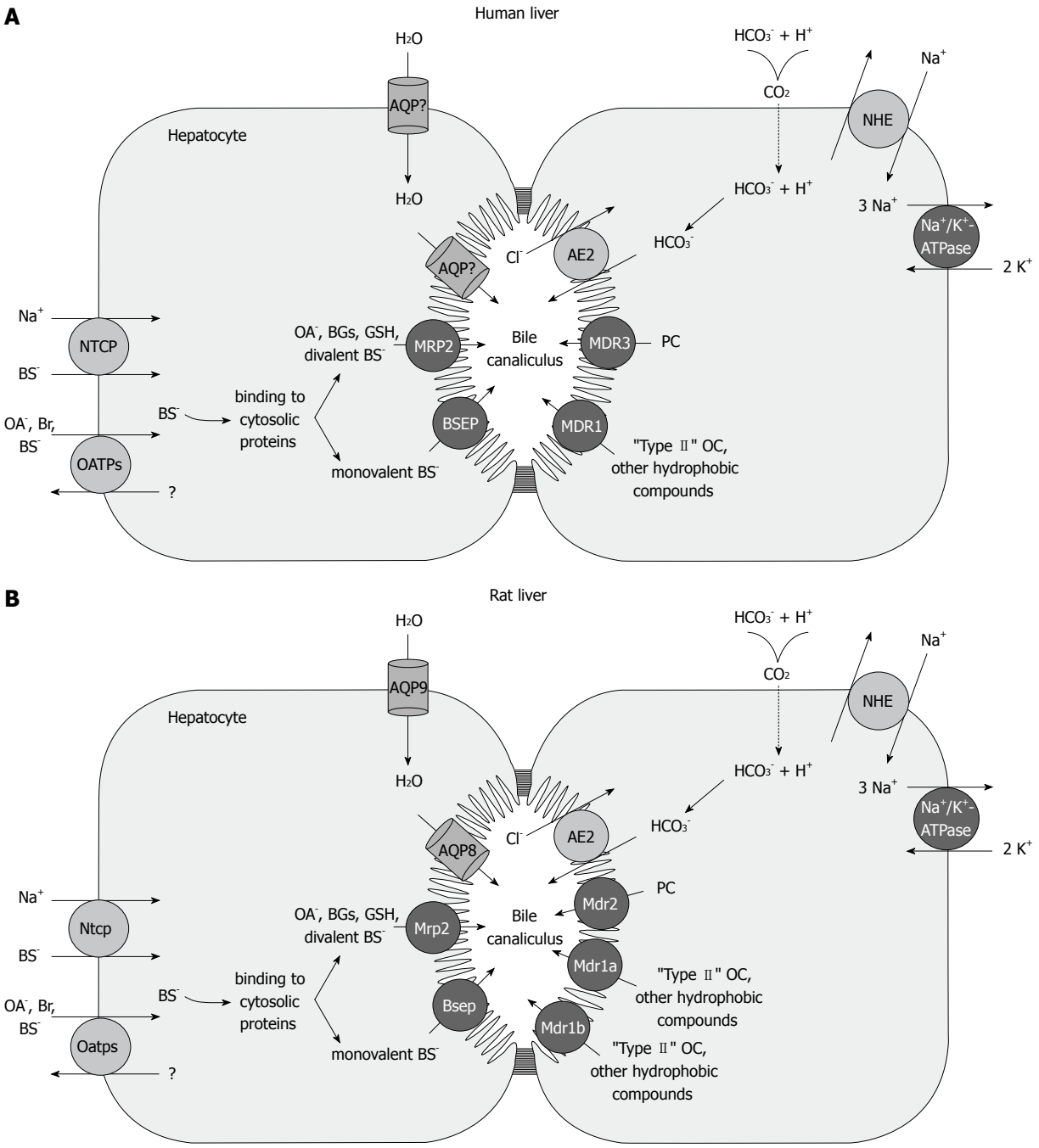

Figure 1 Localization and function of sinusoidal and canalicular hepatocellular transporters. A: humans; B: rodents. The Na^+-dependent sinusoidal uptake of bile salts is mediated by NTCP (human)/Ntcp (rat). The Na^+-independent hepatic uptake of organic anions (OA^-), Bile salts and type Ⅱ organic cations (OC^+) is mediated by members of the OATP/Oatp family. Sinusoidal uptake of type Ⅰ OC^+ is mediated by OCT1/Oct1. Transport across the canalicular membrane is driven mainly by ATP-dependent export pumps (black circles). MDR1/Mdr1a, Mdr1b mediates canalicular excretion of amphiphilic type Ⅱ OC^+ and other hydrophobic compounds. MDR3/Mdr2 functions as a phosphatidylcholine (PC) flippase. BSEP/Bsep mediates apical excretion of BSs. MRP2/Mrp2 transports non-bile-salt organic anions, such as bilirubin glucuronides, GSH, and sulfated/glucuronidated bile salts. Canalicular transport of HCO_3^- is mediated by the Cl^-/HCO_3^- exchanger AE2/Ae2. Aquaporins AQP9 and AQP8 are involved in the transport of water across the rat sinusoidal and the canalicular membrane, respectively. The nature of the water channels in human liver has yet to be characterized.

Monoanionic bile salts are excreted in the canalicular pole by the *bile salt export pump* (BSEP/Bsep; ABCB11/abcb11), another member of the MDR family[13]. In contrast, canalicular efflux of divalent, bipolar sulfated or glucuronidated bile salts is mediated by the multidrug-resistance-associated protein 2 (MRP2/Mrp2; ABCC2/Abcc2)[4,14]. This carrier is also engaged in the biliary excretion of many other organic anions, including glutathione S-conjugates (e.g. of leukotriene C4 or sulphobromophthalein, among others), glucuronides (e.g. of bilirubin and estrogens), and reduced (GSH) and oxidized glutathione (GSSG), the former with low affinity[15,16]. Both GSSG and GSH are major determinants of the so-called "canalicular bile-salt-independent bile flow"[17].

The canalicular membrane domain also contains the electroneutral anion exchanger 2 (AE2/Ae2; SLC4A2/slc4a2), which extrudes HCO_3^- by exchanging the anion

for biliary Cl^-[18]. It functions to regulate intracellular pH when hepatocytes are exposed to an alkaline load[18]. In addition, AE2/Ae2 plays a role in bile flow generation, since HCO_3^- excretion is thought to be an additional primary driving force of the canalicular bile-salt-independent bile flow[18,19]. Both in humans and rats, three transcript variants of AE2/Ae2 have been described, namely the full-length transcript AE2a/Ae2a, expressed from the upstream promoter in most tissues, and the alternative transcripts AE2b1/Ae2b1 and AE2b2/Ae2b2, expressed in a more tissue-restricted fashion (mainly in liver and kidney). AE2b1/2/Ae2b1/2 transcription is driven from overlapping promoter sequences within intron 2, which result in AE2/Ae2 protein isoforms with short N-terminal differences[20,21].

Water transporters

For a solute to drive blood-to-bile vectorial water transport primarily, resultant osmotic forces need to be associated with aquaporin (AQP)-mediated transcellular movement of water molecules from plasma to the bile canaliculus. Both immunochemical and functional studies have demonstrated the constitutive expression of the water channel AQP9 at the basolateral membrane of rat hepatocytes, and the regulated expression of the water channel AQP8 at the hepatocellular canalicular membrane domain[22-24]. As a result of it being inserted in the canalicular membrane on demand, AQP8 is suggested to play a role in bile formation, facilitating the osmotic movement of water under a choleretic stimulus[23,24]. AQP isoforms that mediate polarized water transport in human hepatocytes, if any, remain to be identified.

MECHANISMS OF NORMAL TRAFFICKING OF HEPATOCELLULAR TRANSPORTERS AND ITS REGULATION BY SIGNALING PATHWAYS

Basolateral transporters

NTCP/Ntcp: Basolateral targeting of NTCP is mediated by a sorting pathway that involves translocation of the protein from the endoplasmic reticulum (ER) to the Golgi apparatus, and from there to the plasma membrane, by a trans-Golgi-network-independent pathway[25]. The process may also involve microtubular and microfilamental motor proteins. A role for the cytoskeleton in NTCP translocation has been studied in detail using green fluorescent protein (GFP)-tagged NTCP expressed in the HepG2 cell line[26]. This study showed that targeting of NTCP to the plasma membrane consists of two steps: (1) delivery of NTCP to the region of the plasma membrane *via* microtubules, and (2) insertion of NTCP into the plasma membrane, by a microfilament-mediated mechanism; this actin requirement was also observed in isolated rat hepatocytes[27]. The latter step more likely involves targeting of NTCP from an early (recycling) endosomal compartment[28]. These NTCP/Ntcp-containing vesicles also express the microtubule-based motor proteins dynein and kinesin, and the actin-based motor myosin Ⅱa[28]. This compartment may serve as a reservoir of transporters for their rapid insertion into the sinusoidal membrane under a physiological stimulus that requires their function. It is therefore not surprising that recycling of NTCP/Ntcp from this compartment is a highly regulated process.

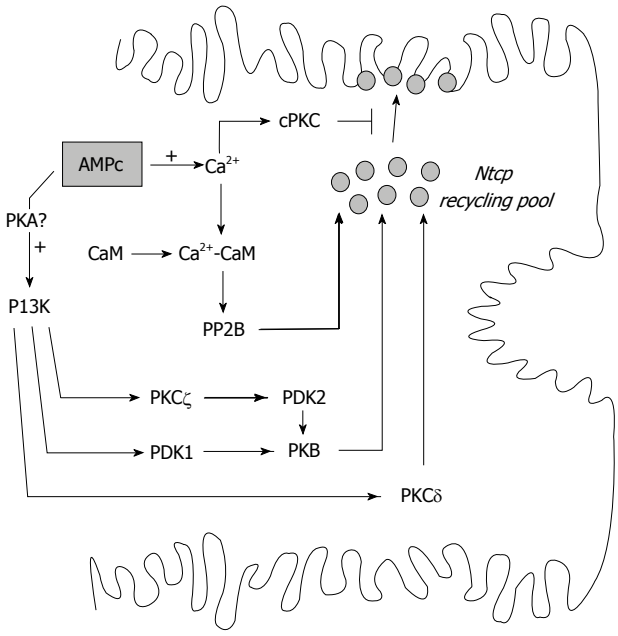

Figure 2 Signaling pathways that regulate the cAMP-induced exocytic insertion of Ntcp into the basolateral membrane. cAMP stimulatory effect involves elevations in cytosolic Ca^{2+} and activation of PI3K-dependent pathway, probably *via* protein kinase A (PKA). CaM complex activates phosphatase 2B (PP2B), which promotes insertion of Ntcp by dephosphorylation. This pathway is counter-regulated by cPKC. cAMP also stimulates Ntcp targeting by PI3K-dependent activation of PDK1 and subsequent PKB activation. Alternatively, PKB is activated by the concerted action of the atypical PKCζ and PDK2. Finally, cAMP/PI3K signaling stimulatory pathway may involve PKCδ.

The cAMP-elevating hormone glucagon and the permeant cAMP analog dibutyryl cAMP stimulate hepatocyte Ntcp maximal transport in rats by insertional exocytosis from intracellular vesicles that contain the transporter[29]. The signaling pathways evoked by cAMP that account for this stimulatory effect are depicted in Figure 2. Protein kinase A (PKA) activation[30], phosphatidylinositol 3-kinase (PI3K) activation[27,31] and elevations of cytosolic Ca^{2+}[30] all mediate the cAMP effect. Although the mechanism of PI3K activation by cAMP has not been elucidated as yet, there is evidence in other cell lines that the cAMP-dependent PKA can activate PI3K by phosphorylation of the PI3K regulatory subunit, p85[32]; if this applies to hepatocytes, this would explain the dual mediation of PKA and PI3K in the cAMP-stimulatory effect. The downstream mediators of the cAMP-PI3K signaling pathway are under debate, and may be multifactorial. The PI3K downstream enzyme, protein kinase B (PKB, also known as Akt), has been implicated[27,31]. Coincidently, hepatocellular swelling, which also evokes the PI3K/

PKB signaling pathway, favors Ntcp translocation to the plasma membrane as well[31,33]. The effect of PI3K/PKB on Ntcp translocation seems to be mediated by the PI3K-dependent activation of atypical protein kinase C zeta (PKCζ)[34]. PKCζ is downstream of PI3K, since PI3K products activate this PKC isoform[35,36]. The requirement of PKCζ for the PKB effect can be explained by PKCζ modulation of activators upstream of PKB. Activation of PKB requires phosphorylation by 3-phosphoinositide phosphate-dependent kinase 1 (PDK1), followed by phosphorylation by a second kinase, PDK2; this latter kinase phosphorylates and activates PKB fully only when associated with PKCζ[36,37]. In addition, a direct, non-PKB-mediated stimulatory role for PKCζ on Ntcp translocation has been suggested[34]. Apart from PKCζ, cAMP-stimulated PI3K phosphorylates the novel protein kinase C delta (PKCδ) at Thr-505, and the resulting activation seems to be involved in Ntcp membrane translocation as well[38]. The molecular target/s phosphorylated by PKB, PKCζ and PKCδ that ultimately account for the translocation of Ntcp are unknown. Ntcp itself seems not to be a target, since cAMP may promote dephosphorylation rather than phosphorylation of the carrier[39-41]. However, studies in transfected COS-7 and Madin-Darby canine kidney (MDCK) cells using GFP-fused Ntcp constructs that lack the cytoplasmic Ntcp tail, which serves as a signal for basolateral sorting, have demonstrated that this moiety has regulatory phosphorylation sites that are essential for cAMP-induced stimulation of Ntcp translocation[42]. The relevance of this finding needs to be tested in a more physiological context. Other possible phosphorylation targets, at least of PKCζ, are the microtubule motors that drive movement of Ntcp-containing vesicles. A majority (75%) of intracellular vesicles containing Ntcp were found to co-localize with PKCζ in rat hepatocytes, and the motility of these vesicles on microtubules, when assessed using an *in vitro* motility assay, was impaired by both PI3K and PKCζ inhibitors, and stimulated by PI3K products[28].

Apart from activating PKA and PI3K, cAMP induces elevations of cytosolic Ca^{2+} in hepatocytes[43,44]. The subsequent formation of the Ca^{2+}-calmodulin (CaM) complex influences Ntcp localization by activating the Ca^{2+}/CaM-dependent serine–threonine phosphatase PP2B (also known as calcineurin)[39]. cAMP promotes both serine and threonine dephosphorylation of Ntcp *via* PP2B[39-41], and dephosphorylated Ntcp is located preferentially in the plasma membrane[45]. Phosphorylated Ser-226 in the third cytoplasmic loop of Ntcp may be the target for cAMP-stimulated dephosphorylation[45]. This cAMP-dependent, Ca^{2+}-mediated pathway may be counter-regulated by activation of "classical" (Ca^{2+}-dependent) PKC (cPKC), since pan-specific activation of PKC with phorbol esters counteracts the cAMP-stimulatory effect[30].

OATP/Oatp: Unlike Ntcp, this family of transporters is not stored in intracellular vesicular compartments, and therefore regulation by trafficking is limited to modulation of its transfer from synthesis sites. Sorting of human OATP-C to the basolateral membrane is mediated by both the Golgi complex- and the vacuolar H^+-ATPase vesicle-mediated membrane sorting pathways, and cAMP positively regulates the first sorting mechanism *via* activation of PKA[46].

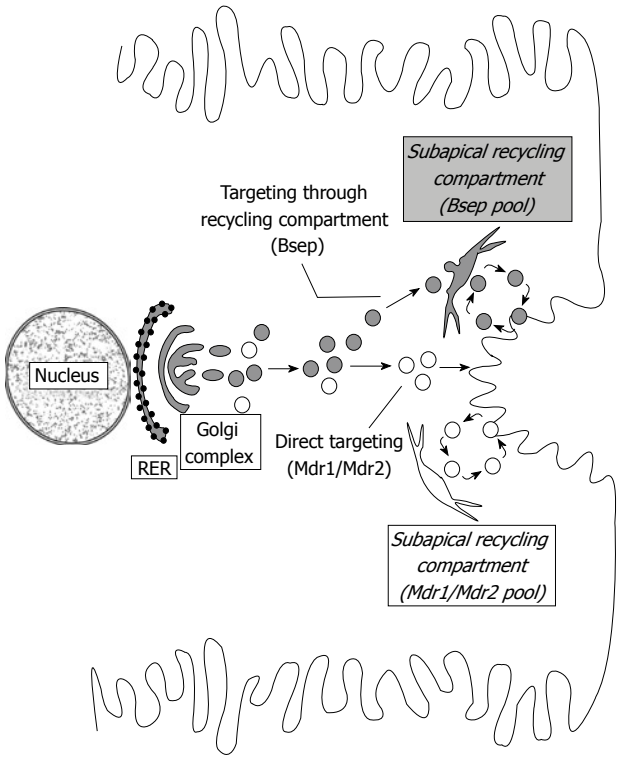

Figure 3 Routes involved in trafficking of canalicular transporters. The trafficking of vesicles delivering Bsep (gray vesicles) or Mdr1/Mdr2 (white vesicles) from the site of synthesis to the canalicular domain is distinct. Mdr1 and Mdr2 are directly targeted to the canalicular membrane, whereas Bsep is indirectly targeted *via* a subapical, endosomal compartment, which allows the recycling of transporters (exocytic insertion/endocytic internalization). Once targeted, Mdr1 and Mdr2 are also able to recycle between the subapical compartment and the canalicular membrane.

Canalicular transporters

ABC canalicular transporters: Vesicle-based trafficking steps of canalicular export pumps are depicted in Figure 3. Once synthesized by the rough ER, *de novo* ABC canalicular transporters belonging to either the MRP or the MDR family traffic *via* the Golgi complex directly to the apical membrane[47-49]. Pulse-chase studies using ^{35}S-methionine followed by immunoprecipitation of the ABC transporters from subcellular fractions have revealed that these transporters are targeted directly to the canalicular membrane, as at no time between passage through Golgi and arrival at the canalicular membrane are the ABC transporters localized at the sinusoidal membrane[49]. However, the post-Golgi trafficking differs among the ABC transporters studied. Mdr1 and Mdr2 are fully delivered to the canalicular membrane 30 min after ^{35}S-methionine administration[49]. This finding was confirmed for Mdr1 in WIF-B cells, a hybrid of rat hepatoma cells and human fibroblasts that has functional bile canaliculi[50]. Contrarily, Bsep only reaches

the canalicular membrane after 2 h, which suggests that, unlike Mdr1/2, Bsep is retained in an intracellular endosomal pool prior to delivery to the canalicular membrane[48]. This intrahepatic, large vesicular pool also serves as a reservoir of ABC transporters, which can be quickly recruited to the canalicular membrane on physiological demand that requires their function (e.g. increased biliary excretion of bile salts for lipid digestion/absorption during the post-prandial period). The recycling process involves exocytic insertion, followed by endocytic internalization once demand is satisfied[47,48].

Compelling evidence in the literature further supports the existence of this recycling compartment for canalicular hepatocellular transporters. Immunogold electron microscopy studies of rat hepatocytes have revealed that distribution of Bsep is not restricted to the canalicular membrane, but is also detected in electron-translucent vacuolar structures close to the apical, but not the basolateral membrane[51]. Pericanalicular localization of Mrp2, Bsep and Mdr1 has also been demonstrated by immunofluorescent staining in isolated rat hepatocyte couplets[52]. Finally, direct visualization of the recycling between the canalicular membrane and subapical endosomes has been observed for Bsep-GDP chimeras in WIF-B cells stably transfected with adenoviral Bsep-GFP constructs[53]. Chimeric Bsep co-localizes with the marker of recycling endosomes Rab11, and its recycling was microtubule- and microfilament-dependent in both ways[53]. On the contrary, and unlike the *de novo* transporter pathway, this recycling does not involve the Golgi complex, since it is unaffected by brefeldin A. This suggests that recycling represents an independent step in the whole trafficking of *de novo* ABC transporters to the canalicular membrane, and that only replenishment of this recycling compartment with newly-synthesized transporters is Golgi-dependent.

This large-range, Golgi-dependent vesicular trafficking of ABC transporters has been characterized by our group and others using the couplet model. Sorting of Mrp2 to the apical membrane has been analyzed by studying the spontaneous retargeting of the transporter after Mrp2 internalization that occurs during the isolation process[54,55]; this vesicle-based trafficking shares the route of newly-synthesized, apically-directed proteins, since it is sensitive to disruption of the Golgi complex function with brefeldin A[55]. Inhibitors of microtubule polymerization diminish, but do not completely block, the restoration of Mrp2 localization[54,55]. Re-establishment of hepatocyte couplet secretory polarity is instead strikingly dependent on microfilament organization[55]. A similar differential cytoskeletal dependency has been suggested to occur for Bsep, as inferred by functional studies upon restoration of the hepatocyte couplet capability to secrete apically the Bsep substrate, cholyl-lysylfluorescein (CLF), and also for Ca^{2+}/Mg^{2+}-ATPase, another canalicular transporter[56]. The vesicle motor protein myosin-Ⅱ may be crucially involved in the actin-dependent targeting of Bsep. Co-immunoprecipitation studies have identified myosin-Ⅱ regulatory light chain as a binding partner of BSEP, and reduced expression of this protein in dominant negative mutant MDCK cells reduces apical membrane BSEP levels[57]. Furthermore, pharmacological inhibition of myosin Ⅱ impedes delivery of newly synthesized transporter to the apical membrane in these cells[57]. These findings suggest that myosin-Ⅱ is required for BSEP trafficking to the apical membrane in polarized epithelial cells.

Trafficking of ABC transporters from their place of synthesis to the canalicular membrane is under signaling modulation. Studies using the re-polarization approach in hepatocyte couplets described above have shown that the spontaneous canalicular targeting of Mrp2 after isolation and culture is Ca^{2+}- but not PKA-dependent[55]. The Ca^{2+}-elevating compound thapsigargin (an inhibitor of the ER Ca^{2+}-ATPase) accelerates, whereas the intracellular Ca^{2+} chelator BAPTA/AM and the CaM inhibitor W7 greatly inhibit this process, which suggests Ca^{2+}-CaM dependency. On the other hand, the PKC-dependent signaling pathway is inhibitory in nature, since the PKC activator phorbol 12,13-dibutyrate inhibits this process, whereas both the pan-specific PKC inhibitor staurosporine and the specific inhibitor cPKC Gö6976 accelerate this process. This indicates that, under basal conditions, cPKC exerts an inhibitory effect on long-range trafficking of ABC transporters to the canalicular pole and that the stimulation induced by Ca^{2+} elevations may generate its own counter-regulatory mechanism, by activating cPKC. In this connection, selective activation of cPKC by administration of thymeleatoxin is associated with retrieval of Bsep and loss of bile salt secretory function in isolated rat perfused liver[58].

Both Roelofsen *et al*[54] and our group[55] have analyzed the influence of cAMP on the time-dependent re-targeting of Mrp2 after isolation-induced Mrp2 internalization. cAMP stimulates this process. This phenomenon is partially inhibited by inhibitors of microtubule polymerization. We have further examined this phenomenon by analyzing the involvement of signaling molecules downstream of cAMP, the cross talk with other signaling pathways, and the dependency of cAMP stimulus on cytoskeleton organization[55] (Figure 4). The cAMP-sensitive stimulatory pathway shares most downstream signaling constituents with the basal, spontaneous pathway described above, i.e. it is not PKA-dependent, but Ca^{2+}-dependent, *via* Ca^{2+}-CaM complex formation. This cAMP-dependent pathway is also counter-regulated by activation of cPKC[55]. Interestingly, a similar counter-regulatory cross-talk between cAMP- and PKC-dependent signaling pathways applies to the trafficking of other transporters, including Ae2[59] and Ntcp[30]. Another candidate to mediate cAMP-stimulatory effects is PI3K. Studies *in vivo* have revealed that cAMP-mediated stimulation of ABC transporter insertion is inhibited by the PI3K inhibitor wortmannin, and restored by phosphoinositide PI3K products[60]. PKCδ has been identified recently

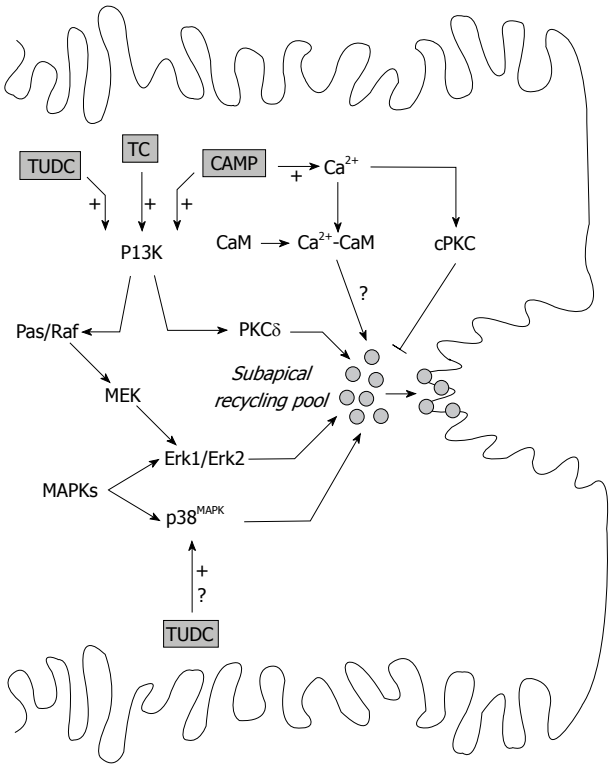

Figure 4 Signaling pathways involved in the exocytic insertion of canalicular transporters promoted by cAMP and by TC and TUDC. cAMP effect involves elevation in cytosolic Ca^{2+} and activation of the PI3K-dependent pathway. Formation of the CaM complex promotes apical insertion of transporters *via* unidentified mediators, and is counter-regulated by activation of cPKC. PI3K promotes exocytic insertion of canalicular transporters by activation of PKCδ and Erk-1 and Erk-2 of MAPK, *via* the Ras/Raf- MAPK kinase (MEK)-Erk-1/2 pathway. TC and TUDC also evoke the PI3K-dependent signaling pathway and promote insertion of canalicular transporters *via* the Ras/Raf-MEK-Erk-1/2 pathway. TUDC also stimulates canalicular carrier insertion by activation of MAPKs of the p38MAPK type, by an unknown mechanism.

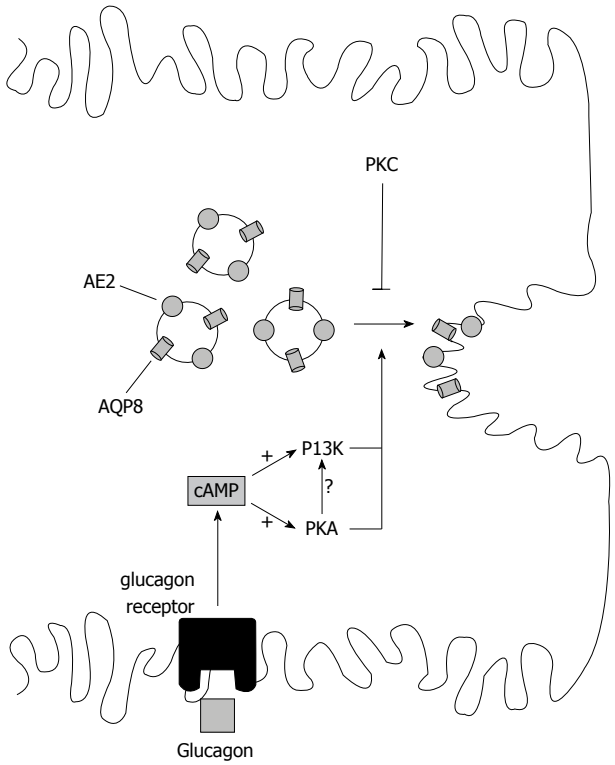

Figure 5 Signaling pathways involved in the co-stimulation of the canalicular targeting of AE2 and AQP8 by cAMP. AE2 and AQP8 are co-localized in the same population of pericanalicular vesicles, thus explaining common signaling modulation. cAMP stimulates AE2 and AQP8 targeting *via* activation of PKA. The PI3K pathway mediates the cAMP-stimulated, PKA-dependent targeting of AQP8, and probably that of AE2. cAMP effect on both transporters is counteracted by activation of PKC.

as a possible effector of the cAMP-dependent, PI3K-mediated pathways that leads to Mrp2 insertion[38]. The endogenous bile salt taurocholate (TC), which, as does cAMP, evokes the PI3K-dependent signaling pathway[61] and activates PKCδ[62], also promotes insertion of ABC transporters into the canalicular membrane in a PI3K-sensitive manner[61].

Another bile salt that stimulates exocytic insertion of canalicular transporters is tauroursodeoxycholate (TUDC)[63], but its action mechanism seems to involve another set of signaling molecules (Figure 4). TUDC activates within minutes mitogen-activated protein kinases (MAPKs) of both the $p38^{MAPK}$ type[63] and of the extracellular signal-regulated kinase (Erk) type (Erk-1 and Erk-2)[64]. These effects are causally linked to increased biliary excretion of bile salts and canalicular insertion of Bsep; the latter event having been demonstrated only for $p38^{MAPK}$[63]. The stimulus induced by TUDC on Erk-1/2, but not on $p38^{MAPK}$, is dependent on the sequential activation of PI3K and Ras/Raf[65]. The two MAPK-dependent pathways seem to act in parallel, and dual activation is required[63]. Studies in human hepatoblastoma HepG2 cells and in rat hepatocytes have shown that TUDC-stimulated insertion of BSEP involves not only increased targeting from the subapical compartment to the canalicular membrane, but also enhanced trafficking from the Golgi complex to the subapical compartment, and that $p38^{MAPK}$ may be a key signaling molecule in mediating this latter effect[66]. Coincidently, hypo-osmotic cell swelling, which shares with TUDC several downstream signaling effectors, also stimulates bile salt excretion by activation of Erk-1/2 and $p38^{MAPK}$[67], and both types of MAPKs are involved in hypotonicity-stimulated, microtubule-sensitive bile salt excretion[68,69].

AE2/Ae2: Apart from its functional localization in the canalicular membrane, the canalicular Cl^-/HCO_3^- exchanger AE2/Ae2 is present in pericanalicular vesicles[21,70], which migrate to the canalicular membrane on demand (Figure 5). The sorting in polarized liver cells of the three Ae2 variants, Ae2a, Ae2b1 and Ae2b2, has been studied using collagen-sandwiched primary rat hepatocytes[21]. After 72-96 h, GFP constructs from each recombinant Ae2 isoform co-localize in the canalicular membrane and in subapical, vesicular structures, and no signal is detected at the basolateral pole. This shared sorting of Ae2 isoforms is sensitive to the microtubule-disrupting agent colchicine, which suggests microtubule-dependent vesicular transport and exocytotic insertion of these transporter isoforms in the canalicular membrane.

Microtubule-dependence of Ae2 trafficking has been confirmed by functional studies. Ae2-mediated Cl^-/HCO_3^- exchange is increased in rat hepatocytes exposed to a bicarbonate-containing medium or in response to cAMP, and this increased activity is blocked with colchicine[59]. The cAMP-elevating hormone glucagon also stimulates this activity through a microtubule- and a cAMP-dependent, PKA-mediated mechanism[71]. The stimulation of Cl^-/HCO_3^- exchange activity by cAMP or glucagon is inhibited by PKC agonists[59,71], which suggests the existence of a counter-regulatory mechanism similar to that occurring for the targeting of Ntcp and ABC canalicular transporters (see above).

AQP8: This water canalicular channel is largely localized in intracellular vesicles in hepatocytes, as demonstrated by both subcellular fractionation[23], confocal immunofluorescence[23] and immunoelectron microscopy studies[72]. As a result of this property, it can be quickly inserted in the canalicular membrane on demand[24,73]. The cell-permeable cAMP analog dibutyryl cAMP induces redistribution of AQP8 to the canalicular membrane, and increases hepatocyte membrane water permeability in a microtubule-dependent manner[22,23]. Further studies in isolated rat hepatocytes[74] have shown that, as with AE2, AQP8 is inserted in the canalicular membrane by the cAMP-elevating hormone glucagon, by a process that involves both PKA and PI3K activation[75]., Immunofluorescent co-staining studies in WIF-B cells have shown intracellular co-localization of AQP8 and AE2, which suggests that these transporters are expressed in the same population of pericanalicular vesicles[76] (Figure 5). This explains the similar behavior of both transporters in response to a similar regulatory stimulus. Thus, apart from modulating the biliary secretion of osmotically-active solutes to the bile canaliculus *via* exocytic insertion of relevant carriers (e.g., BSEP, MRP2, AE2), hepatocytes can also modulate their canalicular membrane water permeability by inserting AQP8, thus facilitating the osmotic movement of water under choleretic stimulus.

ALTERATIONS OF THE DYNAMIC LOCALIZATION OF TRANSPORTERS IN LIVER DISEASE

Endocytic internalization of hepatocellular transporters is a common feature in liver disease. This applies mainly to those liver diseases that involve primary impairment in the capability of hepatocytes to produce bile (hepatocellular cholestasis). In these cases, changes in transporter localization may become a major pathomechanism that explains the secretory failure. Alternatively, changes in carrier localization can occur as a secondary consequence of a cholestatic manifestation caused by mechanical impediments to deliver bile to the duodenum (obstructive cholestasis). In this case, transporter mis-localization may aggravate/perpetuate the primary secretory halt. We summarize here the current evidence in the literature that alterations in the dynamic localization of transporters occur in experimental and human cholestatic liver disease.

Endocytic internalization of transporters in animal models of cholestasis

Endocytic internalization of the main canalicular transporters was first described in experimental models of cholestasis in rodents. Internalization of Mrp2 and Bsep into intracellular vesicles, mainly at the pericanalicular domain, has been shown to occur in experimental models of both obstructive and hepatocellular cholestasis.

Bile duct ligation (BDL): Experimental ligation of the common bile duct in the rat is an accepted model of obstructive cholestasis. BDL leads to a marked alteration in the pattern of staining of both Mrp2 and Bsep, as detected by indirect immunofluorescence microscopy. Paulusma *et al*[77] have found that, 48 h after BDL in rats, immunostaining of these transporters at the canalicular level becomes fuzzy, contrasting with the well-delimited detection in sham-operated controls. The authors have assumed that this represents mis-localization of the transporters to intracellular vesicles at a subapical compartment, next to the canaliculus. These alterations are accompanied by a severe impairment of the biliary excretion of model solutes. For example, Mrp2-mediated transport of the model substrate dinitrophenyl glutathione is substantially impaired in isolated hepatocytes from rats with BDL[77]. Endocytic internalization seems not to be circumscribed to Mrp2 or Bsep, as a similar phenomenon was observed for the canalicular enzymes dipeptidyl peptidase IV[78] and Ca^{2+}/Mg^{2+}-ATPase[79]. Altered localization of Mrp2 and Bsep may represent aggravation of the secretory dysfunction caused by the parallel decrease in the hepatocellular content of the carriers that also occurs in this disease[80,81], or even to be a causal factor of this reduction[77,82-85]. Indeed, Paulusma *et al*[77] have also found that, in contrast to that which is observed for Mrp2 protein content, mRNA levels are preserved after BDL, which suggests post-transcriptional downregulation of Mrp2 expression. They have postulated that endocytic internalization may represent the primary step toward enhanced breakdown of the endocytosed carriers. If maintained with time in chronic cholestatic conditions, this may cause redirection of the protein to the lysosomal compartment, followed by degradation.

The events leading to endocytic internalization of Mrp2 and Bsep in BDL rats remain uncertain. It is likely that accumulation of bile salts or other endogenous, potentially toxic compounds in the liver represents a causal factor. Bile salts are able to trigger oxidative stress[86,87], which in turn may explain the release of pro-inflammatory cytokines in BDL rats[88]. Both events have been involved in canalicular transporter internalization, as described below. We have found that the alteration in the normal pattern of localization of Mrp2, and that of the tight-junctional protein occludin, does not occur until 4 h after BDL in rats[78], in contrast

to the immediate response observed in drug-induced cholestasis (see next section). This suggests that BDL alterations are secondary to intracellular accumulation of deleterious endogenous compounds.

Drug-induced cholestasis: Administration to laboratory animals of drugs known to induce functional, hepatocellular cholestasis, or administration of endogenous compounds thought to be the etiological factors of human cholestatic liver diseases, has been used as an experimental tool to study the mechanisms of the disease. Administration of the cholestatic, naturally-occurring estrogen estradiol-17β-d-glucuronide ($E_2$17G)[89,90], the cholestatic monohydroxylated bile salt taurolithocholate (TLC)[91,92] and the cholestatic immunosuppressor drug cyclosporine A[93] all induce cholestasis in a short-term fashion, accompanied by endocytic internalization of Mrp2 and Bsep.

We have characterized in detail the mechanisms of transporter internalization in E_2-17G-induced cholestasis, an experimental model that reproduces in part pregnancy-induced cholestasis. After a single, i.v. administration of this compound, bile flow decreases in a dose-dependent fashion with a nadir at 20 min, and spontaneously recovers to normality by 2 h post-injection[94]. The cholestatic phase is associated with endocytic internalization of Mrp2 and Bsep, whereas the recovery phase occurs in parallel with the spontaneous re-insertion of subapical vesicles into the canalicular membrane[89,90]. While the internalization process is microtubule-independent, re-insertion is microtubule-dependent, and stimulated by cAMP[95]. We also found that repeated administration of E_2-17G to rats leads to both a deeper internalization of Mrp2 and an abnormal localization of a small fraction to the lateral membrane[78]. The latter phenomenon likely reflects loss of the fence between apical and basolateral domains caused by the simultaneous alteration of the tight-junctional structures[95,96]. Unlike Mrp2 and Bsep, AQP8 has a preserved localization in E_2-17G-induced cholestasis, and, like Mrp2 and Bsep, this water channel has a dual (intracellular plus plasma membrane) localization[97].

Lipopolysaccharide (LPS)-induced cholestasis: LPS is an endotoxin localized in the outer membrane of Gram-negative bacteria. The toxin induces cholestasis mainly by the release of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1 by monocytes/macrophages and, in the liver, Kupffer cells[98]. Administration of LPS to laboratory animals represents, therefore, a good experimental model of inflammatory cholestatic diseases, not only of those caused by endotoxemia, but also those related to hepatitis caused by alcohol, autoimmune disease or drug intake.

LPS administration leads to endocytic internalization of Mrp2 and Bsep, which relocalizes in intracellular vesicular structures[99-101]. The time-dependency of the effect of LPS on Mrp2 internalization has been characterized by Kubitz *et al*[101]. These authors have found that, 3 h after LPS treatment, Mrp2 is found in intracellular vesicles in the vicinity of the canalicular membrane, and that these vesicles are deeply internalized after 6-12 h treatment. Endocytic internalization of ABC canalicular transporters seems to be specific, as localization of the canalicular enzyme dipeptidyl peptidase IV is not affected by the treatment. Mrp2 internalization is reversed by perfusing the liver with a hypo-osmotic buffer, a maneuver known to stimulate exocytic insertion of canalicular transporters under normal conditions[99,102]. However, this rescue of transporters occurs within 3 h of LPS administration, but not later on. It is possible that reversibility of the endocytic process depends on the degree of internalization of Mrp2, and that sustained internalization leads to delivery of the protein to the lysosomal compartment, followed by degradation. LPS effects can be prevented by administration of glucocorticoids[101] or by heat stress[103,104], two maneuvers that cause a decrease in synthesis and/or release of pro-inflammatory cytokines.

Oxidative-stress-induced cholestasis: Oxidative stress is a common feature in most liver diseases[105]. Radical oxygen species induce biliary secretory failure and cholestasis, even at low, pre-necrotic levels[106], and endocytic internalization of canalicular transporters may play a key role. We have shown that Bsep undergoes endocytic internalization into intracellular vesicles in isolated rat hepatocyte couplets when exposed to low levels of the pro-oxidizing compound tert-buthylhydroperoxyde (*t*BOOH)[107]. This is accompanied by a reduced capability to accumulate the fluorescent bile salt analogue CLF in their canalicular vacuoles. A similar phenomenon has been described for Mrp2 after exposure of isolated perfused rat livers to the pro-oxidant agents *t*BOOH[108], chloro-dinitrobenzene[108] and ethacrynic acid[109,110], or after hepatic ischemia-reperfusion[111].

Endocytic internalization of transporters in human cholestatic liver disease

Changes in canalicular export pumps have been shown to occur in many human cholestatic liver diseases. Unlike the situation in rodents, downregulation of the expression of these transporters in human cholestatic disease is mostly post-transcriptional in nature, therefore, internalization of these transporters followed by degradation may represent a crucial mechanism to explain the disease in humans.

Internalization of canalicular export pumps has been observed in virtually all kinds of human cholestasis, including: (1) obstructive extrahepatic cholestasis[112,113]; (2) inflammatory cholestasis associated with autoimmune hepatitis[113]; (3) mixed (obstructive plus inflammatory) cholestatic disease, such as primary biliary cirrhosis[114] and primary sclerosing cholangitis[113]; and (4) acute cholestasis induced by drugs, such as that triggered by

antibiotics, tiopronin, chlorpromazine and non-steroidal anti-inflammatory drugs[113,115]. Patients with obstructive cholestasis that are subjected to percutaneous transhepatic biliary drainage show different degrees of transporter dyslocalization, depending on the efficacy of the biliary drainage[112,113], which points to a central role for retained endogenous compounds in this pathomechanism.

Mechanisms of endocytic internalization in cholestasis: role of signaling pathways

The mechanisms by which endocytosis of canalicular transporters occurs in cholestasis remains poorly understood. At least in part, this may be because they are multifactorial.

Alterations of actin-cytoskeletal integrity by administration of the F-actin poison phalloidin[116], or secondary to the administration of pro-oxidant compounds, such as *t*BOOH[107] or the hydrophobic bile salts taurochenodeoxycholate[117], triggers canalicular transporter endocytosis. This may be related to the fact that actin cytoskeleton is involved in transcytosis processes by operating as a bridge between microtubules and the apical membrane itself, in a coordinated action of the microtubule- and the F-actin-based motor proteins, kinesin and myosin, respectively[118]. However, internalization of canalicular transporters also occurs with preserved actin organization, e.g. in E_2-17G-[89,90] or TLC-[92]induced cholestasis. In these cases, components of the microfilament network other than actin, but associated with it, may be independently affected. Actin can interact with, and possibly regulate, transmembrane proteins *via* binding to plasma membrane actin cross-linking proteins, such as the ezrin-radixin-moesin (ERM) family of proteins, or by binding to interacting-partner proteins, such as PDZK1 and HAX-1. These cytoskeleton-associated proteins are required for the biosynthetic targeting of transmembrane proteins from the *trans*-Golgi network to the proper membrane domain, and for their further cell-surface retention[119-121]. Mice that lack radixin, the main ERM protein in liver, develop conjugated hyperbilirubinemia associated with retrieval of Mrp2[122]. Furthermore, downregulation of radixin using interfering RNA technology in collagen-sandwich-cultured rat hepatocytes disturbed the normal development of canalicular structures, and dissociated canalicular export pumps from their normal location at the apical membrane. Inside the cell, the transporters are found to be largely associated with Rab11-containing endosomes[123]. Furthermore, a disturbed co-localization of MRP2/Mrp2 and radixin associated with endocytic internalization of the carrier is apparent in obstructive and estrogen-induced cholestasis in rats[124], and in several cholestatic liver diseases in humans, including primary biliary cirrhosis stage Ⅲ, drug-induced liver injury, obstructive jaundice, primary sclerosing cholangitis and autoimmune hepatitis[113,114]. On the contrary, alteration in cholestasis of the localization/function of interacting-partner proteins, such as PDZK1 (for Mrp2) and HAX-1 (for Bsep, Mdr2 Mrp1) remains

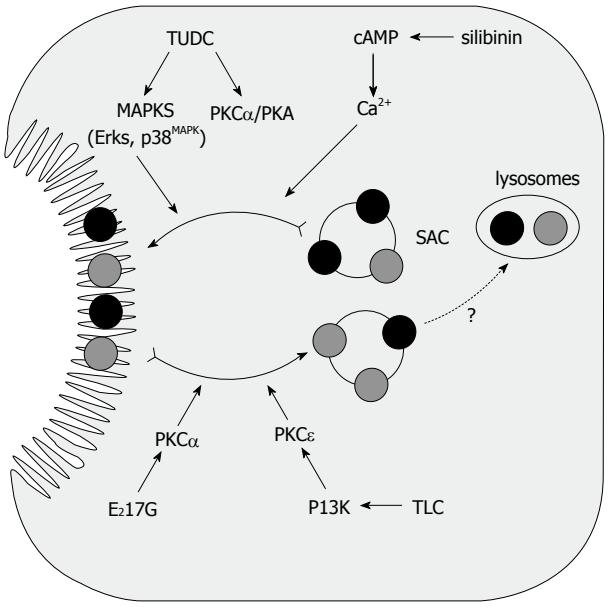

Figure 6 Endocytic internalization of canalicular transporters in $E_2$17G and in TLC-induced cholestasis. Protection from these cholestatic agents by the anticholestatic agents cAMP and TUDC is also shown. $E_2$17G and TLC induce endocytic internalization of canalicular transporters into the subapical compartment (SAC); this may lead to delivery to the lysosomal compartment, followed by degradation. $E_2$17G-induced activation of PKCα and TLC-induced, phosphatidylinositol 3-kinase (PI3K)-dependent activation of PKCε have been proposed to mediate this retrieval. Elevation of intracellular cAMP levels induced by administration of the permeant cAMP analogue DBcAMP, or by the phosphodiesterase inhibitor silibinin, prevents internalization, and accelerates re-insertion, *via* cytosolic Ca^{2+} elevations. On the other hand, TUDC prevents transporter endocytosis probably *via* co-stimulation of PKCα- and PKA-dependent pathways.

to be confirmed. This possibility however exists, since retention of Mrp2[122] and Oatp1a1[125] in the apical and the basolateral membranes, respectively, requires interaction with the PDZ-domain protein, PDZ1. In addition, there is evidence that HAX-1 participates in clathrin-mediated Bsep endocytosis from the canalicular plasma membrane[126].

Accumulating evidence indicates that changes in canalicular transporter localization that occur in cholestasis also depend on activation of critical intracellular signaling pathways (Figure 6). Representative examples are cPKC (mainly, PKCα in hepatocytes). Selective activation of cPKC induces endocytic internalization of Bsep from the canalicular membrane and cholestasis in the isolated perfused rat liver[58]. Coincidently, pan-specific activation of PKC also induces redistribution of MRP2 from the canalicular to the basolateral membrane in HepG2 cells[127]. A critical participation of cPKC in the endocytic internalization of Bsep and the associated bile-salt secretory failure has recently been demonstrated by our group in E_2-17G-induced cholestasis in rats[128]. A similar role for cPKC has also been reported in cholestasis associated with *t*BOOH-induced oxidative stress[107] (Figure 7). However, under oxidative stress, the type of canalicular protein that is internalized and the signaling molecule involved seem to depend on the magnitude of the oxidative challenge. Low concentrations of the oxidizing

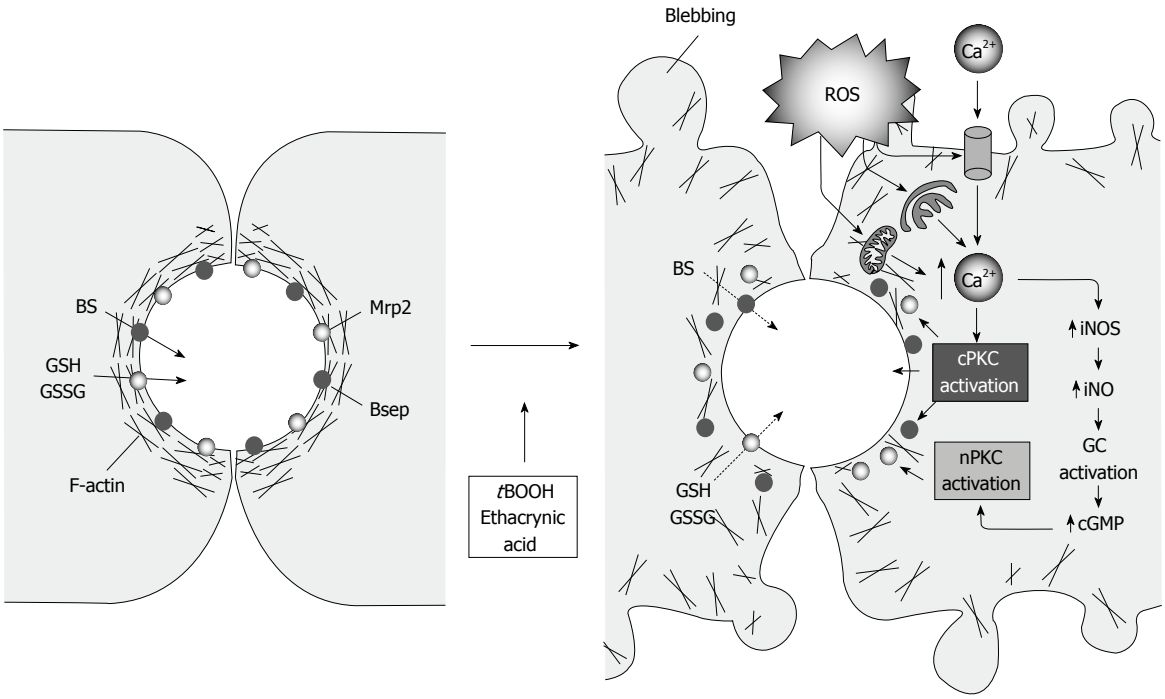

Figure 7 Endocytic internalization of canalicular transporters under oxidative stress. In normal cells, the pericanalicular arrangement of F-actin allows for the appropriate insertion of the canalicular transporters in their membrane domain. Reactive oxygen species produced by the administration of oxidizing compounds, such as tBOOH or ethacrynic acid, induces mobilization of Ca^{2+} across the plasma membrane and membranes of the calciosome (smooth ER and mitochondria), and the subsequent activation of cPKC. cPKC activation induces blebbing and redistribution of F-actin from the pericanalicular region to the cell body. This rearrangement, in turn, leads to canalicular transporter internalization. Moderate Ca^{2+} elevations may also activate iNOS, which induces NO-mediated guanylate cyclase activation and further cGMP-mediated activation of nPKC, which may internalize selectively Mrp2.

compound, ethacrynic acid, does not translocate cPKC, but novel PKC isoforms (nPKC). Under these conditions, the compound internalizes selectively Mrp2 without affecting Bsep, by a mechanism that probably involves Ca^{2+}-dependent activation of inducible nitric oxide (NO) synthase (iNOS), followed by NO-mediated cGMP increase, and further cGMP-activation of nPKC[110]. However, higher doses of ethacrynic acid, sufficient to activate cPKC isoforms, induce internalization of Bsep and Mrp2[110].

The nPKC isoform PKCε is also activated in TLC-induced cholestasis, and has been suggested to be involved in the TLC cholestatic effect[129]. This phenomenon occurs in a PI3K-dependent manner, which is consistent with the finding that PI3K products are potent activators of PKCε[130] (Figure 6). Since PI3K has been also shown to have pro-insertion properties (see above), this may be regarded as paradoxical. However, pro-exocytic and pro-endocytic effects of PI3K have been inferred by using pan-specific inhibitors of PI3K, and different isoforms of this kinase may have accounted for by these different effects.

Anticholestatic therapeutic approaches based upon modulation of dynamic carrier localization

As illustrated above for E2 17G-induced cholestasis, internalization of hepatocellular transporters in cholestasis is spontaneously reversed if the cholestatic insult is transient. This spontaneous recovery occurs by a microtubule-dependent re-targeting of the endocytosed transporters to the canalicular membrane[95]. Some experimental therapeutic approaches have been designed to prevent transporter internalization and/or to accelerate this re-insertion, so as to avoid irreversible consequences of sustained internalization (Figure 6). The therapeutic agents studied include the following.

cAMP: This second messenger partially prevents the impairment of bile flow and internalization of ABC transporters in experimental cholestasis, consistent with its capability to stimulate vesicle-mediated targeting of canalicular transporters[54,55,60]. The drop in bile flow and transport activity of Bsep[90] and Mrp2[89] in the acute phase of E2 17G-induced cholestasis can be partially prevented by cAMP. More significantly, cAMP shortens spontaneous recovery to normality of bile flow, Mrp2 function and Mrp2 localization[89]. A similar acceleration of the re-insertion of endocytosed transporters has been described by our group for Bsep in TLC-induced cholestasis[92]. In isolated rat hepatocyte couplets, a preventive effect of cAMP has been observed in E2 17G-[90,131] and TLC[92,131]-induced Bsep mis-localization. In this case, however, prevention by cAMP is complete. This protective effect is significantly blocked by the Ca^{2+} chelator, BAPTA/AM, but not by the PKA inhibitor, KT5720, which suggests involvement of Ca^{2+}-dependent signaling pathways. A similar anticholestatic mechanism in terms of the signaling modulators involved is afforded by silibinin, the active component of the hepatoprotector silymarin[131]. This most likely

results from the capability of silibinin to inhibit cAMP phosphodiesterase, thus increasing endogenous cAMP intracellular levels[131].

TUDC: This taurine-conjugate bile salt stimulates exocytic insertion of canalicular export pumps as part of its choleretic effect[63], and counteracts endocytic internalization of Bsep[134] and Mrp2[91] in TLC-induced cholestasis (Figure 6). The Ca^{2+}-sensitive, PKC isoform, PKCα, has been proposed to mediate its anticholestatic effect[91], *via* a cooperative PKCα/PKA-dependent mechanism[133]. This is in apparent contradiction with more recent findings that PKCα is cholestatic rather than hepatoprotective[58]. However, the biological response evoked by the interplay between different protein kinases (PKCα/PKA) may be different from that evoked by just one of them (PKCα). Furthermore, TUDC activates Erk[64] and $p38^{MAPK}$[63], and the cholestatic effect of PKCα may be overridden by the choleretic effects of these signal transduction pathways.

4-Phenylbutyrate (4-PBA): This compound has been shown to restore the reduced cell surface expression of cystic fibrosis transmembrane conductance regulator in cystic fibrosis patients, who have mutated forms of the protein, which suggests improved targeting of the transporter to its membrane domain. When the 4PBA-proinserting property was tested for Bsep in normal rats, it was observed that canalicular expression and bile-salt transport function were improved by this compound[134]. A possible mechanism that 4PBA treatment increases the cell-surface-resident Bsep is the interruption of the internalization process from the cell surface to the intracellular compartment, or promotion of recycling from the intracellular compartment back to the cell surface[134]. Stabilization of Bsep in the membrane by 4PBA has also been confirmed in MDCK cells for wild-type Bsep and Bsep with E297G and D482G mutations, which occurs in progressive familiar intrahepatic cholestasis type 2 (PFIC2). Since trafficking of these Bsep-mutated proteins is impaired in PFIC2[135], this agent may be a potential candidate to halt the progression of this genetic disease. Its efficacy in acquired cholestatic diseases remains to be ascertained.

FUTURE DIRECTIONS

The overwhelming progress in molecular biology techniques and the availability of *in vitro*, polarized cell models for the study of hepatobiliary function has greatly facilitated the characterization at a molecular level of the mechanism involved in the sorting of hepatobiliary transport systems from their sites of synthesis, and their recycling from/to endosomal compartments available on demand. However, the increasing number of new cytoskeletal, motor and signaling proteins that are being discovered as a result of these technological developments makes the characterization of their role in transporter trafficking an endless challenge.

Advances in the molecular field have promoted a parallel progress in the understanding of the consequences that the alterations in the mechanisms of trafficking have in liver disease. It is becoming increasingly evident that impairment in the dynamic localization of hepatocellular transporters is a common feature in hepatocellular cholestasis. However, the characterization of the molecular mechanisms that underlie this alteration is in its infancy. Many crucial questions remain to be answered, for example: (1) which are the signaling mediators that trigger endocytosis of canalicular transporters in each kind of cholestasis; (2) which are the molecular targets of these cholestatic mediators that ultimately govern carrier internalization?; and (3) can changes in localization of these transporters be not only prevented but, what is more important from the therapeutic point of view, reversed by factors that counteract these dysfunctions? Satisfactory answers to these questions would allow the design of new therapeutic strategies in cholestatic liver diseases to assure proper localization of transporters in an attempt to prevent their accelerated degradation. We hope that progress in experimental therapeutics based on this current information encourages clinical researchers to apply this knowledge to envisage better, innovative therapeutic alternatives for the treatment of human cholestatic liver disease.

REFERENCES

1 **Reichen J**. The Role of the Sinusoidal Endothelium in Liver Function. *News Physiol Sci* 1999; **14**: 117-121
2 **Hofmann AF**. Bile Acids: The Good, the Bad, and the Ugly. *News Physiol Sci* 1999; **14**: 24-29
3 **Bohan A**, Boyer JL. Mechanisms of hepatic transport of drugs: implications for cholestatic drug reactions. *Semin Liver Dis* 2002; **22**: 123-136
4 **Kullak-Ublick GA**, Stieger B, Hagenbuch B, Meier PJ. Hepatic transport of bile salts. *Semin Liver Dis* 2000; **20**: 273-292
5 **Pellicoro A**, Faber KN. Review article: The function and regulation of proteins involved in bile salt biosynthesis and transport. *Aliment Pharmacol Ther* 2007; **26** Suppl 2: 149-160
6 **Ito K**, Suzuki H, Horie T, Sugiyama Y. Apical/basolateral surface expression of drug transporters and its role in vectorial drug transport. *Pharm Res* 2005; **22**: 1559-1577
7 **Niemi M**. Role of OATP transporters in the disposition of drugs. *Pharmacogenomics* 2007; **8**: 787-802
8 **Müller M**, Jansen PL. Molecular aspects of hepatobiliary transport. *Am J Physiol* 1997; **272**: G1285-G1303
9 **Trauner M**, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* 2003; **83**: 633-671
10 **van Montfoort JE**, Müller M, Groothuis GM, Meijer DK, Koepsell H, Meier PJ. Comparison of "type I" and "type II" organic cation transport by organic cation transporters and organic anion-transporting polypeptides. *J Pharmacol Exp Ther* 2001; **298**: 110-115
11 **Silverman JA**, Raunio H, Gant TW, Thorgeirsson SS. Cloning and characterization of a member of the rat multidrug resistance (mdr) gene family. *Gene* 1991; **106**: 229-236
12 **Crawford AR**, Smith AJ, Hatch VC, Oude Elferink RP, Borst P, Crawford JM. Hepatic secretion of phospholipid vesicles in the mouse critically depends on mdr2 or MDR3

P-glycoprotein expression. Visualization by electron microscopy. *J Clin Invest* 1997; **100**: 2562-2567
13 **Suchy FJ**, Ananthanarayanan M. Bile salt excretory pump: biology and pathobiology. *J Pediatr Gastroenterol Nutr* 2006; **43** Suppl 1: S10-S16
14 **Akita H**, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H, Sugiyama Y. Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. *Biochim Biophys Acta* 2001; **1511**: 7-16
15 **Paulusma CC**, van Geer MA, Evers R, Heijn M, Ottenhoff R, Borst P, Oude Elferink RP. Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. *Biochem J* 1999; **338** (Pt 2): 393-401
16 **Yang B**, Hill CE. Nifedipine modulation of biliary GSH and GSSG/ conjugate efflux in normal and regenerating rat liver. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G85-G94
17 **Ballatori N**, Truong AT. Glutathione as a primary osmotic driving force in hepatic bile formation. *Am J Physiol* 1992; **263**: G617-G624
18 **Banales JM**, Prieto J, Medina JF. Cholangiocyte anion exchange and biliary bicarbonate excretion. *World J Gastroenterol* 2006; **12**: 3496-3511
19 **Hardison WG**, Wood CA. Importance of bicarbonate in bile salt independent fraction of bile flow. *Am J Physiol* 1978; **235**: E158-E164
20 **Medina JF**, Lecanda J, Acín A, Ciesielczyk P, Prieto J. Tissue-specific N-terminal isoforms from overlapping alternate promoters of the human AE2 anion exchanger gene. *Biochem Biophys Res Commun* 2000; **267**: 228-235
21 **Aranda V**, Martínez I, Melero S, Lecanda J, Banales JM, Prieto J, Medina JF. Shared apical sorting of anion exchanger isoforms AE2a, AE2b1, and AE2b2 in primary hepatocytes. *Biochem Biophys Res Commun* 2004; **319**: 1040-1046
22 **Huebert RC**, Splinter PL, Garcia F, Marinelli RA, LaRusso NF. Expression and localization of aquaporin water channels in rat hepatocytes. Evidence for a role in canalicular bile secretion. *J Biol Chem* 2002; **277**: 22710-22717
23 **García F**, Kierbel A, Larocca MC, Gradilone SA, Splinter P, LaRusso NF, Marinelli RA. The water channel aquaporin-8 is mainly intracellular in rat hepatocytes, and its plasma membrane insertion is stimulated by cyclic AMP. *J Biol Chem* 2001; **276**: 12147-12152
24 **Marinelli RA**, Gradilone SA, Carreras FI, Calamita G, Lehmann GL. Liver aquaporins: significance in canalicular and ductal bile formation. *Ann Hepatol* 2004; **3**: 130-136
25 **Sun AQ**, Swaby I, Xu S, Suchy FJ. Cell-specific basolateral membrane sorting of the human liver Na(+)-dependent bile acid cotransporter. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G1305-G1313
26 **Dranoff JA**, McClure M, Burgstahler AD, Denson LA, Crawford AR, Crawford JM, Karpen SJ, Nathanson MH. Short-term regulation of bile acid uptake by microfilament-dependent translocation of rat ntcp to the plasma membrane. *Hepatology* 1999; **30**: 223-229
27 **Webster CR**, Anwer MS. Role of the PI3K/PKB signaling pathway in cAMP-mediated translocation of rat liver Ntcp. *Am J Physiol* 1999; **277**: G1165-G1172
28 **Sarkar S**, Bananis E, Nath S, Anwer MS, Wolkoff AW, Murray JW. PKCzeta is required for microtubule-based motility of vesicles containing the ntcp transporter. *Traffic* 2006; **7**: 1078-1091
29 **Mukhopadhayay S**, Ananthanarayanan M, Stieger B, Meier PJ, Suchy FJ, Anwer MS. cAMP increases liver Na+-taurocholate cotransport by translocating transporter to plasma membranes. *Am J Physiol* 1997; **273**: G842-G848
30 **Grüne S**, Engelking LR, Anwer MS. Role of intracellular calcium and protein kinases in the activation of hepatic Na+/taurocholate cotransport by cyclic AMP. *J Biol Chem* 1993; **268**: 17734-17741
31 **Webster CR**, Srinivasulu U, Ananthanarayanan M, Suchy FJ, Anwer MS. Protein kinase B/Akt mediates cAMP- and cell swelling-stimulated Na+/taurocholate cotransport and Ntcp translocation. *J Biol Chem* 2002; **277**: 28578-28583
32 **Cosentino C**, Di Domenico M, Porcellini A, Cuozzo C, De Gregorio G, Santillo MR, Agnese S, Di Stasio R, Feliciello A, Migliaccio A, Avvedimento EV. p85 regulatory subunit of PI3K mediates cAMP-PKA and estrogens biological effects on growth and survival. *Oncogene* 2007; **26**: 2095-2103
33 **Webster CR**, Blanch CJ, Phillips J, Anwer MS. Cell swelling-induced translocation of rat liver Na(+)/taurocholate cotransport polypeptide is mediated via the phosphoinositide 3-kinase signaling pathway. *J Biol Chem* 2000; **275**: 29754-29760
34 **McConkey M**, Gillin H, Webster CR, Anwer MS. Cross-talk between protein kinases Czeta and B in cyclic AMP-mediated sodium taurocholate co-transporting polypeptide translocation in hepatocytes. *J Biol Chem* 2004; **279**: 20882-20888
35 **Nakanishi H**, Brewer KA, Exton JH. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1993; **268**: 13-16
36 **Miguel BG**, Calcerrada MC, Mata F, Aller P, Clemente R, Catalán RE, Martínez AM. Differential redistribution of protein kinase C isoforms by cyclic AMP in HL60 cells. *Biochem Biophys Res Commun* 2000; **274**: 596-602
37 **Newton AC**. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem J* 2003; **370**: 361-371
38 **Schonhoff CM**, Gillin H, Webster CR, Anwer MS. Protein kinase Cdelta mediates cyclic adenosine monophosphate-stimulated translocation of sodium taurocholate cotransporting polypeptide and multidrug resistant associated protein 2 in rat hepatocytes. *Hepatology* 2008; **47**: 1309-1316
39 **Webster CR**, Blanch C, Anwer MS. Role of PP2B in cAMP-induced dephosphorylation and translocation of NTCP. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G44-G50
40 **Mukhopadhyay S**, Webster CR, Anwer MS. Role of protein phosphatases in cyclic AMP-mediated stimulation of hepatic Na+/taurocholate cotransport. *J Biol Chem* 1998; **273**: 30039-30045
41 **Mukhopadhyay S**, Ananthanarayanan M, Stieger B, Meier PJ, Suchy FJ, Anwer MS. Sodium taurocholate cotransporting polypeptide is a serine, threonine phosphoprotein and is dephosphorylated by cyclic adenosine monophosphate. *Hepatology* 1998; **28**: 1629-1636
42 **Sun AQ**, Arrese MA, Zeng L, Swaby I, Zhou MM, Suchy FJ. The rat liver Na(+)/bile acid cotransporter. Importance of the cytoplasmic tail to function and plasma membrane targeting. *J Biol Chem* 2001; **276**: 6825-6833
43 **Exton JH**. Role of phosphoinositides in the regulation of liver function. *Hepatology* 1988; **8**: 152-166
44 **Staddon JM**, Hansford RG. Evidence indicating that the glucagon-induced increase in cytoplasmic free Ca2+ concentration in hepatocytes is mediated by an increase in cyclic AMP concentration. *Eur J Biochem* 1989; **179**: 47-52
45 **Anwer MS**, Gillin H, Mukhopadhyay S, Balasubramaniyan N, Suchy FJ, Ananthanarayanan M. Dephosphorylation of Ser-226 facilitates plasma membrane retention of Ntcp. *J Biol Chem* 2005; **280**: 33687-33692
46 **Sun AQ**, Ponamgi VM, Boyer JL, Suchy FJ. Membrane trafficking of the human organic anion-transporting polypeptide C (hOATPC). *Pharm Res* 2008; **25**: 463-474
47 **Kipp H**, Arias IM. Trafficking of canalicular ABC transporters in hepatocytes. *Annu Rev Physiol* 2002; **64**: 595-608
48 **Kipp H**, Pichetshote N, Arias IM. Transporters on demand: intrahepatic pools of canalicular ATP binding cassette transporters in rat liver. *J Biol Chem* 2001; **276**: 7218-7224
49 **Kipp H**, Arias IM. Newly synthesized canalicular ABC transporters are directly targeted from the Golgi to the hepatocyte apical domain in rat liver. *J Biol Chem* 2000; **275**:

15917-15925
50 **Sai Y**, Nies AT, Arias IM. Bile acid secretion and direct targeting of mdr1-green fluorescent protein from Golgi to the canalicular membrane in polarized WIF-B cells. *J Cell Sci* 1999; **112** (Pt 24): 4535-4545
51 **Gerloff T**, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF, Meier PJ. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J Biol Chem* 1998; **273**: 10046-10050
52 **Soroka CJ**, Pate MK, Boyer JL. Canalicular export pumps traffic with polymeric immunoglobulin A receptor on the same microtubule-associated vesicle in rat liver. *J Biol Chem* 1999; **274**: 26416-26424
53 **Wakabayashi Y**, Lippincott-Schwartz J, Arias IM. Intracellular trafficking of bile salt export pump (ABCB11) in polarized hepatic cells: constitutive cycling between the canalicular membrane and rab11-positive endosomes. *Mol Biol Cell* 2004; **15**: 3485-3496
54 **Roelofsen H**, Soroka CJ, Keppler D, Boyer JL. Cyclic AMP stimulates sorting of the canalicular organic anion transporter (Mrp2/cMoat) to the apical domain in hepatocyte couplets. *J Cell Sci* 1998; **111** (Pt 8): 1137-1145
55 **Roma MG**, Milkiewicz P, Elias E, Coleman R. Control by signaling modulators of the sorting of canalicular transporters in rat hepatocyte couplets: role of the cytoskeleton. *Hepatology* 2000; **32**: 1342-1356
56 **Gautam A**, Ng OC, Boyer JL. Isolated rat hepatocyte couplets in short-term culture: structural characteristics and plasma membrane reorganization. *Hepatology* 1987; **7**: 216-223
57 **Chan W**, Calderon G, Swift AL, Moseley J, Li S, Hosoya H, Arias IM, Ortiz DF. Myosin II regulatory light chain is required for trafficking of bile salt export protein to the apical membrane in Madin-Darby canine kidney cells. *J Biol Chem* 2005; **280**: 23741-23747
58 **Kubitz R**, Saha N, Kühlkamp T, Dutta S, vom Dahl S, Wettstein M, Häussinger D. Ca2+-dependent protein kinase C isoforms induce cholestasis in rat liver. *J Biol Chem* 2004; **279**: 10323-10330
59 **Benedetti A**, Strazzabosco M, Ng OC, Boyer JL. Regulation of activity and apical targeting of the Cl-/HCO3- exchanger in rat hepatocytes. *Proc Natl Acad Sci USA* 1994; **91**: 792-796
60 **Kagawa T**, Misra S, Varticovski L, Arias IM. The mechanisms whereby cAMP activates PI 3-kinase which regulates canalicular spgp (Abstract). *Hepatology* 2000; **32**: 306A
61 **Misra S**, Ujházy P, Gatmaitan Z, Varticovski L, Arias IM. The role of phosphoinositide 3-kinase in taurocholate-induced trafficking of ATP-dependent canalicular transporters in rat liver. *J Biol Chem* 1998; **273**: 26638-26644
62 **Rao YP**, Stravitz RT, Vlahcevic ZR, Gurley EC, Sando JJ, Hylemon PB. Activation of protein kinase C alpha and delta by bile acids: correlation with bile acid structure and diacylglycerol formation. *J Lipid Res* 1997; **38**: 2446-2454
63 **Kurz AK**, Graf D, Schmitt M, Vom Dahl S, Häussinger D. Tauroursodesoxycholate-induced choleresis involves p38(MAPK) activation and translocation of the bile salt export pump in rats. *Gastroenterology* 2001; **121**: 407-419
64 **Schliess F**, Kurz AK, vom Dahl S, Häussinger D. Mitogen-activated protein kinases mediate the stimulation of bile acid secretion by tauroursodeoxycholate in rat liver. *Gastroenterology* 1997; **113**: 1306-1314
65 **Kurz AK**, Block C, Graf D, Dahl SV, Schliess F, Häussinger D. Phosphoinositide 3-kinase-dependent Ras activation by tauroursodesoxycholate in rat liver. *Biochem J* 2000; **350** Pt 1: 207-213
66 **Kubitz R**, Sütfels G, Kühlkamp T, Kölling R, Häussinger D. Trafficking of the bile salt export pump from the Golgi to the canalicular membrane is regulated by the p38 MAP kinase. *Gastroenterology* 2004; **126**: 541-553
67 **Kim RD**, Darling CE, Cerwenka H, Chari RS. Hypoosmotic stress activates p38, ERK 1 and 2, and SAPK/JNK in rat hepatocytes. *J Surg Res* 2000; **90**: 58-66
68 **Noé B**, Schliess F, Wettstein M, Heinrich S, Häussinger D. Regulation of taurocholate excretion by a hypo-osmolarity-activated signal transduction pathway in rat liver. *Gastroenterology* 1996; **110**: 858-865
69 **Häussinger D**, Schmitt M, Weiergräber O, Kubitz R. Short-term regulation of canalicular transport. *Semin Liver Dis* 2000; **20**: 307-321
70 **Martínez-Ansó E**, Castillo JE, Díez J, Medina JF, Prieto J. Immunohistochemical detection of chloride/bicarbonate anion exchangers in human liver. *Hepatology* 1994; **19**: 1400-1406
71 **Alvaro D**, Della Guardia P, Bini A, Gigliozzi A, Furfaro S, La Rosa T, Piat C, Capocaccia L. Effect of glucagon on intracellular pH regulation in isolated rat hepatocyte couplets. *J Clin Invest* 1995; **96**: 665-675
72 **Calamita G**, Mazzone A, Bizzoca A, Cavalier A, Cassano G, Thomas D, Svelto M. Expression and immunolocalization of the aquaporin-8 water channel in rat gastrointestinal tract. *Eur J Cell Biol* 2001; **80**: 711-719
73 **Portincasa P**, Palasciano G, Svelto M, Calamita G. Aquaporins in the hepatobiliary tract. Which, where and what they do in health and disease. *Eur J Clin Invest* 2008; **38**: 1-10
74 **Gradilone SA**, García F, Huebert RC, Tietz PS, Larocca MC, Kierbel A, Carreras FI, Larusso NF, Marinelli RA. Glucagon induces the plasma membrane insertion of functional aquaporin-8 water channels in isolated rat hepatocytes. *Hepatology* 2003; **37**: 1435-1441
75 **Gradilone SA**, Carreras FI, Lehmann GL, Marinelli RA. Phosphoinositide 3-kinase is involved in the glucagon-induced translocation of aquaporin-8 to hepatocyte plasma membrane. *Biol Cell* 2005; **97**: 831-836
76 **Gradilone SA**, Tietz PS, Splinter PL, Marinelli RA, LaRusso NF. Expression and subcellular localization of aquaporin water channels in the polarized hepatocyte cell line, WIF-B. *BMC Physiol* 2005; **5**: 13
77 **Paulusma CC**, Kothe MJ, Bakker CT, Bosma PJ, van Bokhoven I, van Marle J, Bolder U, Tytgat GN, Oude Elferink RP. Zonal down-regulation and redistribution of the multidrug resistance protein 2 during bile duct ligation in rat liver. *Hepatology* 2000; **31**: 684-693
78 **Mottino AD**, Hoffman T, Crocenzi FA, Sánchez Pozzi EJ, Roma MG, Vore M. Disruption of function and localization of tight junctional structures and Mrp2 in sustained estradiol-17beta-D-glucuronide-induced cholestasis. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G391-G402
79 **Song JY**, Van Marle J, Van Noorden CJ, Frederiks WM. Disturbed structural interactions between microfilaments and tight junctions in rat hepatocytes during extrahepatic cholestasis induced by common bile duct ligation. *Histochem Cell Biol* 1996; **106**: 573-580
80 **Roma MG**, Crocenzi FA, Sánchez Pozzi EA. Hepatocellular transport in acquired cholestasis: new insights into functional, regulatory and therapeutic aspects. *Clin Sci* (Lond) 2008; **114**: 567-588
81 **Trauner M**, Wagner M, Fickert P, Zollner G. Molecular regulation of hepatobiliary transport systems: clinical implications for understanding and treating cholestasis. *J Clin Gastroenterol* 2005; **39**: S111-S124
82 **Trauner M**, Arrese M, Soroka CJ, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, Keppler D, Boyer JL. The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis. *Gastroenterology* 1997; **113**: 255-264
83 **Kamisako T**, Ogawa H. Alteration of the expression of adenosine triphosphate-binding cassette transporters associated with bile acid and cholesterol transport in the rat liver and intestine during cholestasis. *J Gastroenterol Hepatol* 2005; **20**: 1429-1434
84 **Kanno K**, Tazuma S, Niida S, Chayama K. Unique reciprocal changes of hepatocellular membrane transporter expression and fluidity in rats with selective biliary obstruction. *Hepatol Res* 2003; **26**: 157-163

85 **Lee JM**, Trauner M, Soroka CJ, Stieger B, Meier PJ, Boyer JL. Expression of the bile salt export pump is maintained after chronic cholestasis in the rat. *Gastroenterology* 2000; **118**: 163-172

86 **Borgognone M**, Pérez LM, Basiglio CL, Ochoa JE, Roma MG. Signaling modulation of bile salt-induced necrosis in isolated rat hepatocytes. *Toxicol Sci* 2005; **83**: 114-125

87 **Sokol RJ**, Devereaux M, Khandwala R, O'Brien K. Evidence for involvement of oxygen free radicals in bile acid toxicity to isolated rat hepatocytes. *Hepatology* 1993; **17**: 869-881

88 **Liu TZ**, Lee KT, Chern CL, Cheng JT, Stern A, Tsai LY. Free radical-triggered hepatic injury of experimental obstructive jaundice of rats involves overproduction of proinflammatory cytokines and enhanced activation of nuclear factor kappaB. *Ann Clin Lab Sci* 2001; **31**: 383-390

89 **Mottino AD**, Cao J, Veggi LM, Crocenzi F, Roma MG, Vore M. Altered localization and activity of canalicular Mrp2 in estradiol-17beta-D-glucuronide-induced cholestasis. *Hepatology* 2002; **35**: 1409-1419

90 **Crocenzi FA**, Mottino AD, Cao J, Veggi LM, Pozzi EJ, Vore M, Coleman R, Roma MG. Estradiol-17beta-D-glucuronide induces endocytic internalization of Bsep in rats. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G449-G459

91 **Beuers U**, Bilzer M, Chittattu A, Kullak-Ublick GA, Keppler D, Paumgartner G, Dombrowski F. Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. *Hepatology* 2001; **33**: 1206-1216

92 **Crocenzi FA**, Mottino AD, Sánchez Pozzi EJ, Pellegrino JM, Rodríguez Garay EA, Milkiewicz P, Vore M, Coleman R, Roma MG. Impaired localisation and transport function of canalicular Bsep in taurolithocholate induced cholestasis in the rat. *Gut* 2003; **52**: 1170-1177

93 **Román ID**, Fernández-Moreno MD, Fueyo JA, Roma MG, Coleman R. Cyclosporin A induced internalization of the bile salt export pump in isolated rat hepatocyte couplets. *Toxicol Sci* 2003; **71**: 276-281

94 **Meyers M**, Slikker W, Pascoe G, Vore M. Characterization of cholestasis induced by estradiol-17 beta-D-glucuronide in the rat. *J Pharmacol Exp Ther* 1980; **214**: 87-93

95 **Mottino AD**, Crocenzi FA, Pozzi EJ, Veggi LM, Roma MG, Vore M. Role of microtubules in estradiol-17beta-D-glucuronide-induced alteration of canalicular Mrp2 localization and activity. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G327-G336

96 **Kan KS**, Monte MJ, Parslow RA, Coleman R. Oestradiol 17 beta-glucuronide increases tight-junctional permeability in rat liver. *Biochem J* 1989; **261**: 297-300

97 **Mottino AD**, Carreras FI, Gradilone SA, Marinelli RA, Vore M. Canalicular membrane localization of hepatocyte aquaporin-8 is preserved in estradiol-17beta-D-glucuronide-induced cholestasis. *J Hepatol* 2006; **44**: 232-233

98 **Jansen PL**, Müller M. Early events in sepsis-associated cholestasis. *Gastroenterology* 1999; **116**: 486-488

99 **Kubitz R**, D'urso D, Keppler D, Häussinger D. Osmodependent dynamic localization of the multidrug resistance protein 2 in the rat hepatocyte canalicular membrane. *Gastroenterology* 1997; **113**: 1438-1442

100 **Dombrowski F**, Kubitz R, Chittattu A, Wettstein M, Saha N, Häussinger D. Electron-microscopic demonstration of multidrug resistance protein 2 (Mrp2) retrieval from the canalicular membrane in response to hyperosmolarity and lipopolysaccharide. *Biochem J* 2000; **348** Pt 1: 183-188

101 **Kubitz R**, Wettstein M, Warskulat U, Häussinger D. Regulation of the multidrug resistance protein 2 in the rat liver by lipopolysaccharide and dexamethasone. *Gastroenterology* 1999; **116**: 401-410

102 **Schmitt M**, Kubitz R, Lizun S, Wettstein M, Häussinger D. Regulation of the dynamic localization of the rat Bsep gene-encoded bile salt export pump by anisoosmolarity. *Hepatology* 2001; **33**: 509-518

103 **Bolder U**, Schmidt A, Landmann L, Kidder V, Tange S, Jauch KW. Heat stress prevents impairment of bile acid transport in endotoxemic rats by a posttranscriptional mechanism. *Gastroenterology* 2002; **122**: 963-973

104 **Bolder U**, Jeschke MG, Landmann L, Wolf F, de Sousa C, Schlitt HJ, Przkora R. Heat stress enhances recovery of hepatocyte bile acid and organic anion transporters in endotoxemic rats by multiple mechanisms. *Cell Stress Chaperones* 2006; **11**: 89-100

105 **Poli G**. Liver damage due to free radicals. *Br Med Bull* 1993; **49**: 604-620

106 **Roma MG**, Sanchez Pozzi EJ. Oxidative stress: a radical way to stop making bile. *Ann Hepatol* 2008; **7**: 16-33

107 **Pérez LM**, Milkiewicz P, Elias E, Coleman R, Sánchez Pozzi EJ, Roma MG. Oxidative stress induces internalization of the bile salt export pump, Bsep, and bile salt secretory failure in isolated rat hepatocyte couplets: a role for protein kinase C and prevention by protein kinase A. *Toxicol Sci* 2006; **91**: 150-158

108 **Schmitt M**, Kubitz R, Wettstein M, vom Dahl S, Häussinger D. Retrieval of the mrp2 gene encoded conjugate export pump from the canalicular membrane contributes to cholestasis induced by tert-butyl hydroperoxide and chlorodinitrobenzene. *Biol Chem* 2000; **381**: 487-495

109 **Ji B**, Ito K, Sekine S, Tajima A, Horie T. Ethacrynic-acid-induced glutathione depletion and oxidative stress in normal and Mrp2-deficient rat liver. *Free Radic Biol Med* 2004; **37**: 1718-1729

110 **Sekine S**, Ito K, Horie T. Oxidative stress and Mrp2 internalization. *Free Radic Biol Med* 2006; **40**: 2166-2174

111 **Yu QY**, Shu M, Dai JH, Ma JB, Yu Y, Liu DH. [The mechanism of the increase of plasma bilirubin after hepatic ischemia-reperfusion in rats] *Zhonghua Ganzangbing Zazhi* 2007; **15**: 763-766

112 **Shoda J**, Kano M, Oda K, Kamiya J, Nimura Y, Suzuki H, Sugiyama Y, Miyazaki H, Todoroki T, Stengelin S, Kramer W, Matsuzaki Y, Tanaka N. The expression levels of plasma membrane transporters in the cholestatic liver of patients undergoing biliary drainage and their association with the impairment of biliary secretory function. *Am J Gastroenterol* 2001; **96**: 3368-3378

113 **Kojima H**, Sakurai S, Uemura M, Kitamura K, Kanno H, Nakai Y, Fukui H. Disturbed colocalization of multidrug resistance protein 2 and radixin in human cholestatic liver diseases. *J Gastroenterol Hepatol* 2008; **23**: e120-e128

114 **Kojima H**, Nies AT, König J, Hagmann W, Spring H, Uemura M, Fukui H, Keppler D. Changes in the expression and localization of hepatocellular transporters and radixin in primary biliary cirrhosis. *J Hepatol* 2003; **39**: 693-702

115 **Watanabe N**, Takashimizu S, Kojima S, Kagawa T, Nishizaki Y, Mine T, Matsuzaki S. Clinical and pathological features of a prolonged type of acute intrahepatic cholestasis. *Hepatol Res* 2007; **37**: 598-607

116 **Rost D**, Kartenbeck J, Keppler D. Changes in the localization of the rat canalicular conjugate export pump Mrp2 in phalloidin-induced cholestasis. *Hepatology* 1999; **29**: 814-821

117 **Rost D**, Kloeters-Plachky P, Stiehl A. Retrieval of the rat canalicular conjugate export pump Mrp2 is associated with a rearrangement of actin filaments and radixin in bile salt-induced cholestasis. *Eur J Med Res* 2008; **13**: 314-318

118 **Bi GQ**, Morris RL, Liao G, Alderton JM, Scholey JM, Steinhardt RA. Kinesin- and myosin-driven steps of vesicle recruitment for Ca2+-regulated exocytosis. *J Cell Biol* 1997; **138**: 999-1008

119 **Kato Y**, Watanabe C, Tsuji A. Regulation of drug transporters by PDZ adaptor proteins and nuclear receptors. *Eur J Pharm Sci* 2006; **27**: 487-500

120 **Ortiz DF**, Moseley J, Calderon G, Swift AL, Li S, Arias IM. Identification of HAX-1 as a protein that binds bile salt export protein and regulates its abundance in the apical membrane of Madin-Darby canine kidney cells. *J Biol Chem* 2004; **279**: 32761-32770

121 **Gallagher AR**, Cedzich A, Gretz N, Somlo S, Witzgall R. The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton. *Proc Natl Acad Sci USA* 2000; **97**: 4017-4022
122 **Kikuchi S**, Hata M, Fukumoto K, Yamane Y, Matsui T, Tamura A, Yonemura S, Yamagishi H, Keppler D, Tsukita S, Tsukita S. Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. *Nat Genet* 2002; **31**: 320-325
123 **Wang W**, Soroka CJ, Mennone A, Rahner C, Harry K, Pypaert M, Boyer JL. Radixin is required to maintain apical canalicular membrane structure and function in rat hepatocytes. *Gastroenterology* 2006; **131**: 878-884
124 **Kojima H**, Sakurai S, Yoshiji H, Uemura M, Yoshikawa M, Fukui H. The role of radixin in altered localization of canalicular conjugate export pump Mrp2 in cholestatic rat liver. *Hepatol Res* 2008; **38**: 202-210
125 **Wu H**, Parsons JT. Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J Cell Biol* 1993; **120**: 1417-1426
126 **Wang P**, Wang JJ, Xiao Y, Murray JW, Novikoff PM, Angeletti RH, Orr GA, Lan D, Silver DL, Wolkoff AW. Interaction with PDZK1 is required for expression of organic anion transporting protein 1A1 on the hepatocyte surface. *J Biol Chem* 2005; **280**: 30143-30149
127 **Kubitz R**, Huth C, Schmitt M, Horbach A, Kullak-Ublick G, Häussinger D. Protein kinase C-dependent distribution of the multidrug resistance protein 2 from the canalicular to the basolateral membrane in human HepG2 cells. *Hepatology* 2001; **34**: 340-350
128 **Crocenzi FA**, Sánchez Pozzi EJ, Ruiz ML, Zucchetti AE, Roma MG, Mottino AD, Vore M. Ca(2+)-dependent protein kinase C isoforms are critical to estradiol 17beta-D-glucuronide-induced cholestasis in the rat. *Hepatology* 2008; **48**: 1885-1895
129 **Beuers U**, Probst I, Soroka C, Boyer JL, Kullak-Ublick GA, Paumgartner G. Modulation of protein kinase C by taurolithocholic acid in isolated rat hepatocytes. *Hepatology* 1999; **29**: 477-482
130 **Beuers U**, Denk GU, Soroka CJ, Wimmer R, Rust C, Paumgartner G, Boyer JL. Taurolithocholic acid exerts cholestatic effects via phosphatidylinositol 3-kinase-dependent mechanisms in perfused rat livers and rat hepatocyte couplets. *J Biol Chem* 2003; **278**: 17810-17818
131 **Crocenzi FA**, Basiglio CL, Pérez LM, Portesio MS, Pozzi EJ, Roma MG. Silibinin prevents cholestasis-associated retrieval of the bile salt export pump, Bsep, in isolated rat hepatocyte couplets: possible involvement of cAMP. *Biochem Pharmacol* 2005; **69**: 1113-1120
132 **Dombrowski F**, Stieger B, Beuers U. Tauroursodeoxycholic acid inserts the bile salt export pump into canalicular membranes of cholestatic rat liver. *Lab Invest* 2006; **86**: 166-174
133 **Wimmer R**, Hohenester S, Pusl T, Denk GU, Rust C, Beuers U. Tauroursodeoxycholic acid exerts anticholestatic effects by a cooperative cPKC alpha-/PKA-dependent mechanism in rat liver. *Gut* 2008; **57**: 1448-1454
134 **Hayashi H**, Sugiyama Y. 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated bile salt export pumps. *Hepatology* 2007; **45**: 1506-1516
135 **Hayashi H**, Takada T, Suzuki H, Akita H, Sugiyama Y. Two common PFIC2 mutations are associated with the impaired membrane trafficking of BSEP/ABCB11. *Hepatology* 2005; **41**: 916-924

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LIVER CANCER

Inhibitory effect of interferon-α-2b on expression of cyclooxygenase-2 and vascular endothelial growth factor in human hepatocellular carcinoma inoculated in nude mice

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Abstract

AIM: To evaluate the effects of interferon-α-2b (IFN-α-2b) on expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in human hepatocellular carcinoma (HCC) inoculated in nude mice and to study the underlying mechanism of IFN-α-2b against HCC growth.

METHODS: Thirty-two nude mice bearing human HCC were randomly divided into four groups (n = 8). On the 10th day after implantation of HCC cells, the mice in test groups (groups A, B and C) received IFN-α-2b at a serial dose (10 000 IU for group A, 20 000 IU for group B, 40 000 IU for group C sc daily) for 35 d. The mice in control group received normal saline (NS). The growth conditions of transplanted tumors were observed. Both genes and proteins of COX-2 and VEGF were detected by RT-PCR and Western blot. Apoptosis of tumor cells in nude mice was detected by TUNEL assay after treatment with IFN-α-2b.

RESULTS: Tumors were significantly smaller and had a lower weight in the IFN-α-2b treatment groups than those in the control group (P < 0.01), and the tumor growth inhibition rate in groups A, B and C was 27.78%, 65.22% and 49.64%, respectively. The expression levels of both genes and proteins of COX-2 and VEGF were much lower in the IFN-α-2b treatment groups than in the control group (P < 0.01). The apoptosis index (AI) of tumor cells in the IFN-α-2b treatment groups was markedly higher than that in the control group (P < 0.01). Group B had a higher inhibition rate of tumor growth, a lower expression level of COX-2 and VEGF and a higher AI than groups A and C (P < 0.05), but there was no significant difference between groups A and C.

CONCLUSION: The inhibitory effects of IFN-α-2b on implanted tumor growth and apoptosis may be associated with the down-regulation of COX-2 and VEGF expression. There is a dose-effect relationship. The medium dose of IFN-α-2b for inhibiting tumor growth is 20 000 IU/d.

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Key words: Hepatocellular carcinoma; Interferon-α-2b; Cyclooxygenase-2; Vascular endothelial growth factor; Apoptosis

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INTRODUCTION

In recent years, studies have proved the inhibitory effects of interferon-α-2b (IFN-α-2b) on many kinds of tumors[1-3], but the effects of IFN-α-2b on expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in hepatocellular carcinoma (HCC) have not been extensively studied. This study investigated the effects of IFN-α-2b on COX-2 and VEGF expression in human HCC implanted in nude mice and the underlying mechanism of its inhibitory effect on the growth of HCC.

MATERIALS AND METHODS

Reagents

HCC cell line HepG2 was stored in our laboratory. Rabbit anti-human COX-2 polyclonal antibody (Santa Cruz, US) was purchased from Beijing Zhongshan Biotechnology Company, Ltd. Mouse anti-human VEGF polyclonal antibody (Neomarkers, USA), TRIzol and RT-PCR kits were from Jinmei Biotech Company, Ltd. Primers of RT-PCR were synthesized by Alpha Biotechnology Company (Wuhan, China). *In situ* apoptosis detection kits (Boehringer Mannheim, Germany) and ECL chemiluminescence detection kits (Pierce, US) were from Beijing Zhongshan Biotechnology Company, Ltd. IFN-α-2b injection (trade name: Interlong) was from Shenzhen Neptunus Interlong Bio-tech Holdings Company, Ltd (China).

Animal model

A transplantation tumor model of human HCC in nude mice was established. In brief, thirty-two 4-6 wk old female BALB/c nu/nu mice, weighing 18-20 g, bred in SPF rooms, were obtained from the Experimental Animal Center of Tongji Medical University, Huazhong University of Science and Technology (Wuhan, China). The concentration of single cell suspension of HepG2 cells was adjusted to 5×10^9/L at the exponential growth phase. The cell viability was kept above 95% assessed by Typan blue exclusion. Then, the cell suspension was inoculated subcutaneouly in the back of nude mice (0.2 mL/each mouse). The standard of tumor formation was defined as its nodus diameter (up to 0.5 cm). The rate of tumor formation was 100% 10 d after inoculation. The mice meeting the standard of tumor formation were randomly divided into 4 groups (n = 8).

Drugs and groups

Each nude mouse meeting the standard of tumor formation in groups A, B and C received 10 000 IU IFN-α-2b, 20 000 IU and 40 000 IU of IFN-α-2b, respectively, once a day for 35 d. Each nude mouse in control group received only 0.1 mL normal saline (NS), once a day for 35 d. Then, the mice were killed. The maximum and minimum diameters of tumor were measured with a sliding caliper and the weight of tumor was recorded. A certain number of samples were stored at -70℃. The rest samples were fixed in 10% formalin, imbedded in paraffin, and cut into serial sections for assay of *in situ* apoptosis. Volume of tumor (V) = ab2/2, inhibition ratio (%) = [(mean tumor weight of control group-mean tumor weight of test group)/ mean tumor weight of control group] × 100%.

RT-PCR assay

Total RNA was extracted with the TRIzol through one-step method, and its concentration and purity were detected using a DNA/RNA detector (Pharmarcia Company, British). cDNA was synthesized by a reverse transcription reaction of 4 μg of total RNA with oligo-$(dt)_{15}$ primers and retroviridase at 45℃ for 60 min, and stored at -20℃.

The sequences of primers used for semi-quantitative PCR are as follows: sense: 5'-CAAGT CCCTGAGCATCTACG-3' and anti-sense: 5'-CA TTCCTACCACCAGCAACC-3' for COX-2; sense: 5'-TTGCTGCTCTACCTCCAC-3' and anti-sense: 5'-AATGCTTTCTCCGCTCTG-3' for VEGF; sense: 5'-CAGAGCAAGAGAGGCAGCCT-3' and anti-sense: 5'-GGATAGCACAGCCTGGATAG-3' for β-actin. PCR conditions were as follows: 30 cycles at 94℃ for 1 min, at 54℃ for 30 s, and at 72℃ for 1 min for COX-2; 27 cycles at 94℃ for 1 min, at 56℃ for 30 s, and at 72℃ for 1 min for VEGF; 30 cycles at 94℃ for 1 min, at 59℃ for 30 s, and at 72℃ for 1 min for β-actin. The lengths of PCR products of COX-2, VEGF and β-actin were 490 bp, 417 bp and 250 bp, respectively. After separated by electrophoresis on 1.5% agarose gel, the bands of PCR products were scanned and a densitometric analysis was performed with a MUVB-20 gel analysis system (Ultralum Company, USA). The relative expression levels of COX-2 mRNA and VEGF mRNA were represented as absorbance ratios (COX-2/β-actin and VEGF/β-actin).

Western blot assay

The protein was extracted from tumor tissue through three-detergent methods. An equal quantity of protein was separated from each sample by electrophoresis on 10% SDS-PAGE gel, and then transferred to a nitrocellulose filter (NC filter) with a semidry transfer cell. The NC filter containing protein samples was incubated in a blocking buffer containing 5% nonfat dry milk for 2 h, then with the first antibody at a dilution of 1:250 in a blocking buffer overnight at 4℃. After washed with 0.1% TBS, the membrane was incubated at room temperature for 1 h with the second antibodies at a dilution of 1:5000 in a blocking buffer, then washed three times with 0.1% TBS, visualized, photographed and fixed through ECL according to its manufacturer's instructions. The absorbance value of protein bands was assessed with an image analysis system.

TUNEL assay

Paraffin-embedded tumor samples were cut into serial 4-5 μm thick serial sections, heated overnight at 58℃, and routinely deparaffinized and rehydrated. After digested by protease K for 15 min, the sections were incubated with TUNEL at 37℃ for 1 h. After digoxin-marked alkaline phosphatase antibody was added, the sections were colored with BCIP/NBT, dehydrated with alcoholic, air dried, and mounted. Positive cells were defined as hyacinthine particles distributing in nuclei. Five high power fields were randomly selected from each section. Results were expressed as mean values. Apoptosis index (AI) = [the number of apoptosis cells/ (the number of apoptosis cells+ the number of un-apoptosis cells)] × 100%.

Table 1 Inhibitory effect of IFN α-2b on transplanted HCC in nude mice (mean ± SD, *n* = 8)

Groups	Dose (IU/d)	Tumor vol (mm^3)	Tumor Wt (mg)	Inhibition ratio (%)
Control	0	3727.11 ± 745.53	1362.32 ± 272.12	
Group A	10000	2082.18 ± 416.43[a]	983.85 ± 193.76[a]	27.78
Group B	20000	1521.43 ± 313.89[a,d]	473.76 ± 92.57[a,d]	65.22
Group C	40000	1806.35 ± 363.71[a]	686.06 ± 138.21[a]	49.64

[a]$P < 0.01$ *vs* control group; [d]$P < 0.05$ *vs* groups A and C.

Table 2 Expression level of COX-2 and VEGF mRNA and protein in transplanted HCC of nude mice after treatment with IFN α-2b (mean ± SD, *n* = 8)

mRNA				Protein			
Groups	Dose (IU/d)	COX-2	VEGF	Groups	Dose (IU/d)	COX-2	VEGF
Control	0	0.59 ± 0.11	0.61 ± 0.15	Control	0	0.75 ± 0.16	0.82 ± 0.18
Group A	10000	0.44 ± 0.09[a]	0.52 ± 0.12[a]	Group A	10000	0.52 ± 0.09[a]	0.61 ± 0.13[a]
Group B	20000	0.12 ± 0.02[a,d]	0.17 ± 0.03[a,d]	Group B	20000	0.23 ± 0.04[a,d]	0.31 ± 0.05[a,d]
Group C	40000	0.26 ± 0.06[a]	0.31 ± 0.07[a]	Group C	40000	0.38 ± 0.07[a]	0.43 ± 0.08[a]

[a]$P < 0.01$ *vs* control group; [d]$P < 0.05$ *vs* groups A and C.

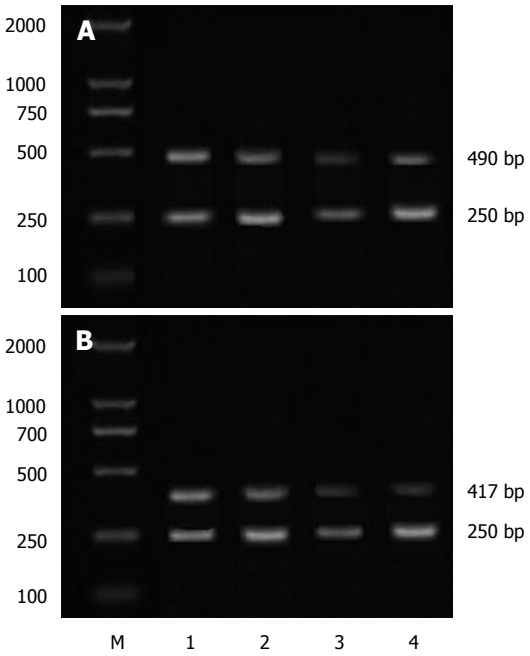

Figure 1 RT-PCR showing expression levels of COX-2 mRNA (A) and VEGF mRNA (B). M: Marker.

Statistical analysis

Data were presented as mean ± SD. SPSS 11.5 was employed to analyze the data. The total difference among groups was analyzed by ANOVA. *Q*-test was used for the comparison between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory effect of IFN-α-2b on transplanted tumors of nude mice

The tumor growth in three IFN-α-2b treatment groups was inhibited at different degrees. The inhibition rate of tumors in groups A, B and C was 27.78%, 65.22% and 49.64%, respectively. The weight and volume of tumors were lower in three IFN-α-2b treatment groups than in control group ($P < 0.01$). The inhibitory effect was the greatest in group B, and significantly different from that in groups A and C ($P < 0.01$), but the difference was not statistically significant between groups A and C (Table 1).

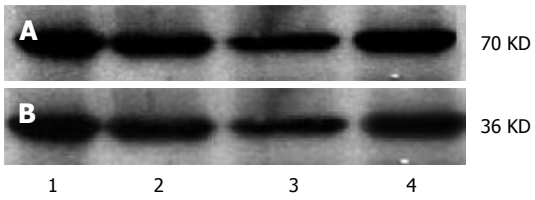

Figure 2 RT-PCR (A) and Western blot (B) showing expression levels of COX-2 and VEGF protein. 1: Control group; 2-4: Groups A, B, and C.

Expression of COX-2 and VEGF mRNA in transplanted tumors of nude mice

RT-PCR showed that the expression levels of COX-2 and VEGF mRNA were lower in three IFN-α-2b treatment groups than in control group ($P < 0.01$). Meanwhile, their expression levels were significantly lower in group B than in groups A and C ($P < 0.05$), but the difference was not statistically significant between groups A and C (Table 2, Figure 1).

Expression of COX-2 and VEGF protein in transplanted tumors of nude mice

Western blot assay showed that the expression levels of COX-2 and VEGF protein were lower in three IFN-α-2b treatment groups than in control group ($P < 0.01$). Statistical analysis revealed similar results of COX-2 and VEGF mRNA in three IFN-α-2b treatment groups (Table 2, Figure 2).

Apoptosis of transplanted tumors of nude mice

TUNEL assay showed that the AI of tumor cells in the

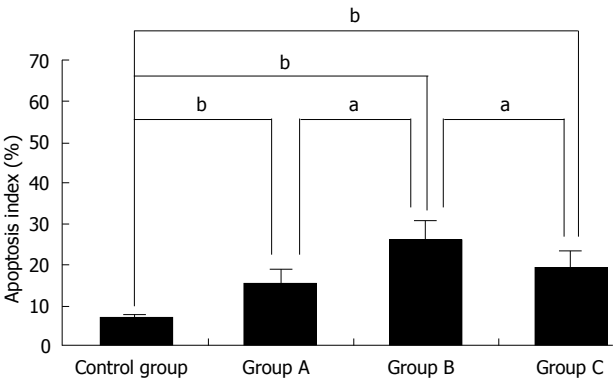

Figure 3 Changes in AI of transplanted HCC cells of nude mice. [a]*P* < 0.05, [b]*P* < 0.01 *vs* groups A and C.

4 groups was 6.39% ± 1.33%, 15.39% ± 3.09%, 25.53% ± 4.98% and 19.28% ± 3.82%, respectively, and was significantly higher in three IFN-α-2b treatment groups than in control group ($P < 0.01$). However, the AI in group B was higher than that in groups A and C ($P < 0.05$), but the difference was not statistically significant between groups A and C (Figures 3-4).

DISCUSSION

HCC is one of the malignant tumors that can lead patients to death[1], but the molecular mechanism of hepatocarcinogenesis has not been well understood. COX can be divided into two subtypes: COX-1 and COX-2. COX-2 is a rate-limiting enzyme of prostaglandin (PG) biosynthesis, encodes for 603 or 604 amino acids including 17 signal peptides of amino acid residues, contains 4 oligoses and 2 bands (molecular weight = 72 KDa and 74 KDa, respectively) in SDS-PAGE. Oshima *et al*[2] showed that the expression of COX-2 is up-regulated in colon tumors and its activity plays an important role in the process of tumorigenesis. Besides, the expression of COX-2 is also higher in liver, breast, tongue, prostatic, cutaneous and mouth dermoid cancers, *etc*[3-5].

It has been recently proved that COX-2 is also expressed in HCC at different degrees and HCC patients with a higher expression level of COX-2 have a higher recurrence rate, early metastasis and worse prognosis[6,7], suggesting that over-expression of COX-2 may be related with a worse prognosis of HCC. The five-year survival rate of HCC patients after operation is only 25%-39%. Some studies indicate that over-expression of COX-2 may promote oncogenesis by regulating the expression of some genes related to cell proliferation and apoptosis[6,7]. The underlying mechanism of COX-2 against oncogenesis may be through influencing many kinds of prostaglandin and thromboxan to bind to their corresponding receptors.

Angiogenesis plays an important role in tumorigenesis and tumor development. If no angiogenesis occurs, the volume of tumor can hardly be larger than 1-2 mm^3. The degree of angiogenesis depends on the rate of angiogenesis and anti-angiogenesis factors in tumor cells and adjacent host cells. The angiogenesis factors mainly include bFGF, VEGF, IL-8, MMP-2, MMP-9, *etc*. Among them, VEGF is most important, and plays a leading role in the growth, generation, recurrence and metastasis of tumors[8-11]. As a dipolymer, glucoprotein has a molecular weight of 34-46 kDa. VEGF exerts its biological effect by binding specifically to Flt1 and KDR/Flk1 exiting on the surface of vascular endothelial cells which belong to RTKⅢ receptors. A recent study showed that angiogenesis and VEGF play an important role in the recurrence and metastasis of HCC[12]. It was reported that the expression of VEGF is up-regulated in HCC and positively correlated with the recurrence and metastasis of HCC after operation[13].

Interferon (IFN) regulates the activity of cytokines which control cell function and replication, and inhibits the activity of tumor cells in many organs and tissues[14-16]. It has been shown that IFN-α decreases the sensitivity of vascular endothelial cells to VEGF and bFGF, as well as the vascularization of tumors[17].

The results of this study indicate that IFN-α-2b could significantly inhibit the growth of HCC in nude mice. The volume and weight of transplanted tumors decreased evidently in IFN-α-2b treatment groups. The expression levels of COX-2 and VEGF mRNA and protein as well as the AI increased significantly in IFN-α-2b treatment groups, indicating that the expression levels of COX-2 and VEGF are closely related with the growth of tumor and apoptosis of tumor cells. IFN-α-2b can down-regulate the expression levels of COX-2 and VEGF, but its mechanism remains unknown. Singer *et al*[18] reported that IFN-α exerts its effects through the NF-κB signal transduction pathway, and inhibitors of NF-κB can decrease the expression level of COX-2. A study about the effect of IFN-α on a signal transduction pathway showed that IFN-α could reduce the activity of NF-κB[19], suggesting that IFN-α-2b can decrease the activity of the NF-κB signal transduction pathway, inhibit the expression of COX-2 and the growth of HCC, and induce apoptosis. It has been shown that IFN-α can down-regulate the expression levels of angiogenesis factors, such as bFGF, VEGF, MMP-9 and IL-8, in different tumors of humans, then inhibit angiogenesis and the growth of tumors[20]. Tsujii *et al*[21] investigated the correlation between COX-2 and angiogenesis of colon carcinoma and found that colon carcinoma cells could secrete angiogenesis factors, such as VEGF, bFGF, TGF-β1 and PDGF, at a high concentration. Inhibitors of COX-2 can significantly depress the expression of angiogenesis factors, indicating that COX-2 can promote the growth of tumors, which is closely related with its effect on promoting angiogenesis. We hold that IFN-α-2b can inhibit HCC growth by inducing apoptosis of tumor cells through down-regulation of COX-2 expression and by inhibiting angiogenesis of tumors through down-regulation of VEGF expression. COX-2 may influence the expression of VEGF. The signal transduction between COX-2 and VEGF still needs to be further studied.

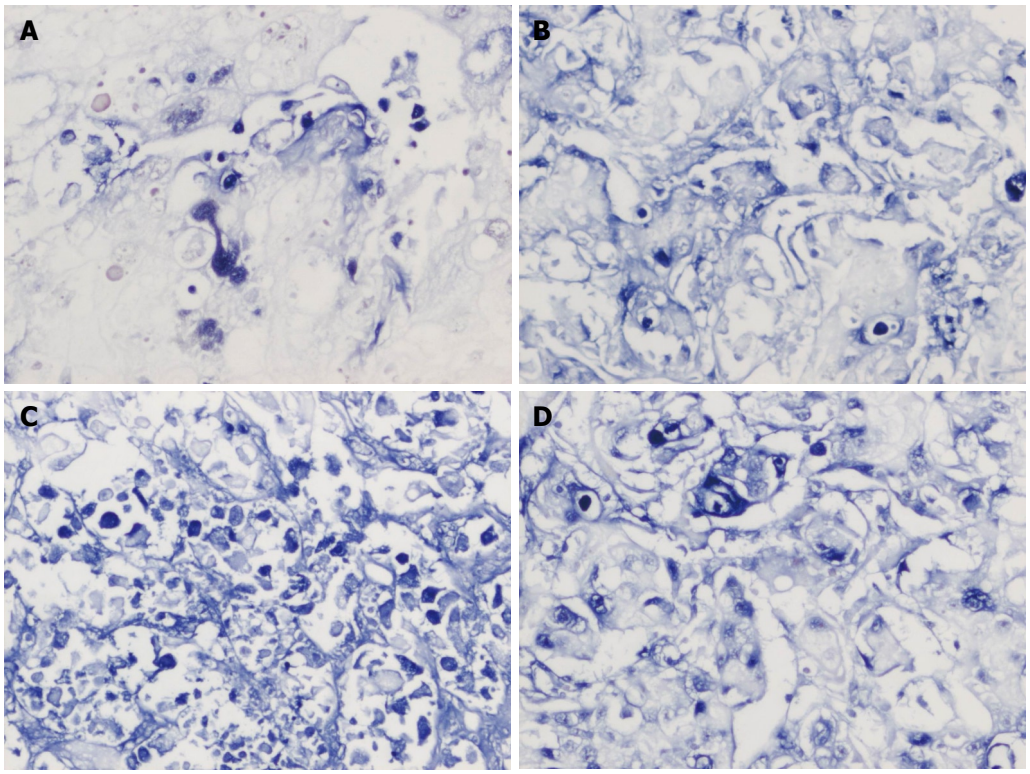

Figure 4 Apoptosis of transplanted HCC (TUNEL × 200). A: Control group; B: Group A; C: Group B; D: Group C.

In this study, the inhibitory effect of IFN-α-2b on HCC growth in nude mice showed a dose-effect relationship. The effect of IFN-α-2b was greater in group B than in groups A and C. However, there was no significant difference between groups A and C. Huang *et al*[22] have reported a similar result in nude mice bearing carcinoma of prostate after treatment with pegylated IFN-α-2b (PEG-IFN-α-2b). At present, no common view is available on the dose of IFN α. Wang *et al*[23] investigated the treatment of nude mice bearing orthotopically transplanted HCC with large doses of IFN-α (3×10^5 U/d and 6×10^5 U/d) and showed that IFN-α could inhibit metastasis and angiogenesis of HCC at a certain degree. Because the signal transduction of IFN is mediated by the JAK/STAT pathway, SOSC protein could down-regulate the signals of IFN by blocking up the JAK/STAT pathway. Although studies are available on the activity of STAT3 inhibited by SOSC3[24-26], further study is needed to show the maximally tolerated dose of IFN-α and its correlation with the signal transduction of STAT1.

It has been shown that nonsteroidal anti-inflammatory drugs (NSAIDs) and INF-α have a synergistic effect on the HCC cell line HepG2. NSAIDs up-regulate the transcription of ISRE, increase the activity and inhibitory effect of IFN-α on tumors, thus providing a new treatment modality for HCC with IFN α[27].

COMMENTS

Background

In recent years, the inhibitory effect of interferon-α-2b (IFN-α-2b) on many tumors has been proved, but its effect on the expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in hepatocellular carcinoma (HCC) has rarely been reported.

Research frontiers

HCC is one of the malignant tumors that can lead to death of patients, but the molecular mechanism of hepatocarcinogenesis remains poorly understood.

Innovations and breakthroughs

Studies have proved the inhibitory effects of interferon-α-2b (IFN-α-2b) on many kinds of tumors. However, the effects of IFN-α-2b on expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in hepatocellular carcinoma (HCC) have not been extensively studied. This study investigated the effects of IFN-α-2b on COX-2 and VEGF expression in human HCC implanted in nude mice and the underlying mechanism of its inhibitory effect on the growth of HCC.

Applications

This study investigated the effects of IFN-α-2b on COX-2 and VEGF expression in human HCC implanted in nude mice and the underlying mechanism of its inhibitory effect on the growth of HCC. The results show that IFN-α-2b can be used as an effective agent in the treatment of HCC.

Peer review

The study is well designed. The results of this study are interesting and seem to be reliable.

REFERENCES

1 **Chen CJ**, Yu MW, Liaw YF. Epidemiological characteristics and risk factors of hepatocellular carcinoma. *J Gastroenterol Hepatol* 1997; **12**: S294-S308
2 **Oshima M**, Murai N, Kargman S, Arguello M, Luk P, Kwong E, Taketo MM, Evans JF. Chemoprevention of intestinal polyposis in the Apcdelta716 mouse by rofecoxib, a specific cyclooxygenase-2 inhibitor. *Cancer Res* 2001; **61**: 1733-1740
3 **Hussain T**, Gupta S, Mukhtar H. Cyclooxygenase-2 and prostate carcinogenesis. *Cancer Lett* 2003; **191**: 125-135
4 **Peng JP**, Su CY, Chang HC, Chai CY, Hung WC. Overexpression of cyclo-oxygenase 2 in squamous cell carcinoma of the hypopharynx. *Hum Pathol* 2002; **33**: 100-104
5 **Sung YK**, Hwang SY, Kim JO, Bae HI, Kim JC, Kim MK. The correlation between cyclooxygenase-2 expression and hepatocellular carcinogenesis. *Mol Cells* 2004; **17**: 35-38
6 **Shiota G**, Okubo M, Noumi T, Noguchi N, Oyama K, Takano Y, Yashima K, Kishimoto Y, Kawasaki H.

Cyclooxygenase-2 expression in hepatocellular carcinoma. *Hepatogastroenterology* 1999; **46**: 407-412
7 **Koga H**, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, Nakashima Y, Nakashima O, Kojiro M, Kurohiji T, Sata M. Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation. *Hepatology* 1999; **29**: 688-696
8 **Folkman J**. Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. *N Engl J Med* 1995; **333**: 1757-1763
9 **Fidler IJ**, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 1994; **79**: 185-188
10 **Hanahan D**, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; **86**: 353-364
11 **Dvorak HF**, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995; **146**: 1029-1039
12 **Yancopoulos GD**, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature* 2000; **407**: 242-248
13 **El-Assal ON**, Yamanoi A, Soda Y, Yamaguchi M, Igarashi M, Yamamoto A, Nabika T, Nagasue N. Clinical significance of microvessel density and vascular endothelial growth factor expression in hepatocellular carcinoma and surrounding liver: possible involvement of vascular endothelial growth factor in the angiogenesis of cirrhotic liver. *Hepatology* 1998; **27**: 1554-1562
14 **Baron S**, Dianzani F. The interferons: a biological system with therapeutic potential in viral infections. *Antiviral Res* 1994; **24**: 97-110
15 **Hertzog PJ**, Hwang SY, Kola I. Role of interferons in the regulation of cell proliferation, differentiation, and development. *Mol Reprod Dev* 1994; **39**: 226-232
16 **Gutterman JU**. Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci USA* 1994; **91**: 1198-1205
17 **Dinney CP**, Bielenberg DR, Perrotte P, Reich R, Eve BY, Bucana CD, Fidler IJ. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. *Cancer Res* 1998; **58**: 808-814
18 **Singer CA**, Baker KJ, McCaffrey A, AuCoin DP, Dechert MA, Gerthoffer WT. p38 MAPK and NF-kappaB mediate COX-2 expression in human airway myocytes. *Am J Physiol Lung Cell Mol Physiol* 2003; **285**: L1087-L1098
19 **Manna SK**, Mukhopadhyay A, Aggarwal BB. IFN-alpha suppresses activation of nuclear transcription factors NF-kappa B and activator protein 1 and potentiates TNF-induced apoptosis. *J Immunol* 2000; **165**: 4927-4934
20 **Slaton JW**, Karashima T, Perrotte P, Inoue K, Kim SJ, Izawa J, Kedar D, McConkey DJ, Millikan R, Sweeney P, Yoshikawa C, Shuin T, Dinney CP. Treatment with low-dose interferon-alpha restores the balance between matrix metalloproteinase-9 and E-cadherin expression in human transitional cell carcinoma of the bladder. *Clin Cancer Res* 2001; **7**: 2840-2853
21 **Tsujii M**, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998; **93**: 705-716
22 **Huang SF**, Kim SJ, Lee AT, Karashima T, Bucana C, Kedar D, Sweeney P, Mian B, Fan D, Shepherd D, Fidler IJ, Dinney CP, Killion JJ. Inhibition of growth and metastasis of orthotopic human prostate cancer in athymic mice by combination therapy with pegylated interferon-alpha-2b and docetaxel. *Cancer Res* 2002; **62**: 5720-5726
23 **Wang L**, Wu WZ, Sun HC, Wu XF, Qin LX, Liu YK, Liu KD, Tang ZY. Mechanism of interferon alpha on inhibition of metastasis and angiogenesis of hepatocellular carcinoma after curative resection in nude mice. *J Gastrointest Surg* 2003; **7**: 587-594
24 **Darnell JE Jr**. STATs and gene regulation. *Science* 1997; **277**: 1630-1635
25 **Alexander WS**, Starr R, Fenner JE, Scott CL, Handman E, Sprigg NS, Corbin JE, Cornish AL, Darwiche R, Owczarek CM, Kay TW, Nicola NA, Hertzog PJ, Metcalf D, Hilton DJ. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* 1999; **98**: 597-608
26 **Suzuki A**, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, Akira S, Matsumoto S, Toyonaga A, Sata M, Yoshimura A. CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. *J Exp Med* 2001; **193**: 471-481
27 **Giambartolomei S**, Artini M, Almerighi C, Moavero SM, Levrero M, Balsano C. Nonsteroidal anti-inflammatory drug metabolism potentiates interferon alfa signaling by increasing STAT1 phosphorylation. *Hepatology* 1999; **30**: 510-516

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BASIC RESEARCH

Failure of P-selectin blockade alone to protect the liver from ischemia-reperfusion injury in the isolated blood-perfused rat liver

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Abstract

AIM: To determine if blockade of P-selectin in the isolated blood-perfused cold *ex vivo* rat liver model protects the liver from ischemia-reperfusion injury.

METHODS: The effect of P-selectin blockade was assessed by employing an isolated blood-perfused cold *ex vivo* rat liver with or without P-selectin antibody treatment before and after 6 h of cold storage in University of Wisconsin solution.

RESULTS: In our isolated blood-perfused rat liver model, pre-treatment with P-selectin antibody failed to protect the liver from ischemia-reperfusion injury, as judged by the elevated aspartate aminotransferase activity. In addition, P-selectin antibody treatment did not significantly reduced hepatic polymorphonuclear leukocyte accumulation after 120 min of perfusion. Histological evaluation of liver sections obtained at 120 min of perfusion showed significant oncotic necrosis in liver sections of both ischemic control and P-selectin antibody-treated groups. However, total bile production after 120 min of perfusion was significantly greater in P-selectin antibody-treated livers, compared to control livers. No significant difference in P-selectin and ICAM-1 mRNAs and proteins, GSH, GSSG, and nuclear NF-κB was found between control and P-selectin antibody-treated livers.

CONCLUSION: In conclusion, we have shown that blockade of P-selectin alone failed to reduced polymorphonuclear leukocyte accumulation in the liver and protect hepatocytes from ischemia-reperfusion injury in the isolated blood-perfused cold-*ex vivo* rat liver model.

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Key words: P-selectin; Ischemia-reperfusion; Antibody-blockade; Liver; Rat

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INTRODUCTION

Ischemia-reperfusion (I/R)[3] injury has been shown to play a major role in clinical and experimental hemorrhagic shock, organ resection, and transplantation[1-5]. The inflammatory component of I/R injury is mediated by pro-inflammatory cytokines such as TNF-α and IL-1β, and cellular adhesion molecules such as β2-integrins, ICAM-1, VCAM-1, and members of the selectin family, P-, E-, and L-selectin[6-8]. The sequence of events currently enjoying the most popularity as the mechanism responsible for I/R injury of the liver is: (1) KC are activated following I/R[9]; (2) During early reperfusion (0-2 h), KC are further activated by complement and produce significant vascular oxidative stress[10]; (3) KC also produce pro-inflammatory cytokines and chemokines, which is dependent on the activation of the redox-sensitive transcription factor NF-κB[11]. Activated hepatocytes and endothelial cells also produce reactive oxygen species (ROS) and contribute to the liver cytokine-chemokine milieu; (4) Cytokine mediated induction of adhesion molecules such as P- and E-selectins, ICAM-1, and VCAM-1 on the liver endothelium occur during reperfusion; (5)

PMNs accumulate in the liver as a result of P- and E-selectin-mediated rolling and margination on the liver endothelium, followed by ICAM-1-dependent firm adhesion. Although PMNs accumulate in the liver during early reperfusion, they do not contribute to liver injury until the latter phase (6-24 h) of I/R injury[10,12,13]; and (6) PMNs transmigrate to the liver parenchyma *via* ICAM-1 and VCAM-1, bind to hepatocytes *via* ICAM-1/β2-integrins (CD11b/CD18), and engage in a sustained production of ROS to produce intracellular oxidative stress in hepatocytes and cell death[14-17].

Following I/R of several organs or tissues, a general mechanism of selectin-dependent rolling of PMNs followed by firmer adhesion to endothelial cells by integrins and ICAM-1 is applicable to their vasculature (heart, lung, intestine, and cremaster muscle). Accordingly, numerous studies reported that anti-P-selectin therapy afforded protection to the liver from I/R injury[18-21]. However, this general mechanism may not be applicable to the liver[13,14]. Numerous reports suggest that P-selectin attenuates I/R injury of the liver by mediating the recruitment of PMNs[18-20], while other reports minimize its role in liver I/R injury and its role in recruiting PMNs in the inflamed liver vasculature[21-26]. Furthermore, hepatic PMNs accumulation, mediated by P-selectin expressed on endothelial cells of postsinusoidal venules, might not contribute significantly to liver injury, because there is no experimental evidence supporting extravasation of these neutrophils to the liver parenchyma[23,26]. In addition, a recent report by Kubes et al. suggest that the protective effect observed in the liver with anti-P-selectin therapy may be mostly secondary to the anti-P-selectin therapy of accompanying intestinal I/R injury[27].

If the above scenario is to hold, then blockade of P-selectin should prevent or attenuate I/R injury, at least during the latter phase of I/R injury in the warm in vivo liver model. Therefore, to investigate if P-selectin blockade alone protects the liver from I/R injury, we employed an antibody to P-selectin and a cold-*ex vivo* I/R rat liver model. The present study demonstrates that while anti-P-selectin treatment may increase total bile flow in livers subjected to I/R, it failed to protect hepatocytes in the isolated blood-perfused rat liver model.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250-350 g) were purchased Charles Rivers, Houston, TX). All an imals used in this study received a nutritionally balanced rodent diet, water ad libitum, and were cared for according to NIH guidelines.

Isolated-Perfused-Rat-Liver (IPRL) model

In brief, animals were anaesthetized with Nembutal (50-60 mg/kg bd. wt., ip, Sigma-Aldrich, St. Louis, MO), and under aseptic conditions, a laparotomy performed to access the liver for mobilization. Livers were carefully isolated from male Sprague-Dawley rats under Nembutal anesthesia after cannulation of the portal vein, common bile duct, and suprahepatic vena cava, while constantly perfused with oxygenated Krebs-Hensleit buffer (pH 7.4) *via* the portal vein[28]. Immediately after isolation, control and treated livers were flushed with 10 mL of pristine UW solution, and stored at 4℃ for 6 h. Livers in the treated group received an additional flush of 1 mL of UW solution containing 420 μg of P-selectin Ab (CD62P, Cat.#553716, PharMingen, San Diego, CA) *via* the portal vein before cold-*ex vivo* ischemia (storage) and immediately before perfusion. This antibody has been show to inhibit the binding of neutrophils to rat P-selectin in both in vitro and in vivo studies. Control livers were also flushed with 1 mL of pristine UW solution immediately before perfusion. At the end of cold storage, livers were perfused with syngenic rat blood (diluted with Krebs-Hensleit buffer (pH 7.4) to a hematocrit of 12%, total volume 100 mL) in a re-circulating perfusion system using a fully-jacketed isolated-perfusion-rat-liver apparatus (RGT #130003, Radnoti Glass Technology, Inc., Monrovia, CA) for 120 min, as previously described[10]. Prior to perfusion, the perfusion apparatus was primed with blood perfusate at 37℃. Oxygenation was done with a membrane-oxygenating chamber (PO_2 held > 250 mm Hg) monitored with inline-digital pressure transducer. Portal vein perfusate flow was continually adjusted to maintain portal pressures between 18 and 23 mm Hg, and monitored with inline-digital pressure transducer. Temperature, pH, and oxygen level were maintained throughout each experiment. Liver sections (snap-frozen in liquid nitrogen), blood perfusate, and bile (collected in pre-weighed eppendorf tubes) were collected every 30 min during perfusion. At the end of each experiment, sections of the liver were snap-frozen or placed in buffered-formalin, for blinded-histological evaluation of hematoxylin and eosin (HE) stained liver sections by a pathologist and determination of PMNs accumulation in the liver.

Plasma aspartate aminotransferase activity

Plasma AST activity was determined with a commercially available kit (#DG158K-U, Sigma Diagnostics, St. Louis, MO).

Histological analysis of liver injury

HE sections from formalin-fixed liver tissues obtained from sham-control and P-selectin Ab-treated livers were randomly selected and blindly analyzed for the degree of necrosis, hepatocellular vacuolization, glycogen depletion, zonal variations, and sinusoidal congestion, as measures of hepatic injury.

Polymorphonuclear leukocyte (PMNs) accumulation in the liver

PMNs accumulation in rat livers during perfusion was determined in formalin-fixed paraffin sections of the

liver obtained at each perfusion-sampling time point as previously described[29]. A commercially available kit (91-C, Sigma-Aldrich, St. Louis, MO) was used to stain for sinusoidal-sequestrated PMNs using the well-established Naphthol AS-D Chloroacetate esterase procedure, according to the manufacturer's directions. At least four random sections from each group were analyzed by viewing (blindly) fifty random high power fields (HPF, × 40) on each section. Results were expressed as number of PMNs/50HPF.

RT-PCR analysis of Liver P-selectin and ICAM-1 mRNAs

Total RNA was extracted from liver tissue using an UltraSpec Total RNA Isolation Kit (#BL-10050, Biotecx Laboratories Inc., Houston, TX). Complementary DNA (cDNA) was transcribed with 4 μg of total RNA, random hexamers, and a SuperScript Ⅱ Preamplification System (#18089-011, GIBCO BRL, Life Technologies, Grand Island, New York) according to the manufacturer's protocol. Using specific primers for p-selectin ICAM-1, and GAPDH, their cDNAs were amplified by polymerase chain reaction (PCR) under the following conditions: P-selectin and ICAM-1 (35 cycles, 94℃ for 60 s, 56℃ for 60 s, and 72℃ for 120 s), and GAPDH, (28 cycles, 94℃ for 60 s, 52℃ for 60 s, and 72℃ for 60 s). PCR reaction primers (Sigma-Genosys, Woodlands, TX) used were as follows: P-selectin forward primer (5'-TGTATCCAGCCTCTTGGGCATTCC-3') and P-selectin reverse primer (5'-TGGGACAGGAAGTGA TGTTACACC-3') to give an 350-bp product; ICAM-1 forward primer (5'-AGGTGTGATATCCGGTAG-3') and ICAM-1 reverse primer (5'-TGGGACAGGAA GTGATGTTACACC-3') to give an 595-bp product; GAPDH forward primer (5'-GCCAAGTATGACAT CAA-3') and GAPDH reverse primer (5'-CCATATT CATTGTCATACCA-3') to give a 203-bp product. All PCR products were electrophoresed on a 2% agarose gel (Fisher Scientific, Fair Lawn, NJ). Bands were visualized by post staining for 30 min with GelStar Nucleic Acid Gel Stain (FMC Bioproducts, Rockland, MA), and photographed. Photographs were digitized and evaluated as stated above. The relative expression of P-selectin and ICAM-1 messenger RNAs (mRNA) were assessed by taking the ratio of the intensity of the DNA bands of P-selectin and ICAM-1 to GAPDH band, and expressed as arbitrary units. To ensure an equal amount of RNA was used for all samples, RNA concentration was determined spectrophotometrically, and its integrity evaluate on agarose gel. DNA bands were digitized (Corel Photohouse 2.0, Ontario, Canada) and evaluated using an image analysis software (Scion Image Beta 3b, NIH Image modified for Windows by Scion Corporation, Frederick MD).

Western blot analysis of liver P-selectin and ICAM-1 proteins

Homogenates and supernatants of liver samples were prepared as described by Vural *et al*[30]. Proteins (P-selectin (14 μg) and ICAM-1 (27 μg)) in liver supernatants were separated on 6% NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Schleicher and Schuel, Dassel). Equal transfer to membranes was confirmed by staining the membranes with Ponceau S (Aldrich-Sigma, St. Louis, MO). P-selectin bands were detected on membranes by incubating with an anti-P-selectin-specific rabbit primary antibody diluted 1:100 (CD62P, PharMingen, San Diego, CA). A horseradish peroxidase-conjugated anti-rabbit secondary antibody (sc-2350, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000 and an enhanced chemiluminescence (ECL) kit (Amersham Life Sciences, Piscataway, NJ) were used to visualize bands. ICAM-1 was detected on membranes by incubating with an anti-ICAM-1-specific mouse primary antibody (MCA1333R, Serotec, Raleigh, NC) diluted 1:50. A horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Life Sciences, Piscataway, NJ) diluted 1:1000 was used to reveal ICAM-1 as described above. To ensure equal loading and normalize Western-blot bands of P-selectin and ICAM-1, membranes were also probed for β-actin. Membranes were immunoblotted for β-actin with an affinity purified goat polyclonal antibody diluted 1:500 (sc-1616, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a horseradish peroxidase-conjugated anti-goat secondary antibody (sc-2350, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (diluted 1:1000). Actin bands were visualized with a commercially available ECL kit, as stated above.

Liver total (GSH + GSSG) and oxidized (GSSG) glutathione levels

Liver GSH + GSSG and GSSG levels were determined by the method of Tietze[31], as previously described by Jaeschke *et al*[14].

Liver nuclear factor-kappa B (NF-κB) activation

Activation of the redox-sensitive transcription factor NF-κB in control and P-selectin Ab-treated livers was measured using a commercially available ELISA kit (Trans-AM™ NF-κB p65 Transcription Factor Assay Kit, Active Motif, Carlsbad, CA). The assay was performed according to the manufacturer's procedure and as described by Renard *et al*[32]. Nuclear proteins were extracted according to the procedure of Osarogiagbon *et al*[33]. Approximately 100 mg of snap-frozen liver tissue was homogenized in 0.4 mL of cold TM buffer (10 mmol/L Tris-HCl, 1 mmol/L $MgCl_2$ (pH 7.0), containing complete™ protease inhibitors (Roche Diagnostics Corp., IN). Homogenates were centrifuged at 2000 r/min for 30 s, and the supernatant mixed with 200 μl of lysis buffer, incubated at 4℃ for 5 min, and centrifuged at 5000 r/min for 10 min. The nuclear pellets were reconstituted with lysis buffer, and centrifuged at 14000 r/min for 20 s at 4℃. Nuclear protein extract (15 μg) of each sample was used to assay for NF-κB activation. To ensure the specificity of the assay, the wild-type consensus oligonucleotide provided by the manufacturer served as a competitor to NF-κB binding.

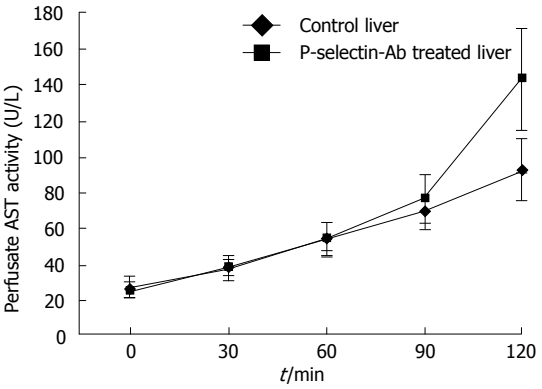

Figure 1 Perfusate aspartate aminotransferase (AST) activity in isolated-blood-perfused control and P-selectin-Ab treated rat livers after 6 h of cold ischemia.

Protein concentration

All protein concentrations were determined according to the method of Lowry *et al*[34].

Statistical analysis

Data were analyzed by unpaired Student's *t*-test or ANOVA. After ANOVA, results were subsequently subjected to Tukey's or Student-Newman Keuls non-parametric test to determined significant differences between groups.

RESULTS

Plasma AST activity, histologic analysis, and PMNs accumulation in the cold ex vivo -perfused liver

No significant difference in AST activity was found between control and P-selectin Ab-treated livers throughout the perfusion period (Figure 1). In addition, after 120 min of perfusion, similar degrees of point necrosis (solid arrows, Figure 2A, B) and inflammation (arrow heads, Figure 2C, D) were found in both cold *ex vivo*-perfused control and P-selectin Ab-treated livers. Similarly, no significant difference in hepatic PMNs accumulation was found between control and P-selectin Ab-treated livers after 120 min of perfusion (Figure 3).

Bile production in cold ex vivo-perfused liver

No significant difference in bile flow was found between control and P-selectin Ab-treated livers at individual sampling time-points throughout the perfusion period (data not shown). However, total bile production after 120 min of perfusion was significantly greater in P-selectin Ab-treated livers, compared to control (Figure 4).

RT-PCR analysis of P-selectin and ICAM-1 mRNAs in the cold-ex vivo perfused liver

No significant difference in P-selectin mRNA and protein was found between cold-*ex-vivo* perfused control and P-selectin Ab-treated livers throughout the entire perfusion period (Figure 5A, B). In contrast, a significant reduction in ICAM-1 mRNA was found between cold-*ex-vivo* perfused control and P-selectin Ab-treated livers at 60 min of perfusion (Figure 5A). However, although a corresponding reduction in ICAM-1 protein was found, it did not reach statistical significance (Figure 5B). No significant difference in ICAM-1 mRNA and protein was found between cold *ex-vivo*-perfused control and P-selectin Ab-treated livers at all other time points of the perfusion period (Figure 5).

Western blot analysis of P-selectin and ICAM-1 proteins in the cold-ex-vivo perfused liver

No significant difference in P-selectin and ICAM-1 proteins was found between cold *ex-vivo*-perfused control and P-selectin Ab-treated livers throughout the entire perfusion period (Figure 6A, B). Although P-selectin expression at 90 min of perfusion is clearly greater in control livers compared to Ab-treated livers, the intra-group variance precluded statistical significance.

GSH + GSSG and GSSG levels in the cold-ex vivo-perfused liver

No significant difference in GSH + GSSG and GSSG levels was found between cold *ex-vivo*-perfused control and P-selectin Ab-treated livers throughout the entire perfusion period (Figure 7).

NF-κB activation in cold ex vivo-perfused liver

No significant difference in NF-κB activation was found between cold *ex-vivo*-perfused control and P-selectin Ab-treated livers throughout the entire perfusion period (Figure 8).

DISCUSSION

Our study demonstrates that antibody-blockade of P-selectin alone failed to protect the rat liver from I/R injury in the IPRL model. However, as stated earlier, considerable evidence exist that suggests that P-selectin plays a major role in I/R injury[18-21]. Existing reports also suggest that P-selectin mediates I/R injury of the liver by mediating initial rolling and margination of PMNs in the liver vasculature[18-20]. However, other reports minimize its role in liver I/R injury, and its role in recruiting PMNs in the inflamed liver vasculature[21-26].

At present, no convincing evidence exist for P-selectin expression on the mouse, human, and rat sinusoidal endothelia under normal and inflammatory conditions[22,35,36], although a more recent study reported immunohistochemical evidence of P-selectin protein expression on the rat liver sinusoid endothelium after cold storage and orthotopic liver transplantation[37]. In the present study, the accumulation of PMNs in livers after P-selectin blockade is probably due to physical trapping, resulting from the swelling of sinusoidal cells (e.g. endothelial and Kupffer cells), direct vasoconstriction, reduced deformability of PMNs exposed to activated complement factors, and the pre-existing intimacy (endothelium massaging) PMNs share with the sinusoidal endothelium, compared to the postsinusoidal endothelium. Once PMNs are slowed in the sinusoids, firm adhesion and transmigration may be

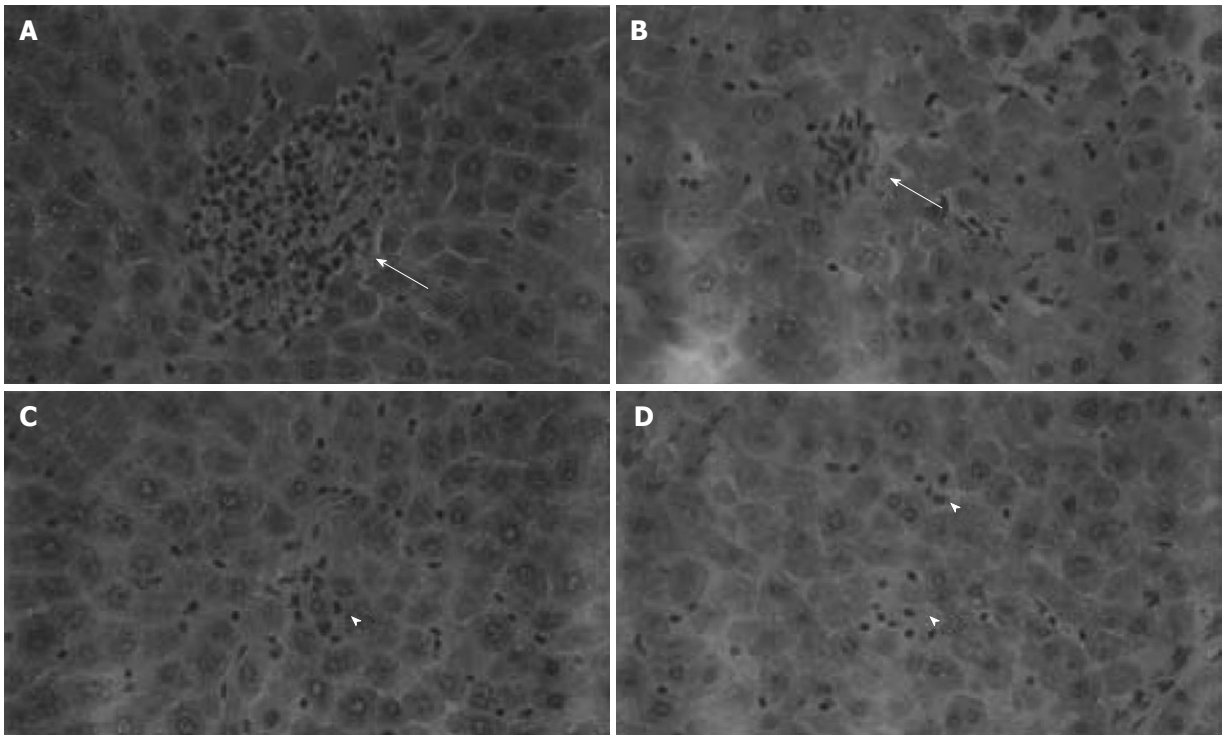

Figure 2 Histological analysis of isolated-blood-perfused control and P-selectin Ab-treated rat liver sections at 120 min perfusion after 6 h of cold ischemia (HE × 400). A, B: Point necroses in livers of control and P-selectin Ab-treated livers at 120 min perfusion (solid arrows); C, D: Inflammation in both control and P-selectin Ab-treated livers at 120 min of perfusion (arrow heads).

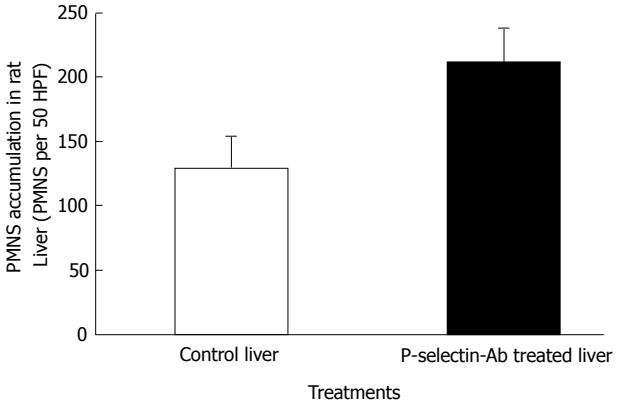

Figure 3 Accumulation of polymorphonuclear leukocytes (PMNs) in isolated-blood-perfused control and P-selectin Ab-treated rat livers at 120 min perfusion after 6 h of cold ischemia.

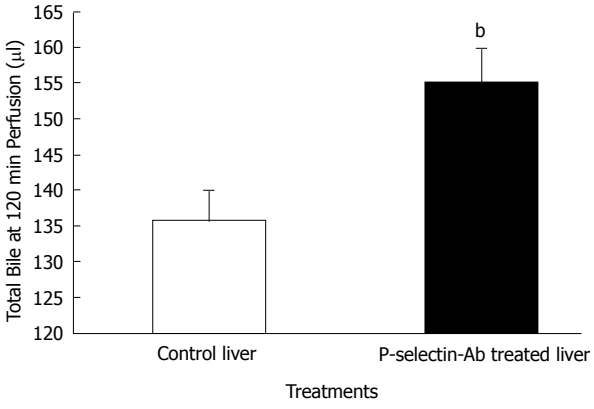

Figure 4 Bile production by isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia. $^{b}P = 0.009$ *vs* control liver.

mediated by ICAM-1/β_2-integrins and VCAM-1/β_1-integrins[23,38]. Alternatively, other, as yet unidentified, adhesion molecules may play a major role in the sequestration and transmigration of PMNs in liver sinusoids.

P-selectin blockade failed to protect the liver from I/R injury in our cold *ex vivo* model, as judged by the time dependent increase in AST activity, PMNs accumulation, and the similar histological-injury pattern found in control and P-selectin Ab-treated livers. Our findings are not in agreement with results of other studies that reported protection of the liver in cold *ex vivo* model with P-selectin blockade alone using the P-selectin ligands sPSGL-1and rPSGL-Ig[19,39] or an antibody to PSGL-1[40]. However, P-selectin blockade did enhance total bile production by 120 min of perfusion, which agrees with earlier reports[39,40]. Exactly how P-selectin blockade enhanced total bile production remains unclear.

P-selectin and ICAM-1 have been reported to be primary mediators of PMNs sequestration in the liver following I/R[19,20,38,41]. However, in this study, P-selectin blockade had little or no effect on P-selectin and ICAM-1 mRNA and protein levels except for the significant decrease in ICAM-1 mRNA found at 60 min perfusion, which had a concomitant decrease in ICAM-1 protein that did not reach statistical significance. These results support our finding that P-selectin blockade did not significantly alter PMNs accumulation in the liver at 120 min perfusion. In fact, P-selectin blockade caused

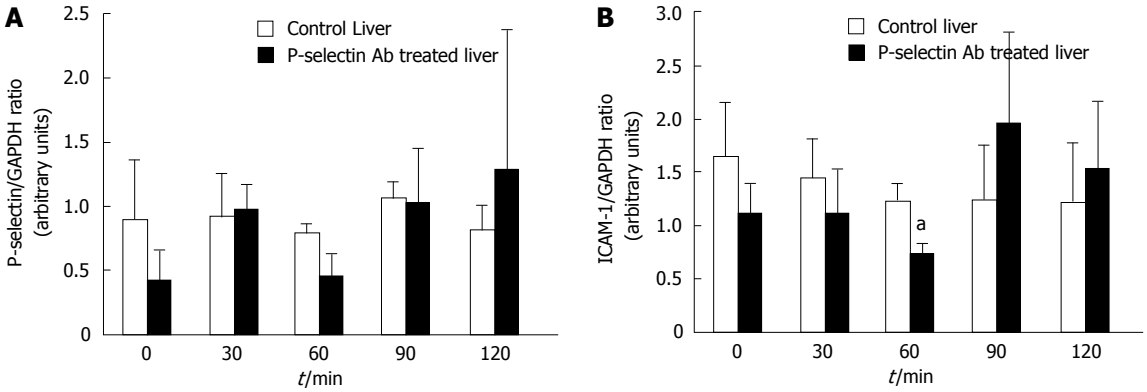

Figure 5 Semi-quantitative RT-PCR analysis of P-selectin and ICAM-1 mRNA levels in isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia. $^{a}P < 0.05$ *vs* control liver.

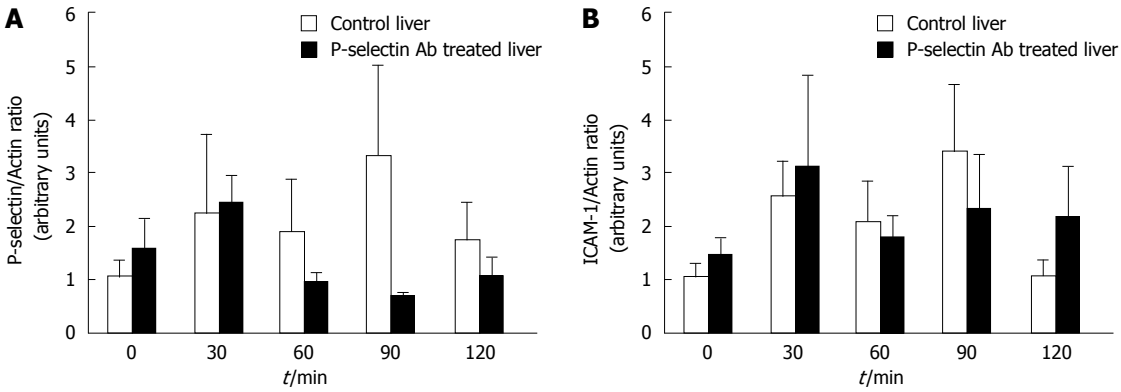

Figure 6 Western blot analysis of P-selectin and ICAM-1 protein levels in isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia.

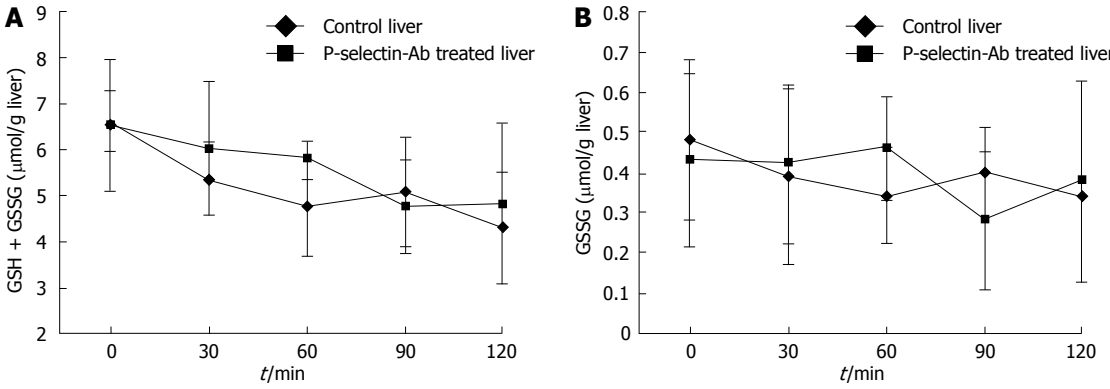

Figure 7 Reduced (GSH + GSSG) and oxidized (GSSG) glutathione levels in isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia.

an increase in PMNs accumulation in the liver, but not to a significant level. In addition, numerous studies have reported that ICAM-1 mediates firm adhesion and transmigration of PMNs in the liver following an inflammatory stimulus[42-47]. However, some studies have questioned the absolute role of ICAM-1 in PMNs sequestration in hepatic vasculature[21,48-52]. Furthermore, although the adhesion molecules ICAM-1 and VCAM-1 are expressed on cells lining the sinusoids, antibodies to selectins, integrins, and CAMs have all failed to prevent accumulation of PMNs in the liver sinusoids.

It is well documented that GSH plays a protective role in liver I/R injury[1,53-57]. GSH can react with ROS such as hydrogen peroxide, peroxynitrite, and hypochlorous acid generated by KC and PMNs. We measured liver GSH and GSSG levels in our cold *ex vivo* model and found no significant difference with P-selectin-blockade treatment. If significant oxidative stress occurred during reperfusion, an increase in liver GSSG should have occurred[1,53]. Although the major oxidative stress observed during perfusion is in the liver vasculature[1,53], we did not measure GSSG levels in the

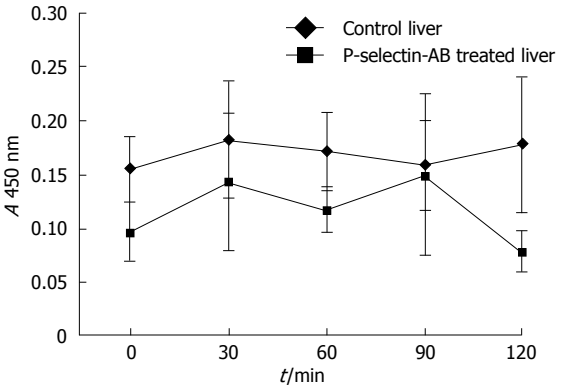

Figure 8 ELISA analysis of nuclear p65 as a measure of liver NF-κB activation in isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia.

perfusate. Nevertheless, recent evidence from livers reperfused after cold storage has shown that hepatocytes may also be a source oxidative changes capable of impairing liver function during reperfusion[58]. Therefore, in this study we should have detected any significant change in GSH or GSSG as an intracellular oxidative stress that occurred during reperfusion. The lack of significant change in liver total GSH and GSSG in our IPRL model may be taken as the absence/attenuation of oxidative stress in the liver[53]. An alternate explanation for our failure to detect liver oxidative stress is that the above study used a more sensitive fluorescence detection compared to the colorimetric method employed in this study.

NF-κB activation in the liver following I/R has been reported[42,43,59]. Its activation has been reported as a requirement for I/R-dependent TNF-α induction in the liver[59]. The inflammatory response following I/R of the liver is primarily mediated by cytokines (e.g. TNF-α, IL-1β) and adhesion molecules P-selectin and ICAM-1, and VCAM-1. Induction of all the above inflammatory mediators requires activation of the transcription factor NF-κB. In addition, existing evidence suggest that redox-sensitive transcription factors NF-κB activation mediates the gene expression of pro-inflammatory cytokines such as TNF-α and IL-1β[11]. Therefore, this study addressed the activation of liver NF-κB in our cold *ex vivo* model, and failed to detect any significant reduction in liver NF-κB activation with P-selectin blockade throughout the perfusion period. This finding supports our results for P-selectin and ICAM-1 expression, with the exception of ICAM-1 mRNA levels at 60 min perfusion. A corresponding decrease in NF-κB activation was found at 60 min perfusion with P-selectin blockade, but did not achieve statistical significance.

Although most studies that characterized the benefit of P-selectin blockade in liver I/R injury used monoclonal antibodies or recombinant P-selectin ligands, in the present study we used a polyclonal antibody. Our use of a polyclonal antibody is not unusual, since other investigators have employed polyclonal antibodies to investigate blockade of mediators involved in I/R injury[60]. It is likely that our results are not in agreement with earlier studies because we employed a polyclonal antibody and at a different dose. Alternately, the antibody may have reacted with activated platelets and complement factors, as was later found for the monoclonal antibody PB1.3 used in the initial report that reported that P-selectin blockade alone protected the liver form I/R injury[20]. Nonetheless, while the general mechanism of selectin-dependent rolling of PMNs followed by firmer adhesion to endothelial cells by integrins and ICAM-1 is applicable to the vasculature of some organs and tissues (heart, lung, intestine, and cremaster muscle), this might not be the case for the entire vasculature of the liver[23,24].

In summary, this study demonstrates that while P-selectin blockade alone increased total bile flow in the IPRL model, it failed to protect the liver from I/R injury.

REFERENCES

1 **Jaeschke H**, Farhood A. Kupffer cell activation after no-flow ischemia versus hemorrhagic shock. *Free Radic Biol Med* 2002; **33**: 210-219
2 **Seekamp A**, Jochum M, Ziegler M, van Griensven M, Martin M, Regel G. Cytokines and adhesion molecules in elective and accidental trauma-related ischemia/reperfusion. *J Trauma* 1998; **44**: 874-882
3 **Kim YI**, Song KE, Ryeon HK, Hwang YJ, Yun YK, Lee JW, Chun BY. Enhanced inflammatory cytokine production at ischemia/reperfusion in human liver resection. *Hepatogastroenterology* 2002; **49**: 1077-1082
4 **Clavien PA**, Yadav S, Sindram D, Bentley RC. Protective effects of ischemic preconditioning for liver resection performed under inflow occlusion in humans. *Ann Surg* 2000; **232**: 155-162
5 **Ilmakunnas M**, Petäjä J, Höckerstedt K, Mäkisalo H, Fernandez JA, Griffin JH, Jansson SE, Repo H, Pesonen EJ. Activation of protein C during reperfusion in clinical liver transplantation. *Transplantation* 2003; **75**: 467-72
6 **Jaeschke H**. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G15-G26
7 **Jaeschke H**, Smith CW, Clemens MG, Ganey PE, Roth RA. Mechanisms of inflammatory liver injury: adhesion molecules and cytotoxicity of neutrophils. *Toxicol Appl Pharmacol* 1996; **139**: 213-226
8 **Lawson JA**, Burns AR, Farhood A, Lynn Bajt M, Collins RG, Smith CW, Jaeschke H. Pathophysiologic importance of E- and L-selectin for neutrophil-induced liver injury during endotoxemia in mice. *Hepatology* 2000; **32**: 990-998
9 **Rymsa B**, Wang JF, de Groot H. O2-. release by activated Kupffer cells upon hypoxia-reoxygenation. *Am J Physiol* 1991; **261**: G602-G607
10 **Jaeschke H**, Farhood A, Bautista AP, Spolarics Z, Spitzer JJ. Complement activates Kupffer cells and neutrophils during reperfusion after hepatic ischemia. *Am J Physiol* 1993; **264**: G801-G809
11 **Lin M**, Rippe RA, Niemelä O, Brittenham G, Tsukamoto H. Role of iron in NF-kappa B activation and cytokine gene expression by rat hepatic macrophages. *Am J Physiol* 1997; **272**: G1355-G1364
12 **Jaeschke H**, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991; **260**: G355-G362
13 **Jaeschke H**, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by Kupffer cells and priming of neutrophils during reperfusion after hepatic ischemia. *Free Radic Res Commun* 1991; **15**: 277-284

14 **Jaeschke H**, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. *FASEB J* 1990; **4**: 3355-3359
15 **Shappell SB**, Toman C, Anderson DC, Taylor AA, Entman ML, Smith CW. Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils. *J Immunol* 1990; **144**: 2702-2711
16 **Jaeschke H**, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by neutrophils and Kupffer cells during in vivo reperfusion after hepatic ischemia in rats. *J Leukoc Biol* 1992; **52**: 377-382
17 **Jaeschke H**, Smith CW. Mechanisms of neutrophil-induced parenchymal cell injury. *J Leukoc Biol* 1997; **61**: 647-653
18 **Yadav SS**, Howell DN, Steeber DA, Harland RC, Tedder TF, Clavien PA. P-Selectin mediates reperfusion injury through neutrophil and platelet sequestration in the warm ischemic mouse liver. *Hepatology* 1999; **29**: 1494-1502
19 **Dulkanchainun TS**, Goss JA, Imagawa DK, Shaw GD, Anselmo DM, Kaldas F, Wang T, Zhao D, Busuttil AA, Kato H, Murray NG, Kupiec-Weglinski JW, Busuttil RW. Reduction of hepatic ischemia/reperfusion injury by a soluble P-selectin glycoprotein ligand-1. *Ann Surg* 1998; **227**: 832-840
20 **Garcia-Criado FJ**, Toledo-Pereyra LH, Lopez-Neblina F, Phillips ML, Paez-Rollys A, Misawa K. Role of P-selectin in total hepatic ischemia and reperfusion. *J Am Coll Surg* 1995; **181**: 327-334
21 **Young CS**, Palma JM, Mosher BD, Harkema J, Naylor DF, Dean RE, Crockett E. Hepatic ischemia/reperfusion injury in P-selectin and intercellular adhesion molecule-1 double-mutant mice. *Am Surg* 2001; **67**: 737-744
22 **Essani NA**, Fisher MA, Simmons CA, Hoover JL, Farhood A, Jaeschke H. Increased P-selectin gene expression in the liver vasculature and its role in the pathophysiology of neutrophil-induced liver injury in murine endotoxin shock. *J Leukoc Biol* 1998; **63**: 288-296
23 **Jaeschke H**. Cellular adhesion molecules: regulation and functional significance in the pathogenesis of liver diseases. *Am J Physiol* 1997; **273**: G602-G611
24 **Jaeschke H**. Is anti-P-selectin therapy effective in hepatic ischemia-reperfusion injury because it inhibits neutrophil recruitment? *Shock* 1999; **12**: 233-234
25 **Wong J**, Johnston B, Lee SS, Bullard DC, Smith CW, Beaudet AL, Kubes P. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. *J Clin Invest* 1997; **99**: 2782-2790
26 **Chosay JG**, Essani NA, Dunn CJ, Jaeschke H. Neutrophil margination and extravasation in sinusoids and venules of liver during endotoxin-induced injury. *Am J Physiol* 1997; **272**: G1195-G1200
27 **Kubes P**, Payne D, Woodman RC. Molecular mechanisms of leukocyte recruitment in postischemic liver microcirculation. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G139-G147
28 **Wyllie S**, Seu P, Gao FQ, Goss JA. Deregulation of iron homeostasis and cold-preservation injury to rat liver stored in University of Wisconsin solution. *Liver Transpl* 2003; **9**: 401-410
29 **Wyllie S**, Seu P, Gao FQ, Gros P, Goss JA. Disruption of the Nramp1 (also known as Slc11a1) gene in Kupffer cells attenuates early-phase, warm ischemia-reperfusion injury in the mouse liver. *J Leukoc Biol* 2002; **72**: 885-897
30 **Vural KM**, Liao H, Oz MC, Pinsky DJ. Effects of mast cell membrane stabilizing agents in a rat lung ischemia-reperfusion model. *Ann Thorac Surg* 2000; **69**: 228-232
31 **Tietze F**. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969; **27**: 502-22
32 **Renard P**, Ernest I, Houbion A, Art M, Le Calvez H, Raes M, Remacle J. Development of a sensitive multi-well colorimetric assay for active NFkappaB. *Nucleic Acids Res* 2001; **29**: E21
33 **Osarogiagbon UR**, Choong S, Belcher JD, Vercellotti GM, Paller MS, Hebbel RP. Reperfusion injury pathophysiology in sickle transgenic mice. *Blood* 2000; **96**: 314-320
34 **Lowry OH**, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-275
35 **Steinhoff G**, Behrend M, Schrader B, Duijvestijn AM, Wonigeit K. Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia. Lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2, and LFA-3. *Am J Pathol* 1993; **142**: 481-488
36 **Wisse E**. An ultrastructural characterization of the endothelial cell in the rat liver sinusoid under normal and various experimental conditions, as a contribution to the distinction between endothelial and Kupffer cells. *J Ultrastruct Res* 1972; **38**: 528-562
37 **Basile J**, Wang L, Tarcsafalvi A, Han R, Boros P, Miller CM. Expression of GMP-140 (P-selectin) correlates with graft viability in cold-preserved rat livers. *Transplantation* 2000; **69**: 2440-2442
38 **Jaeschke H**, Farhood A, Bautista AP, Spolarics Z, Spitzer JJ, Smith CW. Functional inactivation of neutrophils with a Mac-1 (CD11b/CD18) monoclonal antibody protects against ischemia-reperfusion injury in rat liver. *Hepatology* 1993; **17**: 915-923
39 **Amersi F**, Farmer DG, Shaw GD, Kato H, Coito AJ, Kaldas F, Zhao D, Lassman CR, Melinek J, Ma J, Volk HD, Kupiec-Weglinski JW, Busuttil RW. P-selectin glycoprotein ligand-1 (rPSGL-Ig)-mediated blockade of CD62 selectin molecules protects rat steatotic liver grafts from ischemia/reperfusion injury. *Am J Transplant* 2002; **2**: 600-608
40 **Tsuchihashi S**, Fondevila C, Shaw GD, Lorenz M, Marquette K, Benard S, Shen XD, Ke B, Busuttil RW, Kupiec-Weglinski JW. Molecular characterization of rat leukocyte P-selectin glycoprotein ligand-1 and effect of its blockade: protection from ischemia-reperfusion injury in liver transplantation. *J Immunol* 2006; **176**: 616-624
41 **Farhood A**, McGuire GM, Manning AM, Miyasaka M, Smith CW, Jaeschke H. Intercellular adhesion molecule 1 (ICAM-1) expression and its role in neutrophil-induced ischemia-reperfusion injury in rat liver. *J Leukoc Biol* 1995; **57**: 368-374
42 **Zwacka RM**, Zhang Y, Zhou W, Halldorson J, Engelhardt JF. Ischemia/reperfusion injury in the liver of BALB/c mice activates AP-1 and nuclear factor kappaB independently of IkappaB degradation. *Hepatology* 1998; **28**: 1022-1030
43 **Yoshidome H**, Kato A, Edwards MJ, Lentsch AB. Interleukin-10 suppresses hepatic ischemia/reperfusion injury in mice: implications of a central role for nuclear factor kappaB. *Hepatology* 1999; **30**: 203-208
44 **Ghobrial R**, Amersi F, Stecker K, Kato H, Melinek J, Singer J, Mhoyan A, Busuttil RW, Kupiec-Weglinski JW, Stepkowski SM. Amelioration of hepatic ischemia/reperfusion injury with intercellular adhesion molecule-1 antisense oligodeoxynucleotides. *Transplant Proc* 2001; **33**: 538
45 **Viebahn R**, Thoma M, Kinder O, Schenk M, Lauchart W, Becker HD. Analysis of intragraft adhesion molecules and their release in clinical liver transplantation: impact of reperfusion injury. *Transplant Proc* 1998; **30**: 4257-4259
46 **Yadav SS**, Howell DN, Gao W, Steeber DA, Harland RC, Clavien PA. L-selectin and ICAM-1 mediate reperfusion injury and neutrophil adhesion in the warm ischemic mouse liver. *Am J Physiol* 1998; **275**: G1341-G1352
47 **Nakano H**, Nagasaki H, Yoshida K, Kigawa G, Fujiwara Y, Kitamura N, Kuzume M, Takeuchi S, Sasaki J, Shimura H, Yamaguchi M, Kumada K. N-acetylcysteine and anti-ICAM-1 monoclonal antibody reduce ischemia-reperfusion injury of the steatotic rat liver. *Transplant Proc* 1998; **30**: 3763
48 **Colletti LM**, Cortis A, Lukacs N, Kunkel SL, Green M, Strieter RM. Tumor necrosis factor up-regulates intercellular adhesion molecule 1, which is important in the neutrophil-dependent lung and liver injury associated with hepatic

ischemia and reperfusion in the rat. *Shock* 1998; **10**: 182-191
49 **Marubayashi S**, Oshiro Y, Maeda T, Fukuma K, Okada K, Hinoi T, Ikeda M, Yamada K, Itoh H, Dohi K. Protective effect of monoclonal antibodies to adhesion molecules on rat liver ischemia-reperfusion injury. *Surgery* 1997; **122**: 45-52
50 **Rentsch M**, Post S, Palma P, Lang G, Menger MD, Messmer K. Anti-ICAM-1 blockade reduces postsinusoidal WBC adherence following cold ischemia and reperfusion, but does not improve early graft function in rat liver transplantation. *J Hepatol* 2000; **32**: 821-828
51 **Kobayashi A**, Imamura H, Isobe M, Matsuyama Y, Soeda J, Matsunaga K, Kawasaki S. Mac-1 (CD11b/CD18) and intercellular adhesion molecule-1 in ischemia-reperfusion injury of rat liver. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G577-G585
52 **Jaeschke H**, Farhood A, Fisher MA, Smith CW. Sequestration of neutrophils in the hepatic vasculature during endotoxemia is independent of beta 2 integrins and intercellular adhesion molecule-1. *Shock* 1996; **6**: 351-356
53 **Jaeschke H**. Vascular oxidant stress and hepatic ischemia/reperfusion injury. *Free Radic Res Commun* 1991; **12-13** Pt 2: 737-743
54 **Matsui N**, Satsuki I, Morita Y, Inaizumi K, Kasajima K, Kanoh R, Fukuishi N, Akagi M. Xanthine oxidase-derived reactive oxygen species activate nuclear factor kappa B during hepatic ischemia in rats. *Jpn J Pharmacol* 2000; **84**: 363-366
55 **Amersi F**, Nelson SK, Shen XD, Kato H, Melinek J, Kupiec-Weglinski JW, Horwitz LD, Busuttil RW, Horwitz MA. Bucillamine, a thiol antioxidant, prevents transplantation-associated reperfusion injury. *Proc Natl Acad Sci USA* 2002; **99**: 8915-8920
56 **Kumamoto Y**, Suematsu M, Shimazu M, Kato Y, Sano T, Makino N, Hirano KI, Naito M, Wakabayashi G, Ishimura Y, Kitajima M. Kupffer cell-independent acute hepatocellular oxidative stress and decreased bile formation in post-cold-ischemic rat liver. *Hepatology* 1999; **30**: 1454-1463
57 **Jaeschke H**. Role of reactive oxygen species in hepatic ischemia-reperfusion injury and preconditioning. *J Invest Surg* 2003; **16**: 127-140
58 **Grattagliano I**, Vendemiale G, Lauterburg BH. Reperfusion injury of the liver: role of mitochondria and protection by glutathione ester. *J Surg Res* 1999; **86**: 2-8
59 **Ballatori N**, Truong AT, Ma AK, Boyer JL. Determinants of glutathione efflux and biliary GSH/GSSG ratio in perfused rat liver. *Am J Physiol* 1989; **256**: G482-G490
60 **Ono K**, Matsumori A, Furukawa Y, Igata H, Shioi T, Matsushima K, Sasayama S. Prevention of myocardial reperfusion injury in rats by an antibody against monocyte chemotactic and activating factor/monocyte chemoattractant protein-1. *Lab Invest* 1999; **79**: 195-203

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RAPID COMMUNICATION

Cytoreduction and hyperthermic intraperitoneal chemotherapy in the treatment of peritoneal carcinomatosis from pseudomyxoma peritonei

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Abstract

AIM: To investigate the most important aspects of hyperthermic intraperitoneal chemotherapy (HIPEC) that has been accepted as the standard treatment for pseudomyxoma peritonei (PMP), with special regard to morbidity, overall survival (OS) and disease free survival (DFS) over 10 years.

METHODS: Fifty-three patients affected by PMP underwent cytoreduction (CCR) and HIPEC with a "semi-closed" abdomen technique in our institution. The peritonectomy procedure and completeness of CCR were classified according to Sugarbaker criteria. Preoperative evaluation always included thoracic and abdominal CT scan to stage peritoneal disease and exclude distant metastases. Fifty-one patients in our series were treated with a protocol based on administration of cisplatinum 100 mg/m^2 plus mitomycin C 16 mg/m^2, at a temperature of 41.5℃ for 60 min. Anastomoses were always performed at the end of HIPEC. The mean duration of surgery was 12 h including HIPEC. Continuous monitoring of hepatic and renal functions and hydroelectrolytic balance was performed in the postoperative period.

RESULTS: Twenty-four patients presented with postoperative complications: surgical morbidity was observed in 16 patients and 6 patients were re-operated. All complications were successfully treated and no postoperative deaths were observed. Risk factors for postoperative morbidity were considered to be gender, age, body surface, duration of surgery, Peritoneal Cancer Index (PCI) and tumor residual value (CC score). No statistically significant correlation was found during the multivariate analysis: only the CC score was statistically significant. The OS in our experience was 81.8%, with a DFS of 80% at 5 years and of 70% at 10 years.

CONCLUSION: In our experience, even if HIPEC combined with cytoreductive surgery involves a high risk of morbidity, postoperative complications can be resolved favorably in most cases with correct patient selection and adequate postoperative care, thus minimizing mortality. The association of CCR and HIPEC can be considered as the standard treatment for PMP. The OS and DFS results confirm the validity of this combined approach for the treatment of this rare neoplasm. The impact of preoperative chemotherapy on OS, in our opinion, is due to a major aggressiveness of tumors in treated patients.

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Key words: Peritonectomy; Pseudomyxoma peritonei; Hyperthermic perfusion; Hyperthermic intraperitoneal chemotherapy; Peritoneal carcinomatosis; Loco-regional treatment

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INTRODUCTION

Peritoneal carcinomatosis (PC) is one of the most common routes of dissemination of abdominal neoplasms; it may be present at the time of diagnosis of a primary tumor, but more frequently it arises as tumor recurrence after surgical treatment[1].

PC is frequently associated with colorectal cancer, gastric cancer, ovarian carcinoma, and appendiceal cancer. Neoplasms with positive peritoneal cytology

show high rates of peritoneal dissemination[2-6].

Pseudomyxoma peritonei (PMP) is a rare condition, with an incidence of 1/1 000 000 per year, characterized by copious mucus (so-called "jelly belly") containing rare epithelial cells. According to Ronnet, PMP was histologically classified into disseminated peritoneal adenomucinosi (DPAM), peritoneal mucinous carcinomatosis (PMCA) and an intermediate or discordant feature group (ID)[7]. Recent studies show that most cases of PMP originate from ruptured appendiceal tumors with progressive dissemination in the peritoneal cavity of mucin-producing epithelial-cells[8,9]. Lymph-nodal or hematogenous metastases are rare in PMP and evidence suggests it has a poor prognosis.

PC from PMP is generally considered a lethal disease, with a limited response to conventional chemotherapeutic treatments[10]. While systemic chemotherapy has little impact on the treatment of peritoneal disease, some centers have reported encouraging results with intraperitoneal hyperthermic chemoperfusion (HIPEC)[11,12].

This technique is based on surgical cytoreduction (CCR) of the primitive cancer, peritonectomy and HIPEC. The principle of locoregional treatments is to obtain an elevated and persistent drug concentration for the tumor, with a limited systemic concentration. Many studies reported an impact on overall survival (OS) and disease-free survival (DFS) in patients affected by carcinomatosis of mucinous cancers such as PMP[13,14] and in recent trials this combined approach has been proposed as standard treatment for PMP.

In this study we report the results of a 10-year experience with this type of treatment in our institution, where 53 patients with PMP were treated with CCR and HIPEC, with special reference to follow-up and risk factors for postoperative complications.

MATERIALS AND METHODS

For the present study, 53 patients (23 male and 30 female, mean age 58 years, range 32-72) with PMP who underwent surgical treatment and HIPEC between October 1998 and June 2008 at the Department of General Surgery and Surgical Oncology, San Giuseppe Hospital, were considered. Preoperative evaluation always included thoracic and abdominal CT scan to stage peritoneal disease and exclude distant metastases; upper digestive endoscopy and colonoscopy generally completed tumor staging. A careful preoperative evaluation of the patient's general condition was always performed, and included complete blood tests, electrocardiogram, cardiac ultrasound, and spirometry. The presence of hepatic or extra-abdominal metastases, poor general condition or performance status > 2 according to the Eastern Cooperative Oncology Group (ECOG) and an age of > 72 years were generally considered contraindications to the treatment. Informed consent was obtained from all patients[15].

Just after laparotomy, a complete intraoperative staging of peritoneal disease was performed using the peritoneal cancer index (PCI)[16]; the mean PCI was 22.

Surgical technique

The peritonectomy procedure was classified and performed according to Sugarbaker's criteria: (1) Central peritonectomy consists of the removal of previous scars, greater omentectomy (performed by stripping the superficial peritoneal layer of the transverse mesocolon) and a close dissection to the greater curvature of the stomach. Sometimes splenectomy could be necessary *en bloc* with the greater omentum and the left diaphragmatic peritoneum; (2) Left upper quadrant peritonectomy consists of the stripping of the peritoneal tumor tissue from beneath the left hemidiaphragm, left adrenal gland, distal portion of the pancreas, and the cephalad half of Gerota's fascia; (3) Right upper quadrant peritonectomy consists of right hemidiaphragmatic peritoneal stripping, removal of tumor from the right subhepatic space and from the surface of the liver by the stripping of the Glisson's capsule. Peritonectomy is concluded with the removal of the peritoneum covering the right kidney and Morrison's pouch; (4) Lesser omentum peritonectomy is performed after the cholecystectomy, and in this procedure the cancerous tissue which covers the common duct and hepatic artery is stripped from the base of gall bladder bed towards the duodenum. This phase is concluded by the stripping of omental bursa; (5) Pelvic peritonectomy with *en bloc* removal of pelvic peritoneum, sigmoid colon, rectum, uterus and salpingo-oophorectomy; (6) Peritonectomy of the lateral abdominal wall. Implants on the visceral serosa are removed by electrosurgical local dissection and the peritonectomies are variously combined with resections of viscera involved in tumor (total gastrectomy or total colectomy).

The completeness of CCR was also classified according to Sugarbaker's criteria[17] as: CCR-0 (no residual tumor) in 35 cases, CCR-1 (no residual nodule greater than 2.5 mm in diameter) in 18 cases, CCR-2 (no residual nodules greater than 25 mm) in none of the cases and CCR-3 (residual nodules greater than 25 mm) in none of the cases.

HIPEC was performed according to the "semi-closed" abdomen technique[18]. Five drain tubes are placed in the abdominal cavity. There are 2 inflow tubes, and they have multiple holes. They present 2 diffusion lines for the homogeneous distribution of drugs into the abdominal cavity (1 in the sovramesocolic branch, 1 in the pelvis). Three outflow tubes are placed respectively in the pelvis and in the subdiaphragmatic spaces. Backhaus forceps are used to close the cranial and caudal portion of abdominal wound. The skin is then suspended by a self-retaining retractor, placed at more or less 15 cm from the abdomen, by plastic self-blocking strings. This kind of placement creates the virtual cavity needed to perform HIPEC. The central portion of the wound is suspended by the retractor too and covered with a laparoscopic device with sterile drapes on it, with a hole in the middle. The drain tubes are connected to a perfusion system formed by 2 pumps and a heat exchanger to heat the perfusion liquid. The inflow and

outflow pumps are connected through a reservoir, so it is possible to achieve continuous circulation of the perfusate at the speed of more or less 1 L/min. The pumps are controlled by a computerized system that allows the checking of the flow rate and the temperature of the heat exchanger. Three intraperitoneal temperatures are checked by probes; the inflow temperature, outflow temperature, and the patient esophageal temperature. The amount of circulating perfusate required (solution for peritoneal dialysis) is calculated according to the patient's body surface. During perfusion, the surgeon mixes the perfusate by hand through the hole in the sterile drapes. When the ideal intraperitoneal temperature is reached, the drugs are added to the circuit and HIPEC is performed for 60 min. Fifty-one patients in our series were treated with a protocol based on administration of cisplatinum 100 mg/m^2 plus mitomycin C 16 mg/m^2, at a temperature of 41.5℃. Two patients were treated with mitomycin C 35 mg/m^2 for 60 min at a temperature of 40.5℃, according to the Netherland protocol, because of significant side effects from preoperative systemic chemotherapy with platinum. Anastomoses were always performed at the end of HIPEC. The mean duration of surgery was 12 h including HIPEC (range 8-16 h). At the end of the operation, the patient was admitted to the intensive care unit, and then returned to the surgical department when cardiovascular and pulmonary functions became stable. Continuous monitoring of hepatic and renal functions and hydroelectrolytic balance were performed afterwards. The primitive neoplasm was an appendicular adenocarcinoma in 37 patients (69.8%) and an appendicular adenoma in 16 patients (30.2%). Twenty-one patients (39.6%) with histological diagnosis of appendicular adenocarcinoma had been treated with systemic chemotherapy before our operation. Because of the massive involvement of viscera and peritoneum, in some selected patients we performed the treatment in steps. Three patients were treated in 2 steps, and 1 patient was treated in 3 steps. In these cases, we performed the upper abdominal CCR in the first step, then the patient was submitted to systemic chemotherapy for 2 or 3 mo. The second step consisted of lower abdominal CCR and peritoneal perfusion of the entire peritoneal cavity. The details of the CCR procedures are displayed in Table 1.

Table 1 Extent of CCR and HIPEC in 53 patients affected by PMP

Procedures	No.
Greater omentectomy	48
Left colectomy	30
Right colectomy	34
Total abdominal hysterectomy	16
Bilateral salpingo-oophorectomy	20
Splenectomy	40
Cholecystectomy	34
Small bowel partial resection	27
Total gastrectomy	5
Sub-total gastrectomy	3
Distal pancreatectomy	6
Rigth upper quadrant peritonectomy	47
Left upper quadrant peritonectomy	38
Pelvic peritonectomy	43

Statistical analysis

In this study the statistical analyses focused on postoperative complications: the histopathological, clinical and follow-up data were stored in a database. The presence of postoperative complications was considered as the dependent variable whereas gender, age, body mass index, primary tumor, previous systemic chemotherapy, operative time, stage of PCI, and CCR were covariates.

Multivariate analysis of factors was performed by the Cox proportional hazard model. OS was dated from the day of surgery to the time of death due to any causes; progression-free survival (PFS) was dated from the day of the surgery to the time of postoperative disease progression. The survival curves for both OS and PFS were calculated according to the Kaplan-Meier method. The log-rank test was used to assess the significance of the comparison between survival curves.

The Statistical Package for the Social Sciences software (version 11.0) (SPSS, Chicago, IL, USA) was used for statistical analysis: $P < 0.05$ was considered significant.

RESULTS

At the end of follow-up of the 53 patients, 5 and 10 year OS was 94% and 84.6%, respectively (Figure 1A). DFS was 80% and 70% at 5 and 10 years, respectively (Figure 1B).

OS according to the PCI, completeness of CCR (CC-score), histological type, and pre-operative chemotherapy (done *vs* not done) are shown in Figure 2A-D. At the time of the present analysis 48 patients are alive without disease. Two patients died due to systemic disease progression at 16 and 63 mo, respectively, after the operation; 3 patients are alive with disease and intraperitoneal relapse but are not undergoing a further operation, with follow-up of 57, 28, 24, 19, 10 mo, respectively. Three patients had intraperitoneal relapse and were treated with tumor resection followed by HIPEC: 1 of those patients is alive without disease 17 mo after the second surgical procedure; the other 2 patients were treated only with CCR and they are alive without disease after 24 mo of follow-up.

For calculation of the morbidity rate, we considered postoperative complications occurring during the hospital stay or within 30 d of surgery. In 24 patients (45%) we observed postoperative complications: surgical morbidity was observed in 16 patients (3 intestinal fistulas, 2 urinary tract perforations, 2 abdominal abscesses, 4 wound infections, 1 prolonged ileus, 2 postoperative haemorrhages, 1 abdominal wall dehiscence, 1 bleeding from a gastric ulcer) and medical complications were observed in 8 cases (1 arrhythmia, 3 grade 2 hematological toxicities, 1 acute renal failure, 1 cutaneous rash, 2 cases of sepsis). Six patients were

Table 2 Association between morbidity and clinical variables *n* (%)

Variable	No. of cases	With complications	Without complications	*P*-value
Gender				NS
Male	23	11 (46)	12 (41)	
Female	30	13 (54)	17 (59)	
Age (yr)		59 ± 10	56 ± 9	NS
Body mass index (Kg/m^2)		25.4 ± 5.3	26.8 ± 4.7	NS
Previous systemic chemotherapy				NS
Performed	21	10 (42)	11 (38)	
Not performed	32	14 (58)	18 (62)	
PCI				NS
> 16	36	18 (75)	18 (62)	
< 16	17	6 (25)	11 (38)	
Operative time (h)		8.5 ± 3.0	7.1 ± 2.1	NS
Completeness of cancer resection				0.017
CCR-0	35	12 (50)	23 (79)	
CCR-1	18	12 (50)	6 (21)	

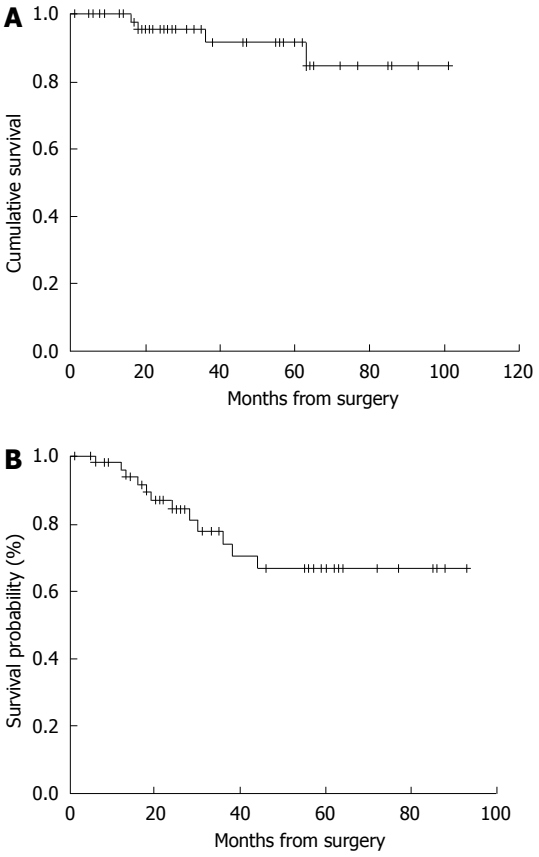

Figure 1 53 cases of PMP. A: Overall survival (Kaplan-Meyer); B: Disease free survival (DFS).

re-operated and 1 patient underwent ureteric stenting. One patient with abdominal abscess was submitted to ultrasound-guided drainage and 1 patient with bleeding from a gastric ulcer was treated by endoscopic haemostasis. All other complications were successfully treated by medical therapy (Table 2). No postoperative deaths were observed. An analysis of risk factors for postoperative morbidity rate was performed. Gender, age, body surface, duration of surgery, PCI and tumor residual value were considered to be risk factors. No statistically significant correlation between the analyzed variables and the incidence of postoperative complications was found except for CC-score ($P < 0.017$) (Table 3).

Table 3 Postoperative complications observed in 24 patients. Multiple complications are included

Complication	No. of cases	Treatment (No. of cases)
Surgical		
Wound infection	4	Drainage
Urinary tract perforation	2	Reoperation (1); Urinary stenting (1)
Intestinal fistula	3	Reoperation
Abdominal abscess	2	US-guided drainage (1); medical (1)
Prolonged ileous	1	Medical
Bleeding from gastric ulcer	1	Endoscopic haemostasis
Intraabdominal bleeding	2	Reoperation
abdominal wall dehiscence	1	Conservative
Medical		
Grade ⩾ 2 hematological toxicity	3	Medical
Acute renal failure	1	Medical
Arrhythmias	1	Medical
Cutaneous rush	1	Medical
Sepsis	2	Medical

Final follow-up data from our experience indicated that survival probability may be good in patients with histological type appendicular adenoma who are optimally cytoreduced (CC-0). An interesting relief was related to whether preoperative chemotherapy was performed or not.

DISCUSSION

HIPEC associated with cytoreductive surgery is becoming a widely accepted procedure for the treatment of PMP.

Like reports in several studies, the results of our experience indicate that, even when combined with an aggressive surgical procedure, HIPEC is associated with an acceptable risk of postoperative complications and mortality[19-24]. The incidence of postoperative complications was similar to that of other reports[25-29]

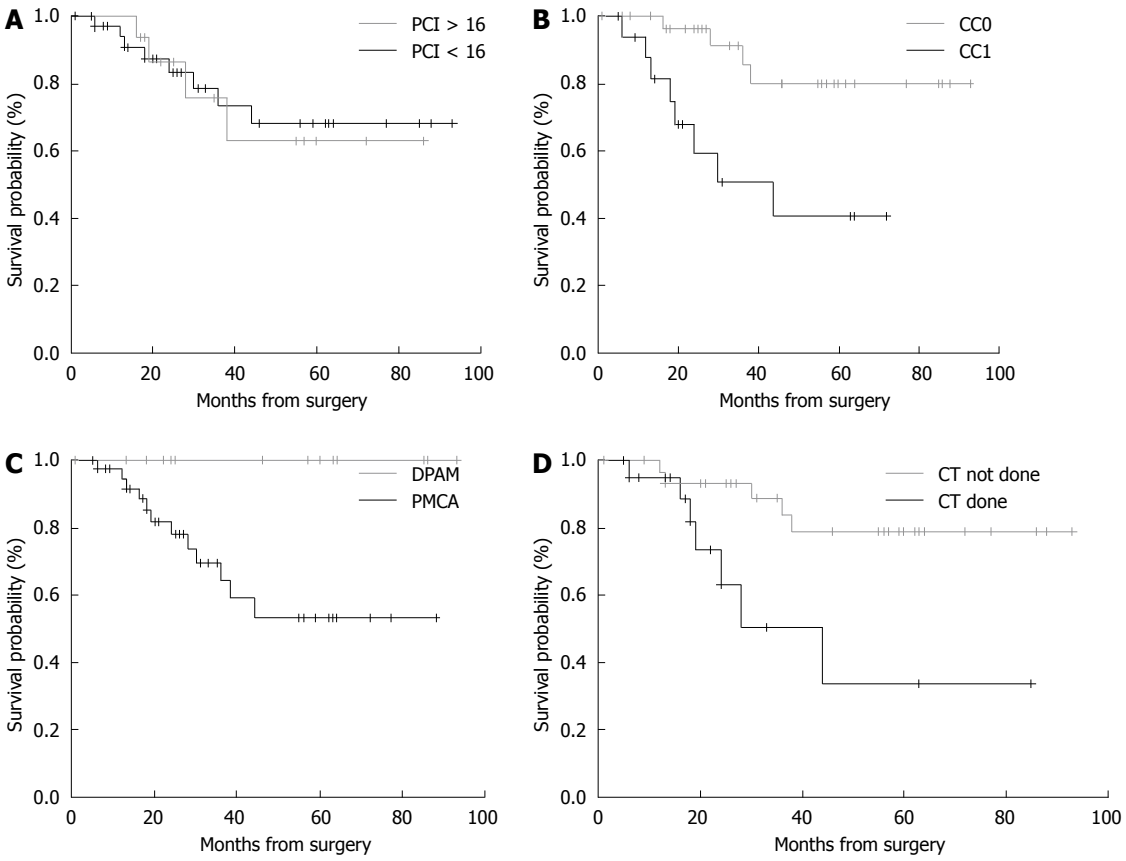

Figure 2 Overall survival (OS) in 53 cases of PMP. A: According to PCI status (P N.S.); B: According to the completeness of CCR ($P < 0.003$); C: According to the histological tumor type ($P < 0.014$); D: According to previous systemic chemotherapy ($P < 0.034$).

and major morbidity occurred in 45% of patients, which is also similar to other recent experiences[27,29,30]. All the complications were successfully treated with surgical or medical therapy. We believe that careful preoperative selection of patients, and adequate postoperative monitoring and care are crucial in order to minimize the incidence of postoperative complications in these patients.

The advanced stage of neoplastic disease and immunodeficiency status of patients previously subjected to chemotherapy were important factors that probably contributed to the occurrence of septic complications after an extended surgical procedure. In our series 1 patient underwent ultrasound-guided drainage of an abdominal abscess and in the others septic complications were successfully treated with medical therapy.

Intestinal fistula has been reported to be an important cause of morbidity and mortality in patients submitted to HIPEC, with an incidence rate ranging from 6% to 27%[21-23, 31-33]. Younan *et al*[32] reported that male gender, duration of surgery, and no previous systemic chemotherapy were independent predictors of bowel complications. The direct effect of HIPEC even in non-resective procedures can be associated with intestinal fistulas in the postoperative period[33,34]. We always performed anastomotic suture following HIPEC but in our series the 3 cases of intestinal fistula were due to intestinal perforation not involving anastomosis.

Recent studies have reported the duration and extent of surgery, visceral resections, PCI and incomplete CCR to be important risk factors for postoperative complications[21,26,31]. In our patients, we did not find this correlation, probably because of the limited number of cases. PCI > 16 was an independent predictor of postoperative morbidity only at univariate analysis and seemed to have an impact on the complication rate, but not on OS.

The largest series of PMP undergoing combined treatment was reported by Sugarbaker[35]: in this series completeness of CCR and Ronnet's criteria were the most important factors correlated with survival and morbidity[36].

Complete CCR was obtained in most of our patients. In our series, we didn't have patients with an elevated CC score (CC-2 or CC-3): as a consequence, even though the morbidity rate was high we did not find a correlation between cytoreductive status and complication rate. However, the CC-score in our experience was strictly correlated to DFS with an evident result between CC-0 and CC-1 patients ($P < 0.003$).

The limited number of patients in our series did not allow further stratifications and for these reasons, the potential impact of other factors on morbidity cannot be excluded.

Survival data indicate that high long-term survival could be achieved in patients with histological type DPAM *vs* PMCA ($P < 0.014$). Furthermore in the present series the adverse prognostic value of preoperative systemic

chemotherapy was a unexpected finding that may be not easy to explain: the patient that received chemotherapy had a poor prognosis compared to those that did not undergo chemotherapy ($P < 0.034$). The same evidence was observed in the series of Baratti *et al*[37].

The hypothesis was that after chemotherapy, mucinous appendiceal tumors change to a more invasive process: it is quite possible that differences in chemotherapy penetration of mucinous and solid tumours may result in persistence and progression of the more solid components of a non uniform tumor. Appendiceal tumors are described as having large areas of adenomucinosis with small, even minute, areas of more aggressive tissue: the penetration of chemotherapy drugs into the mucin that contains adenomatous epithelial cells may eradicate these cells but the small foci of solid tumor may not be completely penetrated by chemotherapy. It is possible that this process selected resistant and more aggressive tumor cell clones but the explanation for the poor results of the treatment in these patients requires further investigation.

In conclusion the present study confirms that an aggressive approach can improve survival in selected patients with PMP. Although HIPEC combined with CCR has a high risk of morbidity, postoperative complications could be resolved favorably in most cases with correct patient selection and adequate postoperative care, thus minimizing mortality. Residual tumor (CC), preoperative chemotherapy and histological type PCMA significantly influence the prognosis of these patients[20,37].

To improve this encouraging survival outcome, it is very important to unify the surgical experience of expertise centers and adequate patient selection. Our results suggest also the need of an integrated approach to this rare neoplasm to identify the biological aspect of PMP that influences the prognosis and the evolution of the disease.

COMMENTS

Background

Peritoneal carcinomatosis is generally considered a lethal disease, with a mean survival time of 6 mo after conventional chemotherapeutic treatments. Systemic chemotherapy has little impact on treatment of peritoneal disease, but some centres have reported encouraging results with intraperitoneal hyperthermic chemoperfusion (HIPEC). Locoregional treatments are considered a new frontier in the management of this condition: it is possible to achieve an elevated and persistent drug concentration in the tumor, with limited systemic effects. Many studies reported an impact on overall survival and disease-free interval in patients affected by carcinomatosis, of mucinous cancers such as pseudomyxoma peritonei (PMP) and in recent trials this combined approach has been proposed as standard treatment for PMP.

Research frontiers

The present study confirms that an aggressive approach can improve survival in selected patients with PMP. Although HIPEC combined with cytoreductive surgery involves a high risk of morbidity, postoperative complications can be resolved favorably in most cases with correct patient selection and adequate postoperative care, thus minimizing mortality. Residual tumor (CC), preoperative chemotherapy and histological type of PMP can be considered as independent variables able to significantly influence the prognosis of these patients. To improve this encouraging survival outcome, it is very important to unify the surgical experience of expertise centres. Our results suggest also the need for an integrated approach to this rare neoplasm to identify the biological aspects of PMP that influence the prognosis and the evolution of the disease.

Innovation and breakthroughs

In this paper we report a very important proof on the integrated approach to PMP. This lethal disease can be treated with good results: in fact 5 and 10 year overall survival was, respectively, 94% and 84.6% in our experience and disease free survival was 80% and 70% at 5 and 10 years, respectively.

Application

On future application, the end point of this approach would be to improve a standard treatment for this particular disease to reduce the surgical risk of major complications. Correct patient selection and adequate postoperative care may minimize the considerable complication rate that is very high (45%).

Peer review

This is a very interesting study on pseudomyxoma peritonei and its treatment with intraperitoneal hyperthermic chemoperfusion.

REFERENCES

1 **Sugarbaker PH**. Peritoneal carcinomatosis: natural history and rational therapeutic interventions using intraperitoneal chemotherapy. *Cancer Treat Res* 1996; **81**: 149-168

2 **Marrelli D**, Roviello F, de Manzoni G, Morgagni P, Di Leo A, Saragoni L, De Stefano A, Folli S, Cordiano C, Pinto E. Different patterns of recurrence in gastric cancer depending on Lauren's histological type: longitudinal study. *World J Surg* 2002; **26**: 1160-1165

3 **Roviello F**, Marrelli D, de Manzoni G, Morgagni P, Di Leo A, Saragoni L, De Stefano A. Prospective study of peritoneal recurrence after curative surgery for gastric cancer. *Br J Surg* 2003; **90**: 1113-1119

4 **Bando E**, Yonemura Y, Takeshita Y, Taniguchi K, Yasui T, Yoshimitsu Y, Fushida S, Fujimura T, Nishimura G, Miwa K. Intraoperative lavage for cytological examination in 1,297 patients with gastric carcinoma. *Am J Surg* 1999; **178**: 256-262

5 **Pestieau SR**, Sugarbaker PH. Treatment of primary colon cancer with peritoneal carcinomatosis: comparison of concomitant vs. delayed management. *Dis Colon Rectum* 2000; **43**: 1341-1346; discussion 1347-1348

6 **Sugarbaker TA**, Chang D, Koslowe P, Sugarbaker PH. Pathobiology of peritoneal carcinomatosis from ovarian malignancy. *Cancer Treat Res* 1996; **81**: 63-74

7 **Ronnett BM**, Zahn CM, Kurman RJ, Kass ME, Sugarbaker PH, Shmookler BM. Disseminated peritoneal adenomucinosis and peritoneal mucinous carcinomatosis. A clinicopathologic analysis of 109 cases with emphasis on distinguishing pathologic features, site of origin, prognosis, and relationship to "pseudomyxoma peritonei". *Am J Surg Pathol* 1995; **19**: 1390-1408

8 **Szych C**, Staebler A, Connolly DC, Wu R, Cho KR, Ronnett BM. Molecular genetic evidence supporting the clonality and appendiceal origin of Pseudomyxoma peritonei in women. *Am J Pathol* 1999; **154**: 1849-1855

9 **Carr NJ**, Emory TS, Sobin LH. Epithelial neoplasms of the appendix and colorectum: an analysis of cell proliferation, apoptosis, and expression of p53, CD44, bcl-2. *Arch Pathol Lab Med* 2002; **126**: 837-841

10 **Sadeghi B**, Arvieux C, Glehen O, Beaujard AC, Rivoire M, Baulieux J, Fontaumard E, Brachet A, Caillot JL, Faure JL, Porcheron J, Peix JL, François Y, Vignal J, Gilly FN. Peritoneal carcinomatosis from non-gynecologic malignancies: results of the EVOCAPE 1 multicentric prospective study. *Cancer* 2000; **88**: 358-363

11 **Fujimoto S**, Takahashi M, Mutou T, Kobayashi K, Toyosawa T, Isawa E, Sumida M, Ohkubo H. Improved mortality rate of gastric carcinoma patients with peritoneal carcinomatosis treated with intraperitoneal hyperthermic chemoperfusion combined with surgery. *Cancer* 1997; **79**: 884-891

12 **Witkamp AJ**, de Bree E, Kaag MM, Boot H, Beijnen

JH, van Slooten GW, van Coevorden F, Zoetmulder FA. Extensive cytoreductive surgery followed by intraoperative hyperthermic intraperitoneal chemotherapy with mitomycin-C in patients with peritoneal carcinomatosis of colorectal origin. *Eur J Cancer* 2001; **37**: 979-984

13 **Sugarbaker PH**, Ronnett BM, Archer A, Averbach AM, Bland R, Chang D, Dalton RR, Ettinghausen SE, Jacquet P, Jelinek J, Koslowe P, Kurman RJ, Shmookler B, Stephens AD, Steves MA, Stuart OA, White S, Zahn CM, Zoetmulder FA. Pseudomyxoma peritonei syndrome. *Adv Surg* 1996; **30**: 233-280

14 **Glehen O**, Kwiatkowski F, Sugarbaker PH, Elias D, Levine EA, De Simone M, Barone R, Yonemura Y, Cavaliere F, Quenet F, Gutman M, Tentes AA, Lorimier G, Bernard JL, Bereder JM, Porcheron J, Gomez-Portilla A, Shen P, Deraco M, Rat P. Cytoreductive surgery combined with perioperative intraperitoneal chemotherapy for the management of peritoneal carcinomatosis from colorectal cancer: a multi-institutional study. *J Clin Oncol* 2004; **22**: 3284-3292

15 **Oken MM**, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, Carbone PP. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982; **5**: 649-655

16 **Sugarbaker PH**, Alderman R, Edwards G, Marquardt CE, Gushchin V, Esquivel J, Chang D. Prospective morbidity and mortality assessment of cytoreductive surgery plus perioperative intraperitoneal chemotherapy to treat peritoneal dissemination of appendiceal mucinous malignancy. *Ann Surg Oncol* 2006; **13**: 635-644

17 **Jacquet P**, Sugarbaker PH. Clinical research methodologies in diagnosis and staging of patients with peritoneal carcinomatosis. *Cancer Treat Res* 1996; **82**: 359-374

18 **De Simone M**, Barone R, Vaira M, Aghemo B, Mioli P, Franco C, Scuderi S, Costamagna D, Dei Poli M. Semi-closed hyperthermic-antiblastic peritoneal perfusion (HAPP) in the treatment of peritoneal carcinosis. *J Surg Oncol* 2003; **82**: 138-140

19 **Glehen O**, Mohamed F, Gilly FN. Peritoneal carcinomatosis from digestive tract cancer: new management by cytoreductive surgery and intraperitoneal chemohyperthermia. *Lancet Oncol* 2004; **5**: 219-228

20 **Roviello F**, Marrelli D, Neri A, Cerretani D, de Manzoni G, Pedrazzani C, Cioppa T, Nastri G, Giorgi G, Pinto E. Treatment of peritoneal carcinomatosis by cytoreductive surgery and intraperitoneal hyperthermic chemoperfusion (IHCP): postoperative outcome and risk factors for morbidity. *World J Surg* 2006; **30**: 2033-2040; discussion 2041-2042

21 **Stephens AD**, Alderman R, Chang D, Edwards GD, Esquivel J, Sebbag G, Steves MA, Sugarbaker PH. Morbidity and mortality analysis of 200 treatments with cytoreductive surgery and hyperthermic intraoperative intraperitoneal chemotherapy using the coliseum technique. *Ann Surg Oncol* 1999; **6**: 790-796

22 **Verwaal VJ**, van Ruth S, de Bree E, van Sloothen GW, van Tinteren H, Boot H, Zoetmulder FA. Randomized trial of cytoreduction and hyperthermic intraperitoneal chemotherapy versus systemic chemotherapy and palliative surgery in patients with peritoneal carcinomatosis of colorectal cancer. *J Clin Oncol* 2003; **21**: 3737-3743

23 **Glehen O**, Mithieux F, Osinsky D, Beaujard AC, Freyer G, Guertsch P, Francois Y, Peyrat P, Panteix G, Vignal J, Gilly FN. Surgery combined with peritonectomy procedures and intraperitoneal chemohyperthermia in abdominal cancers with peritoneal carcinomatosis: a phase II study. *J Clin Oncol* 2003; **21**: 799-806

24 **Ahmad SA**, Kim J, Sussman JJ, Soldano DA, Pennington LJ, James LE, Lowy AM. Reduced morbidity following cytoreductivPilati P, Mocellin S, Rossi CR, Foletto M, Campana L, Nitti D, Lise M. Cytoreductive surgery combined with hyperthermic intraperitoneal intraoperative chemotherapy for peritoneal carcinomatosis arising from colon adenocarcinoma. Ann Surg Oncol 2003; 10: 508-513e surgery and intraperitoneal hyperthermic chemoperfusion. *Ann Surg Oncol* 2004; **11**: 387-392

25 **Pilati P**, Mocellin S, Rossi CR, Foletto M, Campana L, Nitti D, Lise M. Cytoreductive surgery combined with hyperthermic intraperitoneal intraoperative chemotherapy for peritoneal carcinomatosis arising from colon adenocarcinoma. *Ann Surg Oncol* 2003; **10**: 508-513

26 **Verwaal VJ**, van Tinteren H, Ruth SV, Zoetmulder FA. Toxicity of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. *J Surg Oncol* 2004; **85**: 61-67

27 **Cavaliere F**, Perri P, Di Filippo F, Giannarelli D, Botti C, Cosimelli M, Tedesco M, Principi F, Laurenzi L, Cavaliere R. Treatment of peritoneal carcinomatosis with intent to cure. *J Surg Oncol* 2000; **74**: 41-44

28 **Schmidt U**, Dahlke MH, Klempnauer J, Schlitt HJ, Piso P. Perioperative morbidity and quality of life in long-term survivors following cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. *Eur J Surg Oncol* 2005; **31**: 53-58

29 **Elias D**, Blot F, El Otmany A, Antoun S, Lasser P, Boige V, Rougier P, Ducreux M. Curative treatment of peritoneal carcinomatosis arising from colorectal cancer by complete resection and intraperitoneal chemotherapy. *Cancer* 2001; **92**: 71-76

30 **Shen P**, Hawksworth J, Lovato J, Loggie BW, Geisinger KR, Fleming RA, Levine EA. Cytoreductive surgery and intraperitoneal hyperthermic chemotherapy with mitomycin C for peritoneal carcinomatosis from nonappendiceal colorectal carcinoma. *Ann Surg Oncol* 2004; **11**: 178-186

31 **Glehen O**, Osinsky D, Cotte E, Kwiatkowski F, Freyer G, Isaac S, Trillet-Lenoir V, Sayag-Beaujard AC, François Y, Vignal J, Gilly FN. Intraperitoneal chemohyperthermia using a closed abdominal procedure and cytoreductive surgery for the treatment of peritoneal carcinomatosis: morbidity and mortality analysis of 216 consecutive procedures. *Ann Surg Oncol* 2003; **10**: 863-869

32 **Younan R**, Kusamura S, Baratti D, Oliva GD, Costanzo P, Favaro M, Gavazzi C, Deraco M. Bowel complications in 203 cases of peritoneal surface malignancies treated with peritonectomy and closed-technique intraperitoneal hyperthermic perfusion. *Ann Surg Oncol* 2005; **12**: 910-918

33 **Ryu KS**, Kim JH, Ko HS, Kim JW, Ahn WS, Park YG, Kim SJ, Lee JM. Effects of intraperitoneal hyperthermic chemotherapy in ovarian cancer. *Gynecol Oncol* 2004; **94**: 325-332

34 **Makrin V**, Lev-Chelouche D, Even Sapir E, Paran H, Rabau M, Gutman M. Intraperitoneal heated chemotherapy affects healing of experimental colonic anastomosis: an animal study. *J Surg Oncol* 2005; **89**: 18-22

35 **Sugarbaker PH**, Chang D. Results of treatment of 385 patients with peritoneal surface spread of appendiceal malignancy. *Ann Surg Oncol* 1999; **6**: 727-731

36 **González-Moreno S**, Sugarbaker PH. Right hemicolectomy does not confer a survival advantage in patients with mucinous carcinoma of the appendix and peritoneal seeding. *Br J Surg* 2004; **91**: 304-311

37 **Baratti D**, Kusamura S, Nonaka D, Langer M, Andreola S, Favaro M, Gavazzi C, Laterza B, Deraco M. Pseudomyxoma peritonei: clinical pathological and biological prognostic factors in patients treated with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (HIPEC). *Ann Surg Oncol* 2008; **15**: 526-534

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RAPID COMMUNICATION

Effect of propranolol on the splanchnic and peripheral renin angiotensin system in cirrhotic patients

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Abstract

AIM: To evaluate the effect of β-blockade on angiotensins in the splanchnic and peripheral circulation of cirrhotic patients and also to compare hemodynamic parameters during liver transplantation according to propranolol pre-treatment or not.

METHODS: Patients were allocated into two groups: outpatients with advanced liver disease(LD) and during liver transplantation(LT). Both groups were subdivided according to treatment with propranolol or not. Plasma was collected through peripheral venipuncture to determine plasma renin activity(PRA), Angiotensin(Ang) Ⅰ, Ang Ⅱ, and Ang-(1-7) levels by radioimmunoassay in LD group. During liver transplantation, hemodynamic parameters were determined and blood samples were obtained from the portal vein to measure renin angiotensin system(RAS) components.

RESULTS: PRA, Ang Ⅰ, Ang Ⅱ and Ang-(1-7) were significantly lower in the portal vein and periphery in all subgroups treated with propranolol as compared to non-treated. The relationships between Ang-(1-7) and Ang Ⅰ levels and between Ang Ⅱ and Ang Ⅰ were significantly increased in LD group receiving propranolol. The ratio between Ang-(1-7) and Ang Ⅱ remained unchanged in splanchnic and peripheral circulation in patients under β-blockade, whereas the relationship between Ang Ⅱ and Ang Ⅰ was significantly increased in splanchnic circulation of LT patients treated with propranolol. During liver transplantation, cardiac output and index as well systemic vascular resistance and index were reduced in propranolol-treated subgroup.

CONCLUSION: In LD group, propranolol treatment reduced RAS mediators, but did not change the ratio between Ang-(1-7) and Ang Ⅱ in splanchnic and peripheral circulation. Furthermore, the modification of hemodynamic parameters in propranolol treated patients was not associated with changes in the angiotensin ratio.

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Key words: β-blockade; Cirrhosis; Renin angiotensin system; Angiotensin-(1-7)

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INTRODUCTION

The Renin-Angiotensin System(RAS) is a multilayered complex system. Previously, Angiotensin (Ang) Ⅱ was thought to be the principle active peptide, exerting its action through type Ⅰ and type Ⅱ receptors[1]. However, our understanding of the RAS has significantly

grown[1,2]. Additional active RAS peptides have been identified such as Ang Ⅲ, with actions similar to Ang Ⅱ and Ang Ⅳ, which exerts its activity at insulin-regulated amino peptidase receptors, and Ang-(1-7), which acts mainly through Mas receptors[1,2]. There is evidence that the RAS acts at the local tissue and even intracellular level through specific receptors by exerting paracrine, endocrine and intracrine functions[1,2]. Further, the system mediates a variety of opposing physiological actions including vasoconstriction/vasodilation, fibrosis/antifibrosis and inflammation/anti-inflammatory[1,2]. Therefore, the RAS is now viewed as a dual system composed of two arms: a vasoconstrictor arm formed by angiotensin converting enzyme(ACE)-Angiotensin (Ang) Ⅱ-AT1 receptor and a vasodilator arm with ACE2-Ang-(1-7)-Mas receptor. The ACE2-Ang-(1-7)-Mas arm mainly acts as a counter-regulatory mechanism for the vasoconstrictor arm[1]. According to this novel concept, the final functional effect of the RAS may reflect a balance between these two arms[2-5].

This novel view of the RAS makes the evaluation of this system in cirrhosis particularly challenging. In this regard, recent studies have suggested that the RAS seems to be involved in cirrhosis through its two main arms: the first (ACE-Ang Ⅱ-AT1) by inducing liver fibrosis[6] and maintaining the basal vascular tonus in cirrhosis[7] and the second [ACE2-Ang-(1-7)-Mas] by exerting an anti-fibrotic role[8,9] and probably by participating in the vasodilation of cirrhosis[10].

Non-selective β-adrenergic blockers have been widely used in treatment of portal hypertension in cirrhosis. β-blockers lower portal pressure by reducing portal blood flow as a consequence of a decreased cardiac output (β_1-receptor blockade) and arteriolar splanchnic vasoconstriction (β_2-receptor blockade)[11]. β-blockers also inhibit renin secretion[12]. However, the effect of propranolol on RAS mediators has still not been quantified, and neither have the hemodynamic changes that might occur during liver transplantation in cirrhotic patients pre-treated with propranolol. Since non-specific β blockade has been a standard approach to controlling the symptoms of portal hypertension and because the RAS seems to influence the outcome of portal hypertension and cirrhosis, it is reasonable to ask if there is a functional relationship between the RAS and beta-receptor system. For this purpose, we have taken in this study the first steps to understand how β1 and β2 blockade affects the RAS in cirrhotic patients. Thus, the aim of the present study was to compare the levels of plasma renin activity (PRA), Ang Ⅰ, Ang Ⅱ and Ang-(1-7), measured in the splanchnic and peripheral circulations of cirrhotic patients receiving or not propranolol and to evaluate the effect of previous administration of propranolol on hemodynamic parameters during liver transplantation.

MATERIALS AND METHODS

Patients

This cross-sectional study used a convenience sample recruited from either the Alfa Institute of Hepatology/Liver Transplantation or the Clinical Primary Care Center of our institution.

Table 1 Clinical characteristics and casual measurements of advanced liver disease outpatients (LD) treated with propranolol or not

Characteristics and measurements	LD with propranolol (n = 9)	LD without propranolol (n = 7)
Age (yrs)	45 ± 2	54 ± 5
Sex male/female	5 (55.6%)/4 (44.4%)	4 (57%)/3 (43%)
Child Pugh Score	9.8 ± 0.5	11.0 ± 0.8
MELD Score	27.1 ± 1.3	29.3 ± 2.1
Albumin (g/dL)	2.7 ± 0.2	2.4 ± 0.3
Bilirubin (mg/dL)	2.5 (1.3-5.1)	2.5 (1.2-7.1)
Creatinine (mg/dL)	1.2 (0.8-2.3)	1.0 (1.0-1.45)
INR (International Normalized Ratio)	1.62 (1.01-6.15)	1.55 (1.20-2.20)
Serum Na^+ (mEq/L)	133.0 ± 1.6	126.0 ± 2.7[a]

Data are expressed as mean ± SE or median (25 and 75 percentile), except for sex where number of patients and percentages are shown. [a]$P < 0.05$ for the comparison of LD with propranolol and LD without propranolol (unpaired t test for mean comparisons and Mann-Whitney test for median comparisons).

Table 2 Clinical characteristics and casual measurements of patients undergoing liver transplantation (LT) pre-treated with propranolol or not

Characteristics and measurements	LT with propranolol (n = 10)	LT without propranolol (n = 11)
Age (yr)	50.6 ± 3.4	50.0 ± 2.6
Sex male/female	3 (30%)/7 (70%)	7 (63.6%)/4 (36.4%)
Child Pugh Score	10.5 ± 0.4	11.2 ± 0.6
MELD Score	28.0 ± 1.1	29.8 ± 1.6
Albumin (g/dL)	2.81 ± 0.09	2.61 ± 0.15
Bilirubin (mg/dL)	3.38 ± 1.20	3.70 ± 0.87
Creatinine (mg/dL)	1.0 (1.0-1.45)	1.05 (0.75-1.50)
INR (International Normalized Ratio)	1.36 (1.32-1.95)	1.69 (1.24-2.11)
Serum Na^+ (mEq/L)	135.2 ± 1.0	130.1 ± 1.8[a]

Data are expressed as mean ± SE or median (25 and 75 percentile), except for sex where number of patients and percentages are shown. [a]$P < 0.05$ for the comparison of LT with propranolol and LT without propranolol (unpaired t test for mean comparisons and Mann-Whitney test for median comparisons).

Inclusion criteria

Patients diagnosed with hepatic cirrhosis defined through liver histopathology and/or ultrasonography findings were included in this study. Tables 1 and 2 display the Child-Pugh[13] and MELD[14] scores of our patients (all patients were on the waiting list for liver transplantation). The etiology of the liver disease was established in the majority of the subjects (69%), and included alcoholism, virus C, virus B and bile cirrhosis. The cirrhotic patients were allocated to two study groups: one group was composed of patients who had advanced liver disease and were seen in an outpatient clinic (LD, n = 16) and the second group was composed of liver transplant recipients during surgery (LT, n = 21). Each

of these two groups was further divided into patients who received propranolol and those who did not. The assistant physician was the only person responsible for the prescription and indication of propranolol treatment and the study protocol did not interfere with any medical prescriptions and recommendations. Thus, patients who were already on treatment with propranolol were then compared to those that did not receive treatment. As shown in Tables 1 and 2, the two subgroups of patients (treated *vs* non-treated) are comparable in the major demographic characteristics.

The LD group comprised outpatients with ascites and extra-hepatic complications such as encephalopathy and moderate to large esophageal varices (> 5 mm) with risk of bleeding. These patients were using diuretics (furosemide: 40-80 mg/d associated with spironolactone: 25-100 mg/d). Nine of these patients were also receiving propranolol for a mean period of 60 d (40-80 mg/d). The doses of propranolol were titrated to achieve a 20%-25% change in baseline heart rate.

The LT group included hospitalized cirrhotic patients with the same severity of liver disease as compared to LD group based on Child Pugh and MELD scores (Child Pugh: 11.0 ± 0.8 in LD *vs* 11.2 ± 1.2 in LT and MELD: 29.3 ± 2.1 in LD *vs* 29.8 ± 3.2 in LT, $P > 0.05$ for both comparisons). These patients also presented the same clinical and laboratorial features as the LD group and received the same diuretic treatment. The only difference between both groups is the fact that LT patients have been submitted to liver transplantation. Ten of the LT patients were using propranolol (40-80 mg/d) until the time of liver transplantation and their doses were also titrated to achieve a 20%-25% change in baseline heart rate.

Exclusion criteria

Co-morbidities such as diabetes, heart, pulmonary, autoimmune and neurological diseases automatically excluded subjects from the study. Patients receiving chronic treatment with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, renin inhibitors and corticosteroids were also excluded from the study. During liver transplantation, blood collection was suspended whenever the subject presented acute hemodynamic disarrangements and needed to use a vasoconstrictor.

Ethical aspects

The Ethics Committee of the Federal University of Minas Gerais approved the study. Informed consent was obtained from all included subjects. The research protocol did not interfere with any medical recommendations or prescriptions. Subject follow-up was guaranteed even in cases of refusal to participate in the study.

Study protocol

Protocol 1 - Evaluation of circulating RAS in outpatients using or not using propranolol: Blood samples for PRA and angiotensin measurements were obtained from LD patients on a single occasion taking into account the inclusion and exclusion criteria for each group. Due to ethical reasons, no changes to the clinical approach were made for study purposes. Blood samples (10 mL) were collected through peripheral venipuncture in the morning after a fasting period of 8 h. All subjects rested in supine position for at least 30 min before blood sampling.

Protocol 2 - Evaluation of RAS and hemodynamic parameters during pre-anhepatic stage of liver transplantation in patients pre-treated with propranolol or not: In the LT group, blood sampling was performed during the pre-anhepatic stage of liver transplantation and samples were obtained from the portal vein (10 mL) to evaluate RAS mediators. Hemodynamic parameters (cardiac output, cardiac index, systemic vascular resistance and systemic vascular resistance index) were determined simultaneously with the blood sampling. These measurements were obtained through invasive continuous monitoring *via* a Swan-Ganz catheter (CCOMBO/SvO2, 110 cm/7.5 F, Edwards Lifesciences, Irvine, CA, USA), using Dixtal (DX 2020, Dixtal Biomedical, São Paulo, Brazil) and Vigilance (CEDV, Edwards Lifesciences, Irvine, CA, USA) monitors. Anesthesia for liver transplantation was induced by a rapid sequence of etomidate, fentanyl and succinylcholine and maintained by isoflurane (CAM~1.0) and atracurium until the blood sampling.

Blood collection: For all blood collections, samples were drawn into two sets of ice-cooled tubes-one containing 7.5% EDTA for PRA determinations and the other containing a cocktail of protease inhibitors for angiotensin measurements, as previously described[14]. Blood samples were centrifuged at × 2000 *g* for 20 min at 4℃ and plasma stored at -20℃[14].

Plasma extraction and radioimmunoassays: Plasma samples were extracted using Bond-Elut cartridges (Analytichem International, Harbor City, CA), as described elsewhere[15]. PRA as well as Ang Ⅰ, Ang Ⅱ and Ang-(1-7) concentrations were determined through radioimmunoassays, as detailed elsewhere[15]. The recovery of ^{125}I-labeled Ang Ⅰ, Ang Ⅱ, and Ang-(1-7) was 79.2% ± 2.3%, 86.9% ± 0.8% and 83.5% ± 0.9%, respectively. Results were expressed as nanograms of Ang I generated per mL of plasma per hour (ng Ang Ⅰ /mL per hour) for PRA and pg/mL of plasma for Ang measurements.

Statistical analysis

Gaussian distribution of variables was evaluated by the Shapiro normality test. Results were reported as mean ± SE or median, when appropriate. Unpaired *t* test was used for the comparison of means between groups. Mann-Whitney was used to compare non-parametric

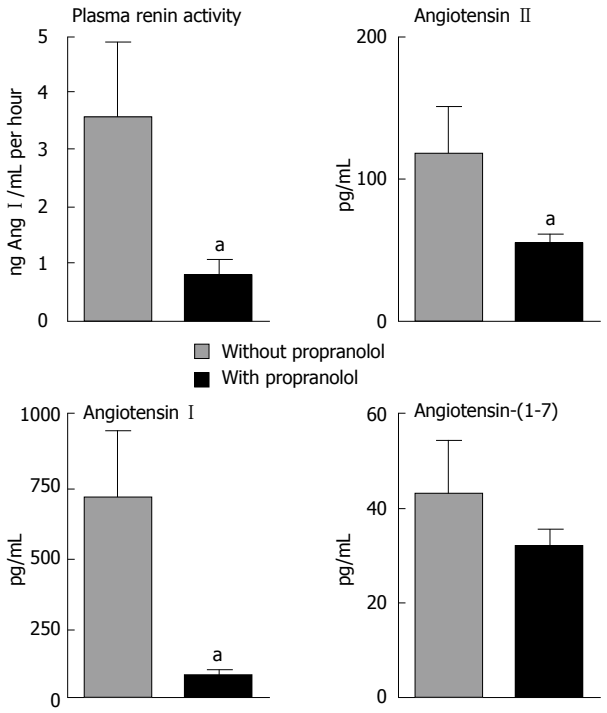

Figure 1 Peripheral circulating RAS profile in advanced liver disease outpatients (LD) receiving propranolol or not. Data are expressed as means ± SE. $^{a}P < 0.05$ for the comparison of LD with propranolol and LD without propranolol (unpaired *t* test).

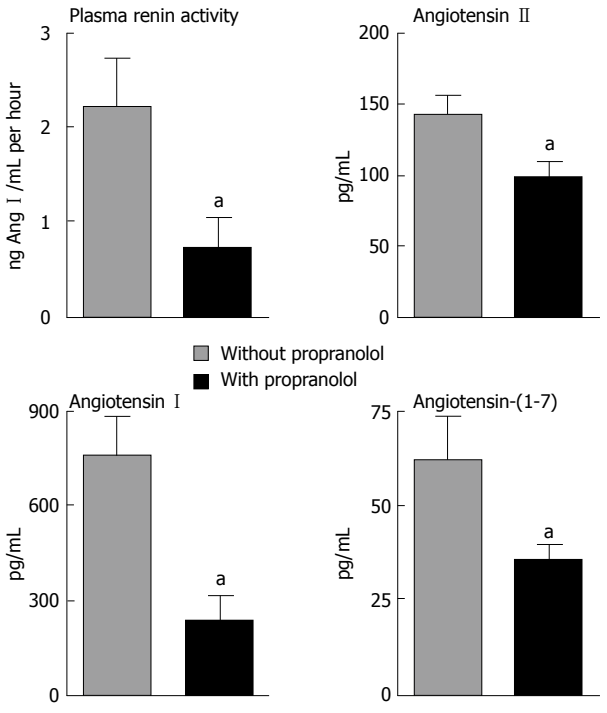

Figure 2 RAS profile of the splanchnic circulation in patients undergoing liver transplantation (LT), pre-treated with propranolol or not. Data are expressed as means ± SE. $^{a}P < 0.05$ for the comparison of LT with propranolol and LT without propranolol (unpaired *t* test).

data. The level of significance was set at $P < 0.05$. Graphpad PRISM was used for the statistical analyses.

RESULTS

Subject characteristics and casual measurements

The outpatients with liver cirrhosis (LD, $n = 16$) consisted of 9 males and 7 females from 44 to 66 years. The etiologies of liver failure in these subjects were: alcoholism in 4, bile cirrhosis in 1, virus C in 5, virus B in 1 and idiopathic causes in 5 patients. Clinical and laboratorial data revealed high Child Pugh and MELD scores that were very similar between the groups of patients using or not using propranolol (Table 1). Only serum sodium levels were significantly reduced in LD patients not receiving propranolol as compared to those treated with propanolol (Table 1).

Cirrhotic patients during liver transplantation (LT, $n = 21$) comprised 10 males and 11 females from 36 to 67 years. The etiologies for primary hepatic diseases in this group included virus C in 9, alcoholism in 4, bile cirrhosis in 2, virus B in 1 and idiopathic in 5 patients. The LT group without propranolol revealed reduced serum sodium levels compared to the LT group receiving propranolol. Other demographic characteristics and liver function scores were similar in both subgroups (Table 2).

Peripheral and splanchnic circulating RAS profile in LD and LT patients pre-treated with propranolol or not

As displayed in Figure 1, PRA and angiotensin I (Ang I) were lower in peripheral circulation of the LD group treated with propranolol in comparison to LD group not receiving propranolol (PRA: 3.54 ± 1.35 ng Ang I /mL per hour *vs* 0.77 ± 0.28 ng Ang I /mL per hour; Ang I: 717.2 ± 39.0 pg/mL *vs* 79.8 ± 24.8 pg/mL, $P < 0.05$ for both comparisons). As shown in Figure 2, the same profile was observed for the LT group pre-treated with propranolol, which also presented a significant reduction of the PRA and Ang I plasma levels in the portal vein when compared to the LT group that was not treated with propranolol (PRA: 2.20 ± 0.51 ng Ang I /mL per hour *vs* 0.74 ± 0.30 ng Ang I /mL per hour; Ang I: 764.2 ± 117.0 pg/mL *vs* 235.0 ± 75.6 pg/mL, $P < 0.05$ for both comparisons).

LD patients receiving propranolol also exhibited a significant reduction in the levels of Ang II in peripheral circulation when compared to LD patients not using propranolol (Ang II: 117.2 ± 33.5 pg/mL *vs* 53.9 ± 6.5 pg/mL, $P < 0.05$, Figure 1). The same reduction of Ang II levels was observed in splanchnic circulation (portal vein) of the LT group under β-blockade in comparison to LT group not treated with propranolol (Ang II: 143.4 ± 13.3 pg/mL *vs* 96.9 ± 12.6 pg/mL, $P < 0.05$, Figure 2). Plasma levels of Ang-(1-7) were also reduced in splanchnic circulation of the LT group that was previously treated with propranolol in comparison to non-treated LT group (62.3 ± 10.9 pg/mL *vs* 35.5 ± 3.8 pg/mL, $P < 0.05$, Figure 2), whereas plasma Ang-(1-7) in peripheral circulation of the LD group did not differ significantly despite treatment or not with propranolol (Figure 1).

Ratios between Ang-(1-7) and Ang I levels, between Ang II and Ang I, and between Ang-(1-7) and Ang II in LD and LT groups are displayed in Tables 3 and 4, respectively. The ratio between Ang-(1-7) and Ang I indirectly reflects ACE2 activity, whereas the

Table 3 Ratios between angiotensins in peripheral circulation of advanced liver disease outpatients (LD) treated with propranolol or not

Angiotensin ratios	LD with propranolol (n = 9)	LD without propranolol (n = 7)
Ang Ⅱ/Ang Ⅰ	1.24 ± 0.30	0.21 ± 0.05[a]
Ang-(1-7)/Ang Ⅰ	0.74 ± 0.19	0.09 ± 0.03[a]
Ang-(1-7)/Ang Ⅱ	0.62 ± 0.06	0.42 ± 0.09

Data are expressed as mean ± SE. [a]P < 0.05 for the comparison of NLD with propranolol and NLD without propranolol (unpaired t test).

Table 4 Ratios between angiotensins in splanchnic circulation of patients undergoing liver transplantation (LT) pre-treated with propranolol or not

Angiotensin ratios	LT with propranolol (n = 10)	LT without propranolol (n = 11)
Ang Ⅱ/Ang Ⅰ	0.46 (0.26-2.64)	0.19 (0.13-0.24)[a]
Ang-(1-7)/Ang Ⅰ	0.27 (0.06-0.91)	0.05 (0.04-0.11)
Ang-(1-7)/Ang Ⅱ	0.43 ± 0.08	0.52 ± 0.12

Data are expressed as mean ± SE or median (25 and 75 percentile). [a]P < 0.05 for the comparison of LT with propranolol and LT without propranolol (unpaired t test for mean comparisons and Mann-Whitney test for median comparisons).

ratio between Ang Ⅱ and Ang Ⅰ indirectly estimates ACE activity. Both ratios were significantly increased in peripheral circulation of the LD group using propranolol in comparison to LD patients not receiving the β-blocker (P < 0.01, Table 3). Only Ang Ⅱ/Ang Ⅰ ratio was increased in splanchnic circulation of the LT group pre-treated with propranolol in comparison to LT patients that had not received propranolol (P < 0.01, Table 4), whereas Ang-(1-7)/Ang Ⅰ did not significantly differ in splanchnic circulation of LT patients despite the previous treatment or not with propranolol. More importantly, the ratio between Ang-(1-7) and Ang Ⅱ, which could represent the final functional relationship between RAS mediators, did not differ in either peripheral circulation of the LD group or in splanchnic circulation of the LT group, independently of the previous use or not of the β-blocker.

Hemodynamic parameters during pre-anhepatic stage of liver transplantation in LT pre-treated with propranolol or not

In order to demonstrate that our LT patients pre-treated with propranolol were adequately β blocked, we measured hemodynamic parameters during pre-anhepatic stage of liver transplantation. Accordingly, in LT patients pre-treated with propranolol, the cardiac output (8.9 ± 0.9 L/min *vs* 5.6 ± 0.6 L/min) and cardiac index (4.7 ± 0.4 L/min per m^2 *vs* 3.2 ± 0.3 L/min per m^2) were reduced and the systemic vascular resistance (604.2 ± 65.0 × 877.5 ± 106.1 dyn.s/cm^5) and its index [(1036 ± 86) × (1399 ± 147)] dyn.s/cm^5 per m^2) were increased in comparison to patients that had not previously received propranolol (P < 0.05 for all comparisons, Figure 3).

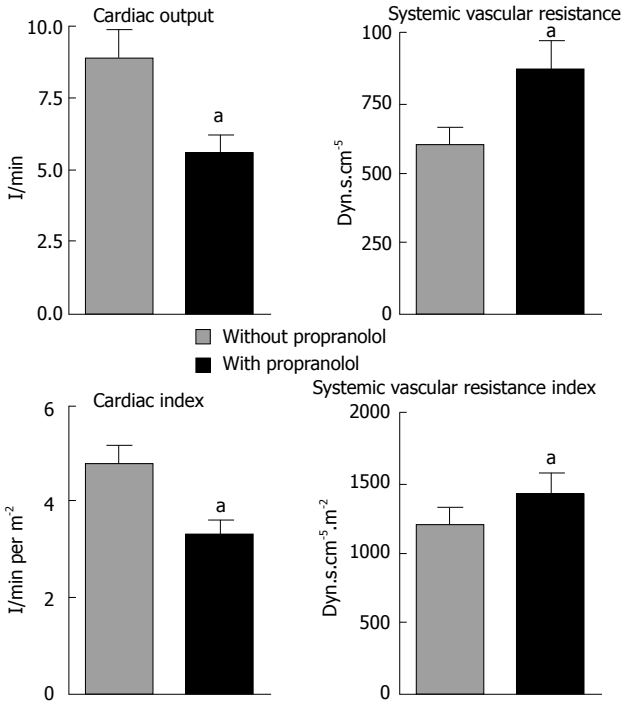

Figure 3 Hemodynamic parameters from cirrhotic patients during liver transplantation (LT) pre-treated with propranolol or not. Data are expressed as means ± SE. [a]P < 0.05 for the comparison of LT with propranolol and LT without propranolol (unpaired t test).

DISCUSSION

In general, our data showed that chronic treatment with propranolol in cirrhotic patients is characterized by marked changes in the precursors of RAS cascade (Renin and Ang Ⅰ) with repercussion in RAS two main arms at the splanchnic and peripheral circulation. On the other hand, no changes were detected in the ratio between the two main RAS mediators [Ang-(1-7)/Ang Ⅱ], which has been used to evaluate the final functional effect of the RAS. In parallel, the chronic use of propranolol produced hemodynamic changes, which were probably able to control the hyperdynamic circulation of cirrhotic patients. Taken together, these findings suggest that the reduction of hyperdynamic circulation produced by chronic treatment with propranolol in cirrhotic patients was associated with an overall RAS inhibition, but it was not due to changes in the balance between the two RAS arms: ACE-Ang-AT1 (vasoconstrictor) *versus* ACE2-Ang-(1-7)-Mas (vasodilator).

In splanchnic and peripheral circulation, the β-blockade in cirrhotic patients was characterized by reduced PRA and Ang Ⅰ levels. These RAS components can lead to the synthesis of both Ang Ⅱ and Ang-(1-7)[5,16]. The cirrhotic patients receiving propranolol have reduced Ang Ⅱ levels in the splanchnic and in the peripheral circulation as well as reduced Ang-(1-7) levels in the splanchnic circulation. In this regard, Blumenfeld *et al*[12] previously suggested that β-blockade reduced Ang Ⅱ levels and PRA in normotensive and hypertensive subjects by inhibiting prorenin processing to renin.

Ratios between angiotensins, especially the relationship between Ang-(1-7) and Ang Ⅱ, have been used to estimate the final functional RAS effect[2,3,4]. In this study, propranolol use was not able to change the ratio between Ang-(1-7) and Ang Ⅱ in splanchnic and peripheral circulation of non-compensated cirrhotic patients, although there was an absolute decrease in both angiotensins. In parallel, systemic vascular resistance (SVR) and its index increased and cardiac output (CO) and its index decreased in non-compensated cirrhotic patients treated with propranolol. Similar hemodynamic changes in cirrhotic patients receiving propranolol have already been reported[17-19] and attributed to β-adrenergic blockade[20]. Since the relationship between Ang-(1-7) and Ang Ⅱ remained unchanged in splanchnic and peripheral circulation of our cirrhotic patients, we could hypothesize that propranolol was not able to interfere with the final functional RAS effect upon vascular tone. Our data also suggest that the activity of the two main RAS enzymes, ACE and ACE2, were probably not reduced by propranolol use in cirrhotic patients, since the ratios between Ang Ⅱ and Ang I and between Ang-(1-7) and Ang I were increased in peripheral circulation and the ratio between Ang Ⅱ and Ang I was also elevated in splanchnic circulation. However, we can not exclude the possibility that other factors such as changes in the catabolism of Ang Ⅱ or Ang-(1-7) could have contributed to the reduction in absolute levels of each peptide.

In cirrhotic patients, arteriolar vasodilation and diuretic administration cause a decreased effective arterial blood volume that stimulates vasopressor systems leading to high levels of PRA, circulating norepinephrine, and vasopressin[21-23]. In this context, propranolol inhibits renin secretion and reduces vasodilation (SVR increase) in cirrhosis[17], leading to a reduction of the relative arterial hypovolemia. These actions could oppose the activated vasopressor systems (RAS, sympathetic nervous system and vasopressin) and may be involved in the amelioration of the hyperdynamic circulation observed in our cirrhotic patients.

It should also be pointed out that we are aware of the limitations of our study design. For example, peripheral blood samples generally represent the cumulative expression of RAS in multiple tissues and may not reliably reflect molecular activity in the splanchnic circulation. For this reason, we did manage to collect samples from the portal vein during liver transplantation. However, it is still difficult to compare these findings to the samples collected in peripheral blood from outpatients. Nevertheless, some aspects of this study may increase the strength of our findings, such as the utilization of strictly defined inclusion and exclusion criteria and the well-established protocol for the measurements of PRA and angiotensins[15].

In conclusion, results obtained with propranolol treatment in cirrhotic patients have been controversial[20,24]. While in advanced liver disease with significant reduction of the hepatic venous pressure gradient propanolol treatment decreased the risk of ascites, spontaneous bacterial peritonitis, hepatorenal syndrome and death[24], in unselected cirrhotic patients the same β-blocker was not able to prevent varices and was associated with an increased number of adverse events[20]. We believe that the use of propranolol in cirrhosis could change the prognosis of patients with hyperdynamic circulation and relative hypovolemia, but it is probably not able to interfere with potentially reversible liver fibrosis. Indeed, the use of propranolol did not alter the balance between the activity of the anti-fibrotic arm of the RAS, ACE2-Ang-(1-7)-Mas[8], and of the pro-fibrotic arm, ACE-Ang Ⅱ-AT1[6]. For this purpose, many studies have suggested that ACE inhibitors and AT1 receptor blockers seemed to be effective[25-29]. Their mechanisms of action probably involve not only the inhibition of Ang Ⅱ formation or action but also the augmentation of Ang-(1-7) levels or effects[5,29]. On the other hand, it should be mentioned that, mostly in advanced stages of cirrhosis, the ACE-Ang Ⅱ-AT1 arm contributes to the maintenance of basal vascular tonus[7] and therefore the use of AT1 receptor blockers or ACE inhibitors as antifibrotic therapies could not be well tolerated. Since propranolol administration seems to improve only the extrahepatic complications of the advanced cirrhotic patients, a possible therapeutic approach for human cirrhosis at this stage could be the combination of AT1 receptor blockers or ACE inhibitors with propranolol. Future studies with more powerful designs are obviously necessary to evaluate whether the use of propranolol at this stage of cirrhosis would enable the administration of AT1 receptor blockers or ACE inhibitors or even receptor Mas agonists to reduce liver fibrosis.

COMMENTS

Background

Recent studies have suggested that the Renin Angiotensin System (RAS) seems to be involved in cirrhosis. Non-selective β-adrenergic blockers have been widely used in treatment of portal hypertension in cirrhosis. However, the effect of propranolol on RAS mediators has still not been quantified. Since non-specific β blockade has been a standard approach to controlling the symptoms of portal hypertension and because the RAS seems to influence the outcome of portal hypertension and cirrhosis, it is reasonable to ask if there is a functional relationship between the RAS and beta-receptor system.

Research frontiers

This study represents an initial approach in understanding how non-specific β blockade affects RAS in cirrhotic patients by comparing the levels of plasma renin activity, Angiotensin (Ang) Ⅰ, Ang Ⅱ and Ang-(1-7), measured in the splanchnic and peripheral circulations of cirrhotic patients receiving or not receiving propranolol and by evaluating the effect of previous administration of propranolol on hemodynamic parameters during liver transplantation.

Innovations and breakthroughs

Chronic treatment with propranolol in cirrhotic patients is characterized by marked changes in the precursors of RAS cascade (Renin and Ang Ⅰ) with repercussion in RAS two main arms in the splanchnic and peripheral circulation. On the other hand, no changes were detected in the ratio between the two main RAS mediators [Ang-(1-7)/Ang Ⅱ]. Additionally, the treatment with propranolol seemed to be able to control the hyperdynamic circulation of cirrhotic patients probably due to an overall RAS inhibition, but without changes in the balance between the two RAS arms: ACE-Ang-AT1 (vasoconstrictor) *versus* ACE2-Ang-(1-7)-Mas (vasodilator).

Applications

Our data suggest that a possible therapeutic approach for advanced human cirrhosis could be the combination of AT1 receptor blockers or ACE inhibitors

with propranolol. Future studies with more powerful designs are obviously necessary to evaluate whether the use of propranolol at this stage of cirrhosis would enable the administration of AT1 receptor blockers or ACE inhibitors or even receptor Mas agonists to reduce liver fibrosis.

Peer review

In the current study, the investigators have taken the first steps to understand how β1 and β2 blockade affects RAS in patients. This is important because non-specific β blockade has been a standard approach to controlling the symptoms of portal hypertension.

REFERENCES

1 **Santos RA**, Ferreira AJ, Simões E Silva AC. Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis. *Exp Physiol* 2008; **93**: 519-527

2 **Simões E Silva AC**, Pinheiro SV, Pereira RM, Ferreira AJ, Santos RA. The therapeutic potential of Angiotensin-(1-7) as a novel Renin-Angiotensin System mediator. *Mini Rev Med Chem* 2006; **6**: 603-609

3 **Matsui T**, Tamaya K, Matsumoto K, Osajima Y, Uezono K, Kawasaki T. Plasma concentrations of angiotensin metabolites in young male normotensive and mild hypertensive subjects. *Hypertens Res* 1999; **22**: 273-277

4 **Nogueira AI**, Souza Santos RA, Simões E Silva AC, Cabral AC, Vieira RL, Drumond TC, Machado LJ, Freire CM, Ribeiro-Oliveira A Jr. The pregnancy-induced increase of plasma angiotensin-(1-7) is blunted in gestational diabetes. *Regul Pept* 2007; **141**: 55-60

5 **Warner FJ**, Lubel JS, McCaughan GW, Angus PW. Liver fibrosis: a balance of ACEs? *Clin Sci* (Lond) 2007; **113**: 109-118

6 **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218

7 **Helmy A**, Jalan R, Newby DE, Hayes PC, Webb DJ. Role of angiotensin II in regulation of basal and sympathetically stimulated vascular tone in early and advanced cirrhosis. *Gastroenterology* 2000; **118**: 565-572

8 **Pereira RM**, Dos Santos RA, Teixeira MM, Leite VH, Costa LP, da Costa Dias FL, Barcelos LS, Collares GB, Simões E Silva AC. The renin-angiotensin system in a rat model of hepatic fibrosis: evidence for a protective role of Angiotensin-(1-7). *J Hepatol* 2007; **46**: 674-681

9 **Herath CB**, Warner FJ, Lubel JS, Dean RG, Jia Z, Lew RA, Smith AI, Burrell LM, Angus PW. Upregulation of hepatic angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1-7) levels in experimental biliary fibrosis. *J Hepatol* 2007; **47**: 387-395

10 **Paizis G**, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, Shaw T, Warner FJ, Zuilli A, Burrell LM, Angus PW. Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. *Gut* 2005; **54**: 1790-1796

11 **Dib N**, Oberti F, Cales P. Current management of the complications of portal hypertension: variceal bleeding and ascites. *CMAJ* 2006; **174**: 1433-1443

12 **Blumenfeld JD**, Sealey JE, Mann SJ, Bragat A, Marion R, Pecker MS, Sotelo J, August P, Pickering TG, Laragh JH. Beta-adrenergic receptor blockade as a therapeutic approach for suppressing the renin-angiotensin-aldosterone system in normotensive and hypertensive subjects. *Am J Hypertens* 1999; **12**: 451-459

13 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649

14 **Wiesner RH**, McDiarmid SV, Kamath PS, Edwards EB, Malinchoc M, Kremers WK, Krom RA, Kim WR. MELD and PELD: application of survival models to liver allocation. *Liver Transpl* 2001; **7**: 567-580

15 **Simões E Silva AC**, Diniz JS, Regueira Filho A, Santos RA. The renin angiotensin system in childhood hypertension: selective increase of angiotensin-(1-7) in essential hypertension. *J Pediatr* 2004; **145**: 93-98

16 **Pagliaro P**, Penna C. Rethinking the renin-angiotensin system and its role in cardiovascular regulation. *Cardiovasc Drugs Ther* 2005; **19**: 77-87

17 **Andreu V**, Perello A, Moitinho E, Escorsell A, García-Pagán JC, Bosch J, Rodés J. Total effective vascular compliance in patients with cirrhosis. Effects of propranolol. *J Hepatol* 2002; **36**: 356-361

18 **Bañares R**, Moitinho E, Piqueras B, Casado M, Garcia-Pagan JC, de Diego A, Bosch J. Carvedilol, a new nonselective beta-blocker with intrinsic anti- Alpha1-adrenergic activity, has a greater portal hypotensive effect than propranolol in patients with cirrhosis. *Hepatology* 1999; **30**: 79-83

19 **Moller S**, Bendtsen F, Henriksen JH. Effect of beta-adrenergic blockade on elevated arterial compliance and low systemic vascular resistance in cirrhosis. *Scand J Gastroenterol* 2001; **36**: 653-657

20 **Groszmann RJ**, Garcia-Tsao G, Bosch J, Grace ND, Burroughs AK, Planas R, Escorsell A, Garcia-Pagan JC, Patch D, Matloff DS, Gao H, Makuch R. Beta-blockers to prevent gastroesophageal varices in patients with cirrhosis. *N Engl J Med* 2005; **353**: 2254-2261

21 **Henriksen JH**, Moller S. Liver cirrhosis and arterial hypertension. *World J Gastroenterol* 2006; **12**: 678-685

22 **Arroyo V**, Fernandez J, Gine P. Pathogenesis and treatment of hepatorenal syndrome. *Semin Liver Dis* 2008; **28**: 81-95

23 **Ginès P**, Cárdenas A. The management of ascites and hyponatremia in cirrhosis. *Semin Liver Dis* 2008; **28**: 43-58

24 **Bosch J**, Abraldes JG, Groszmann R. Current management of portal hypertension. *J Hepatol* 2003; **38** Suppl 1: S54-S68

25 **Terui Y**, Saito T, Watanabe H, Togashi H, Kawata S, Kamada Y, Sakuta S. Effect of angiotensin receptor antagonist on liver fibrosis in early stages of chronic hepatitis C. *Hepatology* 2002; **36**: 1022

26 **Wei YH**, Jun L, Qiang CJ. Effect of losartan, an angiotensin II antagonist, on hepatic fibrosis induced by CCl4 in rats. *Dig Dis Sci* 2004; **49**: 1589-1594

27 **Yoshiji H**, Noguchi R, Fukui H. Combined effect of an ACE inhibitor, perindopril, and interferon on liver fibrosis markers in patients with chronic hepatitis C. *J Gastroenterol* 2005; **40**: 215-216

28 **Debernardi-Venon W**, Martini S, Biasi F, Vizio B, Termine A, Poli G, Brunello F, Alessandria C, Bonardi R, Saracco G, Rizzetto M, Marzano A. AT1 receptor antagonist Candesartan in selected cirrhotic patients: effect on portal pressure and liver fibrosis markers. *J Hepatol* 2007; **46**: 1026-1033

29 **Lubel JS**, Herath CB, Burrell LM, Angus PW. Liver disease and the renin-angiotensin system: recent discoveries and clinical implications. *J Gastroenterol Hepatol* 2008; **23**: 1327-1338

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RAPID COMMUNICATION

Pre-operative predictive factors for gallbladder cholesterol polyps using conventional diagnostic imaging

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Abstract

AIM: To determine the clinical data that might be useful for differentiating benign from malignant gallbladder (GB) polyps by comparing radiological methods, including abdominal ultrasonography (US) and computed tomography (CT) scanning, with postoperative pathology findings.

METHODS: Fifty-nine patients underwent laparoscopic cholecystectomy for a GB polyp of around 10 mm. They were divided into two groups, one with cholesterol polyps and the other with non-cholesterol polyps. Clinical features such as gender, age, symptoms, size and number of polyps, the presence of a GB stone, the radiologically measured maximum diameter of the polyp by US and CT scanning, and the measurements of diameter from postoperative pathology were recorded for comparative analysis.

RESULTS: Fifteen of the 41 cases with cholesterol polyps (36.6%) were detected with US but not CT scanning, whereas all 18 non-cholesterol polyps were observed using both methods. In the cholesterol polyp group, the maximum measured diameter of the polyp was smaller by CT scan than by US. Consequently, the discrepancy between those two scanning measurements was greater than for the non-cholesterol polyp group.

CONCLUSION: The clinical signs indicative of a cholesterol polyp include: (1) a polyp observed by US but not observable by CT scanning, (2) a smaller diameter on the CT scan compared to US, and (3) a discrepancy in its maximum diameter between US and CT measurements. In addition, US and the CT scan had low accuracy in predicting the polyp diameter compared to that determined by postoperative pathology.

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Key words: Cholesterol; Polyps; Gallbladder; Computed tomography; Ultrasonography

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INTRODUCTION

The development of radiological diagnostic tools such as ultrasonography (US) and computed tomography (CT) scanning has led to an increased frequency of the diagnosis of gallbladder (GB) lesions, such as GB polyps[1-3]. Because of the poor prognosis of GB malignancies, it is very important to distinguish between benign and malignant GB polyps so that malignant disease can be treated as soon as possible. Currently, clinical data such as the size and number of GB polyps and the age of the patient are used to help distinguish benign from malignant disease. Improved diagnostic methods are needed to differentiate between benign and malignant disease, and to determine which GB polyps

require surgical intervention[4-6].

Therefore, we evaluated clinical data to determine which factors would help distinguish benign from malignant GB polyps. We retrospectively analyzed the preoperative US and CT findings in patients with GB polyps and compared the results with their postoperative gross and microscopic findings.

MATERIALS AND METHODS

Fifty-nine patients who underwent laparoscopic cholecystectomy for a GB polyp of around 10 mm between January 2006 and August 2007 were enrolled in this study. We divided these patients into two groups, a cholesterol polyp group and a non-cholesterol polyp group. Data were collected for clinical features such as gender, age, symptoms, size and number of polyps, presence of a GB stone, radiological data from the preoperative US and CT scanning, and postoperative pathology data.

We compared the radiologically measured maximum diameters of the GB polyps obtained by one radiologist with the postoperatively obtained pathologic measurements of maximum diameters obtained by one pathologist. Results are reported as the mean ± standard deviation. For statistical analysis, a Chi-square, *t*-test and Fisher's Exact Test were used (SPSS version 15.0 software). A *P*-value < 0.05 was considered statistically significant.

RESULTS

Pathologic findings of the GB polyps

Of the 59 cases, 46 (78%) were pseudo-polyps such as a cholesterol polyp, inflammatory or hyperplastic polyp. Of these 46 pseudo-polyps, 41 (69.5%) were cholesterol polyps. True polyps were observed in 13 cases. Among the true polyps, 10 cases (17%) were adenomatous polyps and three cases (5.0%) were malignant.

Clinical findings of the GB polyps

Of the 59 patients, 37 patients were male and 22 were female. No difference was observed in gender ratios for the cholesterol polyp group (M:F = 25:16) and the non-cholesterol polyp group (M:F = 12:6 $P > 0.05$). The mean ages for each group were 40.98 ± 9.41 for the cholesterol polyp group and 48.39 ± 16.87 for the non-cholesterol polyp group. The former group had a significantly lower mean age ($P = 0.044$).

Five patients had presenting symptoms, which included three cases of indigestion, one case of right upper quadrant pain and discomfort and one case with fever suggesting cholecystitis. Three cases of cholesterol polyps (7.3%) and two cases of non-cholesterol polyps (11.1%) were associated with a GB stone. The factors associated with the metabolic syndrome were analyzed in the two groups. The mean body mass index (BMI) was 24.83 ± 2.92 kg/m^2 in the cholesterol polyp group and 23.80 ± 3.23 in the non-cholesterol polyp group; the mean homeostasis model assessment of insulin resistance (HOMA-IR) was 1.49 ± 1.85 and 1.26 ± 1.35 respectively, the mean HbA1c was 5.58 ± 0.74 (%) and 5.41 ± 0.23, respectively. The mean values for all these factors were slightly higher in the cholesterol polyp group but they were not statistically significant ($P > 0.05$) (Table 1).

Table 1 Clinical and laboratory characteristics of patients with gallbladder polyps (mean ± SD)

Histologic finding	Cholesterol polyp	Non cholesterol polyp	*P*-value
Age (yr)	40.98 ± 9.41	48.39 ±16.87	< 0.05
Sex (male/female)	25/16	12/6	0.677
Height (m)	1.6703 ± 0.09	1.65 ± 0.07	0.595
Weight (kg)	69.39 ± 13.26	64.85 ± 10.73	0.207
Cholelithiasis(case)	3	2	0.63
BMI (kg/m^2)	24.83 ± 2.92	23.80 ± 3.23	0.245
Fasting glucose (mg/dL)	97.05 ± 21.76	96.94 ± 15.0	0.985
Insulin (μIU/mL)	10.54 ± 3.63	9.47 ± 2.71	0.412
Homa-IR	1.49 ± 1.85	1.26 ± 1.35	0.64
HbA1c (%)	5.58 ± 0.74	5.41 ± 0.23	0.553
US size (mm)	9.95 ± 2.31	11.94 ± 4.02	< 0.05
CT size (mm)	6.77 ± 2.65	9.78 ± 5.19	< 0.05
Pathology size (mm)	4.83 ± 2.97	11.06 ± 5.11	< 0.01

Table 2 The number of polyps in cases with cholesterol and non-cholesterol polyps *n*(%)

		Cholesterol polyp	Non-cholesterol polyp	Total
Ultrasonographic findings ($P < 0.01$)				
Polyp number	Solitary	19 (46.3)	16 (88.8)	35 (59.3)
	Multiple	22 (53.7)	2 (11.1)	24 (40.7)
Total		41 (69.5)	18 (30.5)	59 (100.0)
Pathologic finding ($P < 0.01$)				
Polyp number	Solitary	11 (26.8)	15 (83.3)	26 (44.1)
	Multiple	30 (73.2)	3 (16.7)	33 (55.9)
Total		41 (69.5)	18 (30.5)	59 (100.0)

The number of GB polyps

In US, a single GB polyp was observed in 35 cases (59.3%) and multiple GB polyps were observed in 24 cases (40.7%). The proportion of multiple polyps in the cholesterol polyp group was 53.7% (22 out of 41 cases), which was higher than in the non-cholesterol polyp group (11.1%: 2 out of 18 cases, $P = 0.002$). For the postoperative pathology examinations, these proportions increased; 73.2% (30 out of 41 cases) and 16.7% (3 out of 18 cases), respectively ($P < 0.001$) (Table 2).

The discrepancy in maximum diameter between US and CT scanning

The preoperative mean maximum diameters measured by US in the cholesterol polyp group and the non-cholesterol polyp group were 9.95 ± 2.31 mm and 11.94 ± 4.02 mm, respectively, whereas for the CT scan they were 6.77 ± 2.65 mm and 9.78 ± 5.19 mm, respectively. The mean values for CT scanning tended to be smaller than for US.

The discrepancies in maximum diameters between US and CT scanning were 5.66 ± 3.87 mm in the

Table 3 The difference in the maximum polyp size between cholesterol and non-cholesterol polyps (mean ± SD)

	Cholesterol polyp	Non-cholesterol polyp	*P*-value
US-CT size difference (mm)	5.66 ± 3.87	2.17 ± 2.12	0
US size > CT size[1]	40/41	12/18	0.002
CT undetectable rate(%)	15/41 (36.6)	0/18 (0)	0.001
US-pathologic size difference (mm)	5.12 ± 3.42	0.89 ± 3.69	0

[1]Indicates number of patients having a larger size with US than CT.

Table 4 The correlation of size between cholesterol and non-cholesterol polyps

Correlation coefficients		Pathologic size	US size	CT size
Pathologic size	Non-cholesterol polyp	1	0.698[b]	0.746[b]
	Cholesterol polyp	1	0.181	0.324
US size	Non-cholesterol polyp	0.698[b]	1	0.925[d]
	Cholesterol polyp	0.181	1	0.427[c]
CT size	Non-cholesterol polyp	0.746[b]	0.925[d]	1
	Cholesterol polyp	0.324	0.427[c]	1

[b]$P < 0.01$ *vs* Pathologic size, [b]$P < 0.05$ *vs* US size.

cholesterol polyp group and 2.17 ± 2.12 mm in the non-cholesterol polyp group and this difference was statistically significant ($P < 0.01$). In 40 out of 41 cholesterol polyps (97.6%) and 12 out of 18 non-cholesterol polyps (66.6%) the diameters were smaller with CT scanning than with US ($P < 0.01$).

All 18 cases in the non-cholesterol polyp group were detected both by US and CT whereas 15 cases in the cholesterol polyp group among 41 (36.6%) were detected by US but not by CT scanning ($P < 0.01$, Table 3).

The discrepancy between preoperatively and postoperatively measured maximum polyp diameters

The pathologically measured mean maximum diameters were 4.83 ± 2.97 mm in the cholesterol polyp group and 11.06 ± 5.11 mm in the non-cholesterol polyp group ($P < 0.01$). When we compared these values with the preoperatively US measurements the discrepancies between preoperative and postoperative measurements were 5.12 ± 3.42 mm in the cholesterol polyps and 0.89 ± 3.69 mm in the non-cholesterol polyps ($P < 0.01$, Table 3).

The correlation between radiologically measured and pathologically measured polyp diameters

The non-cholesterol polyps showed statistically significant linear correlations between the actual maximum diameter from the pathology examination and the preoperative US measured diameter (correlation coefficient 0.698) and the CT measured diameter (correlation coefficient 0.746, $P < 0.01$). The cholesterol polyps, however, did not show this correlation ($P > 0.05$, Table 4).

DISCUSSION

The correct diagnosis of cholesterol polyps, which account for most of the pseudo-polyps of the GB, will help prevent unnecessary surgery and follow-up examinations. In this study, we attempted to characterize the features of the cholesterol polyp and determine accurate radiological predictive factors. Age is known to have a significant association with malignant polyps and is considered an independent risk factor[5-7]. This study also found that patients with non-cholesterol polyps had a higher mean age than did the patients in the cholesterol polyp group. Metabolic syndrome is also known to have a close relationship with the development of cholesterol polyps[2,8,9]. Although the patients with cholesterol polyps had higher levels of the BMI, HOMA-IR, and HbA1c, the differences did not reach statistical significance. The sample size might have been too small to detect any differences.

Regarding the number of polyps in the GB, it is also known that a single polyp is more likely to be a malignant polyp, which prompts the need for more aggressive interventions when a single polyp is identified compared to multiple polyps[5,10]. We found a similar tendency among our study population. The patients with cholesterol polyps more frequently had multiple polyps than did the patients with non-cholesterol polyps. It is well known that the size of a GB polyp is related to malignancy. Many studies have reported that a GB polyp ⩾ 10 mm has a high risk of being a malignancy and this size is one of the criteria for surgical intervention[4,11-13]. However, we also have observed that a benign polyp, such as a cholesterol polyp, can be as large as 10 mm. Therefore, size may not afford an accurate distinction between benign and malignant polyps[14,15].

In cases with a cholesterol polyp, we observed discrepancies in the size and number of polyps between the preoperative radiological measurements and the postoperative pathology measurements. The postoperative pathology of cholesterol polyps had a smaller size and higher multiplicity than did the preoperative radiological studies. A possible explanation for this finding is that the cholesterol polyp might be damaged during the laparoscopic cholecystectomy or during handling of the GB tissue considering its histological fragility and weakness. The cholesterol polyp had low correlation coefficients in the comparisons between the pathologically measured size after surgery and the radiologically measured sizes prior to surgery. Therefore, the radiological studies are limited in obtaining the correct measurements for cholesterol polyps.

In conclusion, the cholesterol polyp has a tendency to be observed more frequently in younger patients and has higher multiplicity. The predictive signs for a cholesterol polyp, a benign tumor, include: a polyp observable by US but not CT scanning, a discrepancy ⩾ 5 mm in the maximum diameter of the polyp between the US and CT measurements, a smaller diameter of the polyp by CT compared to US, and a low correlation between the diameter of the polyp from postoperative pathology and

the preoperative radiological measurements.

We suggest that it would be more efficient to make a flexible and tailored follow up plan or treatment plan for GB polyps based on the above mentioned signs rather than fixed or inflexible guidelines. In addition, the preoperative radiological measurement of diameter is of predictive value for the postoperatively measured actual diameter only for non-cholesterol polyps. For cholesterol polyps, the preoperative radiological measurements are limited in their prediction of postoperative pathology diameter. Therefore, methods that are more accurate for the preoperative diagnosis of cholesterol polyps are needed.

COMMENTS

Background

The development of radiological diagnostic tools has led to an increased frequency of the diagnosis of gallbladder (GB) lesions, such as GB polyps.

Research frontiers

It is very important to distinguish between benign and malignant GB polyps because of the poor prognosis of GB malignancies. Improved diagnostic methods are needed to differentiate benign from malignant disease, and to determine which GB polyps require surgical intervention.

Innovation and breakthroughs

The predictive signs for a cholesterol polyp, the most common benign GB polyp, include: a polyp observable by ultrasonography (US) but not computed tomography (CT) scanning, a discrepancy $\geqslant$ 5 mm in the maximum diameter of the polyp between US and CT measurements, a smaller diameter of the polyp by CT than by US.

Applications

This study should help to distinguish a cholesterol polyp from a non-cholesterol polyp. It would be more efficient to make a flexible and tailored follow up plan or treatment plan for GB polyps.

Peer review

The concept of this study is useful.

REFERENCES

1 **Chen CY**, Lu CL, Chang FY, Lee SD. Risk factors for gallbladder polyps in the Chinese population. *Am J Gastroenterol* 1997; **92**: 2066-2068

2 **Segawa K**, Arisawa T, Niwa Y, Suzuki T, Tsukamoto Y, Goto H, Hamajima E, Shimodaira M, Ohmiya N. Prevalence of gallbladder polyps among apparently healthy Japanese: ultrasonographic study. *Am J Gastroenterol* 1992; **87**: 630-633

3 **Onoyama H**, Yamamoto M, Takada M, Urakawa T, Ajiki T, Yamada I, Fujita T, Saitoh Y. Diagnostic imaging of early gallbladder cancer: retrospective study of 53 cases. *World J Surg* 1999; **23**: 708-712

4 **Koga A**, Watanabe K, Fukuyama T, Takiguchi S, Nakayama F. Diagnosis and operative indications for polypoid lesions of the gallbladder. *Arch Surg* 1988; **123**: 26-29

5 **Yeh CN**, Jan YY, Chao TC, Chen MF. Laparoscopic cholecystectomy for polypoid lesions of the gallbladder: a clinicopathologic study. *Surg Laparosc Endosc Percutan Tech* 2001; **11**: 176-181

6 **Terzi C**, Sökmen S, Seçkin S, Albayrak L, Uğurlu M. Polypoid lesions of the gallbladder: report of 100 cases with special reference to operative indications. *Surgery* 2000; **127**: 622-627

7 **Jørgensen T**, Jensen KH. Polyps in the gallbladder. A prevalence study. *Scand J Gastroenterol* 1990; **25**: 281-286

8 **Sahlin S**, Granström L, Gustafsson U, Ståhlberg D, Backman L, Einarsson K. Hepatic esterification rate of cholesterol and biliary lipids in human obesity. *J Lipid Res* 1994; **35**: 484-490

9 **Sandri L**, Colecchia A, Larocca A, Vestito A, Capodicasa S, Azzaroli F, Mazzella G, Mwangemi C, Roda E, Festi D. Gallbladder cholesterol polyps and cholesterolosis. *Minerva Gastroenterol Dietol* 2003; **49**: 217-224

10 **Doh YW**, Lee JH, Lim HM, Chi KC, Park YG. Polypoid lesions of gallbladder: clinicopathological features and indication of operation. *J Korean Surg Soc* 2005; **69**: 245-251

11 **Akatsu T**, Aiura K, Shimazu M, Ueda M, Wakabayashi G, Tanabe M, Kawachi S, Kitajima M. Can endoscopic ultrasonography differentiate nonneoplastic from neoplastic gallbladder polyps? *Dig Dis Sci* 2006; **51**: 416-421

12 **Mainprize KS**, Gould SW, Gilbert JM. Surgical management of polypoid lesions of the gallbladder. *Br J Surg* 2000; **87**: 414-417

13 **Yang HL**, Sun YG, Wang Z. Polypoid lesions of the gallbladder: diagnosis and indications for surgery. *Br J Surg* 1992; **79**: 227-229

14 **Pandey M**, Sood BP, Shukla RC, Aryya NC, Singh S, Shukla VK. Carcinoma of the gallbladder: role of sonography in diagnosis and staging. *J Clin Ultrasound* 2000; **28**: 227-232

15 **Levy AD**, Murakata LA, Abbott RM, Rohrmann CA Jr. From the archives of the AFIP. Benign tumors and tumorlike lesions of the gallbladder and extrahepatic bile ducts: radiologic-pathologic correlation. Armed Forces Institute of Pathology. *Radiographics* 2002; **22**: 387-413

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RAPID COMMUNICATION

Micronucleus analysis in patients with colorectal adenocarcinoma and colorectal polyps

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Abstract

AIM: To determine, by counting micronucleus (MN) frequencies, whether chromosomal or DNA damage have an effect on the pathogenesis of early colorectal adenocarcinoma (CRC).

METHODS: We analyzed MN frequencies in 21 patients with CRC, 24 patients with colon polyps [10 neoplastic polyps (NP) and 14 non-neoplastic polyps (NNP)] and 20 normal controls.

RESULTS: MN frequency was significantly increased in CRC patients and in NP patients compared with controls (3.72 ± 1.34, 3.58 ± 1.21 *vs* 1.97 ± 0.81, $P < 0.001$). However, there was no difference in the MN frequency between CRC patients and NP patients ($P > 0.05$). Similarly, there was no difference in the MN frequency between NNP patients (2.06 ± 0.85) and controls ($P > 0.05$).

CONCLUSION: Our results suggest increased chromosome/DNA instabilities may be associated with the pathogenesis of early CRC.

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Key words: Colorectal adenocarcinoma; Colon polyp; Micronucleus; Genetic instability

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INTRODUCTION

Genomic instability plays an essential role in the development and progression of human colorectal cancer (CRC)[1]. Two major types of genetic instability have been described in CRC: chromosomal instability and microsatellite instability[1]. About 60% of CRCs develop through the chromosomal instability pathway, which is characterized by losses and gains of chromosomes (aneuploidy), as well as losses of heterozygosity[1].

CRC progresses through four distinct clinical stages that are described as dysplastic crypts, small benign tumors, malignant tumors invading surrounding tissues, and finally metastatic cancer. This progression involves several genetic changes such as inactivation of tumor suppressor genes and activation of oncogenes[2]. Mutations of the adenomatous polyposis coli (*APC*) gene are considered the earliest[3] and most prevalent genetic changes in colorectal tumorigenesis. More than 85% of colon cancers are estimated to have a somatic mutation of *APC*[4]. Furthermore, a large number of genes that trigger chromosomal instability have been identified in yeast in the past[5]. The underlying mechanisms leading to chromosomal instability in colorectal cancer remain to be characterized. The DNA double-strand break (DSB) is regarded as the most critical of all DNA lesions[6,7], and it has been shown that defects in the cellular response to DSBs can lead to genetic alteration, chromosomal instability, and ultimately malignant transformation[8].

The genome damage to the lymphocytes of peripheral blood has been widely used as a biomarker of genotoxic environmental factors, and long-term studies have demonstrated its validity and high clinical productivity[9]. Micronucleus (MN) is an acentric chromosome fragment or whole chromosome that is left behind during mitotic cellular division and appears in the cytoplasm of interphasic cells as a

small additional nucleus[10]. The formation of MN in dividing cells is the result of chromosome breakage due to unrepaired or mis-repaired DNA lesions, or chromosome malsegregation due to mitotic malfunction. These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell cycle checkpoint and/or DNA repair genes, as well as deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation machinery[11-14]. All these events can cause the formation of MN through chromosomal rearrangements, altered gene expression or aneuploidy, effects associated with the chromosome instability phenotype often seen in cancer[15,16].

The MN frequency test, widely accepted for *in vitro* and *in vivo* genotoxicity investigations, is a sensitive marker of genomic damage[17,18]. The presence of an association between MN induction and cancer development is supported by a number of observations. The most substantiated include: the high frequency of MN in untreated cancer patients and in subjects affected by cancer-prone congenital diseases, e.g. Bloom syndrome or ataxia telangiectasia[15,19], the presence of elevated MN frequencies in oral mucosa, used as a surrogate biomarker of cancer in clinical chemo-prevention trials[20], the correlation existing between genotoxic MN-inducing agents and carcinogenicity, e.g. ionizing and ultraviolet radiation[21,22].

A major question in cancer genetics is to what extent chromosome or genetic instability is an early event and thus a driving force of tumorigenesis?[23,24]. The aim of this study was to determine, by counting MN frequencies, whether chromosomal or DNA damage has an effect on the pathogenesis of early CRC.

MATERIALS AND METHODS

Patients

This study was conducted between May 2008 and September 2008 in the Erzurum Training and Research Hospital. Twenty-one patients with colorectal adenocarcinoma and 24 patients with colorectal polyps were studied. The study was conducted using colonoscopic specimens from subjects with the established diagnosis of colorectal polyps or colorectal adenocarcinoma in histologic analysis. Specimens were separated for each level and placed in 10% formalin solution. The pathologic specimens were reviewed independently by two pathologists.

Pathologists were blinded to the subject's clinical history, the colonoscopic findings, and the results of the Hematoxylin-Eosin staining assay. Pathologic reading was determined for each biopsy slide with an overall pathologic diagnosis determined for each subject.

We performed MN analysis in 21 (12 females and 9 males; mean age: 57.62 ± 10.84 years) patients with CRC, in 10 (4 females and 6 males; mean age: 52.44 ± 8.36 years) patients with NP, in 14 (6 females, 8 males; mean age: 52.92 ± 9.14 years) patients with NNP and in 20 (8 females and 12 males; mean age: 50.25 ± 9.38 years) healthy controls. The patients were selected from non-smoking and nonalcoholic subjects. None of the subjects had a history of viral infection, bacterial infection or any metabolic diseases. The patients had not been treated with chemotherapy or radiotherapy during the last 4 mo. The patient and control groups were chosen for their similar habits. The hospital Ethical Committee approved the human study. All patients were analyzed prior to treatment.

Micronucleus analysis

For MN analysis, 2 mL of heparinized blood was drawn from each individual. Lymphocyte cultures were established by adding 0.5 mL of whole blood to 5 mL karyotyping medium (Biological Industries, Beit Haemek, Israel) with 2% phytohaemagglutinin M (PHA; Biological Industries) according to standard techniques. The culture was kept at 37℃ for 72 h. Cytochalasin B (6 μg/mL, Sigma, USA) was added after 44 h of culture to block cytokinesis, allowing the identification of lymphocytes dividing in culture. Cells that had undergone the first mitosis were thus recognized as binucleated cells and were selectively screened for the presence of MN. The cells were then treated hypotonically with 0.075 mol/L KCl for 5 min at room temperature, and fixed in methanol/acetic acid (3:1). Cells were dropped onto slides and stained with 5% Giemsa in phosphate buffer (pH 6.8) for 5 min A thousand binucleated cells from each case were examined for MN by an experienced observer[25].

Statistical analysis

The MN rates were analyzed statistically by student's *t*-test. To evaluate the correlations between the age, sex, and MN rates, the coefficients of Spearman ρ correlation were calculated. A P value less than 0.05 was considered to be significant.

RESULTS

MN frequencies and clinical data obtained from the patient and control groups are shown in Table 1. According to these results, the mean MN frequency was significantly increased in CRC patients compared with controls (3.72 ± 1.34 *vs* 1.97 ± 0.81, $P < 0.001$). Similarly, the mean MN frequency was significantly increased in NP patients compared with controls (3.58 ± 1.21 *vs* 1.97 ± 0.81, $P < 0.001$). However, there was no difference in the mean MN frequency between CRC patients, and NP patients ($P > 0.05$). Similarly, there was no difference in mean MN frequency between NNP patients and controls (2.06 ± 0.85 *vs* 1.97 ± 0.81, $P > 0.05$). On the other hand, the mean MN frequencies did not correlate with patients' age or sex in the CRC patients (for each, $P > 0.05$). Similarly, the mean MN frequencies did not correlate with patients' age or sex in the colon polyp patients (for each, $P > 0.05$).

DISCUSSION

CRCs progress through a series of clinical and his-

Table 1 Micronucleus (MN) results of the patients with colorectal cancer and colon polyps and healthy controls (mean ± SE)

	Sex (F/M)	Age (yr)	Age at diagnosis (yr)	MN/1000 BN
CRC patients (n = 21)	12/9	57.62 ± 10.84	56.98 ± 9.45	3.72 ± 1.34
NP patients (n = 10)	4/6	52.44 ± 8.36	51.68 ± 8.54	3.58 ± 1.21
NNP patients (n = 14)	6/8	52.92 ± 9.14	50.48 ± 8.29	2.06 ± 0.85
Controls (n = 20)	8/12	50.25 ± 9.38		1.97 ± 0.81

CRC: Colorectal adenocarcinoma; NP: Neoplastic polyp; NNP: Non-neoplastic polyp.

topathological stages ranging from dysplastic crypts through small benign tumors to malignant cancers. This progression is the result of a series of genetic changes that involve activation of oncogenes and inactivations of tumor suppressor genes[26]. In colorectal cancer, chromosomal instability is the major form of genetic instability[27]. It is generally agreed that colorectal cancers develop as a consequence of accumulation of mutations in key genes such as *K-Ras, Apc*, and *p53* that are critical for regulating cell proliferation or cell cycle checkpoint control. In humans, the development from early adenomas to metastatic carcinomas takes somewhere from 20 to 40 years; it is believed that genetic instability plays a key role in accelerating the rate of mutation in cancerous cells[28].

CRCs exhibit a defect in chromosome segregation, leading to frequent gains or losses of chromosomes ($> 10^2$ per chromosome per generation)[28]. Chromosome instability has been detected in the smallest adenoma, suggesting that chromosome instability may occur at very early stages of colorectal carcinogenesis[29]. Extensive research during the past has led to the identification of genes that play a major role in the development of colorectal cancer. For example, mutations or deletions of the adenomatous polyposis coli (*APC*) gene, encoding a 310-kDa cytoplasmic protein[30,31], are commonly found in inherited familial adenomatous polyposis patients and in sporadic colorectal cancers[32,33]. Such mutations appear to be an early event during colorectal tumorigenesis[4].

The most commonly affected gene in sporadic colon cancer with defective DNA mismatch repair (MMR) is *hMLH1*, with the primary mechanism of gene inactivation being hypermethylation of the promoter[34]. These tumors account for approximately 15% of sporadic colon cancers. The majority of sporadic colon cancers (85%), however, are proficient in DNA MMR but show another form of genomic instability at the gross chromosomal level, which has been called chromosomal instability. Such chromosomal instability represents the end result of a number of processes, including mutations in mitotic checkpoint genes, microtubule spindle defects, and telomere dysfunction[35].

Two types of genetic instability have been identified, with chromosomal instability predominating[1,36]. The molecular basis for chromosomal instability is just beginning to be explored[37]. A large number of gene alterations can give rise to chromosomal instability in *Saccharomyces cerevisiae*[5,38]. These genes include those involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function, and microtubule formation and dynamics as well as checkpoints that monitor the progress of the cell cycle. To date, the only genes implicated in aneuploidy in human tumor cells are those of the latter class. Heterozygous mutations in the mitotic spindle checkpoint gene *hBUB1* were detected in a small portion of colorectal tumors with the chromosomal instability[39].

The identification of aneuploidy at early stages of tumor formation in *MYH*- and *APC*-mutant polyps is interesting also in view of previous reports showing that loss of *APC* function in primary mouse cell lines results in chromosomal instability due to a kinetochore attachment defect at mitosis[36]. It is generally accepted that *APC*'s main tumor suppressing activity resides in its capacity to bind and regulate Wnt/β-catenin signal transduction[40]. However, additional *APC* functions in cytoskeletal organization, mitotic spindle assembly, cell migration, and apoptosis may play important roles in tumor progression and malignant transformation[40,41].

It has been demonstrated that chromosomes display nonrandom changes in cancer cells. These include structural rearrangements, e.g. deletions, amplifications or translocations that arise from breaks in DNA, as well as alterations in the number of intact chromosomes, known as whole-chromosome missegregations, originating from errors in cell division (mitosis). As a result of the accumulation of such processes, chromosomal instability is thought to play a key role in tumor development[40].

In the present study, we investigated whether cytogenetic abnormalities participate in the pathogenesis of early CRC. Cytogenetic endpoints are sensitive biomarkers that are widely accepted to evaluate chromosome damage[42,43]. MN assay provides a measure of both chromosome breakage and chromosome loss or nondisjunction in clastogenic and aneugenic events, respectively[11,13].

MN assay is a sensitive indicator of exogenously or endogenously caused genetic damage and MN frequency has become an important end point in genotoxicity testing both *in vivo* and *in vitro*[17,18]. Elevated levels of MN are indicative of defects in DNA repair and chromosome segregation which could result in generation of daughter cells with altered gene dosage, or deregulation of gene expression that could lead to the evolution of the chromosome instability phenotype often seen in cancer[10,11,15,21]. These considerations give

mechanistic support to a possible causal association between MN frequency and the risk of cancer. A recently published cohort study linking the frequency of micronuclei in lymphocytes of healthy subjects to the risk of cancer reported stomach cancer among the sites most specifically associated with micronuclei frequency[44]. Similar findings have also been reported for preneoplastic lesions of colon[45], esophagus[46] and cervix[47]. In particular, the higher risks noted for stomach and intestinal cancers, are in agreement with the literature, which emphasizes the role of chromosome rearrangements in the early stages of these tumours[47,48].

Our study, which showed increased MN frequencies in the lymphocytes of CRC and colon polyp patients, could support these observations, as the induction of changes in DNA that lead to mutations plays a role in carcinogenicity. Establishment of inherited susceptibility factors is important in recognizing individuals at a higher risk of developing CRC, so that they may benefit from early detection and prevention programs. Many investigators have demonstrated genomic instability and abnormalities in patients with CRC[49-51]. Further, experimental evidence shows that early colorectal adenomas have allelic imbalance[52]. *hCDC4* mutations have been shown to occur early in colorectal tumorigenesis[53].

An association between MN and cancer has been reported[19]. The causes of this association may be structural chromosomal aberrations and aneuploidy[19]. The presence of an association between the frequency of micronuclei in lymphocytes and cancer risk has been suggested[13,44]. Our findings of a high level of MN frequency in patients with CRC or NP seem to support this association. Thus, MN assay may be performed in lymphocytes as an indicator of genomic instability relevant to colorectal tumorigenesis.

In conclusion, our results indicate that the increased MN frequency in lymphocytes of patients with CRC and NP may reflect genomic instability or deficiency of DNA repair capacity. Further, these results suggest increased chromosome/DNA instabilities may be associated with the pathogenesis of early CRC.

COMMENTS

Background

It is known there is an increased micronucleus (MN) frequency rate in neoplastic disease. Colorectal adenocarcinoma (CRC) is a common cause of cancer-related deaths worldwide, despite improved diagnostic and therapeutic implications. Hence, early diagnosis has critical importance. The aim of this study was to determine, by counting MN frequencies, whether chromosomal or DNA damage has an effect on the pathogenesis of early CRC.

Research frontiers

The MN frequency test, widely accepted for in vitro and in vivo genotoxicity investigations, is a sensitive marker of genomic damage. Therefore, in this study, we aimed to determine, by assessing MN rates, whether genetic impairment and DNA damage have an effect on the pathogenesis of CRC.

Innovations and breakthroughs

Our results suggest increased genomic instability may be associated with the pathogenesis of early CRC. The identification of increased MN frequency rate in patients with colorectal lesions may be helpful in the early diagnosis of CRC.

Applications

MN analysis has come into use as a sensitive means of monitoring DNA damage. MN analysis may be used as a marker to estimate the risk of CRC.

Terminology

Micronucleus (MN): MN is an acentric chromosome fragment or whole chromosome that is left behind during mitotic cellular division and appears in the cytoplasm of interphasic cells as a small additional nucleus.

Peer review

This study indicated genetic impairment and genetic instability may play an important role in CRC. Further, MN frequency is a promising biomarker for assessing the risk of neoplastic progression in colorectal adenocarcinoma.

REFERENCES

1 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998; **396**: 643-649
2 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767
3 **The Genetic Basis for Human Cancer**. 1st ed. In: Vogelstein B, Kinzler KW (eds). Toronto: McGraw Hill, 1998
4 **Powell SM**, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. APC mutations occur early during colorectal tumorigenesis. *Nature* 1992; **359**: 235-237
5 **Kolodner RD**, Putnam CD, Myung K. Maintenance of genome stability in Saccharomyces cerevisiae. *Science* 2002; **297**: 552-557
6 **van Gent DC**, Hoeijmakers JH, Kanaar R. Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet* 2001; **2**: 196-206
7 **Zhou BB**, Elledge SJ. The DNA damage response: putting checkpoints in perspective. *Nature* 2000; **408**: 433-439
8 **Mills KD**, Ferguson DO, Alt FW. The role of DNA breaks in genomic instability and tumorigenesis. *Immunol Rev* 2003; **194**: 77-95
9 **Hagmar L**, Stromberg U, Bonassi S, Hansteen IL, Knudsen LE, Lindholm C, Norppa H. Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts. *Cancer Res* 2004; **64**: 2258-2263
10 **Kirsch-Volders M**, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate M Jr, Kirchner S, Lorge E, Morita T, Norppa H, Surrallés J, Vanhauwaert A, Wakata A. Report from the in vitro micronucleus assay working group. *Mutat Res* 2003; **540**: 153-163
11 **Fenech M**, Holland N, Chang WP, Zeiger E, Bonassi S. The HUman MicroNucleus Project--An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat Res* 1999; **428**: 271-283
12 **Umegaki K**, Fenech M. Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils. *Mutagenesis* 2000; **15**: 261-269
13 **Mateuca R**, Lombaert N, Aka PV, Decordier I, Kirsch-Volders M. Chromosomal changes: induction, detection methods and applicability in human biomonitoring. *Biochimie* 2006; **88**: 1515-1531
14 **Fenech M**, Baghurst P, Luderer W, Turner J, Record S, Ceppi M, Bonassi S. Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, beta-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability--results from a dietary intake and micronucleus index survey in South Australia. *Carcinogenesis* 2005; **26**: 991-999
15 **Fenech M**. Chromosomal biomarkers of genomic instability relevant to cancer. *Drug Discov Today* 2002; **7**: 1128-1137
16 **Fenech M**, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus

assay using isolated human lymphocyte cultures. *Mutat Res* 2003; **534**: 65-75
17 **Fenech M**. The in vitro micronucleus technique. *Mutat Res* 2000; **455**: 81-95
18 **Miller B**, Pötter-Locher F, Seelbach A, Stopper H, Utesch D, Madle S. Evaluation of the in vitro micronucleus test as an alternative to the in vitro chromosomal aberration assay: position of the GUM Working Group on the in vitro micronucleus test. Gesellschaft for Umwelt-Mutations-forschung. *Mutat Res* 1998; **410**: 81-116
19 **Fenech M**, Holland N, Chang WP, Zeiger E, Bonassi S. The HUman MicroNucleus Project--An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat Res* 1999; **428**: 271-283
20 **Van Schooten FJ**, Besaratinia A, De Flora S, D'Agostini F, Izzotti A, Camoirano A, Balm AJ, Dallinga JW, Bast A, Haenen GR, Van't Veer L, Baas P, Sakai H, Van Zandwijk N. Effects of oral administration of N-acetyl-L-cysteine: a multi-biomarker study in smokers. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 167-175
21 **Chang WP**, Hwang BF, Wang D, Wang JD. Cytogenetic effect of chronic low-dose, low-dose-rate gamma-radiation in residents of irradiated buildings. *Lancet* 1997; **350**: 330-333
22 **Bettega D**, Calzolari P, Doneda L, Belloni F, Tallone L, Redpath JL. Differential effectiveness of solar UVB subcomponents in causing cell death, oncogenic transformation and micronucleus induction in human hybrid cells. *Int J Radiat Biol* 2003; **79**: 211-216
23 **Nowak MA**, Komarova NL, Sengupta A, Jallepalli PV, Shih IeM, Vogelstein B, Lengauer C. The role of chromosomal instability in tumor initiation. *Proc Natl Acad Sci USA* 2002; **99**: 16226-16231
24 **Michor F**, Iwasa Y, Komarova NL, Nowak MA. Local regulation of homeostasis favors chromosomal instability. *Curr Biol* 2003; **13**: 581-584
25 **Fenech M**, Morley AA. Measurement of micronuclei in lymphocytes. *Mutat Res* 1985; **147**: 29-36
26 **Cruz-Bustillo Clarens D**. Molecular genetics of colorectal cancer. *Rev Esp Enferm Dig* 2004; **96**: 48-59
27 **Chung DC**. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000; **119**: 854-865
28 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997; **386**: 623-627
29 **Rajagopalan H**, Nowak MA, Vogelstein B, Lengauer C. The significance of unstable chromosomes in colorectal cancer. *Nat Rev Cancer* 2003; **3**: 695-701
30 **Groden J**, Thliveris A, Samowitz W, Carlson M, Gelbert L, Albertsen H, Joslyn G, Stevens J, Spirio L, Robertson M. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* 1991; **66**: 589-600
31 **Kinzler KW**, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB, Smith KJ, Preisinger AC, Hedge P, McKechnie D. Identification of FAP locus genes from chromosome 5q21. *Science* 1991; **253**: 661-665
32 **Bodmer W**, Bishop T, Karran P. Genetic steps in colorectal cancer. *Nat Genet* 1994; **6**: 217-219
33 **Bodmer WF**, Bailey CJ, Bodmer J, Bussey HJ, Ellis A, Gorman P, Lucibello FC, Murday VA, Rider SH, Scambler P. Localization of the gene for familial adenomatous polyposis on chromosome 5. *Nature* 1987; **328**: 614-616
34 **Cunningham JM**, Christensen ER, Tester DJ, Kim CY, Roche PC, Burgart LJ, Thibodeau SN. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res* 1998; **58**: 3455-3460
35 **Grady WM**. Genomic instability and colon cancer. *Cancer Metastasis Rev* 2004; **23**: 11-27
36 **Sen S**. Aneuploidy and cancer. *Curr Opin Oncol* 2000; **12**: 82-88
37 **Maser RS**, DePinho RA. Connecting chromosomes, crisis, and cancer. *Science* 2002; **297**: 565-569
38 **Nasmyth K**. Segregating sister genomes: the molecular biology of chromosome separation. *Science* 2002; **297**: 559-565
39 **Cahill DP**, Lengauer C, Yu J, Riggins GJ, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B. Mutations of mitotic checkpoint genes in human cancers. *Nature* 1998; **392**: 300-303
40 **Fodde R**, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer* 2001; **1**: 55-67
41 **Fodde R**. The multiple functions of tumour suppressors: it's all in APC. *Nat Cell Biol* 2003; **5**: 190-192
42 **Kirsch-Volders M**. Towards a validation of the micronucleus test. *Mutat Res* 1997; **392**: 1-4
43 **Norppa H**. Cytogenetic biomarkers. *IARC Sci Publ* 2004; 179-205
44 **Bonassi S**, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, Ban S, Barale R, Bigatti MP, Bolognesi C, Cebulska-Wasilewska A, Fabianova E, Fucic A, Hagmar L, Joksic G, Martelli A, Migliore L, Mirkova E, Scarfi MR, Zijno A, Norppa H, Fenech M. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis* 2007; **28**: 625-631
45 **Cardoso J**, Molenaar L, de Menezes RX, van Leerdam M, Rosenberg C, Moslein G, Sampson J, Morreau H, Boer JM, Fodde R. Chromosomal instability in MYH- and APC-mutant adenomatous polyps. *Cancer Res* 2006; **66**: 2514-2519
46 **Doak SH**, Jenkins GJ, Parry EM, D'Souza FR, Griffiths AP, Toffazal N, Shah V, Baxter JN, Parry JM. Chromosome 4 hyperploidy represents an early genetic aberration in premalignant Barrett's oesophagus. *Gut* 2003; **52**: 623-628
47 **Olaharski AJ**, Sotelo R, Solorza-Luna G, Gonsebatt ME, Guzman P, Mohar A, Eastmond DA. Tetraploidy and chromosomal instability are early events during cervical carcinogenesis. *Carcinogenesis* 2006; **27**: 337-343
48 **Stewenius Y**, Gorunova L, Jonson T, Larsson N, Hoglund M, Mandahl N, Mertens F, Mitelman F, Gisselsson D. Structural and numerical chromosome changes in colon cancer develop through telomere-mediated anaphase bridges, not through mitotic multipolarity. *Proc Natl Acad Sci USA* 2005; **102**: 5541-5546
49 **Sieber OM**, Heinimann K, Gorman P, Lamlum H, Crabtree M, Simpson CA, Davies D, Neale K, Hodgson SV, Roylance RR, Phillips RK, Bodmer WF, Tomlinson IP. Analysis of chromosomal instability in human colorectal adenomas with two mutational hits at APC. *Proc Natl Acad Sci USA* 2002; **99**: 16910-16915
50 **Little MP**, Wright EG. A stochastic carcinogenesis model incorporating genomic instability fitted to colon cancer data. *Math Biosci* 2003; **183**: 111-134
51 **Komarova NL**, Lengauer C, Vogelstein B, Nowak MA. Dynamics of genetic instability in sporadic and familial colorectal cancer. *Cancer Biol Ther* 2002; **1**: 685-692
52 **Rajagopalan H**, Jallepalli PV, Rago C, Velculescu VE, Kinzler KW, Vogelstein B, Lengauer C. Inactivation of hCDC4 can cause chromosomal instability. *Nature* 2004; **428**: 77-81
53 **Shih IM**, Zhou W, Goodman SN, Lengauer C, Kinzler KW, Vogelstein B. Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. *Cancer Res* 2001; **61**: 818-822

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RAPID COMMUNICATION

Serum neopterin levels in children with hepatitis-B-related chronic liver disease and its relationship to disease severity

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Abstract

AIM: To evaluate serum neopterin levels and their correlations with liver function tests and histological grade in children with hepatitis-B-related chronic liver disease.

METHODS: The study population comprised 48 patients with chronic active hepatitis B, 32 patients with hepatitis-B-related active liver cirrhosis and 40 normal controls. Serum neopterin was measured using an enzyme-linked immunosorbent assay.

RESULTS: The mean ± SD serum neopterin levels were 14.2 ± 5.6 nmol/L in patients with chronic hepatitis, 20.3 ± 7.9 nmol/L in patients with liver cirrhosis and 5.2 ± 1.4 nmol/L in control group. Serum neopterin levels were significantly higher in patients with chronic hepatitis (P = 0.005) and cirrhosis patients (P = 0.008), than in control subjects. Cirrhotic patients had significantly higher serum neopterin levels than patients with chronic hepatitis (P = 0.004). There was a positive correlation between serum neopterin levels and alanine aminotransferase levels in patients with chronic hepatitis (r = 0.41, P = 0.004) and cirrhotic patients (r = 0.39, P = 0.005). Positive correlations were detected between serum neopterin levels and inflammatory score in patients with chronic hepatitis (r = 0.51, P = 0.003) and cirrhotic patients (r = 0.49, P = 0.001).

CONCLUSION: Our results suggest that serum neopterin levels can be considered as a marker of inflammatory activity and severity of disease in children with hepatitis-B-related chronic liver disease.

INTRODUCTION

Neopterin is a pteridine derivative produced by macrophages activated under the control of gamma-interferon and released from T-cells by the activation of the cellular immune system[1]. It has been demonstrated that there is a relation between neopterin levels in biological materials, the changes in their elimination rates and various pathological conditions. In addition to its association with activation of cell-mediated immunity and with cell expansion, significant changes were seen in neopterin levels and elimination rates in viral diseases (for example viral hepatitis)[2,3], atypical phenylketonuria[4], organ and tissue rejection[5], autoimmune diseases (such as rheumatoid arthritis and systemic lupus erythematosus[6]), genital cancer and hematologic neoplastic disorders[7,8]. In all these cases, enhanced concentrations of neopterin have been shown to have prognostic significance[9].

In chronic active hepatitis, necrosis is observed as disseminated to the parenchyma and the perilobular consisting of lymphocytes and plasma cells. The gamma-interferon released from T-lymphocytes in the area stimulates and activates macrophages[10]. Lymphocytic cell infiltration was shown, in addition to the macrophages within the fibrous bands in the liver of patients with cirrhosis resulting from various etiologies[11]. Thus, it is suggested that neopterin secreted from the inflammation-activated macrophages can be an indication of the inflammation in the liver in chronic

liver diseases[10-12].

Studies in adult patients with acute hepatitis, chronic hepatitis and cirrhosis have shown that serum neopterin levels are elevated and this elevation is correlated with the severity of disease. However, there is no data about serum neopterin concentrations in children with chronic hepatitis B and liver cirrhosis. Therefore, we investigated serum neopterin concentrations in children with hepatitis-B-related chronic liver disease and correlated these concentrations with liver function tests and inflammatory activity of the liver. The aim of this study was to demonstrate a possible relationship between serum neopterin levels and severity of the disease.

MATERIALS AND METHODS

The study population comprised 48 patients with chronic active hepatitis B, 32 patients with hepatititis-B-related active liver cirrhosis and 40 normal controls. The control group consisted of otherwise healthy, age- and sex-matched children whose biochemical tests were also within normal limits. The study was performed according to the Declaration of Helsinki, and all parents gave informed consent for the participation of their children in the study.

Chronic active hepatitis B was diagnosed on the basis of Hepatitis B surface antigen (HbsAg) and Hepatitis B e antigen (HbeAg) positivity in serum for a period over 6 mo, positive HBV-DNA determined at least twice in one-month intervals (> 5 pg/mL), and evidence of chronic active hepatitis B in a liver biopsy carried out within the last 6 mo. None of the patients in this group had Hepatitis C or Hepatitis D infections, decompensated liver disorder, autoimmune hepatitis, α_1-antitrypsin deficiency, Wilson disease or any other liver disease. The patients were evaluated before no treatment was initiated.

Hepatitis-B-related active liver cirrhosis was diagnosed by clinical, serological, and biochemical tests as well as histopathological investigation of liver biopsy. The cirrhotic patients were classified by the Child-Pugh classification defined by Pugh *et al*[13].

Liver function tests (serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphase (AP) and albumin) were also performed in all subjects using an autoanalyzer.

Serum neopterin levels were examined using an enzyme-linked immunosorbent assay (ELISA) kit (Neopterin ELISA, IBL Immuno-Biological-Laboratories, Hamburg).

All patients with chronic hepatitis and liver cirrhosis underwent liver biopsy. Liver biopsy was performed according to the Menghini technique. The biopsy material was kept in 10% formaldehyde solution and evaluated by a pathologist experienced in liver pathology. In the samples obtained from the patients with chronic hepatitis and liver cirrhosis, histological activity index (HAI) score was defined as suggested by Knodell *et al*[14] and modified by Desmet *et al*[15]. It was graded 0-18 by adding the scores for periportal ± bridging necrosis (0-10), intralobular degeneration and focal necrosis (0-4) and portal inflammation (0-4).

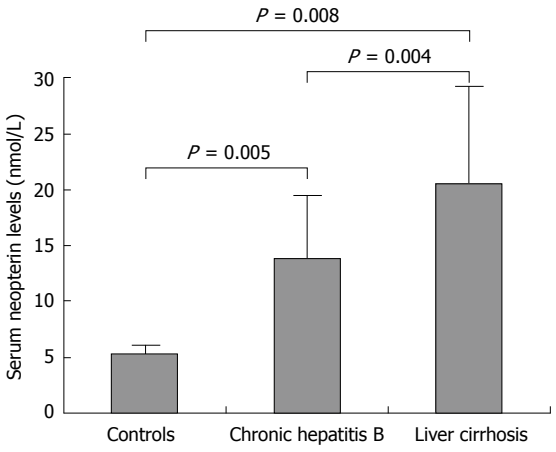

Figure 1 Serum neopterin levels in normal controls and patients with chronic hepatitis B and liver cirrhosis.

All data was shown in mean ± SD values. A Chi square test was used to analyze the categorical data, whereas an ANOVA test was used to compare the numerical data of the groups. The homogeneity of the intergroup variance was tested by the Levene method. If the variance was homogenous ($P > 0.05$), then the Tukey test was used, and if not ($P > 0.05$), then the Tamhane test was used to evaluate the significance of the difference between the groups. The correlations between serum neopterin levels and biochemical and histological parameters were determined by the Pearson correlation test. The differences between the groups were taken statistically significant if it is $P < 0.05$.

RESULTS

The characteristics of all the subjects are shown in Table 1. There were no significant differences between the three groups in terms of sex and age. Serum neopterin levels (mean ± SD) were found to be 14.2 ± 5.6 nmol/L (range 2.7-32) in patients with chronic active hepatitis B, 20.3 ± 7.9 nmol/L (range 12-41) in patients with hepatitis-B-related active liver cirrhosis and 5.2 ± 1.4 nmol/L (range 2.2-6.8) in controls. Serum neopterin levels were significantly elevated in patients with chronic hepatitis B ($P = 0.005$) and liver cirrhosis ($P = 0.008$) than in healthy controls. Patients with liver cirrhosis had higher serum neopterin levels compared to patients with chronic hepatitis B ($P = 0.008$) (Figure 1).

There was a positive significant correlation between serum neopterin levels and ALT levels ($r = 0.41$, $P = 0.004$). No significant correlations were found between serum neopterin levels and AST ($r = 0.18$, $P = 0.22$), GGT ($r = 0.33$, $P = 0.07$), AP ($r = 0.09$, $P = 0.64$), and albumin ($r = -0.34$, $P = 0.06$) levels in patients with chronic hepatitis B. A positive significant correlation was observed between neopterin levels and HAI ($r = 0.52$, $P = 0.001$) (Figure 2A).

According to the Child-Pugh classification, all of the 32 patients with liver cirrhosis were in stage A. In the liver

Table 1 Demographic, biochemical and histological characteristics of all subjects (mean ± SD)

	Chronic hepatitis B	Liver cirrhosis	Control
n	48	32	40
Sex (M/F)	22/26	15/17	19/21
Age (yr)	8.2 ± 3.4 (2-17)	7.4 ± 5.3 (1-16)	7.1 ± 2.2 (2-16)
ALT (IU/L)	68.8 ± 52.6 (14-280)	108.9 ± 76.8 (24-240)	26.1 ± 7 (14-36)
AST (IU/L)	64.6 ± 51.4 (21-276)	153.4 ± 147.6 (39-580)	27.5 ± 6.7 (18-38)
GGT (IU/L)	23.4 ± 21.6 (14-162)	115.4 ± 30.3 (88-439)	36.8 ± 8.8 (18-48)
AP (IU/L)	528 ± 193.5 (199-1055)	705.7 ± 487.4 (228-1851)	167.1 ± 50.7 (98-240)
Albumin (mg/dL)	3.6 ± 0.7 (2.5-4.7)	3.2 ± 1 (2-5.1)	4.4 ± 0.6 (3.7-5.4)
Neopterin (nmol/L)	14.2 ± 5.6 (2.7-32)	20.5 ± 8.6 (13-38)	5.2 ± 1.4 (2.2-6.8)
HAI	6.2 ± 2.9 (1-12)	9.1 ± 2.2 (5-12)	-

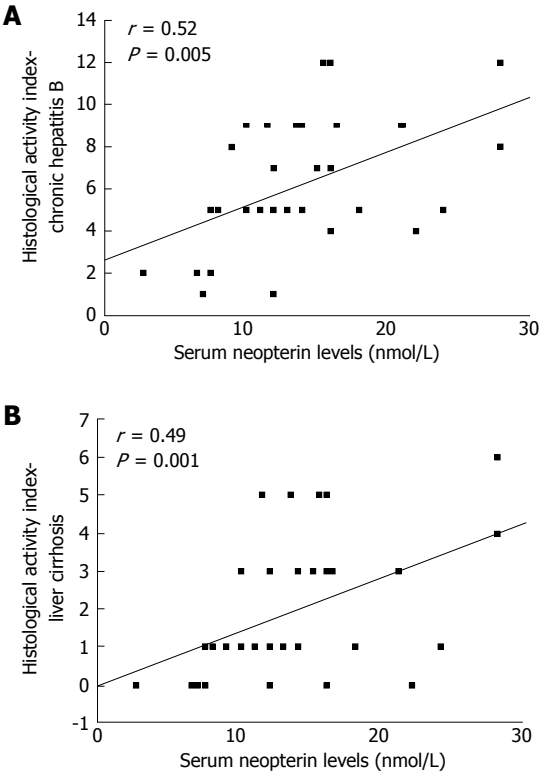

Figure 2 Correlation between serum nopterin levels and histological activity index. A: Chronic hepatitis B; B: Hepatitis-B-related liver cirrhosis.

cirrhosis group, serum neopterin levels were significantly related to ALT values ($r = 0.39$, $P = 0.005$) and HAI ($r = 0.49$, $P = 0.001$) (Figure 2B). There were no correlations between serum neopterin levels and AST ($r = 0.20$, $P = 0.517$), GGT ($r = 0.35$, $P = 0.263$), AP ($r = 0.09$, $P = 0.772$), and albumin ($r = -0.11$, $P = 0.731$) levels.

DISCUSSION

It has been reported that neopterin levels increase in body fluids and change in parallel to the activity of the disease in many infectious diseases and various malign disorders in which activation of the cellular immune system plays an important role in the pathogenesis[7,16,17]. It has been shown that the activation of cellular immune system stimulates the secretion of γ-interferon from the T lymphocytes, and γ-interferon is an effective inducer of neopterin release by monocytes/macrophages[10]. Gamma-interferon is produced by the stimulation of T-lymphocytes by several specific antigens, primarily viral antigens, thus it was found that the neopterin levels were especially elevated in viral infections.[3,17].

Evidence of elevation in neopterin levels in body fluids due to the activation of immune system, which was also supported by several studies involved in diseases leading to activation of the immune system, suggests that elevated neopterin can also be a marker for the follow-up of chronic liver disorders, especially of viral liver disorders[9]. However, since no such data is available related to children, our results can only be compared to results obtained from the studies carried out with adult patients.

It is suggested that serum neopterin levels can be used as a significant parameter for the differential diagnosis of non-infectious hepatitis and viral hepatitis[18]. Serum neopterin levels of the patients with acute and chronic hepatitis B were found to be significantly higher than the donors' serum. The relation between neopterin levels and severity of the disease has been proved, and it can be used in combination with clinical data as a prognostic evidence for the progress of the disease[19]. In asymptomatic HbsAg carriage, acute hepatitis, chronic inactive hepatitis, chronic active hepatitis, liver cirrhosis, hepatocellular carcinoma and alcoholic liver disease, serum and urine neopterin levels were found to be higher than in controls. The most elevated neopterin levels were seen in patients with acute hepatitis[20].

In adult patients with liver cirrhosis, serum neopterin levels were more elevated than non-cirrhotic patients and control groups[9,21] whereas out of non-cirrhotic patients, patients with chronic hepatitis B had also elevated neopterin levels[9].

Serum neopterin levels were elevated in patients with alcoholic cirrhosis[22]. Neopterin measurement was reported to be beneficial for the differential diagnosis of viral and alcoholic liver diseases, and it has been shown that patients with viral hepatitis had higher neopterin concentrations compared to patients with alcoholic liver diseases[20].

Serum and urine neopterin levels were elevated from baseline after the initiation of interferon therapy in HbeAg positive patients with chronic hepatitis B, and they remained markedly elevated during the treatment. However, the neopterin levels were restored rapidly to baseline values after the end of the therapy. Therefore

it was suggested that serum and urine neopterin levels could be a good marker of the cellular immunity during interferon treatment in the chronic hepatitis B infection[23].

In our study, serum neopterin levels was found to be markedly higher in the pediatric patients with chronic hepatitis B and liver cirrhosis than in healthy controls, which is in agreement with the data obtained from the adult patients. It was also higher in patients with cirrhosis when compared with chronic hepatitis B patients. The patients in the cirrhotic stage, independent of their etiology, have elevated concentrations of serum neopterin levels released from the activated macrophages. In those patients, substances that are considered to stimulate the macrophages such as immune complexes or endotoxins, increase in blood due to the lack of peptide clearance by the liver[24]. These mechanisms explain the highest concentrations of serum neopterin in patients with cirrhosis.

Although no correlation was found between serum neopterin levels and ALT, AST and AP levels in adults with various chronic liver diseases of various etiologies, a negative correlation was found with albumin[9]. While neopterin levels were found correlated with liver function tests in patients with acute hepatitis, this correlation was not verified in patients with chronic liver diseases[20]. However, in other studies, a correlation was found between serum neopterin levels and biochemical tests or liver inflammatory grading in patients with chronic hepatitis C and B[9,25]. We found a significant correlation between serum neopterin levels and ALT or HAI in children with hepatitis-B-related chronic hepatitis B and liver cirrhosis. This data agrees with the data obtained from the adult patients.

In conclusion, these results suggest that measurement of serum neopterin levels can be considered as a marker of inflammatory activity and severity of disease in children with hepatitis-B-related chronic liver disease. However, this needs to be further studied in children.

REFERENCES

1 **Rigby AS**, Chamberlain MA, Bhakta B. Behcet's disease. *Baillieres Clin Rheumatol* 1995; **9**: 375-395
2 **Reibnegger G**, Auhuber I, Fuchs D, Hausen A, Judmaier G, Prior C, Werner ER, Wachter H. Urinary neopterin levels in acute viral hepatitis. *Hepatology* 1988; **8**: 771-774
3 **Denz H**, Fuchs D, Hausen A, Huber H, Nachbaur D, Reibnegger G, Thaler J, Werner ER, Wachter H. Value of urinary neopterin in the differential diagnosis of bacterial and viral infections. *Klin Wochenschr* 1990; **68**: 218-222
4 **Kaufman S**. Hyperphenylalaninaemia caused by defects in biopterin metabolism. *J Inherit Metab Dis* 1985; **8** Suppl 1: 20-27
5 **Bron D**, Wouters A, Barekayo I, Snoeck R, Stryckmans P, Fruhling P. Neopterin: an useful biochemical marker in the monitoring of allogeneic bone marrow transplantation. *Acta Clin Belg* 1988; **43**: 120-126
6 **Reibnegger G**, Egg D, Fuchs D, Gunther R, Hausen A, Werner ER, Wachter H. Urinary neopterin reflects clinical activity in patients with rheumatoid arthritis. *Arthritis Rheum* 1986; **29**: 1063-1070
7 **Abate G**, Comella P, Marfella A, Santelli G, Nitsch F, Fiore M, Perna M. Prognostic relevance of urinary neopterin in non-Hodgkin's lymphomas. *Cancer* 1989; **63**: 484-489
8 **Zeitler HJ**, Andondonskaja-Renz B. Evaluation of pteridines in patients with different tumors. *Cancer Detect Prev* 1987; **10**: 71-79
9 **Wilmer A**, Nolchen B, Tilg H, Herold M, Pechlaner C, Judmaier G, Dietze O, Vogel W. Serum neopterin concentrations in chronic liver disease. *Gut* 1995; **37**: 108-112
10 **Huber C**, Batchelor JR, Fuchs D, Hausen A, Lang A, Niederwieser D, Reibnegger G, Swetly P, Troppmair J, Wachter H. Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma. *J Exp Med* 1984; **160**: 310-316
11 **Woloszczuk W**, Troppmair W, Leiter E, Flener R, Schwarx M, Kovarik J, Pohanka E, Margreiter R, Huber C. Relationship of interferon-gamma and neopterin levels during stimulation with alloantigens in vivo and in vitro. *Tranplantation* 1986; **41**: 716-719
12 **Thomas HC**. Immunological mechanisms in chronic liver disease. In: Zakim D, Boyer TD, eds. Hepatology. 2nd ed. Philadelphia: WB Saunders, 1990: 1144-1126
13 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649
14 **Knodell RG**, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981; **1**: 431-435
15 **Desmet VJ**, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513-1520
16 **Wachter H**, Fuchs D, Hausen A, Reibnegger G, Werner ER. Neopterin as marker for activation of cellular immunity: immunologic basis and clinical application. *Adv Clin Chem* 1989; **27**: 81-141
17 **Reibnegger G**, Fuchs D, Hausen A, Werner ER, Werner-Felmayer G, Wachter H. Neopterin and viral infections: diagnostic potential in virally induced liver disease. *Biomed Pharmacother* 1989; **43**: 287-293
18 **Prior C**, Fuchs D, Hausen A, Judmaier G, Reibnegger G, Werner ER, Vogel W, Wachter H. Potential of urinary neopterin excretion in differentiating chronic non-A, non-B hepatitis from fatty liver. *Lancet* 1987; **2**: 1235-1237
19 **Samsonov MIu**, Golban TD, Nasonov EL, Masenko VP. [Serum neopterin in hepatitis B] *Klin Med* (Mosk) 1992; **70**: 40-42
20 **Daito K**, Suou T, Kawasaki H. Clinical significance of serum and urinary neopterin levels in patients with various liver diseases. *Am J Gastroenterol* 1992; **87**: 471-476
21 **Fernandez E**, Rodrigo L, Riestra S, Carcia S, Gutierrez F, Ocio G. Adenosine deaminase isoenzymes and neopterin in liver cirrhosis. *J Clin Gastroenterol* 2000; **30**: 181-186
22 **Antonio DR**, Gernot PT, Francısco G. Neopterin and soluble tumor necrosis factor receptor type1 in alcohol-induced cirrhosis. *Hepatology* 1995; **21**: 976-978
23 **Daito K**, Suou T, Kawasaki H. Serum and urinary neopterin levels in patients with chronic active hepatitis B treated with interferon. *Res Commun Chem Pathol Pharmacol* 1994; **83**: 303-316
24 **Kovacs EJ**. Fibrogenic cytokines: the role of immune mediators in the development of scar tissue. *Immunol Today* 1991; **12**: 17-23
25 **Zwirska-Korczala K**, Dziambor AP, Wiczkowski A, Berdowska A, Gajewska K, Stolarz W. [Hepatocytes growth factor (HGF), leptin, neopterin serum concentrations in patients with chronic hepatitis C] *Przegl Epidemiol* 2001; **55** Suppl 3: 164-169

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RAPID COMMUNICATION

Induction of IgA and sustained deficiency of cell proliferative response in chronic hepatitis C

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Abstract

AIM: In the present study, antibody and peripheral blood mononuclear cells (PBMC) proliferative responses against hepatitis C virus (HCV) antigens were evaluated in HCV chronically infected patients.

METHODS: Paired serum and PBMC samples were taken six months apart from 34 individuals, either treated or not, and tested by enzyme-linked immunosorbent assay (ELISA) and carboxyfluorescein succinimidyl ester staining.

RESULTS: Over 70% of the patients showed specific IgG and IgM against capsid, E1 and NS3, while HVR-1 was recognized by half of the patients. An increase in the levels of the anti-capsid IgM (P = 0.027) and IgG (P = 0.0006) was observed in six-month samples, compared to baseline. Similarly, a significantly higher percent of patients had detectable IgA reactivity to capsid (P = 0.017) and NS3 (P = 0.005) after six months, compared to baseline. Particularly, IgA against structural antigens positively correlated with hepatic damage (P = 0.036). IgG subclasses evaluation against capsid and NS3 revealed a positive recognition mediated by IgG1 in more than 80% of the individuals. On the contrary, less than 30% of the patients showed a positive proliferative response either of CD4+ or CD8+ T cells, being the capsid poorly recognized.

CONCLUSION: These results confirm that while the cellular immune response is narrow and weak, a broad and vigorous humoral response occurs in HCV chronic infection. The observed correlation between IgA and hepatic damage may have diagnostic significance, although it warrants further confirmation.

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Key words: Hepatitis C; Antibody response; Lymphoproliferation; Core; Envelope

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INTRODUCTION

Hepatitis C virus (HCV) constitutes a major health problem, since it is infecting an estimated 170 million people worldwide. Probably, the most characteristic feature of this virus is its propensity to cause chronic infection, which is established in the majority of cases and has become a leading indication to liver transplantation in Western countries[1]. The burden of HCV infection is even more dramatic due to the absence of preventive or therapeutic vaccines. Additionally, the best available treatments, based on pegylated interferon plus ribavirin, are generally effective only in 50% of cases[2]. Thus, the development of new treatments and prophylactic interventions is currently a priority.

Despite almost two decades of intense research, since its description by Choo and coworkers[3], correlates of protection have not been entirely established for HCV infection. The specific humoral response to acute infection is considered of relatively low titer and delayed in time from the moment of infection[4,5]. Antibodies directed to the capsid protein are the first ones to be detected[4], and have been determined to be principally of IgG and, secondly, of IgM classes in chronic infection[6]. Particularly, the significance of IgM anti-HCV capsid in chronic infection has been studied[7,8]. Results indicate that IgM anti-HCV capsid occurrence is directly related to viremia levels[8] and these antibodies have been found to decrease or disappear in patients in disease remission and increase when the disease reactivates after therapy[6]. Another feature reported as characteristic of HCV-specific antibody response is its restriction, except for the capsid, to the IgG1 isotype, the rest of the subclasses being poorly prevalent[4].

Evidence for a significant role of antibody responses in viral clearance seems conflicting, since it has been observed that subjects with antibody deficiencies may spontaneously clear HCV infection[9]. Several epitopes of the envelope glycoproteins have been identified as targets of neutralizing antibodies[10]. However, in the majority of the patients, chronic infection is established in spite of their presence[11], probably due to their absence or low titers in early phases of the infection[12].

On the other hand, the importance of a sustained, multispecific CD4+, as well as CD8+, T cell responses, targeting numerous epitopes early in infection, has been highlighted[13-16]. In persistently infected patients, CD4+ and CD8+ T cells are found at low frequencies in peripheral blood, but seem to be compartmentalized in the liver[17]. It has been demonstrated that, in most subjects, a detectable cell-mediated immune response is generated at the onset of acute infection, but this response progressively disappears in those where HCV infection becomes persistent[18]. Impaired production of interferon-gamma (IFN-γ) and interleukin- (IL-) 2, as well as incapability to proliferate *in vitro*, have been demonstrated for CD4+ T cells of chronically infected patients[18-20]. Similarly, CD8+ T cells of persistently infected subjects fail to produce IFN-γ and tumor necrosis factor-alpha (TNF-α) in functional assays[21,22].

In this work, we aimed at evaluating HCV-specific immune response in chronically infected patients, treated or untreated, using paired blood samples taken 6 months apart. IgG, IgM and IgA levels, as well as IgG1-4 subclasses and peripheral blood mononuclear cells proliferative responses against HCV core, envelope and NS3 antigens were measured. Additionally, we studied the relationship between immune parameters and patients' demographic characteristics.

MATERIALS AND METHODS

Study population

The study cohort included 34 patients with chronic HCV genotype 1 infection. The enrolment of patients was conducted at the National Institute of Gastroenterology (Havana, Cuba). Written informed consent was obtained from every patient prior to start of the study. All procedures were conducted in accordance with the national ethics guidelines and the Declaration of Helsinki, as revised in 1996. A patient with chronic HCV infection was defined as an individual with detectable HCV RNA and sustained liver injury for more than 6 mo, as monitored by liver function tests [alanine aminotransferase (ALT)/aspartate aminotransferase (AST)] and/or liver biopsy, scored according to the Ishak system. Patients were either treatment naïve or had been treated with the interferon-α (IFN-α) and ribavirin combination during the study period, except one subject who had received IFN-α monotherapy. Blood samples were taken at baseline (T = 0) and 6 mo later (T = 6). Demographic data of patients involved in the study are shown in Table 1.

Table 1 Demographic characteristics of patients with chronic hepatitis C

Characteristic	Value
Age (yr)	
Mean ± SD	48 ± 11
Median (Interquartile range)	49 (39-54)
Race (*n*/%)	
Caucasian	31/91.2
Black	2/5.8
Mixed	1/2.9
Body Mass Index (kg/m^2)	
Mean ± SD	26.4 ± 4.5
Median (Interquartile range)	26.4 (23.3-28.9)
Gender (*n*/%)	
Female	22/64.7
Male	12/35.3
Possible source of infection (*n*/%)	
Transfusion/surgery	27/79.4
Unknown	7/20.6
Treatment (*n*/%)	
IFN-α + ribavirin	23/67.6
IFN-α	1/2.9
Untreated	10/29.4
Hepatic damage[1] (*n*/%)	
Undetermined	9/26.5
Mild	15/44.1
Moderate	7/20.6
Severe	3/8.8
Alcohol consumption (*n*/%)	
Yes	4/11.7
No	30/88.2

[1]Necro-inflammatory activity.

Antigens

The recombinant proteins Co.120[23], E1.340[24] and NS3[25] are expressed in modified *Escherichia coli* and purified to 90%, except E1.340 which is purified to 85%. E2.680 recombinant protein is expressed in modified *Picchia pastoris* yeast and purified to 85%[26]. The HVR-1 peptide comprises amino acids 384-414 (TGTYVTGGTAARGVSQFTGLFTSGPSQKIQL) of the E2 protein[27]. All the recombinant proteins and the

HVR-1 synthetic peptide correspond to a genotype 1b strain. Peptide pools individually comprising the whole sequence of the capsid, E1 and E2 proteins of HCV-1a strain were also used for Peripheral blood mononuclear cells (PBMC) proliferation assays. These peptides were 18 amino acids in length, overlapping adjacent peptides by 10 amino acids. Peptide pools were kindly donated by Dr Naglaa Shoukry (Centre de Recherche du CHUM, Montreal, Canada).

Evaluation of antibody response against HCV antigens

To detect human antibodies to HCV structural antigens, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 100 μL of Co.120 (10 μg/mL), E1.340 (10 μg/mL), HVR-1 synthetic peptide (2 μg/mL) or NS3 (5 μg/mL) diluted in coating buffer (50 mmol/L carbonate buffer, pH 9.6) followed by 16-h incubation at 4℃. The wells were washed four times with 0.1% Tween 20 in phosphate buffered saline (0.14 mol/L NaCl, 0.003 mol/L KCl, 0.01 mol/L Na_2HPO_4, 0.001 mol/L KH_2PO_4, pH 7.5) (PBST) and blocked with 200 μL of PBST containing 2% skim milk (Oxoid Ltd, England) and 5% goat normal serum (blocking solution) for 1 h at 25℃. After four washes with PBST, each well received 100 μL of a 1:10 dilution of human sera in blocking solution and the plates were incubated at 37℃ for 1 h. Sera were diluted 1:80 in blocking solution for the evaluation of the specific response against E1.340. The plates were washed four times with PBST. Then, 100 μL of horseradish peroxidase-conjugated goat anti-human IgM, IgA or IgG secondary antibodies (Sigma, St Louis, USA), 1:10 000, 1:25 000 and 1:30 000 diluted, respectively, in PBST plus 2% skim milk, were added and the plates were incubated at 37℃ for 1 h, followed by four washes with PBST. IgG subclasses were evaluated with the secondary biotinylated antibodies against human IgG1, IgG2, IgG3 and IgG4 (Sigma-Aldrich, St Louis, USA) respectively diluted 1:24 000, 1:5000, 1:5000 and 1:1000 in blocking solution. After four washes with PBST, an additional 1 h incubation step at 37℃ with extravidin-peroxidase conjugate (Sigma, St Louis, USA), 1:1000 diluted in PBST plus 2% skim milk, was carried out followed by four washes with PBST. In every case, positive reactions were visualized with o-phenylenediamine (Sigma-Aldrich, St Louis, USA) 0.05% in substrate buffer (0.1 mol/L citric acid, 0.2 mol/L NaH_2PO_4, pH 5.0) with 0.015% H_2O_2 (Merck, Germany) as substrate. Reactions were stopped with 50 μL of 2.5 mol/L H_2SO_4. Measurement of absorbance (A) at 492 nm was made in a SensIdent Scan reader plate (Merck, Darmstadt, Germany). At least two human sera, anti-HCV negative by UMELISA (Center for Immunoassay, Cuba), were used as negative controls in each experiment. Anti-HCV positive human sera (as tested by UMELISA, Center for Immunoassay, Cuba), having a known antibody titre of at least 1:150 against the corresponding antigen, served as positive controls. The cut-off value to consider a positive antibody (Ab) response was established as twice the mean A_{492nm} of the negative control sera.

PBMC preparation

Blood anticoagulated with acid citrate dextrose (1:9) was processed within 2 h after sample collection. PBMC from HCV patients and a healthy individual were isolated using Ficoll-Paque PLUS density gradients (Amersham, Oslo, Norway), and adjusted to 5-10 × 10^6 cells/mL in freezing medium consisting of nine parts of foetal bovine serum (FBS; Hyclone) and one part of DMSO (Sigma, Deisenhofen, Germany). PBMC were stored for 16 h in 1℃ freezing containers (Nalgene Nunc International, Rochester, New York, USA) at 80℃ and then transferred into liquid nitrogen until use.

Evaluation of CD4+ and CD8+ T cell proliferative response against HCV antigens

T cell proliferation assays were used to analyze HCV specific T cell responses against proteins Co.120, E1.340, E2.680 and NS3 or peptide pools covering core, E1 and E2 HCV proteins, depending on patients' PBMC availability. Cryopreserved PBMC were thawed quickly in a 37℃ water bath, and washed twice with R10 medium. After a 16-h resting period at 37℃ and 50 mL/L of CO_2, cells were washed twice with PBS, adjusted to 20 × 10^6 cells/mL and labeled with 4 μmol/L of carboxyfluorescein succinimidyl ester (CFSE) for 8 min at room temperature in the dark. The reaction was stopped by adding 1 volume of human AB serum (Sigma-Aldrich, St. Louis, USA). Next, PBMC were washed twice with PBS and once with RPMI 1640 medium (Sigma-Aldrich, St. Louis, USA). Cells were finally adjusted to 2 × 10^6 cells/mL and stimulated or not with the peptide pools (1 μg/mL) and proteins (2 μg/mL, except for NS3, of which a concentration of 5 μg/mL was used) for 6 days at 37℃ and 50 mL/L of CO_2. Cells incubated with media alone were considered as negative control. Concanavalin A (ConA, Sigma-Aldrich, St. Louis, USA, 5 μg/mL) was used as positive control. Cells were harvested, stained with surface antibodies and analyzed by flow cytometry. Anti-CD4 allophycocyanin (APC) (clone # 11 830), anti-CD8 phycoerythrin (PE) (clone # 37 006) and anti-CD8 APC (clone # 37 006) monoclonal antibodies, 4 μg/mL, 2.5 μg/mL and 5 μg/mL respectively, were from R&D Systems (R&D Systems, Minneapolis, USA). The stimulation index (SI) was calculated by dividing the proliferative frequency (%) in the presence of antigen by the proliferative frequency (%) without antigen. The stimulation index was considered positive if $\geqslant$ 2.5 after peptide stimulation and $\geqslant$ 3 after protein stimulation.

Statistical analysis

GraphPad Prism version 4.00 statistical software (GraphPad Software, San Diego, CA) was generally used to carry out statistical analysis. Unpaired t test (for data sets with a Gaussian distribution and equal variances) and Mann Whitney test (for data sets with non-Gaussian distribution or different variances) were used to compare the magnitude of a given response between the two evaluated time points. For comparison of the number of

Table 2 Reactivity of the main antibody classes against core, E1, HVR-1 and NS3 antigens in sera taken at baseline

Isotypes	Percentage of patients with a positive response against the indicated antigen			
	Core	E1	HVR-1	NS3
IgG	91.1	70.8	51.5	88.2
IgM	76.4	78.7	5.8	79.4
IgA	52.9	39.3	12.1	50

Table 3 Reactivity of IgG subclasses against core and NS3 antigens in sera taken at baseline

IgG Subclasses	Percentage of patients with a positive response against the indicated antigen	
	Core	NS3
IgG1	85.2	82.3
IgG2	17.6	39.3
IgG3	11.7	12.1
IgG4	48.4	78.7

positive samples at the two evaluated moments, Fisher's exact test was used. Correlations between variables were analyzed by Spearman's rank correlation coefficient, using SPSS 11.5.1 Software for Windows. Significant differences were considered when $P < 0.05$.

RESULTS

Study subjects

The present study was designed to evaluate the specific immune response against HCV in genotype 1 chronically infected patients, in a 6-mo follow-up period. Of the 34 patients initially enrolled in the study, only 31 could be contacted for a second blood extraction 6 mo later. In this cohort, 73.5% of the patients were over 40 years of age, 67.6% had been treated with the standard combined therapy with IFN-α and ribavirin and only 11.7% of the individuals reported to consume alcohol. Over 73% of the patients had undergone a liver biopsy and of them, 64.7% showed a mild to moderate necro-inflammatory activity, as measured by Ishak scores. Table 1 summarizes the principal demographic characteristics.

Humoral immune response to HCV antigens

The reactivity to HCV antigens was assessed in serum samples from 34 patients chronically infected with HCV. All the patients displayed a positive antibody response against several of the evaluated antigens. At baseline, IgG and IgM reactivities were present in more than 70% of the individuals against Co.120, E1.340 and NS3 proteins (Table 2). Regarding the reactivity towards HVR-1, IgG could only be detected in half of the individuals; still, it was dominant over the rest of the evaluated classes. IgA was generally less frequently detected than IgG and IgM against all antigens.

The assessment of the reactivity of the IgG subclasses, at baseline, against the highly conserved capsid and NS3 antigens, revealed that their recognition was mediated by IgG1 in more than 80% of the patients (Table 3). At the same time, in 78.7% of the individuals a positive IgG4 response could be detected against NS3, while it was detectable only in nearly half of the samples against Co.120. IgG2 and IgG3 were detected in a small percent of the tested samples against both antigens (Table 3).

Table 4 Correlation between demographic variables and aspects of humoral response

	Alcohol consumption	Specific treatment	Hepatic damage[1]
IgM[2]	$R = -0.53^{b}$	$R = 0.313$	$R = 0.107$
	$P = 0.01$	$P = 0.071$	$P = 0.609$
IgA	$R = -0.402^{a}$	$R = 0.238$	$R = 0.422^{a}$
	$P = 0.019$	$P = 0.176$	$P = 0.036$
IgG[3]	$R = -0.383^{a}$	$R = 0.257$	$R = 0.100$
	$P = 0.028$	$P = 0.149$	$P = 0.635$
IgG4	$R = -0.717^{b}$	$R = 0.418^{a}$	$R = 0.401^{a}$
	$P = 0.000$	$P = 0.014$	$P = 0.047$

[1]Necro-inflammatory activity; [2]IgM positive response to HCV structural antigens; [3]IgG positive response to HCV HVR-1 peptide; R = Spearman's correlation coefficient; [a]Significant correlation at 0.05 level; [b]Significant correlation at 0.01 level.

The reactivity of the main classes IgM, IgA and IgG against Co.120 and NS3 was not only assessed at baseline (T = 0), but also 6 months later (T = 6) (Figure 1). The comparison of these two time points revealed that there was a significantly higher percentage of individuals with a positive IgA response against both antigens at T = 6 (Co.120 52.9% *vs* 88.4%, $P = 0.017$; NS3 50.5% *vs* 88.4%, $P = 0.005$). Interestingly, none of the patients lost the specific IgA reactivity from baseline to the end of the study: instead, the observed increase was totally due to *de novo* responses at T = 6. Regarding the magnitude of the response, a statistically significant difference was observed in the IgG and IgM classes against Co.120 (Figure 1A). The mean reactivity was higher at T = 6 when compared to baseline (IgM 0.6419 *vs* 0.9099, $P = 0.027$; IgG 0.7802 *vs* 0.9532, $P = 0.0006$).

Correlation analyses between demographic variables and humoral responses revealed that alcohol consumption was negatively correlated with the responses of the main classes IgM ($P = 0.01$), IgA ($P = 0.019$) and IgG ($P = 0.028$), as well as IgG4 ($P = 0.00000182$) (Table 4). On the other hand, positive IgG4 positively correlated with the fact of being treated ($P = 0.014$) and the grade of hepatic damage ($P = 0.047$). Additionally, the hepatic damage, expressed as necro-inflammatory activity, also correlated with IgA ($P = 0.036$), while fibrosis did not ($P = 0.487$).

T cell proliferative response to HCV antigens

PBMC were analyzed in a proliferation assay for their capacity to expand in response to stimulation with HCV antigens. Samples from both T = 0 and T = 6 were evaluated. Depending on patients PBMC availability, the response to the structural antigens was evaluated by stimulation with the corresponding recombinant protein and a peptide pool. Cells were surface-stained with anti-

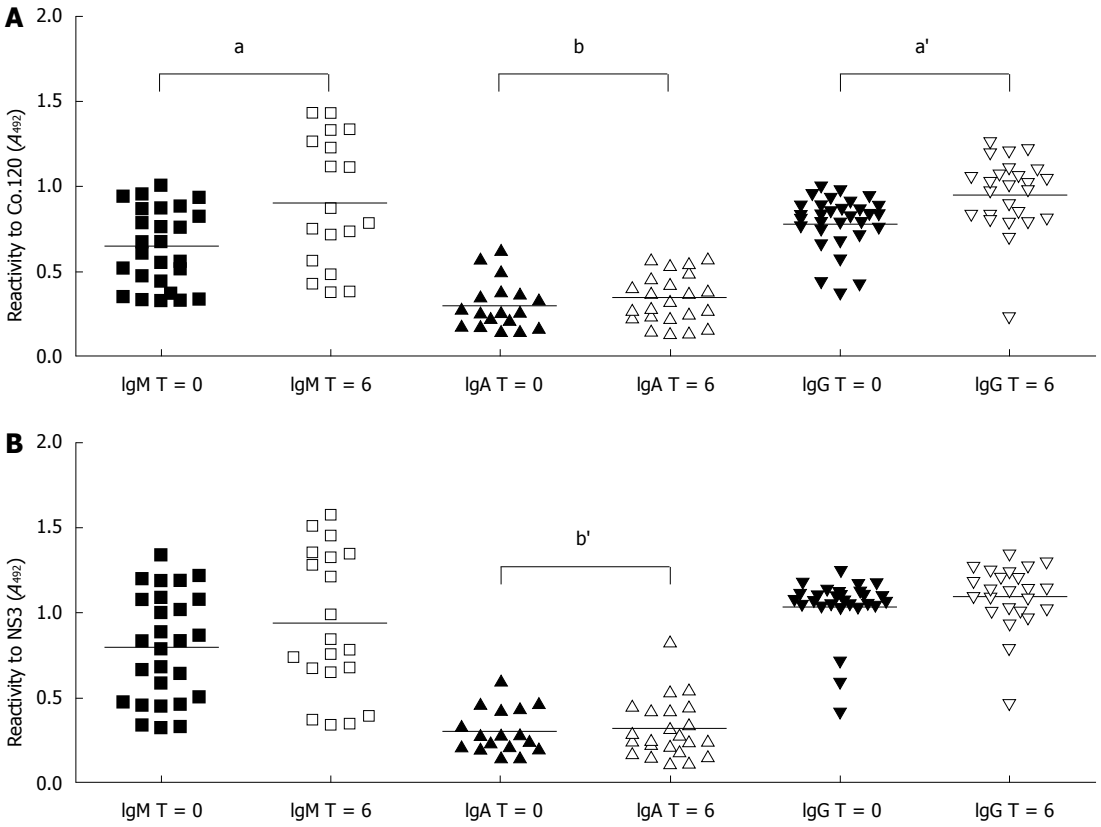

Figure 1 Comparison of antibody reactivity to HCV recombinant Co.120 (A) and NS3 (B) proteins in serum samples from baseline (T = 0) and 6 mo (T = 6) of follow-up. Symbols represent individual values. Only samples showing a positive reactivity are displayed. The horizontal lines represent mean values. Letters over brackets indicate statistical significance (a denotes differences in response magnitude, $^{a}P = 0.027$, $^{a}P = 0.0006$; b denotes differences in the number of positive samples, $^{b}P = 0.017$, $^{b}P = 0.005$).

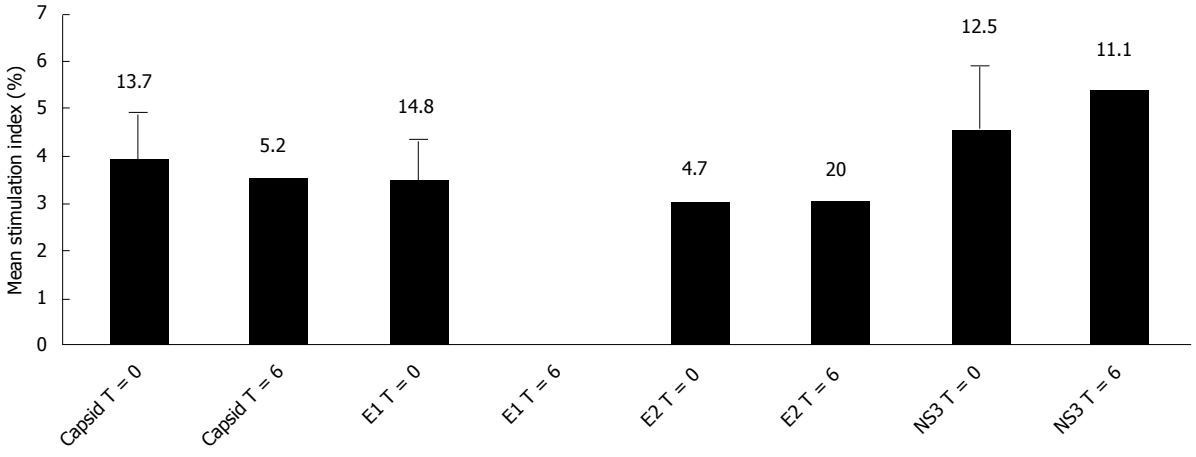

Figure 2 Comparison of CD8+ T cell proliferative response to HCV structural and non-structural antigens in baseline and six months follow up samples. Bars represent mean stimulation index of positive samples only. Error bars represent standard deviation of the mean. Numbers above bars indicate percent of positive samples.

CD4 to evaluate whether the CD4+ cell subpopulation proliferated when stimulated with specific HCV antigens. Particularly, a specific proliferative response was neither detected against the capsid and NS3 proteins at T = 0 nor to E2 at T = 6 (data not shown). Only 10% of the evaluated samples showed a positive response to the capsid at the end of the study, being this antigen the less frequently recognized. The highest percent of positive samples (25%) was detected towards E2 at baseline, followed by NS3 at T = 6 (20%), while E1 protein was always recognized by 14% of the individuals. None of the patients showed a positive proliferative response against more than one antigen.

On the other hand, the analysis of CD8+ cells allowed the identification of specific responses to all the tested antigens, except to E1 at T = 6 (Figure 2). However, these responses were present in less than 15% of the patients against each antigen. Two patients

showed a positive proliferative response simultaneously against the capsid and E1 proteins at T = 0, while another individual recognized E1 at baseline and also E2, 6 months later. CD8+ cells of only one individual proliferated against all tested antigens (capsid, envelope and NS3 proteins) at T = 0; unfortunately, this patient could not be contacted for a second blood sample, and therefore, the persistence of this peculiar response could not be assessed. Taking into account the results of both CD4+ and CD8+ cell proliferation, we did not detect any patient in which the two time-point samples were consistently positive throughout the study.

DISCUSSION

In this study, we assessed HCV-specific immune responses in a group of HCV chronically infected patients at two different time-points six months apart. The evaluation of the antibody response against viral antigens revealed that all the patients displayed a positive antibody response against several of the evaluated antigens. Specifically, in the majority of patients the reactivity was dominated by IgG and IgM, both in terms of number of positive patients and the magnitude of the response. Particularly, the presence of IgM anti-HCV-capsid antibodies have been regarded as a negative prognostic marker of response to treatment[28] and a factor associated to recurrence of hepatitis and its severity in HCV-infected liver transplant recipients[29]. Therefore, their presence in our cohort of chronic patients reinforces the notion of their inefficacy in this phase of the infection. Our results are in agreement with previous works reporting elevated prevalence of both immunoglobulin classes in chronically infected patients[7,30]. These studies only refer to the specific response to the capsid protein; our results extend this notion to other antigens such as E1 and NS3.

Among the IgG subtypes, IgG1 has been, by far, the most frequently found against all tested viral antigens[5,30]. Moreover, it has been reported that the antibody response to most HCV antigens is highly restricted to this subclass[31], the rest of the subclasses being rarely detected. This response restriction to IgG1 has lead studies aiming to find relations of antibody production to long-term outcome after therapy. In fact, it has been observed that IgG1 specific to an N-terminal epitope of the capsid protein decrease in complete responders, while remain unchanged in non-responders. Therefore, these antibodies have been proposed as markers of the efficacy of IFN-α therapy[30]. Our results showed a high prevalence of IgG1 against the capsid, and also against NS3, HCV's most conserved antigens. Nevertheless, 78.7% of the patients also showed a positive IgG4 response against NS3. It is of general knowledge that in chronic viral infections in humans, viral proteins generally elicit the IgG1 and IgG3 subtypes and to a lesser extent, IgG2 and IgG4. IgG4 has been frequently found dominating in responses to prolonged antigenic stimulation[32] and has been identified as a major component of circulating immune-complexes in chronic hepatitis B virus-infected individuals[33]. Given that antibodies of IgG4 subclass do not activate the complement system through the classic pathway and have a low affinity to Fc-γ receptors, their presence is considered a factor that may contribute to chronicity.

The assessment of correlations between immunological and demographic variables in our study revealed that IgM to structural antigens, IgG to the HVR-1, IgA and IgG4 responses negatively correlated with alcohol consumption, indicating that this habit may dampen the potentiality for generating a diverse immune response. In contrast, the fact of having been treated with the standard therapy positively correlated with the presence of IgG4 responses. Regarding the involvement of IgG4, it has been observed that the nonselective modulatory effect of IFN-α treatment may contribute to widen the diversity of specific IgG subclasses profiles in hepatitis B virus infection, contributing to the high participation of IgG4[34]. To our knowledge, this effect of antiviral therapy has not been assessed specifically in HCV infection, but seems a plausible hypothesis supporting our findings.

Another positive correlation that could be detected was that between hepatic damage, expressed as necro-inflammatory activity, and IgA response. It has been observed that TGF-β1, which is produced by hepatic stellate cells and Kupffer's cells, induces the isotype switching to IgA in B lymphocytes proliferating *in vitro*[35]. This cytokine is a prominent profibrogenic factor during inflammation, tissue regeneration and fibrogenesis[36] and in line with this, it has been demonstrated that HCV patients have elevated levels of circulating TGF-β1 *versus* controls[37]. To our knowledge, whether there is a direct relation between serum IgA and TGF-β1 circulating levels in HCV chronic patients has not been explored so far. Nevertheless, our results warrant further studies, although they do not point out to a direct correlation with fibrosis, but rather with the necro-inflammatory activity. On the other hand, the liver plays an important role in IgA clearance, and the loss of hepatic function due to chronic inflammation and damage may reduce normal IgA catabolism, and contribute to its accumulation in serum[38]. Therefore, the observed correlation might be probably indicating that IgA increase is a consequence, rather than a cause, of hepatic damage.

Additionally, IgG4 responses positively correlated with hepatic damage. As previously discussed, IgG4 has not been usually found as a dominant component of HCV specific immune response. Therefore, the implications of the presence of this subclass have not been explored so far in patients chronically infected with HCV. Nevertheless, a series of inflammatory and autoimmune diseases, in which IgG4 have a strong participation, have been described: these are known as IgG4-related sclerosing diseases[39]. In general, these disorders are characterized by high serum IgG4 levels, which are closely associated with disease activity, in a context of chronic lymphocyte infiltration and fibrosis of the affected organs[39]. Although the role of

IgG4 in these disorders remains obscure, it has been suggested that the formation and accumulation of immune complexes and the activation of the alternative complement pathway contribute to disease activity[39]. Further studies with larger cohort of patients are needed to definitively discern the real role of IgG4 in chronic hepatitis C as well as in other chronic inflammatory disorders.

We also evaluated the proliferative response of PBMC of chronic patients against stimulation with HCV antigens, in paired samples taken six months apart. Only a small percent of samples showed a positive response against each of the tested antigens. Moreover, the great majority of the patients displayed a detectable proliferation only to a single antigen and this response was never constant in the two evaluated time points. These results are in agreement with previous works reporting instability, low frequencies and a small number of targeted epitopes by both CD4+ and CD8+ T cells in peripheral blood of chronic patients[14,16,40]. The characteristic asymptomatic course of this disease hinders the accumulation of immunological data regarding the very early phase of the infection; therefore, it has been difficult, so far, to discriminate between primary T cell failure and early T cell exhaustion or deletion, once chronic infection has already been established. In fact, both phenomena seem to operate in different patients and equally lead to persistence[41]. Many mechanisms are postulated to be involved in T cell failure, namely impaired antigen presentation[42,43], reduced cytokine secretion by antigen presenting cells[44], immunomodulatory effects exerted by different viral factors[45,46] and increased levels of CD4 + $CD25^{high}$ Treg cells[47].

In summary these results confirm that in patients chronically infected with HCV, either naïve or non-responders to the standard therapy with IFN-α plus ribavirin, cellular proliferative responses are rarely detected, usually weak, not sustained and narrowly directed. On the other hand, the humoral response is characterized by a broad representation of antibody classes and subclasses, some of which have not been demonstrated to contribute to viral clearance, but rather to persistence. Particularly, the association of specific IgA response to necro-inflammatory activity paves the way to further studies to confirm its utility as an easy-to-measure marker of increased histological activity.

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COMMENTS

Background

Correlates of protection against hepatitis C virus (HCV) are extensively pursued in nowadays research. Early, vigorous and sustained peripheral blood mononuclear cells (PBMC) proliferative responses specific to HCV have been regarded as pivotal for viral clearance. On the other hand, antibody responses' contribution is still controversial and the significance of specific antibody classes during chronic infection has been investigated.

Research frontiers

So far, in HCV infection, the most extensively studied antibodies are those directed to the capsid protein. Several data indicate that IgM anti-HCV capsid occurrence is directly related to viremia levels. Additionally, HCV-specific antibody response is regarded as restricted to the IgG1 isotype, except for the capsid. The rest of the classes and IgG subclasses have been found very rarely represented, and therefore their significance in acute and chronic HCV is unclear.

Innovations and breakthroughs

Correlation analysis between demographic variables and humoral response confirmed the negative influence of alcohol consumption on the immune response, particularly on responses of the main immunoglobulin classes. On the other hand, IgG4, an IgG subclass characteristic of chronic antigenic stimulation, positively correlated with the grade of necro-inflammatory activity and the fact of being treated with the standard therapy; the latter already demonstrated for hepatitis B virus (HBV), but not for HCV. Additionally, for the first time a positive correlation between necro-inflammatory activity with HCV-specific IgA was found.

Applications

Particularly, the association of specific IgA response to necro-inflammatory activity paves they way to further studies to confirm its utility as a non invasive, easy-to-measure marker of increased histological activity in chronic HCV infection.

Peer review

An interesting study assessing the humoral response in chronic HCV patients. The association of elevated IgG and globulins with fibrosis have been described as referenced. It is an interesting observation to find IgA specific to HCV antigens.

REFERENCES

1 **Hoofnagle JH**. Course and outcome of hepatitis C. *Hepatology* 2002; **36**: S21-S29
2 **Yuan HJ**, Lee WM. Nonresponse to treatment for hepatitis C: current management strategies. *Drugs* 2008; **68**: 27-42
3 **Choo QL**, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359-362
4 **Chen M**, Sällberg M, Sönnerborg A, Weiland O, Mattsson L, Jin L, Birkett A, Peterson D, Milich DR. Limited humoral immunity in hepatitis C virus infection. *Gastroenterology* 1999; **116**: 135-143
5 **Netski DM**, Mosbruger T, Depla E, Maertens G, Ray SC, Hamilton RG, Roundtree S, Thomas DL, McKeating J, Cox A. Humoral immune response in acute hepatitis C virus infection. *Clin Infect Dis* 2005; **41**: 667-675
6 **Nagayama R**, Miyake K, Tsuda F, Okamoto H. IgM antibody to a hepatitis C virus core peptide (CP14) for monitoring activity of liver disease in patients with acute or chronic hepatitis C. *J Med Virol* 1994; **42**: 311-317
7 **Quiroga JA**, van Binsbergen J, Wang CY, Pardo M, Navas S, Trines C, Herrero M, Carreño V. Immunoglobulin M antibody to hepatitis C virus core antigen: correlations with viral replication, histological activity, and liver disease outcome. *Hepatology* 1995; **22**: 1635-1640
8 **Yuki N**, Hayashi N, Ohkawa K, Hagiwara H, Oshita M,

Katayama K, Sasaki Y, Kasahara A, Fusamoto H, Kamada T. The significance of immunoglobulin M antibody response to hepatitis C virus core protein in patients with chronic hepatitis C. *Hepatology* 1995; **22**: 402-406

9 **Chapel HM**, Christie JM, Peach V, Chapman RW. Five-year follow-up of patients with primary antibody deficiencies following an outbreak of acute hepatitis C. *Clin Immunol* 2001; **99**: 320-324

10 **Zeisel MB**, Fafi-Kremer S, Fofana I, Barth H, Stoll-Keller F, Doffoel M, Baumert TF. Neutralizing antibodies in hepatitis C virus infection. *World J Gastroenterol* 2007; **13**: 4824-4830

11 **Meunier JC**, Engle RE, Faulk K, Zhao M, Bartosch B, Alter H, Emerson SU, Cosset FL, Purcell RH, Bukh J. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. *Proc Natl Acad Sci USA* 2005; **102**: 4560-4565

12 **Pestka JM**, Zeisel MB, Bläser E, Schürmann P, Bartosch B, Cosset FL, Patel AH, Meisel H, Baumert J, Viazov S, Rispeter K, Blum HE, Roggendorf M, Baumert TF. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci USA* 2007; **104**: 6025-6030

13 **Grüner NH**, Gerlach TJ, Jung MC, Diepolder HM, Schirren CA, Schraut WW, Hoffmann R, Zachoval R, Santantonio T, Cucchiarini M, Cerny A, Pape GR. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis* 2000; **181**: 1528-1536

14 **Day CL**, Lauer GM, Robbins GK, McGovern B, Wurcel AG, Gandhi RT, Chung RT, Walker BD. Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection. *J Virol* 2002; **76**: 12584-12595

15 **Thimme R**, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, Govindarajan S, Purcell RH, Chisari FV. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* 2002; **99**: 15661-15668

16 **Cox AL**, Mosbruger T, Lauer GM, Pardoll D, Thomas DL, Ray SC. Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology* 2005; **42**: 104-112

17 **Grabowska AM**, Lechner F, Klenerman P, Tighe PJ, Ryder S, Ball JK, Thomson BJ, Irving WL, Robins RA. Direct ex vivo comparison of the breadth and specificity of the T cells in the liver and peripheral blood of patients with chronic HCV infection. *Eur J Immunol* 2001; **31**: 2388-2394

18 **Folgori A**, Spada E, Pezzanera M, Ruggeri L, Mele A, Garbuglia AR, Perrone MP, Del Porto P, Piccolella E, Cortese R, Nicosia A, Vitelli A. Early impairment of hepatitis C virus specific T cell proliferation during acute infection leads to failure of viral clearance. *Gut* 2006; **55**: 1012-1019

19 **Ulsenheimer A**, Gerlach JT, Gruener NH, Jung MC, Schirren CA, Schraut W, Zachoval R, Pape GR, Diepolder HM. Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. *Hepatology* 2003; **37**: 1189-1198

20 **Semmo N**, Day CL, Ward SM, Lucas M, Harcourt G, Loughry A, Klenerman P. Preferential loss of IL-2-secreting CD4+ T helper cells in chronic HCV infection. *Hepatology* 2005; **41**: 1019-1028

21 **Wedemeyer H**, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, Liang TJ, Alter H, Rehermann B. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 2002; **169**: 3447-3458

22 **Spangenberg HC**, Viazov S, Kersting N, Neumann-Haefelin C, McKinney D, Roggendorf M, von Weizsäcker F, Blum HE, Thimme R. Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology* 2005; **42**: 828-837

23 **Dueñas-Carrera S**, Morales J, Acosta-Rivero N, Lorenzo LJ, García C, Ramos T, Guerra I, Peña M. Variable level expression of hepatitis C virus core protein in a prokaryotic system. Analysis of the humoral response in rabbit. *Biotecnología Aplicada* 1999; **16**: 226-231

24 **Lorenzo LJ**, García O, Acosta-Rivero N, Dueñas-Carrera S, Martínez G, Alvarez-Obregón J, Pichardo D, Ramos A, Guerra I, Morales J. Expression and immunological evaluation of the Escherichia coli-derived hepatitis C virus envelope E1 protein. *Biotechnol Appl Biochem* 2000; **32** (Pt 2): 137-143

25 **Pentón N**, Musacchio A, Rivera JM, Roca J, Ponce M, Rodríguez D, Caballero A, Tallo YI, Narciandi RE. Antigenicity of a recombinant NS3 protein representative of ATPase/helicase domain from hepatitis C virus. *Clin Biochem* 2003; **36**: 41-49

26 **Martinez-Donato G**, Acosta-Rivero N, Morales-Grillo J, Musacchio A, Vina A, Alvarez C, Figueroa N, Guerra I, Garcia J, Varas L, Muzio V, Dueñas-Carrera S. Expression and processing of hepatitis C virus structural proteins in Pichia pastoris yeast. *Biochem Biophys Res Commun* 2006; **342**: 625-631

27 **Dueñas-Carrera S**, Viña A, Garay HE, Reyes O, Alvarez-Lajonchere L, Guerra I, González LJ, Morales J. Immunological evaluation of Escherichia coli-derived hepatitis C virus second envelope protein (E2) variants. *J Pept Res* 2001; **58**: 221-228

28 **Papatheodoridis GV**, Delladetsima JK, Katsoulidou A, Sypsa V, Albrecht M, Michel G, Hatzakis A, Tassopoulos NC. Significance of IgM anti-HCV core level in chronic hepatitis C. *J Hepatol* 1997; **27**: 36-41

29 **Bizollon T**, Ahmed SN, Guichard S, Chevallier P, Adham M, Ducerf C, Baulieux J, Trepo C. Anti-hepatitis C virus core IgM antibodies correlate with hepatitis C recurrence and its severity in liver transplant patients. *Gut* 2000; **47**: 698-702

30 **Hirayama M**, Maruyama T, Mitsui H, Maekawa H, Yamada H, Hashimoto N, Koike K, Kimura S, Yasuda K, Iino S, Green J. IgG1 anti-P2 as a marker of response to interferon in patients with chronic hepatitis C. *Clin Exp Immunol* 2001; **126**: 92-100

31 **Chen M**, Sällberg M, Sönnerborg A, Weiland O, Mattsson L, Jin L, Birkett A, Peterson D, Milich DR. Limited humoral immunity in hepatitis C virus infection. *Gastroenterology* 1999; **116**: 135-143

32 **Aalberse RC**, van der Gaag R, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J Immunol* 1983; **130**: 722-726

33 **Rath S**, Devey ME. IgG subclass composition of antibodies to HBsAg in circulating immune complexes from patients with hepatitis B virus infections. *Clin Exp Immunol* 1988; **72**: 164-167

34 **Gregorek H**, Madaliński K, Woynarowski M, Mikolajewicz J, Syczewska M, Socha J. IgG subclass distribution of hepatitis B surface antigen antibodies induced in children with chronic hepatitis B infection after interferon-alpha therapy. *J Infect Dis* 2000; **181**: 2059-2062

35 **Zan H**, Cerutti A, Dramitinos P, Schaffer A, Casali P. CD40 engagement triggers switching to IgA1 and IgA2 in human B cells through induction of endogenous TGF-beta: evidence for TGF-beta but not IL-10-dependent direct S mu-->S alpha and sequential S mu-->S gamma, S gamma-->S alpha DNA recombination. *J Immunol* 1998; **161**: 5217-5225

36 **Schuppan D**, Krebs A, Bauer M, Hahn EG. Hepatitis C and liver fibrosis. *Cell Death Differ* 2003; **10** Suppl 1: S59-S67

37 **Peterson MC**. Elevated circulating transforming growth factor beta-1 may explain poorer renal survival in type II diabetics with chronic hepatitis C. *Med Sci Monit* 2007; **13**: RA81-RA85

38 **Woof JM**, Kerr MA. The function of immunoglobulin A in immunity. *J Pathol* 2006; **208**: 270-282

39 **Nakanuma Y**, Zen Y. Pathology and immunopathology of immunoglobulin G4-related sclerosing cholangitis: The latest addition to the sclerosing cholangitis family. *Hepatol Res* 2007; **37** Suppl 3: S478-S486

40 **Lauer GM**, Barnes E, Lucas M, Timm J, Ouchi K, Kim AY, Day CL, Robbins GK, Casson DR, Reiser M, Dusheiko G, Allen TM, Chung RT, Walker BD, Klenerman P. High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 2004; **127**: 924-936

41 **Thimme R**, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; **194**: 1395-1406

42 **Bain C**, Inchauspé G. [Dendritic cells and hepatitis C virus] *Pathol Biol (Paris)* 2001; **49**: 464-465

43 **Lee CH**, Choi YH, Yang SH, Lee CW, Ha SJ, Sung YC. Hepatitis C virus core protein inhibits interleukin 12 and nitric oxide production from activated macrophages. *Virology* 2001; **279**: 271-279

44 **Anthony DD**, Yonkers NL, Post AB, Asaad R, Heinzel FP, Lederman MM, Lehmann PV, Valdez H. Selective impairments in dendritic cell-associated function distinguish hepatitis C virus and HIV infection. *J Immunol* 2004; **172**: 4907-4916

45 **Zhu N**, Ware CF, Lai MM. Hepatitis C virus core protein enhances FADD-mediated apoptosis and suppresses TRADD signaling of tumor necrosis factor receptor. *Virology* 2001; **283**: 178-187

46 **Accapezzato D**, Francavilla V, Rawson P, Cerino A, Cividini A, Mondelli MU, Barnaba V. Subversion of effector CD8+ T cell differentiation in acute hepatitis C virus infection: the role of the virus. *Eur J Immunol* 2004; **34**: 438-446

47 **Smyk-Pearson S**, Golden-Mason L, Klarquist J, Burton JR Jr, Tester IA, Wang CC, Culbertson N, Vandenbark AA, Rosen HR. Functional suppression by FoxP3+CD4+CD25(high) regulatory T cells during acute hepatitis C virus infection. *J Infect Dis* 2008; **197**: 46-57

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RAPID COMMUNICATION

Hepatoma cells up-regulate expression of programmed cell death-1 on T cells

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Abstract

AIM: To investigate the effect of hepatoma cells on up-regulation of programmed cell death-1 (PD-1), and the function of PD-1 on T cells.

METHODS: HepG2 or HepG2.2.1.5 cells were co-cultured with a lymphoma cell line-Jurkat cells. PD-1 expression was detected by flow cytometry. IL-2, INF-γ and IL-10 in culture supernatant were detected by enzyme-linked immunosorbent assay (ELISA). Cytotoxic action of T cells was determined by MTT reduction assay-direct mononuclear cell cytotoxicity assay.

RESULTS: The PD-1 expression on Jurkat cells increased by 16.17% ± 2.5% and 17.43% ± 2.2% after HepG2 or HepG2.2.1.5 cells were co-cultured for 48 h. The levels of IL-2, INF-γ and IL-10 in the culture supernatant were 202.9 ± 53.0 pg/mL, 88.6 ± 4.6 pg/mL and 63.7 ± 13.4 pg/mL respectively, which were significantly higher than those (102.9 ± 53 pg/mL, 39.3 ± 4.2 pg/mL, and 34.6 ±13.7 pg/mL) in the control group ($P < 0.05$). The OD value for MTT assay in the blocking group (0.29 ± 0.06) was significantly higher than that (0.19 ± 0.09) in the control group ($P < 0.05$).

CONCLUSION: PD-1 expression on Jurkat cells is up-regulated by hepatoma cells, cytokines and cytotoxic action are elevated after PD-1/PD-L1 is blocked.

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Key words: Hepatoma cell; Programmed cell death-1; Protein expression; T cell function; Cytokine

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INTRODUCTION

Programmed cell death-1 (PD-1), a member of the CD28 family, was first isolated from T cell hybridoma by subtractive hybridization in 1992[1], and is expressed on activated T and B cells[2]. PD-1 has been recently found to play a role in immunity regulation as an inhibitory co-signaling molecule[3]. Upon its ligands (PD-L1 and PD-L2) are ligated, PD-1 decreases T cell receptor (TCR)-mediated proliferation, cytokine production and cytolytic activity[4-7]. Barber *et al*[8] showed that up-regulation of PD-1 expression on T cells is associated with the exhaustion of T cells in lymphocytic choriomeningitis virus (LCMV) infection. The number of effective T cells increases and their function markedly improves when the interaction of PD-1 and PD-L is blocked. It was reported that PD-1 also plays a significant role in some autoimmune diseases[9-11] and viral infectious diseases, especially in chronic infectious diseases caused by human immunodeficiency virus (HIV)[12-14], hepatitis C virus (HCV)[15,16] and hepatitis B virus (HBV)[17-19]. There is evidence that PD-1 suppresses immune activation in PD-1-deficient mice with autoimmune disease[20]. Single-nucleotide polymorphisms at the PD-1 locus have also been identified in patients with autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes[21].

Recent evidence from studies on tumors shows that the PD-1/PD-1 pathway might play a critical role in tumor immunity evasion[22-24]. PD-1 is up-regulated on tumor specific T cells and PD-L is up-regulated in tumor tissue[25-27]. As a result, the function of tumor specific T cells is suppressed, leading to the immune escaping of tumor cells, and the level of mRNA and PD-L1

protein can be detected in tumor tissues[25-27]. In addition, PD-1/PD-L interaction promotes apoptosis of T cells, induces clearance of specific T cells, and ultimately inhibits the anti-tumor immunity response[27]. Blocking the interaction of PD-1/PD-L with anti-PD-l antibody partially recovers the function of tumor specific T cells.

It was reported that the expression of PD-L can be induced by virus and cytokines such as IFN-α and INF-γ[28]. However, no evidence for the induced expression of PD-1 is available. Liver, an important immune organ, plays a critical role in immune regulation although it is prone to induce immune tolerance in many cases. The present study was designed to investigate whether hepatoma cells (HepG2 and HepG2.2.1.5) induce expression of PD-1 in T cells. The functional role of PD-1/PD-L interaction was also studied.

MATERIALS AND METHODS

Cell culture

Jurkat cells were cultured in RPMI 1640 medium (Gibco, USA), supplemented with 10% fetal calf serum, 300 μg/mL glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. HepG2.2.1.5 cells or HepG2 cells provide by Mountsinai Medical Center were cultured in complete DMEM (Gibco, USA) containing 380 μg/L G418.

Co-culture system

HepG2 and HepG2.2.1.5 cells were cultured in 6-well plates (5×10^5 cells/well) for 24 h and Jurkat cells (5×10^5 cells/well) were added and co-cultured for 48 h. The suspended cells were collected for analysis of PD-1. Jurkat cells were cultured solitarily for 48 h as controls.

Analysis of PD-1 expression

Cell surface expression of PD-1 was detected by flow cytometry after incubated with allophycocyanin (APC)-conjugated anti-PD-1 antibodies (eBioscience). Cells were collected and suspended in PBS containing 1% fetal calf serum (FCS). The cell density was adjusted to 1×10^6 cells/vial. After incubated with anti-PD-1 antibodies or matching isotype controls at 37℃ for 30 min, cells were washed with PBS and fixed in 2% paraformaldehyde for analysis. A total of 20000 gated cells were analyzed on Becton Dickinson FACS (Becton Dickinson, USA) using the CELLQuest™ software.

Blockade of PD-1/PD-L1 interaction

Antibody against human PD-L1 (eBioscience) was used to block PD-L1. Jurkat cells were activated with phytohemagglutinin (PHA) (2 μg/mL, Sigma, USA) and co-cultured with HepG2 or HepG2.2.1.5 cells. Anti-PD-L1 antibodies (25 μg/mL) were added into the culture to block the interaction of PD-1 and PD-L1 for 48 h. Mouse IgG, as a control antibody, was used in the control group. After cultured for 48 h, the supernatants of co-cultures were collected and stored at -80℃.

Analysis of cytokine secretion

Levels of IL-2, IL-10 and INF-γ in the supernatants were measured by ELISA (Ucytech, Netherlands) following its manufacturer's instructions.

Analysis of cytolytic activity

Cytolytic activity of Jurkat cells was detected by MTT assay. After co-cultured for 48 h, Jurkat cells were isolated and MTT was added to incubate HepG2.2.1.5 cells for 4 h, and bleached with DMSO. Finally, the cytolytic effect of Jurkat cells on HepG2.2.1.5 cells was analyzed on Bio-Rad 450 (USA).

Statistical analysis

Results were expressed as mean ± SD or percentage. Comparison between groups was made using Student's unpaired *t*-test. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS 13.0 for Windows.

Table 1 Up-regulated expression of PD-1 on T cells (n = 24, mean ± SD)

Groups		PD-1(%)
Controls		0.70 ± 0.03
Co-cultured	HepG2	16.17 ± 2.5[a]
	HepG2.2.1.5	17.43 ± 2.2[b]

[a]P = 0.000, [b]P = 0.000 *vs* control group.

RESULTS

Enhancement of PD-1 expression on Jurkat cells

PD-1 expression on Jurkat cells was determined by FACS analysis at 48 h after co-cultured with HepG2 or HepG2.2.1.5 cells. Jurkat cells were also cultured solitarily as controls. The expression of PD-1 was induced on Jurkat cells after co-culture with HepG2 or HepG2.2.1.5 cells for 48 h, which was significantly higher on Jurkat cells co-cultured with hepatoma cells than on controls (P = 0.000, Table 1).

Function restoration of T cells

Supernatants were collected from the blocking and control groups. To investigate the influence of cytokine production after PD-1/PD-L1 was blocked, the levels of IL-2, IL-10 and INF-γ were measured. After PD-L1 was blocked with specific antibodies, the levels of IL-2, IL-10 and INF-γ were much higher in the blocking group than in the control group (P = 0.000, Table 2).

Furthermore, the effect of the PD-1/PD-L1 pathway on cytolytic activity of T cells was also investigated by MTT assay. The A value (0.29 ± 0.06) in the blocking group was much higher than that (0.19 ± 0.09) in the control group (P = 0.000).

DISCUSSION

Activation of resting lymphocytes triggers expression of several products of the immunoglobulin superfamily of genes. These activation-induced antigens are involved in

Table 2 Secretion of cytokines by T cells (pg/mL, n = 24, mean ± SD)

Groups	IL-2	IFN-γ	IL-10
Control group	102.9 ± 53.0	39.3 ± 4.2	34.6 ± 13.7
Blocking group	202.9 ± 53.0[c]	88.6 ± 4.6[d]	63.7 ± 13.4[e]

[c]P = 0.000, [d]P = 0.000, [e]P = 0.000 *vs* control group.

many physiological and pathological processes including cell proliferation (IL-2R), functional differentiation (CTLA-4), and apoptosis (Fas)[29,30]. The expression patterns of these antigens are cell-specific, and have different regulation functions in different cells. PD-1, a member of the CD28 family, which was isolated from apoptosis-induced T cell hybridoma in 1992[1], is expressed on activated T and B cells[2].

Agata *et al*[2] showed that PD-1 expresses on activated T and B cells. Anti-CD3 and concanavalin A (ConA) can stimulate its expression on thymocytes and T cells in spleen, and anti-IgM antibody can stimulate its expression on B cells in spleen. Vibhakar *et al*[31] also demonstrated that PD-1 mRNA and protein levels in Jurkat cells are up-regulated in a time-dependent manner during phorbo ester (TPA)-induced differentiation, indicating that lymphocyte activators can up-regulate PD-1 expression on lymphocytes. Since PHA is another T cell activating agent, the expression of PD-1 in T cells can be detected after stimulation of PHA. A time-dependent up-regulation of hPD-1 was also observed during PHA induction (data not shown), and was used as a stimulus of Jurkat cells in our blocking experiment.

It was reported that, as an inhibitory co-stimulating molecular, PD-1 plays a role in immune regulation and is associated with the exhaustion of effective T cells[32-34]. Barber *et al*[8] showed that, in chronic viral infection diseases, PD-1 is highly expressed on the exhausted LCMV-specific CD8 T cells and blocking the PD-1/PD-L1 interaction during the chronic phase of infection can efficiently reanimate the exhausted CD8 T cells and promote clearance of the persisting virus. In contrast, PD-1 expression is transiently induced and declines quickly to its basal level in acute LCMV-Armstrong infection, thus promoting studies on other diseases associated with immune. Up-regulation of PD-1 expression on effective T cells leads to suppression of immune, which might be the underlying mechanism of immune evasion. PD-L1/PD-L2 expression in a variety of tumor cells has been detected in human tumors[35,25-27], while PD-1 over-expression on tumor specific T cells has also been observed[23]. Interaction of PD-1/PD-L1 promotes apoptosis of T cells, inhibits anti-tumor immune response of T cells, and stimulates growth of tumors[27]. Obstructing the interaction of PD-1/PD-L1 enhances the function of T cells, hampers development of tumors[22,23,26]. In the present study, tumor cells induced expression of PD-1 on T cells. After co-cultured with hepatoma cells, PD-1 was expressed on T cells, but not expressed on Jurkat cells after cultured for 48 h solitarily. PD-1 was expressed on T cells after Jurkat cells were co-cultured with the supernatant of hepatoma cells (data not shown), suggesting that hepatoma cells can up-regulate the expression of PD-1.

These findings lead to the clinical use of PD-1 blockers in the treatment of tumors. In chronic infectious diseases, virus can induce the expression of PD-1 on T cell. However, the precise mechanism PD-1 blockers still remains unclear. We observed the effect of tumor cells on PD-1 expression in T cells. Jurkat cells, a kind of $CD4^+$ T cells, can be used as target cells co-cultured with hepatoma cells. FACS analysis showed that tumor cells could induce PD-1 expression on T cells. In this study, HepG2.2.1.5 cells transferring HBV genome and HepG2 cells not transferring HBV genome could induce PD-1 expression, suggesting that HBV has no effect on PD-1 expression on T cells.

In addition, the function of PD-1 on T cells was also observed. Anti-PD-L1 antibody was used to block the interaction of PD-1 and PD-L1. PD-1 was induced by PHA, and PD-L1 was expressed in HepG2.2.1.5 cells identified by FACS (data not shown). Jurkat cells after activated by PHA were co-cultured with HepG2.2.1.5cells. Antibodies against human PD-L1 were added into the co-culture system as a blocking group, while mouse IgG was added as a control group. The levels of cytokines including IL-2, INF-γ and IL-10 in culture supernatant and the cytolytic activity of T cells were detected, which were significantly elevated in blocking group compared to the control group, suggesting that both Th1 and Th2 have immune responses can be restored and PD-1/PD-L that negatively regulates the immune reaction by blocking the PD-1/PD-L pathway can recover the function of T cells, which introduces a new theory of tumor immune evasion. This new mechanism of tumor immunology might provide a novel target for therapy.

COMMENTS

Background

Programmed cell death-1 (PD-1), originally isolated from apoptotic T cells, is a 55-kDa transmembrane protein with an extracellular IgV-like domain and a 97-amino acid cytoplasmic tail containing an immunotyrosine inhibitory motif (ITIM) and an immunotyrosine switch motif (ITSM). PD-1 has two ligands: PD-L1 and PD-L2, which are members of the CD28/B7 superfamily. The expressions of CD28, CTLA-4, and ICOS are limited in T cells. PD-1 can be expressed on activated T, B and myeloid cells. The expression of PD-L1 has been detected in many organ tissues, such as heart, lung, pancreas, muscle, and placenta, including lymphocytes and non-lymphocytes. The expression of PD-L2 is restricted in DC and macrophages.

Research frontiers

Recent findings suggest that PD-1/PD-L pathway plays a role in regulating tolerance and autoimmunity. The role of PD-1 and its ligands in regulating human autoimmune disease, infectious diseases and tumors has been investigated. Interaction of PD-1 and PD-L has been found to be important for controlling effective T cells. Significantly increased expression of PD-1 and PD-L1 in T and B macrophages/dendritic cells and tumor cells, associated with T-cell exhaustion and disease progression, immobilized auto-antibodies to PD-L, can stimulate T cell proliferation, cytokine production, and programmed cell death. The up-regulation of PD-L1 in tumors and PD-1 in T cells can lead to immune tolerance.

Innovations and breakthroughs

The present study demonstrated the effect of hepatoma cells on up-regulating

the expression of PD-1 in T cells. In addition, the function of PD-1 in T cells was assessed, showing that the cytokine production and cytotoxicity of T cells can be elevated by blocking the interaction of PD-1 and PD-L1.

Applications

The up-regulation of PD-1 in T cells by hepatoma cells has led to a new hypothesis that the PD-1 and PD-L pathway may be a means by which tumors evade T cell recognition. Manipulation of the PD-1 pathway can enhance immune response, and may become a novel strategy for the treatment of tumors.

Terminology

MTT is a direct mono-nuclear cell direct cytotoxicity assay used to detect the cytotoxicity of T cells. PHA is a T cell stimulus that can activate T cells. G418, a kind of antibiotics, is used in selective cell culture medium. G418 must be added into culture medium of HepG2.2.1.5 cells to support the selective survival of HepG2.2.1.5 cells.

Peer review

This is a very interesting study, showing that HCC-induced modulation of PD-1 expression in T-cells might contribute to immune evasion. This concept might hold its promise for new therapeutic interventions. The experiments support the authors' claim.

REFERENCES

1 **Ishida Y**, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* 1992; **11**: 3887-3895

2 **Agata Y**, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 1996; **8**: 765-772

3 **Dong H**, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999; **5**: 1365-1369

4 **Ishida M**, Iwai Y, Tanaka Y, Okazaki T, Freeman GJ, Minato N, Honjo T. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. *Immunol Lett* 2002; **84**: 57-62

5 **Parry RV**, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, Linsley PS, Thompson CB, Riley JL. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol* 2005; **25**: 9543-9553

6 **Chemnitz JM**, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 2004; **173**: 945-954

7 **Kim HK**, Guan H, Zu G, Li H, Wu L, Feng X, Elmets C, Fu Y, Xu H. High-level expression of B7-H1 molecules by dendritic cells suppresses the function of activated T cells and desensitizes allergen-primed animals. *J Leukoc Biol* 2006; **79**: 686-695

8 **Barber DL**, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; **439**: 682-687

9 **Zhu B**, Guleria I, Khosroshahi A, Chitnis T, Imitola J, Azuma M, Yagita H, Sayegh MH, Khoury SJ. Differential role of programmed death-ligand 1 [corrected] and programmed death-ligand 2 [corrected] in regulating the susceptibility and chronic progression of experimental autoimmune encephalomyelitis. *J Immunol* 2006; **176**: 3480-3489

10 **Liang SC**, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, Sharpe AH. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol* 2003; **33**: 2706-2716

11 **Hatachi S**, Iwai Y, Kawano S, Morinobu S, Kobayashi M, Koshiba M, Saura R, Kurosaka M, Honjo T, Kumagai S. CD4+ PD-1+ T cells accumulate as unique anergic cells in rheumatoid arthritis synovial fluid. *J Rheumatol* 2003; **30**: 1410-1419

12 **Day CL**, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; **443**: 350-354

13 **Trautmann L**, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, Routy JP, Haddad EK, Sekaly RP. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 2006; **12**: 1198-1202

14 **Petrovas C**, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, Precopio ML, Schacker T, Roederer M, Douek DC, Koup RA. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* 2006; **203**: 2281-2292

15 **Penna A**, Pilli M, Zerbini A, Orlandini A, Mezzadri S, Sacchelli L, Missale G, Ferrari C. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology* 2007; **45**: 588-601

16 **Urbani S**, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, Ferrari C. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 2006; **80**: 11398-11403

17 **Boni C**, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, Zerbini A, Cavalli A, Missale G, Bertoletti A, Ferrari C. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 2007; **81**: 4215-4225

18 **Peng G**, Li S, Wu W, Tan X, Chen Y, Chen Z. PD-1 upregulation is associated with HBV-specific T cell dysfunction in chronic hepatitis B patients. *Mol Immunol* 2008; **45**: 963-970

19 **Chen L**, Zhang Z, Chen W, Zhang Z, Li Y, Shi M, Zhang J, Chen L, Wang S, Wang FS. B7-H1 up-regulation on myeloid dendritic cells significantly suppresses T cell immune function in patients with chronic hepatitis B. *J Immunol* 2007; **178**: 6634-6641

20 **Ansari MJ**, Salama AD, Chitnis T, Smith RN, Yagita H, Akiba H, Yamazaki T, Azuma M, Iwai H, Khoury SJ, Auchincloss H Jr, Sayegh MH. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med* 2003; **198**: 63-69

21 **Prokunina L**, Alarcon-Riquelme M. The genetic basis of systemic lupus erythematosus--knowledge of today and thoughts for tomorrow. *Hum Mol Genet* 2004; **13**: R143-R148

22 **Iwai Y**, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci USA* 2002; **99**: 12293-12297

23 **Blank C**, Kuball J, Voelkl S, Wiendl H, Becker B, Walter B, Majdic O, Gajewski TF, Theobald M, Andreesen R, Mackensen A. Blockade of PD-L1 (B7-H1) augments human tumor-specific T cell responses in vitro. *Int J Cancer* 2006; **119**: 317-327

24 **Loos M**, Giese NA, Kleeff J, Giese T, Gaida MM, Bergmann F, Laschinger M, Buchler M, Friess H. Clinical significance and regulation of the costimulatory molecule B7-H1 in pancreatic cancer. *Cancer Lett* 2008; **268**: 98-109

25 **Wintterle S**, Schreiner B, Mitsdoerffer M, Schneider D, Chen L, Meyermann R, Weller M, Wiendl H. Expression of the B7-related molecule B7-H1 by glioma cells: a potential mechanism of immune paralysis. *Cancer Res* 2003; **63**: 7462-7467

26 **Strome SE**, Dong H, Tamura H, Voss SG, Flies DB, Tamada K, Salomao D, Cheville J, Hirano F, Lin W, Kasperbauer JL, Ballman KV, Chen L. B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Cancer Res* 2003; **63**: 6501-6505

27 **Dong H**, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E,

Chen L. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002; **8**: 793-800

28 **Muhlbauer M**, Fleck M, Schutz C, Weiss T, Froh M, Blank C, Scholmerich J, Hellerbrand C. PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *J Hepatol* 2006; **45**: 520-528

29 **Cotner T**, Williams JM, Christenson L, Shapiro HM, Strom TB, Strominger J. Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. *J Exp Med* 1983; **157**: 461-472

30 **Lindsten T**, Lee KP, Harris ES, Petryniak B, Craighead N, Reynolds PJ, Lombard DB, Freeman GJ, Nadler LM, Gray GS. Characterization of CTLA-4 structure and expression on human T cells. *J Immunol* 1993; **151**: 3489-3499

31 **Vibhakar R**, Juan G, Traganos F, Darzynkiewicz Z, Finger LR. Activation-induced expression of human programmed death-1 gene in T-lymphocytes. *Exp Cell Res* 1997; **232**: 25-28

32 **Latchman Y**, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001; **2**: 261-268

33 **Freeman GJ**, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000; **192**: 1027-1034

34 **Seo SK**, Seo HM, Jeong HY, Choi IW, Park YM, Yagita H, Chen L, Choi IH. Co-inhibitory role of T-cell-associated B7-H1 and B7-DC in the T-cell immune response. *Immunol Lett* 2006; **102**: 222-228

35 **Ohigashi Y**, Sho M, Yamada Y, Tsurui Y, Hamada K, Ikeda N, Mizuno T, Yoriki R, Kashizuka H, Yane K, Tsushima F, Otsuki N, Yagita H, Azuma M, Nakajima Y. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin Cancer Res* 2005; **11**: 2947-2953

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RAPID COMMUNICATION

Effects of recombinant human growth hormone on enterocutaneous fistula patients

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Abstract

AIM: To explore the effects of recombinant human growth hormone (rhGH) on intestinal mucosal epithelial cell proliferation and nutritional status in patients with enterocutaneous fistula.

METHODS: Eight patients with enterocutaneous fistulas received recombinant human growth hormone (10 μg/d) for 7 d. Image analysis and immunohistochemical techniques were used to analyse the expression of proliferating cell nuclear antigen (PCNA) in intestinal mucosal epithelial cells in biopsy samples from the patients who had undergone an endoscopic biopsy through the fistula at day 0, 4 and 7. Body weights, nitrogen excretion, serum levels of total proteins, albumin, prealbumin, transferrin and fibronectin were measured at day 0, 4 and 7.

RESULTS: Significant improvements occurred in the expression of PCNA in the intestinal mucosal epithelial cells at day 4 and 7 compared to day 0 (24.93 ± 3.41%, 30.46 ± 5.24% *vs* 12.92 ± 4.20%, $P < 0.01$). These changes were accompanied by the significant improvement of villus height (500.54 ± 53.79 μm, 459.03 ± 88.98 μm *vs* 210.94 ± 49.16 μm, $P < 0.01$), serum levels of total proteins (70.52 ± 5.13 g/L, 74.89 ± 5.16 g/L *vs* 63.51 ± 2.47 g/L, $P < 0.01$), albumin (39.44 ± 1.18 g/L, 42.39 ± 1.68 g/L *vs* 35.74 ± 1.75 g/L, $P < 0.01$) and fibronectin (236.3 ± 16.5 mg/L, 275.8 ± 16.9 mg/L *vs* 172.5 ± 21.4 mg/L, $P < 0.01$) at day 4 and 7, and prealbumin (286.38 ± 65.61 mg/L *vs* 180.88 ± 48.28 mg/L, $P < 0.05$), transferrin (2.61 ± 0.12 g/L *vs* 2.41 ± 0.14 g/L, $P < 0.05$) at day 7. Nitrogen excretion was significantly decreased at day 7 (3.40 ± 1.65 g/d *vs* 7.25 ± 3.92 g/d, $P < 0.05$). No change was observed in the body weight.

CONCLUSION: Recombinant human growth hormone could promote intestinal mucosal epithelial cell proliferation and protein synthesis in patients with enterocutaneous fistula.

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Key words: Recombinant human growth hormone; Enterocutaneous fistula; Intestinal; Epithelial cell; Proliferating cell nuclear antigen

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INTRODUCTION

From the mid 1980s, recombinant human growth hormone (rhGH) has been applied clinically. Growth hormone is a peptide hormone which stimulates proliferation and differentiation of many kinds of cells. It also has anabolic effects on the modulation of energy and substance metabolism. Previous animal experiments[1-3] have demonstrated it could promote the structural repair of the intestinal mucosa in short bowel rats, but few studies have made direct observations of the effects of rhGH on intestinal mucosa in human. The objective of this study was to explore the effects of rhGH on intestinal mucosal proliferation and nutritional status in patients with enterocutaneous fistula.

Table 1 General state of the patients

Age (Yr)	Sex	Weight (kg)	Distance from ligament of tretiz (cm)	Rest energy expenditure (KJ)	Enteral nutrition energy (KJ)
65	F	52	40	4276	6276
42	M	68	80	5648	8368
30	M	80	100	6192	9205
32	M	61	120	4384	6276
18	M	48	60	4322	6276
72	F	56	90	5773	8368
38	M	77	135	6158	9205
27	F	49	85	4359	6276

MATERIALS AND METHODS

Study protocol

Eight patients (Table 1) with enterocutaneous fistula were injected with rhGH (10 U/d) for 7 d. Intestinal mucosa biopsies were performed by endoscopy through the fistula at 20 cm proximal to the fistula at day 0, 4 and 7. All the patients gave informed consent to participate in the study. This study was approved by the Ethical Committee of Jinling Hospital, Nanjing University. Biological tests revealed no signs of inflammation, metabolic disturbances or hepatic, renal and cardiac dysfunction before the patients were enrolled into the study. The subjects had a mean body mass index of 14.37 kg/m^2 (range 11.09-17.65 kg/m^2). For all the subjects, enteral nutrition (Peptisorb, Nutricia, Holland) was prescribed and taken by nasogastric or nasointestinal tube to maintain the metabolic balance. The formula contained 1 kcal/mL, and the total calories given according to the energy expenditure was determined by indirect calorimetry (MedGraphics, USA). Endoscopic biopsies were fixed in formalin for histological assessment.

Recombinant human growth hormone (rhGH)

rhGH (Saizen) was provided by Serono China Pte. Ltd, China. The dose of rhGH was 10 U/d administered once a day (8:00 pm) as a subcutaneous injection to an upper limb, beginning on day 1 and continued for 7 d.

Immunohistochemical staining

To assess the degree of cell proliferation, an immunohistochemical technique based on the proliferating cell nuclear antigen (PCNA) was used. Sections from tissue samples were dewaxed, taken through alcohol and then immersed for 10 min in 25% phosphate-buffered saline in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Sections were subsequently taken to water and immunostained using the Vectastain ABC peroxidase kit (Vecta Laboratories, Burlingame, CA). 0.4% diaminobenzidine (DAB, Aldrich Co.) was employed as a chromogen and a light haematoxylin counterstain was used. Counts were carried out in 30 crypts per preparation under microscope (40 ×), using an automatic image analysis system (HPLAS-1000, Tongji qianping Ltd). A proliferation index was determined based on the ratio between PCNA-positive cells and the total number of cells per longitudinal crypt section at the base of the crypt. This index is equal to the quotient of the number of proliferating cells and the total number of cells multiplied by 100.

Table 2 Changes of intestinal mucosal villus height, PCNA and nitrogen excretion before and after treatment with rhGH

	The days when treated with rhGH		
	0	4	7
Villus height (μm)	210.94 ± 49.16	500.54 ± 53.79^b	459.03 ± 88.98^b
PCNA labelling index (%)	12.92 ± 4.20	24.93 ± 3.41^b	30.46 ± 5.24^b
Nitrogen excretion (g/d)	7.25 ± 3.92	4.64 ± 1.95	3.40 ± 1.65^a

All values are expressed as mean ± SD. $^aP < 0.05$, $^bP < 0.01$ *vs* day 0.

Mucosal height

Sections from tissue samples were fixed in 4% paraformaldehyde, dehydrated with alcohol and then paraffin-embedded. The formatted specimens were cut by sliding microtome and stained with hematoxylin and eosin. Samples were analyzed with the automatic image analysis device (HPLAS-1000, Tongji qianping Ltd), using a microscope at 10 ×. The total mucosal height from the base of the crypt to the villous tip was measured (10 measures per preparation, in the 10 highest villi of each sample, and the base of the crypts measurement reached the muscularis mucosae).

Biochemical assays and nitrogen excretion

Serum albumin, prealbumin, transferrin and fibronectin concentrations were determined by automatic biochemical analysis device (Beckman Coulter, USA).

Daily urinary and fecal nitrogen excretion was determined by the Kjeldahl method at day 0, 4 and 7.

Statistical analysis

Data were analyzed using a statistical software package for Windows (SPSS version 10.0, SPSS Inc, Chicago, IL, USA). All variables of each group were described by common statistical methods. Results are presented as mean ± SD. One-way ANOVA for repeated measures was performed in order to evaluate the differences among the three states of the study. The level of significance was set at P value of 0.05 or less.

RESULTS

Villus height and proliferative activity

Compared with the baseline, significant improvement occurred in the intestinal mucosal villus height at day 4 and 7 (both $P < 0.01$), which was accompanied by the increase of proliferative activity of epithelial cells assessed by the PCNA labelling index (both $P < 0.01$) (Table 2, Figure 1).

Nitrogen excretion, body weight and serum levels of protein

Nitrogen excretion was significantly decreased at day 7 (P

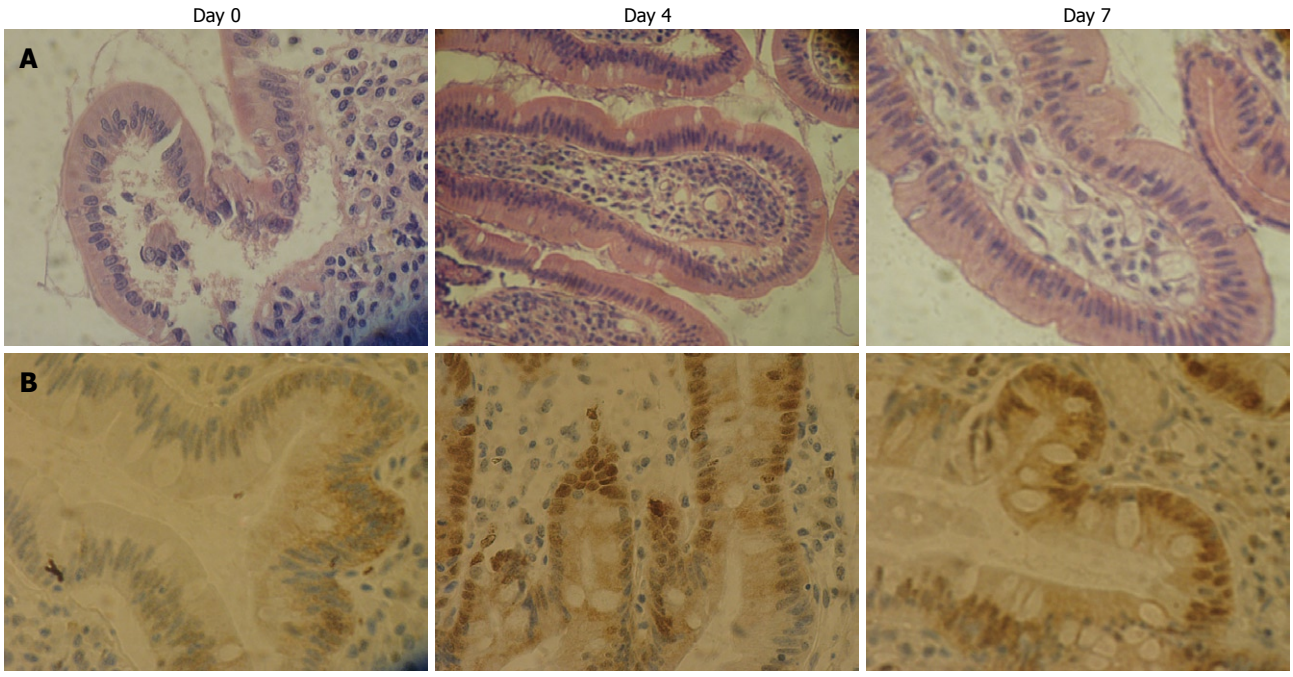

Figure 1 Villus height and proliferative activity. Significant improvements occurred in villus heights and in the expression of PCNA on the intestinal mucosal epithelial cells at day 4 and 7 ($P < 0.01$). A: Villus heights; B: PCNA labelling index.

Table 3 Changes in body weight and serum proteins before and after treatment with rhGH

	The days when treated with rhGH		
	0	4	7
Body weight (kg)	37.39 ± 12.48	38.64 ± 12.84	39.13 ± 12.19
Body mass index (kg/m^2)	14.37 ± 3.28	15.21 ± 3.41	15.40 ± 3.24
Total proteins (g/L)	63.51 ± 2.47	70.52 ± 5.13^b	74.89 ± 5.16^b
Albumin (g/L)	35.74 ± 1.75	39.44 ± 1.18^b	42.39 ± 1.68^b
Prealbumin (mg/L)	180.88 ± 48.28	231.38 ± 52.31	286.38 ± 65.61^a
Transferrin (g/L)	2.41 ± 0.14	2.49 ± 0.12	2.61 ± 0.12^a
Fibronectin (mg/L)	172.5 ± 21.4	236.3 ± 16.5^b	275.8 ± 16.9^b

All values are expressed as mean ± SD. $^aP < 0.05$, $^bP < 0.01$ *vs* day 0.

< 0.05) (Table 2). Serum levels of total proteins, albumin and fibronectin were significantly increased at day 4 and 7 (both $P < 0.01$). The levels of prealbumin and transferrin were increased at day 7 ($P < 0.05$) (Table 3). No change was observed in the body weight.

DISCUSSION

Previous studies have shown that GH stimulates bowel growth[1-4]. Administration of GH improves gut mucosal structure in animals with short bowel syndrome[5-7] and may promote the structural repair of the graft[8,9]. Experiments *in vitro* have also demonstrated that GH is involved in the regulation of crypt cell proliferation in the human small intestine[10-13].

The therapeutic efficacy of rhGH has been suggested by results of animal studies. In the present study the effects of rhGH in situ on the intestine of patients with enterocutaneous fistula were observed. Our results revealed that significant improvement occurred in the intestinal mucosal villus height at day 4 and 7, which was accompanied by the increase of proliferative activity of epithelial cells assessed by the PCNA labelling index.

Nutrient malabsorption often occurs in patients with gastrointestinal fistula[14-17], and it causes body weight loss, barrier damage, followed by bacterial translocation from the gastrointestinal tract to the mesenteric lymph nodes, and even blood. Administration of glutamine and growth hormone synergistically reduces bacterial translocation in sepsis[18,19]. Hormonal therapy with GH can improve weight gain in a rat model of severe short bowel syndrome. This improvement in weight gain was associated with an increase in nutrient transport at the cellular level and variable increases in villus size[20,21]. GH treatment increased [^{14}C] glucose and [^{3}H] palmitic acid plasma concentration after oral nutrient tolerance tests[22]. Clinical trials also showed that GH could promote positive nitrogen balance and protein synthesis[23-25]. However, there are some conflicting results: no improvement was observed in the absorption of total energy, carbohydrate, fat, nitrogen, or wet weight of stool or stool electrolytes compared with baseline and placebo measurements[26-28]. In the present study the body weights of the eight patients were maintained at normal level. All the patients showed positive nitrogen balance and the nitrogen excretion was significantly decreased at day 7. Serum levels of total proteins, albumin and fibronectin were significantly increased at day 4 and 7. And the levels of prealbumin and transferrin were increased at day 7.

GH stimulated the formation and deposition of collagen in both skin incisional wounds and in colonic anastomoses in rats[29,30].

After the trial all the eight patients underwent surgery to close the fistula and they recovered very well, and no fistula recurred.

In summary, our study shows that rhGH can promote intestinal mucosal epithelial cell proliferation and protein synthesis in patients with enterocutaneous fistula.

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COMMENTS

Background

In some medical literature, hormonal therapy with GH has been shown to improve weight gain in a model of severe short bowel syndrome. This improvement in weight gain was associated with an increase in nutrient transport at the cellular level and variable increases in villus size. But there are some conflicting results: no improvement was observed in the absorption of total energy, carbohydrate, fat, nitrogen, or wet weight of stool or stool electrolytes compared with baseline and placebo measurements.

Research frontiers

This study has been carefully designed to investigate whether recombinant human growth hormone (rhGH) could increase the proliferative activity of epithelial cells and nutrient absorption in human. The results showed that rhGH could promote intestinal mucosal epithelial cell proliferation and protein synthesis in humans.

Innovations and breakthroughs

Few studies have made direct observations of the effects of rhGH on intestinal mucosa in humans. In this study, the effects of rhGH on intestinal mucosa proliferation were directly observed. Intestinal mucosal biopsies were performed by endoscopy through enterocutaneous fistula.

Applications

This study suggests that rhGH may reasonably be applied in a clinical setting.

Peer review

Although this is a very interesting study, it is just a preliminary observation. It should be verified in the future. Authors should comment on possible adverse effects of this drug.

REFERENCES

1 **Gu Y**, Wu ZH, Xie JX, Jin DY, Zhuo HC. Effects of growth hormone (rhGH) and glutamine supplemented parenteral nutrition on intestinal adaptation in short bowel rats. *Clin Nutr* 2001; **20**: 159-166
2 **Byrne TA**, Morrissey TB, Nattakom TV, Ziegler TR, Wilmore DW. Growth hormone, glutamine, and a modified diet enhance nutrient absorption in patients with severe short bowel syndrome. *JPEN J Parenter Enteral Nutr* 1995; **19**: 296-302
3 **Byrne TA**, Wilmore DW, Iyer K, Dibaise J, Clancy K, Robinson MK, Chang P, Gertner JM, Lautz D. Growth hormone, glutamine, and an optimal diet reduces parenteral nutrition in patients with short bowel syndrome: a prospective, randomized, placebo-controlled, double-blind clinical trial. *Ann Surg* 2005; **242**: 655-661
4 **Byrne TA**, Persinger RL, Young LS, Ziegler TR, Wilmore DW. A new treatment for patients with short-bowel syndrome. Growth hormone, glutamine, and a modified diet. *Ann Surg* 1995; **222**: 243-254; discussion 254-255
5 **Eizaguirre I**, Aldazabal P, Barrena MJ, Garcia-Arenzana JM, Ariz C, Candelas S, Tovar JA. Effect of growth hormone, epidermal growth factor, and insulin on bacterial translocation in experimental short bowel syndrome. *J Pediatr Surg* 2000; **35**: 692-695
6 **Benhamou PH**, Canarelli JP, Richard S, Cordonnier C, Postel JP, Grenier E, Leke A, Dupont C. Human recombinant growth hormone increases small bowel lengthening after massive small bowel resection in piglets. *J Pediatr Surg* 1997; **32**: 1332-1336
7 **Jeschke MG**, Herndon DN, Finnerty CC, Bolder U, Thompson JC, Mueller U, Wolf SE, Przkora R. The effect of growth hormone on gut mucosal homeostasis and cellular mediators after severe trauma. *J Surg Res* 2005; **127**: 183-189
8 **Zhang X**, Li J, Li N. Growth hormone improves graft mucosal structure and recipient protein metabolism in rat small bowel transplantation. *Chin Med J* (Engl) 2002; **115**: 732-735
9 **Shulman DI**, Hu CS, Duckett G, Lavallee-Grey M. Effects of short-term growth hormone therapy in rats undergoing 75% small intestinal resection. *J Pediatr Gastroenterol Nutr* 1992; **14**: 3-11
10 **Wheeler EE**, Challacombe DN. The trophic action of growth hormone, insulin-like growth factor-I, and insulin on human duodenal mucosa cultured in vitro. *Gut* 1997; **40**: 57-60
11 **Challacombe DN**, Wheeler EE. Trophic action of epidermal growth factor on human duodenal mucosa cultured in vitro. *Gut* 1991; **32**: 991-993
12 **Challacombe DN**, Wheeler EE. The trophic action of human growth hormone on human duodenal mucosa cultured in vitro. *J Pediatr Gastroenterol Nutr* 1995; **21**: 50-53
13 **Chen JY**, Liang DM, Gan P, Zhang Y, Lin J. In vitro effects of recombinant human growth hormone on growth of human gastric cancer cell line BGC823 cells. *World J Gastroenterol* 2004; **10**: 1132-1136
14 **Wang GF**, Ren JA, Jiang J, Fan CG, Wang XB, Li JS. Catheter-related infection in gastrointestinal fistula patients. *World J Gastroenterol* 2004; **10**: 1345-1348
15 **Wang XB**, Ren JA, Li JS. Sequential changes of body composition in patients with enterocutaneous fistula during the 10 days after admission. *World J Gastroenterol* 2002; **8**: 1149-1152
16 **Fan CG**, Ren JA, Wang XB, Li JS. Refeeding syndrome in patients with gastrointestinal fistula. *Nutrition* 2004; **20**: 346-350
17 **Ren JA**, Mao Y, Wang GF, Wang XB, Fan CG, Wang ZM, Li JS. Enteral refeeding syndrome after long-term total parenteral nutrition. *Chin Med J* (Engl) 2006; **119**: 1856-1860
18 **Jung SE**, Youn YK, Lim YS, Song HG, Rhee JE, Suh GJ. Combined administration of glutamine and growth hormone synergistically reduces bacterial translocation in sepsis. *J Korean Med Sci* 2003; **18**: 17-22
19 **Scopa CD**, Koureleas S, Tsamandas AC, Spiliopoulou I, Alexandrides T, Filos KS, Vagianos CE. Beneficial effects of growth hormone and insulin-like growth factor I on intestinal bacterial translocation, endotoxemia, and apoptosis in experimentally jaundiced rats. *J Am Coll Surg* 2000; **190**: 423-431
20 **Sigalet DL**, Martin GR. Hormonal therapy for short bowel syndrome. *J Pediatr Surg* 2000; **35**: 360-363; discussion 364
21 **Zhang W**, Frankel WL, Adamson WT, Roth JA, Mantell MP, Bain A, Ziegler TR, Smith RJ, Rombeau JL. Insulin-like growth factor-I improves mucosal structure and function in transplanted rat small intestine. *Transplantation* 1995; **59**: 755-761
22 **Zhou X**, Li YX, Li N, Li JS. Glutamine enhances the gut-trophic effect of growth hormone in rat after massive small bowel resection. *J Surg Res* 2001; **99**: 47-52
23 **Vara-Thorbeck R**, Guerrero JA, Rosell J, Ruiz-Requena E, Capitan JM. Exogenous growth hormone: effects on the catabolic response to surgically produced acute stress and on postoperative immune function. *World J Surg* 1993; **17**: 530-537; discussion 537-538
24 **Seguy D**, Vahedi K, Kapel N, Souberbielle JC, Messing B. Low-dose growth hormone in adult home parenteral nutrition-dependent short bowel syndrome patients: a positive study. *Gastroenterology* 2003; **124**: 293-302

25 **Byrne TA**, Cox S, Karimbakas M, Veglia LM, Bennett HM, Lautz DB, Robinson MK, Wilmore DW. Bowel rehabilitation: an alternative to long-term parenteral nutrition and intestinal transplantation for some patients with short bowel syndrome. *Transplant Proc* 2002; **34**: 887-890

26 **Szkudlarek J**, Jeppesen PB, Mortensen PB. Effect of high dose growth hormone with glutamine and no change in diet on intestinal absorption in short bowel patients: a randomised, double blind, crossover, placebo controlled study. *Gut* 2000; **47**: 199-205

27 **Vanderhoof JA**, Kollman KA, Griffin S, Adrian TE. Growth hormone and glutamine do not stimulate intestinal adaptation following massive small bowel resection in the rat. *J Pediatr Gastroenterol Nutr* 1997; **25**: 327-331

28 **Park JH**, Vanderhoof JA. Growth hormone did not enhance mucosal hyperplasia after small-bowel resection. *Scand J Gastroenterol* 1996; **31**: 349-354

29 **Jorgensen PH**, Oxlund H. Growth hormone increases the biomechanical strength and collagen deposition rate during the early phase of skin wound healing. *Wound Repair Regen* 1996; **4**: 40-47

30 **Oxlund H**, Christensen H, Seyer-Hansen M, Andreassen TT. Collagen deposition and mechanical strength of colon anastomoses and skin incisional wounds of rats. *J Surg Res* 1996; **66**: 25-30

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CASE REPORT

Johanson-Blizzard syndrome with mild phenotypic features confirmed by *UBR1* gene testing

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Abstract

Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive condition associated with exocrine pancreatic insufficiency, and is characterized by hypoplastic nasal alae, mental retardation, sensorineural hearing loss, short stature, scalp defects, dental abnormalities and abnormal hair patterns. Growth hormone deficiency, hypopituitarism, and impaired glucagon secretion response to insulin-induced hypoglycemia have been reported. Congenital heart defects have also been described in this condition. Mental retardation is typically moderate to severe in patients with JBS; however, normal intelligence can occur. In the pancreas, there is a selective defect of acinar tissue, whereas the islets of Langerhans and ducts are preserved. Diabetes has been reported in older children, suggesting the progressive nature of pancreatic disease. The molecular basis of JBS has recently been mapped to chromosome 15q15-q21 with identified mutations in the *UBR1* gene. We report the case of a 7-year-old female with pancreatic insufficiency and mild phenotypic features, in whom the diagnosis of JBS was established using recently described molecular testing for the *UBR1* gene.

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Key words: Johanson-Blizzard syndrome; Pancreatic insufficiency; Sensorineural hearing loss; *UBR1* gene

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INTRODUCTION

Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive multisystem disorder in which the most characteristic feature is exocrine pancreatic insufficiency. Other common abnormalities include an abnormal facial appearance with a small beak-like nose, dental abnormalities, sensorineural hearing loss, midline scalp defects, hypothyroidism, genitourinary abnormalities, varying degrees of mental retardation, and growth failure[1,2]. In 2005 the disease-associated locus in individuals with this syndrome was mapped to chromosome 15q15-21 with identified mutations in the gene *UBR1* encoding a ubiquitin ligase of the N-end rule pathway[3]. We report the case of a 7-year-old patient recently diagnosed with JBS, confirmed by genetic testing, who has been followed for longstanding pancreatic insufficiency of unknown etiology, but with only mild phenotypic features of JBS, mild sensorineural hearing loss, and who is of normal intelligence.

CASE REPORT

The patient is a 7-year-old girl who was initially evaluated at 18 mo of age for a history of growth failure and increased stool frequency. She was a term infant, birth weight 3230 g, born to non-consanguineous parents. She was initially breast-fed and transitioned to a soy-based formula at 3 mo of age and lactose-free milk at 1 year. She tolerated the introduction of solid foods at 4 mo of age. She had a history of 3-5 large bulky stools per day that contained partially undigested food and were described as being occasionally oily. Her growth was below the

Table 1 Initial evaluation

Complete blood count
Hemoglobin 11.5 g/dL (normal, 10.5-13.5)
WBC 7420/μL (normal, 5.5-15.5)
ANC 1460/μL
Otherwise unremarkable
Complete metabolic panel
Total protein 6.6 g/dL (normal, 6-8.4)
Albumin 4.1 g/dL (normal, 3.5-5)
Alk phos 369 U/L (normal, 80-340)
Otherwise unremarkable
Lipase 8 U/L (normal, 12-70)
Amylase 49 U/L (normal, 0-137)
PT 11.6 s
Vitamin A and D levels were normal
Vitamin E 0.1 mg/dL (normal, 0.5-2)
Celiac antibody testing negative
Serum trypsinogen < 1.2 ng/mL (normal, 10-57)
TSH and free T4 were normal
Stool ova and parasites negative
Seventy-two fecal fat
Fecal fat quant 10.6 g/24 h (normal, < 7)
Coefficient of fat absorption 83%
Stool chymotrypsin < 3 U/10 g (normal, > 9)

Sweat test: 45 mmol/L (normal, 8-45); CFTR gene mutation analysis: No known deleterious mutations; Shwachman-Diamond gene analysis: Negative.

3rd percentile for weight and height. She was otherwise healthy, with the exception of having five ear infections between the ages of 6 and 18 mo, which ultimately required pressure-equalizing tube placement. Her developmental history was normal, other than starting to walk independently at 18 mo of age. Family history was negative for cystic fibrosis, celiac disease, chronic diarrhea and growth failure. Her physical examination was remarkable for a weight of 9.3 kg (7% ile) and a height of 77.6 cm (< 5% ile). Her initial laboratory evaluation is shown in Table 1. She had evidence of significant fat malabsorption; however, testing was negative for celiac disease, cystic fibrosis, and Shwachman-Diamond syndrome. Based on the presumed diagnosis of pancreatic insufficiency, she was started on pancreatic enzyme replacement and fat soluble vitamin supplementation. She gained weight and grew along the 5%-10% ile for height and weight on this regimen. She was otherwise healthy and did not have recurrent or frequent infections. Her development was normal.

At 5 years of age, she failed her routine kindergarten hearing screen. Her parents had previously not noted any problem with her hearing or speech. An audiology evaluation was abnormal demonstrating a mild-to-moderate bilateral asymmetric sensorineural hearing loss, greater on the left than the right. She was referred for genetic evaluation given the known association between JBS and sensorineural hearing loss with her history of pancreatic insufficiency. At that time, mild phenotypic features of JBS were identified, including an abnormal hair pattern, hypoplasia of the nasal alae, small teeth and a narrow upper lip (Figure 1). A computed tomography (CT) scan of her abdomen demonstrated complete fatty replacement of the pancreas with no visualized

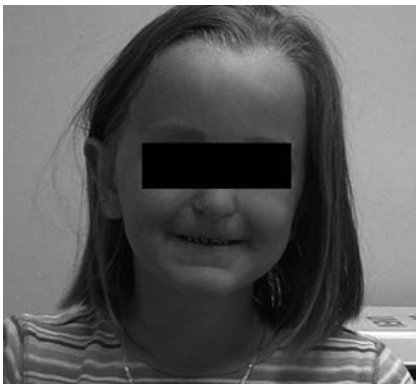

Figure 1 Phenotypic features of JBS in our patient: abnormal hair pattern, nasal alae hypoplasia, small teeth and narrow upper lip (with permission from parents).

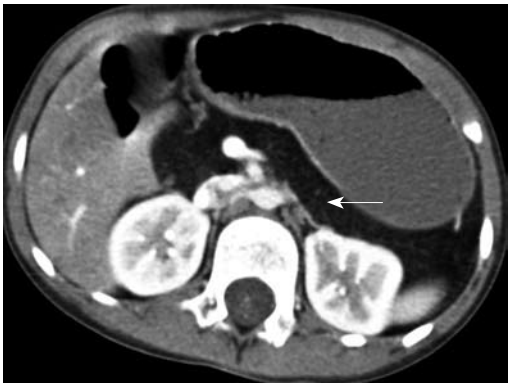

Figure 2 CT scan of the abdomen demonstrating fatty replacement of the pancreas (arrow).

gland residing in the pancreatic bed (Figure 2). Renal ultrasound to evaluate genitourinary abnormalities was negative.

Research testing for mutations in *UBR1* revealed two novel mutations that molecularly confirmed the diagnosis of JBS, IVS1+5G>C (c.81+G>C) a splice site mutation (paternally inherited, father had a mosaic mutation present in only a subset of cells) and exon 17, c.1979_1981delTTG (p.V660del, which is a deletion of a highly conserved valine that was maternally inherited (Figure 3). While abnormal splicing at the splice donor site of exon 1 is predicted to lead to no expression of a functional protein (functional null allele), the maternally inherited deletion may represent a hypomorphic mutation, conferring partial residual function. Indeed, analysis of mRNA from lymphoblastoid cells from the patient by RT-PCR and sequencing indicated that mRNA from the allele with the splice site mutation underwent early degradation (Figure 3). However, we cannot exclude the possibility that some production of a functional UBR1 protein may also result from a low level of normal splicing despite the splice donor mutation at position +5. Our patient has continued to do well on pancreatic enzyme replacement and is being followed by otolaryngology for her hearing loss. She was referred to dental services for her tooth abnormalities.

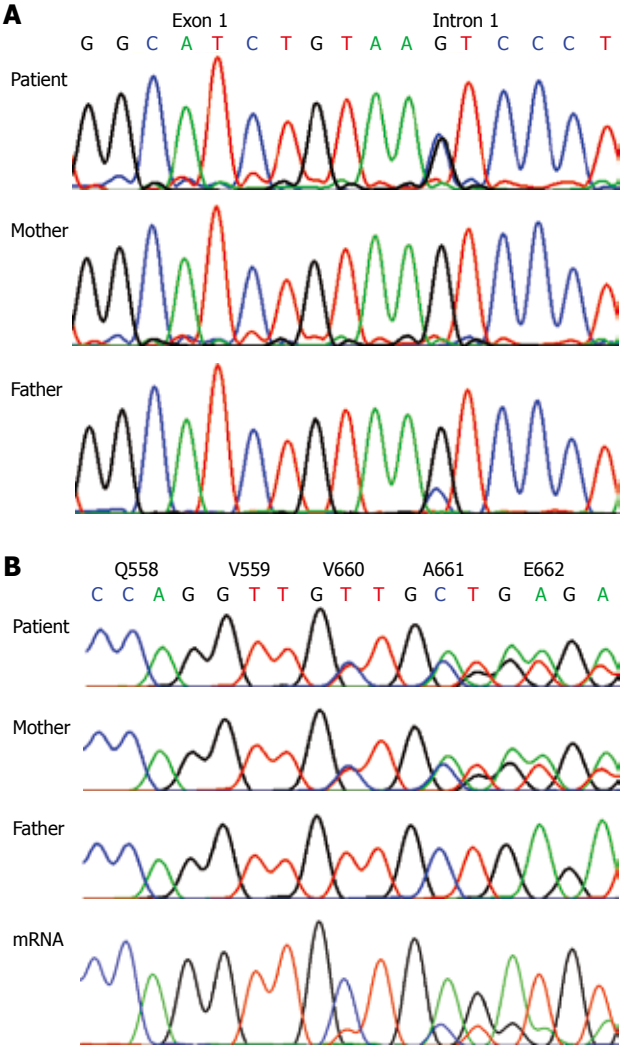

Figure 3 Diagram of the *UBR1* gene mutations in our patient and her parents. A: Exon 1-intron 1 transition showing a heterozygous nucleotide exchange at position +5 in the patient, IVS1+5G>C (c.81+G>C). Note the small peak in the father, indicating that he is mosaic for this mutation. B: Section of exon 17 showing a heterozygous 3 bp deletion in the patient and her mother, c.1978_1981delTTG (p.V660del), predicting the deletion of a highly conserved valine. In mRNA from lymphoblastoid cells from the patient the deletion is the predominant allele, indicating that mRNA from the allele with the splice site mutation is largely degradated.

DISCUSSION

JBS is a rare autosomal recessive multisystem disorder. The most prominent feature of this syndrome is exocrine pancreatic insufficiency. Other abnormalities include a characteristic facial appearance with a small beak-like nose (secondary to aplasia or hypoplasia of the nasal alae), long and narrow upper lip, small pointed chin, abnormalities of both deciduous and permanent teeth, sparse coarse hair/midline scalp defects, short stature in > 80%, hypothyroidism in 40%, sensorineural hearing loss in > 80%, mental retardation in 77%, imperforate anus in 39%, and genitourinary abnormalities in 38%[4]. Growth hormone deficiency, hypopituitarism, and impaired glucagon secretion response to insulin-induced hypoglycemia have been reported[5-7]. Congenital heart defects including atrial septal defect, ventricular septal defect, and dextrocardia with transposition of the great vessels have also been described in this condition[8]. Mental retardation is typically moderate to severe in patients with JBS, however, normal intelligence can occur[9]. Growth failure in patients with JBS typically begins in the intrauterine period and continues throughout childhood. Pancreatic hypoplasia with resultant exocrine insufficiency and malabsorption is thought to be responsible. In the pancreas of patients with this condition there is a selective defect of acinar tissue, whereas the islets of Langerhans and ducts are preserved[10,11]. This results in an almost complete absence of zymogens from duodenal juice, whereas bicarbonate secretion is much less impaired[10]. Diabetes has been reported in older children, suggesting the progressive nature of pancreatic disease[12,13].

The molecular basis of JBS has recently been mapped to chromosome 15q15-q21 with identified mutations in the *UBR1* gene[3,4]. *UBR1* expression is highest in predominantly skeletal muscle and pancreatic acinar cells. *UBR1* encodes one of several E3 ubiquitin ligases of the N-end rule pathway, an ubiquitin-dependent proteolytic pathway. Ubiquitylation and subsequent degradation of proteins at the proteasome is the universal mechanism for regulated protein degradation and the control of many intracellular protein levels[14-16]. *UBR1* is considered to play a critical role in the development and maintenance of acinar cells. In patients with JBS, destruction of acinar tissue which may begin *in utero* results in the development of exocrine pancreatic insufficiency and fatty replacement of the pancreas. Since the initial description of JBS in 1971, more than 60 cases have been reported[4]. The majority of these reports include children with significant pancreatic insufficiency, markedly abnormal facial features and moderate to severe mental retardation.

Our patient presented with pancreatic insufficiency and initially unrecognized mild phenotypic features of JBS. This diagnosis was only suspected when she failed a routine screening hearing test, without prior suspicion of hearing loss. In contrast to previous findings of biallelic *UBR1* mutations predicting complete loss of function in the majority of patients with JBS[3], in our patient, the maternally inherited deletion is thought to be a hypomorphic mutation conferring partial residual function and explaining the more subtle phenotype. This is the first evidence for genotype-phenotype correlation in JBS. The purpose of this report is to highlight the broader spectrum of this syndrome which may have been previously unrecognized prior to the availability of specialized genetic testing. Once the diagnosis of JBS is established, patients with this condition need to be screened for renal anomalies, referred for dental evaluation, monitored for the development of hypothyroidism and diabetes and provided with appropriate genetic counseling.

JBS is a rare cause of pancreatic insufficiency, usually associated with typical phenotypic features. The genetic basis for this syndrome has been recently identified, and is related to UBR1 deficiency which leads to perturbation of pancreatic acinar cells as well as other

organs. Gastroenterologists should be aware of the availability of genetic testing for JBS. Recognition of more subtle presentations of this syndrome may help to identify other patients with this autosomal recessive condition, previously thought to have idiopathic pancreatic insufficiency.

REFERENCES

1 **Johanson A**, Blizzard R. A syndrome of congenital aplasia of the alae nasi, deafness, hypothyroidism, dwarfism, absent permanent teeth, and malabsorption. *J Pediatr* 1971; **79**: 982-987

2 **Hurst JA**, Baraitser M. Johanson-Blizzard syndrome. *J Med Genet* 1989; **26**: 45-48

3 **Zenker M**, Mayerle J, Lerch MM, Tagariello A, Zerres K, Durie PR, Beier M, Hulskamp G, Guzman C, Rehder H, Beemer FA, Hamel B, Vanlieferinghen P, Gershoni-Baruch R, Vieira MW, Dumic M, Auslender R, Gil-da-Silva-Lopes VL, Steinlicht S, Rauh M, Shalev SA, Thiel C, Ekici AB, Winterpacht A, Kwon YT, Varshavsky A, Reis A. Deficiency of UBR1, a ubiquitin ligase of the N-end rule pathway, causes pancreatic dysfunction, malformations and mental retardation (Johanson-Blizzard syndrome). *Nat Genet* 2005; **37**: 1345-1350

4 **Zenker M**, Mayerle J, Reis A, Lerch MM. Genetic basis and pancreatic biology of Johanson-Blizzard syndrome. *Endocrinol Metab Clin North Am* 2006; **35**: 243-253, vii-viii

5 **Sandhu BK**, Brueton MJ. Concurrent pancreatic and growth hormone insufficiency in Johanson-Blizzard syndrome. *J Pediatr Gastroenterol Nutr* 1989; **9**: 535-538

6 **Kristjansson K**, Hoffman WH, Flannery DB, Cohen MJ. Johanson-Blizzard syndrome and hypopituitarism. *J Pediatr* 1988; **113**: 851-853

7 **Takahashi T**, Fujishima M, Tsuchida S, Enoki M, Takada G. Johanson-blizzard syndrome: loss of glucagon secretion response to insulin-induced hypoglycemia. *J Pediatr Endocrinol Metab* 2004; **17**: 1141-1144

8 **Alpay F**, Gul D, Lenk MK, Ogur G. Severe intrauterine growth retardation, aged facial appearance, and congenital heart disease in a newborn with Johanson-Blizzard syndrome. *Pediatr Cardiol* 2000; **21**: 389-390

9 **Moeschler JB**, Lubinsky MS. Johanson-Blizzard syndrome with normal intelligence. *Am J Med Genet* 1985; **22**: 69-73

10 **Jones NL**, Hofley PM, Durie PR. Pathophysiology of the pancreatic defect in Johanson-Blizzard syndrome: a disorder of acinar development. *J Pediatr* 1994; **125**: 406-408

11 **Daentl DL**, Frias JL, Gilbert EF, Opitz JM. The Johanson-Blizzard syndrome: case report and autopsy findings. *Am J Med Genet* 1979; **3**: 129-135

12 **Steinbach WJ**, Hintz RL. Diabetes mellitus and profound insulin resistance in Johanson-Blizzard syndrome. *J Pediatr Endocrinol Metab* 2000; **13**: 1633-1636

13 **Trellis DR**, Clouse RE. Johanson-Blizzard syndrome. Progression of pancreatic involvement in adulthood. *Dig Dis Sci* 1991; **36**: 365-369

14 **Ciechanover A**, Schwartz AL. The ubiquitin system: pathogenesis of human diseases and drug targeting. *Biochim Biophys Acta* 2004; **1695**: 3-17

15 **Pickart CM**. Back to the future with ubiquitin. *Cell* 2004; **116**: 181-190

16 **Hershko A**, Ciechanover A, Varshavsky A. Basic Medical Research Award. The ubiquitin system. *Nat Med* 2000; **6**: 1073-1081

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CASE REPORT

Endoscopic Ultrasound-guided drainage of an abdominal fluid collection following Whipple's resection

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Author contributions: Jah A collected the data on the patient; Jah A and Praseedom R wrote the manuscript; Jamieson N and Huguet E critically reviewed the manuscript; Griffiths W and Carroll N performed the endoscopic ultrasound guided drainage.
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Abstract

Percutaneous aspiration and drainage of post-operative abdominal fluid collections is a well established standard technique. However, some fluid collections are not amenable to percutaneous drainage either due to location or the presence of surrounding visceral structures. Endoscopic Ultrasound (EUS) has been widely used for the drainage of pancreatitis-related abdominal fluid collections. However, there are no reports on the use of this technique in the post-operative setting. We report a case where the EUS-guided technique was used to drain a percutaneously inaccessible post-operative collection which had developed after Whipple's resection.

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Key words: Endoscopic ultrasound; Pancreatectomy; Whipple's resection

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INTRODUCTION

Drainage of post-operative abdominal fluid collections is generally carried out percutaneously under radiological guidance. However, some of these collections may be inaccessible to percutaneous drainage because of their location and surrounding vital structures.

We report a case where a post-operative fluid collection which developed after a Whipple's resection was successfully drained endoscopically under endoscopic ultrasound (EUS) guidance. Although widely used in pancreatitis, this is the first report of the use of this technique in a post-operative setting.

CASE REPORT

A 45 year old lady underwent Whipple's resection for cholangiocarcinoma of the distal common bile duct. Pancreatico-jejunostomy and hepatico-jejunostomy were performed on to a 70 cm Roux-en-Y jejunal loop and a gastrojejunostomy was fashioned using the end of the main jejunal segment continuous with the rest of the intestine.

Her initial recovery was uneventful but on the 9th post-operative day she developed pyrexia and vomiting suggestive of gastric outlet obstruction. A contrast-enhanced computerized tomogram (CT) showed a deep-seated fluid collection measuring 5.1 × 3.3 cm which was compressing and stretching the efferent jejunal loop distal to the gastrojejunostomy (Figure 1). Percutaneous drainage was deemed unsafe due to the surrounding bowel loops and blood vessels. However, its proximity to the gastrojejunostomy made it accessible endoscopically.

An EUS-endoscope (GF-UCT240, Olympus, UK) was passed through the gastrojejunostomy and the sero-sanguinous collection was aspirated to dryness under EUS guidance using a 19-gauge Echotip-Ultra needle (Wilson-Cook, Ireland) (Figure 2). The amylase level in the aspirate was normal and all the cultures were sterile. Following aspiration of the fluid collection the

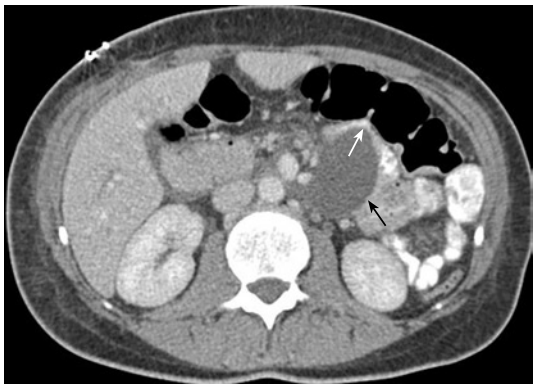

Figure 1 Contrast-enhanced CT showing the fluid collection (black arrow) compressing the efferent jejunal loop (white arrow) just distal to the gastrojejunostomy.

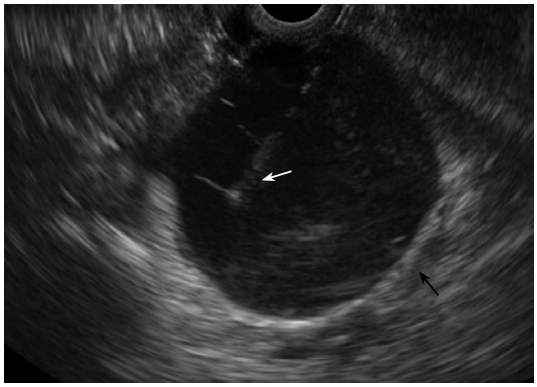

Figure 2 EUS showing the fluid collection (black arrow) with the needle in position for aspiration (white arrow).

vomiting and pyrexia settled, and the patient was able to recommence oral intake. There were no complications related to this procedure and she was discharged from hospital after three days. She was asymptomatic at her routine surgical outpatient review 6 wk later.

DISCUSSION

Abdominal fluid collections are common after Whipple's resection and are frequently situated in the pancreatic bed. They may be related to an anastomotic leak (generally pancreatic) and often cause mechanical and/or infective complications. These collections are generally deep-seated and are surrounded by major blood vessels. This combined with altered post-surgical anatomy (presence of Roux loops *etc*) often makes a direct percutaneous route for drainage impossible to find. In this patient, it was important to exclude a pancreatic leak and to resolve the gastric outlet obstruction. In such cases, a laparotomy would normally be necessary in the absence of a safe percutaneous option.

In contrast, endoscopic transmural drainage or aspiration with EUS guidance has greatly reduced the need for surgery in pancreatitis-related fluid collections and has now become a standard procedure for this condition in our unit. The safety of this technique and high success rates have been widely reported[1-3]. This experience prompted us to use the EUS-guided technique as an alternative route for drainage of this post-operative fluid collection where percutaneous approach was deemed unsafe.

In a patient with a visceral fluid collection, the feasibility of EUS-guided drainage should first be assessed with dual contrast-enhanced CT. Close proximity to the stomach or jejunum provides an easy endoscopic access to these collections. The technique of EUS-guided aspiration is well established[1-3].

We anticipate that the main role of EUS-guided aspiration of collections in the post-operative situation would be to allow much-needed sampling of fluid for microbiological and biochemical analysis. In patients with sepsis or mechanical complications such as obstruction secondary to collections, this technique would also be therapeutic. In stable patients this could eliminate the need for a laparotomy which would otherwise carry a much higher risk of morbidity and mortality. Thus, we conclude that EUS-guided aspiration/drainage should be considered in patients with otherwise inaccessible post-operative fluid collections.

REFERENCES

1 **Baron TH**. Endoscopic drainage of pancreatic fluid collections and pancreatic necrosis. *Gastrointest Endosc Clin N Am* 2003; **13**: 743-764

2 **Hookey LC**, Debroux S, Delhaye M, Arvanitakis M, Le Moine O, Devière J. Endoscopic drainage of pancreatic-fluid collections in 116 patients: a comparison of etiologies, drainage techniques, and outcomes. *Gastrointest Endosc* 2006; **63**: 635-643

3 **Lopes CV**, Pesenti C, Bories E, Caillol F, Giovannini M. Endoscopic-ultrasound-guided endoscopic transmural drainage of pancreatic pseudocysts and abscesses. *Scand J Gastroenterol* 2007; **42**: 524-529

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CASE REPORT

Complete hepatocellular carcinoma necrosis following sequential porto-arterial embolization

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Abstract

Most patients with hepatocellular carcinoma (HCC) are not eligible for curative treatment, which is resection or transplantation. Two recent series have emphasized the potential benefits of preoperative arterio-portal embolization prior to surgical resection of such tumours. This preoperative strategy offers a better disease free survival rate and a higher rate of total tumor necrosis. In case of non resectable HCC it is now widely accepted that transarterial chemoembolization (TACE) leads to a better survival when compared to conservative treatment. Thus, the question remains whether combined portal vein embolization (PVE) may enhance the proven efficiency of TACE in patients with unresectable HCC. We herein report the case of a 56-year-old cirrhotic woman with a voluminous HCC unsuitable for surgical resection. Yet, complete tumour necrosis and prolonged survival could be achieved after a combined porto-arterial embolization. This case emphasizes the potential synergistic effect of a combined arterio-portal embolization and the hypothetical survival benefit of such a procedure, in selected patients, with HCC not suitable for surgery or local ablative therapy.

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INTRODUCTION

Despite a close observation of patients with liver cirrhosis, hepatocellular carcinoma (HCC) is often diagnosed at an advanced stage where no optimal treatment has been established[1-3] only few patients (20%-25%) will benefit of resection or liver transplantation[4], only chance to improve life expectancy. Actually, 80% to 90%[5] of HCC develop in cirrhotic patients with impaired liver function, limiting the possibility of safe major liver resections. If liver resection has shown a survival benefit, in case of cirrhosis, it's a risky procedure with a high prevalence of postoperative liver failure and should not be performed if the future liver remnant (FLR) is estimated to be less than 40%[6]. In those patients who are not suitable for surgery, treatment is palliative and survival is poor and correlated to TNM stage (TNM classification of primary liver cancer by the International Hepato-Pancreato-Biliary Association[7]) and liver damage severity[8]. Despite previous inconclusive randomized controlled trials comparing transarterial chemoembolization (TACE) to conservative treatment in unresectable HCC[9-11], Liovet *et al*[12] ultimately demonstrated that TACE led to an increased survival in selected patients with preserved liver function. TACE is now widely accepted as the procedure of choice in selected patients who are not eligible for

resection or local ablative therapy. However, the question remains whether combined portal embolization may enhance the proven efficiency of TACE in patients with unresectable hepatocellular carcinoma (HCC).

We herein report the case of a cirrhotic patient with advanced HCC in whom complete tumour necrosis and prolonged survival were observed following a combined porto-arterial embolization.

CASE REPORT

A 56-year-old woman with alcoholic cirrhosis, Child-Pugh A6 presented with an 80 mm HCC stage Ⅲ. The tumor developed in the right hepatic lobe, impinging on the median hepatic vein and in contact with the right glissonian pedicle (Figure 1A). There were two satellite nodules but the left hepatic lobe was free of tumor. The right portal vein was patent. The patient had stopped alcohol intake for 3 mo before admission. There was no past history of encephalopathy, ascites or upper gastro-intestinal bleeding despite stage I oesophageal varices. The clinical examination was normal. Liver biochemistry showed: A 74% prothrombin time, normal albumin level, bilirubin 50 μmol/L (N < 17), ASAT/ALAT: 59/66 UI/L (N < 40), gamma glutamyl transferase: 204 UI/L (N < 140), alkaline phosphatase: 105 UI/L (N < 80), platelet count was 126 000/mm^3. Alpha-foeto protein level was 108 μg/L (N < 5). The diagnosis of established alcoholic cirrhosis was confirmed by a percutaneous liver biopsy. As the surgical strategy was a right hepatectomy removing the median hepatic vein and the patient underwent a right portal vein embolization (PVE) prior to surgery (Figure 2A and B). After 4 wk the left lobe had gained 40%. During surgery, intra-abdominal exploration revealed moderate to severe portal hypertension with an enlarged spleen, mild ascites and dilated splanchnic veins. The liver appeared cirrhotic with regeneration nodules. Intraoperative ultrasound confirmed an 8 cm HCC, mainly involving segment V and Ⅷ, invading the median hepatic vein and close to the right glissonian pedicle. Peroperative observation precluded liver resection and separate biopsy of both tumor and liver parenchyma was done before abdominal closure. In view of previous right PVE, intra-arterial chemoembolization was thought to be unsafe and a supportive medical care was decided. During follow-up, a 20 mm intra-tumoral aneurysm of right arterial branch was diagnosed on computer tomography (CT) scan (Figure 1B), most probably related to an arterial trauma during intraoperative tumor biopsy. A supra-selective arterial embolization with coils was then undertaken with complete obliteration of the arterial aneurysm on control angiography (Figure 3A and B). A control CT scan performed 3 mo later showed complete necrosis of the tumor (Figure 1C) as suggested by return of alpha-foeto protein to normal value (6.8 μg/L). Disease-free survival lasted for two years. Multiple intra-hepatic and bone recurrence was diagnosed on progressive increase of alpha foeto-protein level. The patient ultimately died after a follow-up of three years.

DISCUSSION

In up to 90%, HCC develop in cirrhotic patients whose impaired liver function precludes major liver resections if FLR is less than 40%[6]. First reported by Makuuchi *et al*[13], PVE can be performed safely[14] in order to induce a homolateral liver parenchyma atrophy and a hypertrophy of the FLR, allowing resection in patients with large tumors or abnormal liver function[15]. Yet, this technique was initially described for patients with Klatskin tumors[13] and its' application to cirrhotic patients with HCC is debated by some authors who rather recommend a preoperative combined arterio-portal embolization[16]. HCC being hypervascular tumours mainly fed by an arterial blood flow, cessation of the portal's flow leads to a compensatory increased flow in the corresponding arterial territory[17] that may cause the tumor progress.

Recently, Ogata *et al* [18] have reported in a controlled trial the feasibility and efficacy of a sequential arterio-portal embolization, TACE followed by PVE after a 3 wk delay, before major liver resection in cirrhotic patients with HCC. When compared to PVE alone, this procedure offers a significantly higher rate of complete tumor necrosis (83% *vs* 5%, P < 0.001), a higher 5-year disease-free survival rate (37% *vs* 19%, P = 0.041) with a similar rate of morbidity. In this report the authors confirmed that complete tumor necrosis can be obtained by its complete blood flow privation (arterial and portal) and highlight the potential benefit of this sequence in term of prognosis. Moreover, in their report, the Beaujon's group[18] suggests that sequential embolization could effectively be an appropriate treatment itself in patients in whom surgery is precluded due to a poor degree of liver hypertrophy.

In case of unresectable HCC, efficacy of TACE is now admitted[19,20] with a benefit in survival when compared to conservative treatment. Yet, TACE alone leads to around 50% of complete tumour necrosis[21-23] whereas this rate is over 80% after sequential arterio-portal embolization and this may have an impact on survival curves[16,18]. Aoki *et al*[16] obtained a necrosis rate superior to 70% in 12/17 (71%) whereas Yamakada *et al*[24] observed a complete tumoral necrosis in 7/9 (78%) of the resected specimen after a sequential arterio-portal procedure with 1, 3 and 5 years survival rates of 87%, 72% and 51%, respectively.

Even though portal vein thrombosis, which is a frequent complication of HCC, is considered to be an absolute contraindication to TACE, due to increased risk of post procedure liver failure or infarction, efficacy and safety of TACE in such cases have been reported[25] in selected patients. Taken together, these observations suggest a good tolerance of liver parenchyma to ischemia when interval between portal and arterial occlusion is delayed[18]. Ogata *et al*[18] who have suggested a minimum of 3 wk between both procedures had lesser morbidity and aminotransferase levels as compared to Aoki *et al*[16] who performed both embolizations within a period of 7 d.

In the case herein reported, although our patient

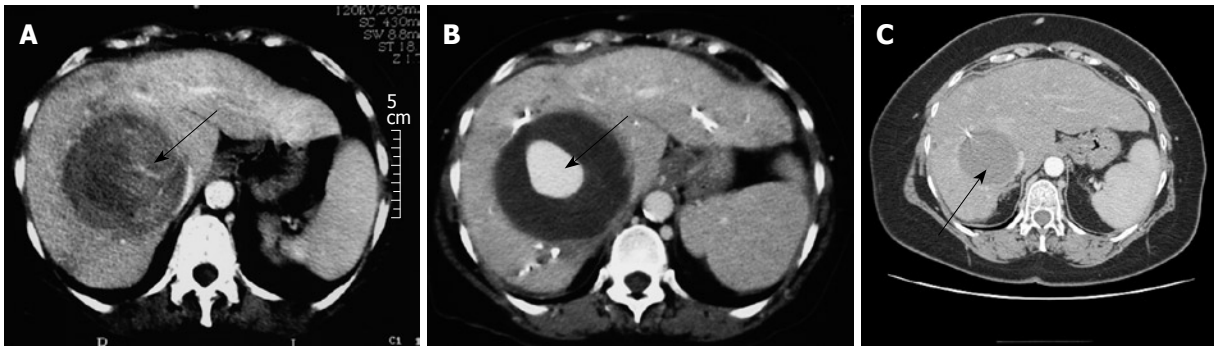

Figure 1 Enhanced CT scan. A: Showing an eighty millimetre HCC (black arrow) developed in the right hepatic lobe, driving back the median hepatic vein, in contact with the right glissonian pedicle; B: Showing an arterial aneurysm (black arrow) due to the main tumour artery traumatism during biopsy; C: Showing complete tumour necrosis after combined portoarterial embolization (black arrow).

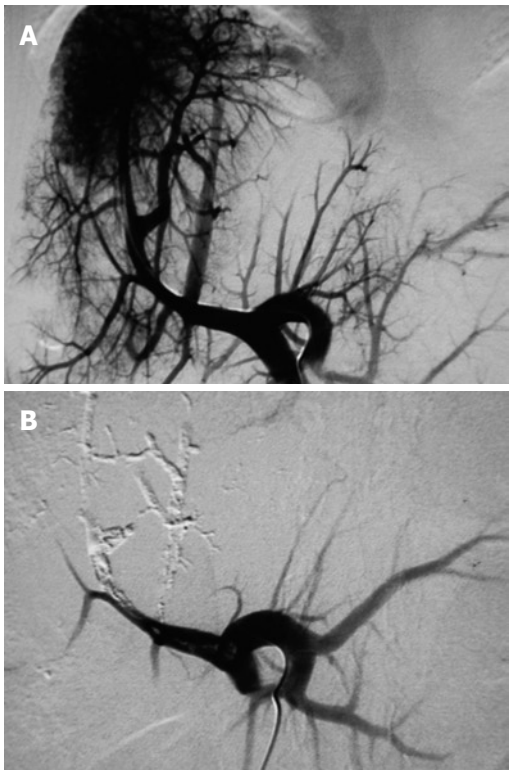

Figure 2 Portography. A: Prior to embolization; B: After embolization.

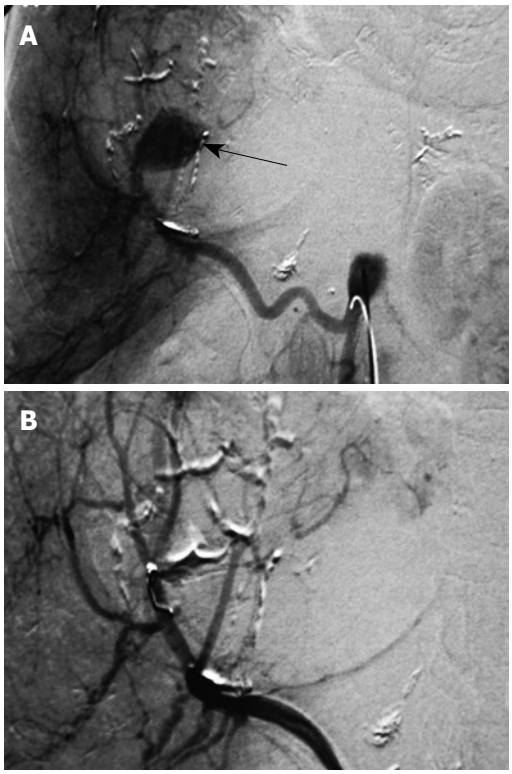

Figure 3 Arteriography. A: The arterial aneurysm developed on the HCC main feeding artery (black arrow), prior to embolization with coils; B: After complete arterial occlusion with coils.

was planned to have a major liver resection we did not perform a preoperative TACE before PVE, which is now systematic in our department. In this particular case, arterial embolization was performed after portal embolization and was indicated to treat a traumatic arterial aneurysm following a peroperative fine needle biopsy. Yet, it was well tolerated, had no consequence on liver function and led to a complete tumor necrosis with a prolonged survival. We agree that a porto-arterial sequence is unusual and do not recommend it that way, but this case fully illustrates the synergy of a combined embolization in term of tumor necrosis. We rather recommend a sequential arterio-portal sequence combining TACE and PVE as previously described[18], with a 3 wk delay between both procedures. In this view, we assume that tolerance is related to the sequence and its timing whereas efficacy is related to both, arterial and portal, HCC vascular exclusion.

Our report aims at giving further support to the combination of TACE and portal embolization in the treatment of voluminous HCC that cannot be treated by surgery or alternative therapy such as radiofrequency as previously hypothesized by others[18]. We assume that cirrhotic liver parenchyma has a relatively good tolerance to arterial and portal ischemia when the interval between both vascular occlusions is delayed (at least 3 wk). Proven efficacy of TACE might be enhanced by a combined sequential PVE. Patients With large HCC, not suitable for surgery or local ablative therapy, could effectively be treated with combined arterio-portal embolization with a limited morbidity and a likely benefit in survival.

REFERENCES

1 **Bruix J**, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. *Hepatology* 2002; **35**: 519-524
2 **Trinchet JC**, Beaugrand M. Treatment of hepatocellular carcinoma in patients with cirrhosis. *J Hepatol* 1997; **27**: 756-765
3 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodes J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430
4 **Lai EC**, Fan ST, Lo CM, Chu KM, Liu CL, Wong J. Hepatic resection for hepatocellular carcinoma. An audit of 343 patients. *Ann Surg* 1995; **221**: 291-298
5 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917
6 **Fan ST**. Methods and related drawbacks in the estimation of surgical risks in cirrhotic patients undergoing hepatectomy. *Hepatogastroenterology* 2002; **49**: 17-20
7 **Makuuchi M**, Belghiti J, Belli G, Fan ST, Lau JW, Ringe B, Strasberg SM, Vauthey JN, Yamaoka Y, Yamasaki S. IHPBA concordant classification of primary liver cancer: working group report. *J Hepatobiliary Pancreat Surg* 2003; **10**: 26-30
8 **Takayasu K**, Arii S, Ikai I, Omata M, Okita K, Ichida T, Matsuyama Y, Nakanuma Y, Kojiro M, Makuuchi M, Yamaoka Y. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. *Gastroenterology* 2006; **131**: 461-469
9 A comparison of lipiodol chemoembolization and conservative treatment for unresectable hepatocellular carcinoma. Groupe d'Etude et de Traitement du Carcinome Hepatocellulaire**.** *N Engl J Med* 1995; **332**: 1256-1261
10 **Bruix J**, Llovet JM, Castells A, Montana X, Bru C, Ayuso MC, Vilana R, Rodes J. Transarterial embolization versus symptomatic treatment in patients with advanced hepatocellular carcinoma: results of a randomized, controlled trial in a single institution. *Hepatology* 1998; **27**: 1578-1583
11 **Pelletier G**, Ducreux M, Gay F, Luboinski M, Hagege H, Dao T, Van Steenbergen W, Buffet C, Rougier P, Adler M, Pignon JP, Roche A. Treatment of unresectable hepatocellular carcinoma with lipiodol chemoembolization: a multicenter randomized trial. Groupe CHC. *J Hepatol* 1998; **29**: 129-134
12 **Llovet JM**, Real MI, Montana X, Planas R, Coll S, Aponte J, Ayuso C, Sala M, Muchart J, Sola R, Rodes J, Bruix J. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002; **359**: 1734-1739
13 **Makuuchi M**, Thai BL, Takayasu K, Takayama T, Kosuge T, Gunven P, Yamazaki S, Hasegawa H, Ozaki H. Preoperative portal embolization to increase safety of major hepatectomy for hilar bile duct carcinoma: a preliminary report. *Surgery* 1990; **107**: 521-527
14 **Di Stefano DR**, de Baere T, Denys A, Hakime A, Gorin G, Gillet M, Saric J, Trillaud H, Petit P, Bartoli JM, Elias D, Delpero JR. Preoperative percutaneous portal vein embolization: evaluation of adverse events in 188 patients. *Radiology* 2005; **234**: 625-630
15 **Kubota K**, Makuuchi M, Kusaka K, Kobayashi T, Miki K, Hasegawa K, Harihara Y, Takayama T. Measurement of liver volume and hepatic functional reserve as a guide to decision-making in resectional surgery for hepatic tumors. *Hepatology* 1997; **26**: 1176-1181
16 **Aoki T**, Imamura H, Hasegawa K, Matsukura A, Sano K, Sugawara Y, Kokudo N, Makuuchi M. Sequential preoperative arterial and portal venous embolizations in patients with hepatocellular carcinoma. *Arch Surg* 2004; **139**: 766-774
17 **Nagino M**, Nimura Y, Kamiya J, Kanai M, Hayakawa N, Yamamoto H. Immediate increase in arterial blood flow in embolized hepatic segments after portal vein embolization: CT demonstration. *AJR Am J Roentgenol* 1998; **171**: 1037-1039
18 **Ogata S**, Belghiti J, Farges O, Varma D, Sibert A, Vilgrain V. Sequential arterial and portal vein embolizations before right hepatectomy in patients with cirrhosis and hepatocellular carcinoma. *Br J Surg* 2006; **93**: 1091-1098
19 **Llovet JM**, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 2003; **37**: 429-442
20 **Camma C**, Schepis F, Orlando A, Albanese M, Shahied L, Trevisani F, Andreone P, CraxiA, Cottone M. Transarterial chemoembolization for unresectable hepatocellular carcinoma: meta-analysis of randomized controlled trials. *Radiology* 2002; **224**: 47-54
21 **Zhang Z**, Liu Q, He J, Yang J, Yang G, Wu M. The effect of preoperative transcatheter hepatic arterial chemoembolization on disease-free survival after hepatectomy for hepatocellular carcinoma. *Cancer* 2000; **89**: 2606-2612
22 **Harada T**, Matsuo K, Inoue T, Tamesue S, Inoue T, Nakamura H. Is preoperative hepatic arterial chemoembolization safe and effective for hepatocellular carcinoma? *Ann Surg* 1996; **224**: 4-9
23 **Adachi E**, Matsumata T, Nishizaki T, Hashimoto H, Tsuneyoshi M, Sugimachi K. Effects of preoperative transcatheter hepatic arterial chemoembolization for hepatocellular carcinoma. The relationship between postoperative course and tumor necrosis. *Cancer* 1993; **72**: 3593-3598
24 **Yamakado K**, Nakatsuka A, Tanaka N, Matsumura K, Takase K, Takeda K. Long-term follow-up arterial chemoembolization combined with transportal ethanol injection used to treat hepatocellular carcinoma. *J Vasc Interv Radiol* 1999; **10**: 641-647
25 **Georgiades CS**, Hong K, D'Angelo M, Geschwind JF. Safety and efficacy of transarterial chemoembolization in patients with unresectable hepatocellular carcinoma and portal vein thrombosis. *J Vasc Interv Radiol* 2005; **16**: 1653-1659

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CASE REPORT

Cystic lymphangioma of the pancreas

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Abstract

Lymphangioma of the pancreas is an extremely rare benign tumour of lymphatic origin, with fewer than 60 published cases. Histologically, it is polycystic, with the cysts separated by thin septa and lined with endothelial cells. Though congenital, it can affect all age groups, and occurs more frequently in females. Patients usually present with epigastric pain and an associated palpable mass. Complete excision is curative, even though, depending on the tumour location, surgery may be simple or involve extensive pancreatic resection and anastomoses. The authors present a 49-year-old woman in whom a polycystic septated mass, 35 mm x 35 mm in size, was discovered by ultrasonography (US) in the body of the pancreas during investigations for epigastric pain and nausea. At surgery, a well circumscribed polycystic tumor was completely excised, with preservation of the pancreatic duct. The postoperative recovery was uneventful. Histology confirmed a microcystic lymphangioma of the pancreas. Immunohistochemistry showed cystic endothelial cells reactivity to factor Ⅷ -RA (++), CD31 (+++) and CD34 (-). Postoperatively, abdominal pain disappeared and the patient remained symptomfree for 12 mo until now. Although extremely rare, lymphangioma of the pancreas should be taken into consideration as a differential diagnosis of a pancreatic cystic lesion, especially in women.

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Key words: Pancreas; Cystic lymphangioma; Local surgical excision

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INTRODUCTION

Lymphangiomas are rare benign cystic tumours that probably occur as a result of congenital malformations of the lymphatics leading to the obstruction of local lymph flow and the development of lymphangiectasia. Histopathologically, they are composed of dilated cystic spaces containing proteinaceous eosinophilic fluid, seperated by fine septa and lined with endothelial cells[1]. These tumours present most frequently in childhood[2] and have an associated broad spectrum of clinical symptoms, depending on the disease location. They are most commonly found in the neck (75%) and the axillae (20%), though a variety of other sites have been described including the mediastinum, pleura, pericardium, groin, bones and the abdomen[2,3].

Lymphangioma of the pancreas is extremely rare accounting for less than 1% of these tumours[4], and with only 60 previously reported cases. We present the rare case of an adult with lymphangioma of the pancreas and review the literature.

CASE REPORT

A 49-year-old women presented with increasing upper abdominal pain and nausea in November 2007. She had a past medical history of a uterine myomectomy in 1997, and a hysterectomy and left oophorectomy in 2006. On examination, she was

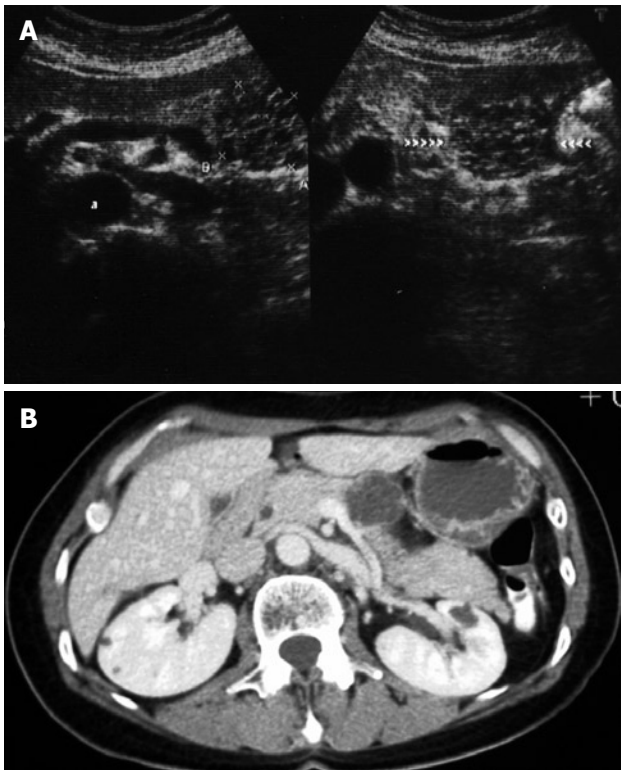

Figure 1 A well-circumscribed 35 mm polycystic lesion in the body of the pancreas, with thin septa within the lesion. A: US scan demonstrating the polycystic tumour of the body of the pancreas; B: CT scan showing the cystic tumour with fine septa.

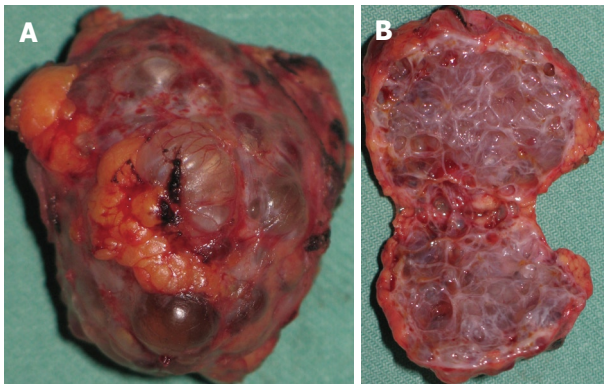

Figure 2 Tumour surrounded by normal pancreatic tissue. A: The excised polycystic mass; B: The tumour after sectioning.

found to be slightly tender at the epigastrium, and laboratory analyses were all within normal limits. An ultrasonography (US) and computer tomography (CT) scan revealed a well-circumscribed 35 mm polycystic lesion in the body of the pancreas, with thin septa within the lesion (Figure 1).

At laparotomy the lesion was found in the lower part of the body of the pancreas, and did not involve the main pancreatic duct. The lesion was completely excised and the main pancreatic duct was preserved. No other pathology was found within the abdomen, and the postoperative recovery was uneventful. Abdominal pain disappeared postoperatively and the patient has been doing well for the following 12 mo until now.

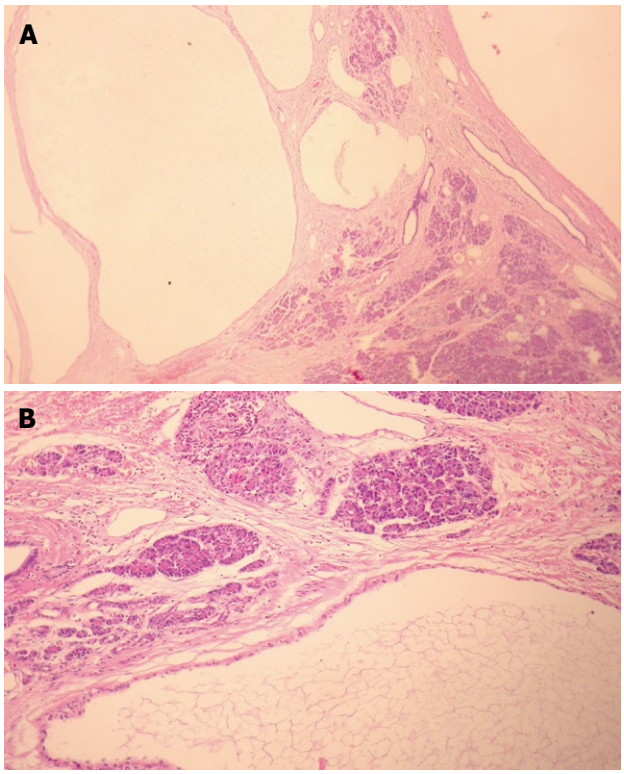

Figure 3 Microscopically all the sections (HE stain). A: Vascular spaces of the pancreatic cystic Lymphangioma containing predominantly clear fluid with few erythrocytes or lymphocytes (x 13); B: The cysts and dilated lymphatics in the surrounding pancreatic tissue are lined with a thin endothelial layer (x 64).

The tumour, measuring 34 mm × 32 mm × 29 mm, had a nodular, gray-blue surface and was surrounded by normal pancreatic tissue (Figure 2A). On sectioning, it had a honeycomb appearance with 1-7 mm polycystic spaces filled with murky haemorrhagic yellowish fluid (Figure 2B). Microscopically, all the sections showed a polycystic structure composed of ectatic lymphatics lined with endothelial cells (Figure 3). The cysts were separated by thin hypocellular septa similar in appearance to the thin capsule surrounding the tumour mass itself. No cell atypia was found. Immunohistochemistry showed immunoreactivity to the factor Ⅷ-R antigen (++), CD 31 positivity (+++) and CD 34 negativity (-). The final histological diagnosis was of microcystic lymphangioma of the pancreas.

DISCUSSION

Lymphangioma of the pancreas is rare, accounting for less than 1% of lymphangiomas[4]. It occurs more frequently in females and is often located in the distal pancreas[5]. The tumour size may vary between 3 and 20 cm in diameter (average 12 cm)[6]. Patients usually present with abdominal pain[5] and an associated palpable abdominal mass[7-9], although an acute abdomen has also been described[10]. Pancreatitis, weight loss, and laboratory abnormalities are not usual disease manifestations[1]. US typically shows a polycystic tumour, and calcifications, which are typical for cystadenomas of the pancreas, are very rare[11]. On CT, the tumour

is a well-circumscribed, encapsulated, water-isodense, polycystic tumour with thin septa, similar in appearance to cystadenomas, which occur far more frequently[1,12].

Differential diagnoses include pancreatic pseudocysts, mucinous and serous cystadenomas, other congenital cysts and pancreatic ductal carcinoma with cystic degeneration[1,13,14]. The final diagnosis is histological[1], with the endothelial cells showing immunohistochemical reactivity to factor VIII/R antigen, CD 31 (+) positivity[6,8] and CD 34 (-) negativity[6], as seen in our patient.

A complete surgical excision is curative[6,8,15], with incomplete excision being the only reason for recurrent disease[7]. Depending on the tumour location and size, complete excision may involve a simple marginal tumorectomy[10] or may require larger pancreatic resections with anastomoses.

REFERENCES

1 **Gray G**, Fried K, Iraci J. Cystic lymphangioma of the pancreas: CT and pathologic findings. *Abdom Imaging* 1998; **23**: 78-80

2 **Khandelwal M**, Lichtenstein GR, Morris JB, Furth EE, Long WB. Abdominal lymphangioma masquerading as a pancreatic cystic neoplasm. *J Clin Gastroenterol* 1995; **20**: 142-144

3 **Kullendorff CM**, Malmgren N. Cystic abdominal lymphangioma in children. Case report. *Eur J Surg* 1993; **159**: 499-501

4 **Leung TK**, Lee CM, Shen LK, Chen YY. Differential diagnosis of cystic lymphangioma of the pancreas based on imaging features. *J Formos Med Assoc* 2006; **105**: 512-517

5 **Gui L**, Bigler SA, Subramony C. Lymphangioma of the pancreas with "ovarian-like" mesenchymal stroma: a case report with emphasis on histogenesis. *Arch Pathol Lab Med* 2003; **127**: 1513-1516

6 **Paal E**, Thompson LD, Heffess CS. A clinicopathologic and immunohistochemical study of ten pancreatic lymphangiomas and a review of the literature. *Cancer* 1998; **82**: 2150-2158

7 **Daltrey IR**, Johnson CD. Cystic lymphangioma of the pancreas. *Postgrad Med J* 1996; **72**: 564-566

8 **Igarashi A**, Maruo Y, Ito T, Ohsawa K, Serizawa A, Yabe M, Seki K, Konno H, Nakamura S. Huge cystic lymphangioma of the pancreas: report of a case. *Surg Today* 2001; **31**: 743-746

9 **Murao T**, Toda K, Tomiyama Y. Lymphangioma of the pancreas. A case report with electron microscopic observations. *Acta Pathol Jpn* 1987; **37**: 503-510

10 **Itterbeek P**, Vanclooster P, de Gheldere C. Cystic lymphangioma of the pancreas: an unusual cause of the acute surgical abdomen. *Acta Chir Belg* 1997; **97**: 297-298

11 **Hanelin LG**, Schimmel DH. Lymphangioma of the pancreas exhibiting an unusual pattern of calcification. *Radiology* 1977; **122**: 636

12 **Koenig TR**, Loyer EM, Whitman GJ, Raymond AK, Charnsangavej C. Cystic lymphangioma of the pancreas. *AJR Am J Roentgenol* 2001; **177**: 1090

13 **Schneider G**, Seidel R, Altmeyer K, Remberger K, Pistorius G, Kramann B, Uder M. Lymphangioma of the pancreas and the duodenal wall: MR imaging findings. *Eur Radiol* 2001; **11**: 2232-2235

14 **Casadei R**, Minni F, Selva S, Marrano N, Marrano D. Cystic lymphangioma of the pancreas: anatomoclinical, diagnostic and therapeutic considerations regarding three personal observations and review of the literature. *Hepatogastroenterology* 2003; **50**: 1681-1686

15 **Letoquart JP**, Marcorelles P, Lancien G, Pompilio M, Denier P, Leveque J, Procyk S, Haffaf Y, Mambrini A. [A new case of cystic lymphangioma of the pancreas] *J Chir* (Paris) 1989; **126**: 650-658

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CASE REPORT

A patient with unresectable advanced pancreatic cancer achieving long-term survival with Gemcitabine chemotherapy

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Abstract

A 68-year-old female visited a local clinic with epigastralgia. A routine laboratory test revealed jaundice and liver dysfunction. She was referred to this hospital. Abdominal computed tomography (CT) and endoscopic retrograde cholangio-pancreatography (ERCP) revealed that the density of the entire pancreas had decreased, and showed dilatation of the common bile duct (CBD) and the main pancreatic duct (MPD). Pancreatic cancer was diagnosed by cytological examination analyzing the pancreatic juice obtained by ERCP. When jaundice had decreased the tumor was observed *via* laparotomy. No ascites, liver metastasis, or peritoneal dissemination was observed. The entire pancreas was a hard mass, and a needle biopsy was obtained from the head, body and tail of the pancreas. These biopsies diagnosed a poorly differentiated adenocarcinoma. Hepaticojejunostomy was thus performed, and postoperative progress was good. Chemotherapy with 1000 mg/body per week of gemcitabine was administered beginning 15 d postoperatively. However, the patient suffered relatively severe side effects, and it was necessary to change the dosing schedule of gemcitabine. Abdominal CT revealed a complete response (CR) after 3 treatments. Therefore, weekly chemotherapy was stopped and was changed to monthly administration. To date, for 4 years after chemotherapy, the tumor has not reappeared.

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Key words: Gemcitabine; Long-term survival; Unresectable advanced pancreatic cancer

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INTRODUCTION

Only 10%-20% of patients with pancreatic cancer present with localized or potentially resectable disease at the time of diagnosis, and the majority of patients are diagnosed at either an unresectable or metastatic stage[1]. The prognosis of patients with advanced unresectable pancreatic cancer remains very poor. When pancreatic cancer is diagnosed, the disease progresses and curability remains unsatisfactory, especially in stage Ⅳb pancreatic cancer[2]. The Japanese Pancreatic Cancer Registry reported that the mean survival of patients with stage Ⅳb pancreatic cancer is 6.2 mo[3], and palliation for the relief of jaundice, duodenal obstruction, or pain is required for these patients. Recently, gemcitabine was developed for the treatment of advanced pancreatic cancer, and current studies have reported improvements in survival as well as clinical benefit in patients[4]. Burris et al. reported that gemcitabine was more effective than 5-fluorouracil in the alleviation of some disease-related symptoms and improved survival was seen in patients with advanced pancreatic cancer[5].

This report describes a case of advanced pancreatic cancer which was initially diagnosed during laparotomy as unresectable; however, the patient achieved long-term survival of over 4 years, following a treatment regimen of gemcitabine chemotherapy.

CASE REPORT

A 68-year-old female visited a local clinic complaining of epigastralgia. Routine laboratory tests revealed that she had jaundice (T. bil 6.5 mg/dL) and liver dysfunction

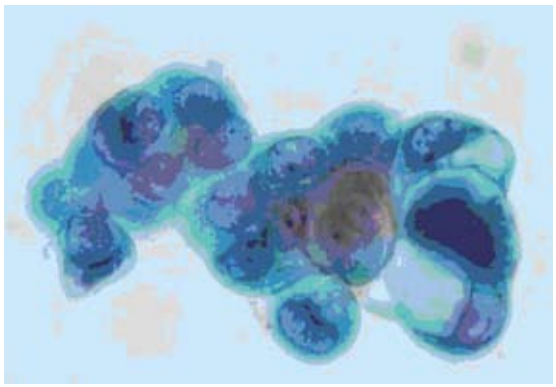

Figure 1 Cytological findings from pancreatic fluid. An adenocarcinoma was indicated.

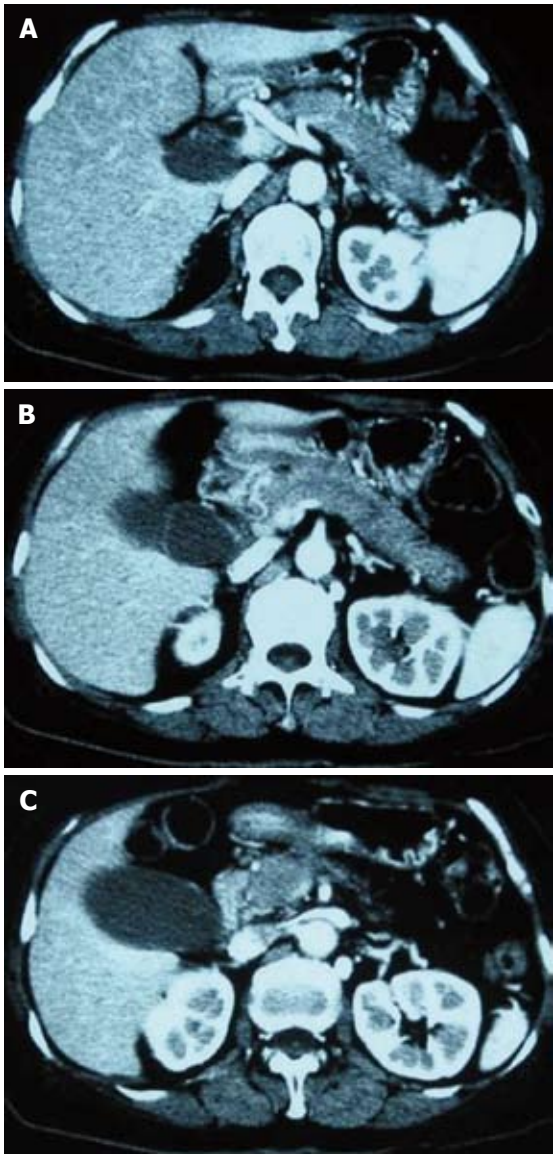

Figure 2 Abdominal CT before surgery. A low density area was observed throughout the entire pancreas, the common bile duct and main pancreatic duct were dilated, the intra-hepatic biliary duct was not dilated. CT: Computed tomography.

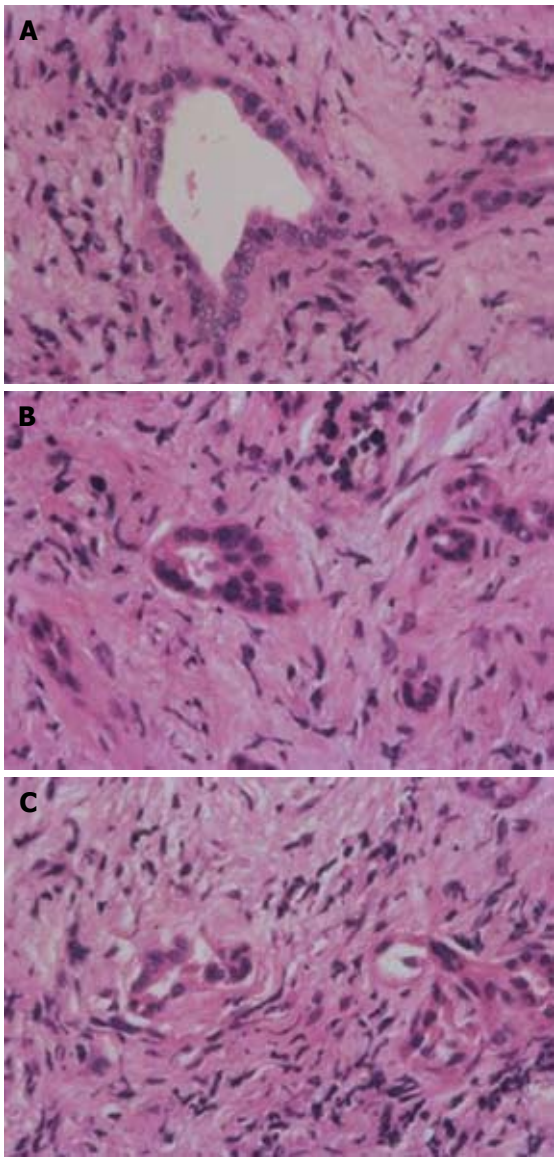

Figure 3 Needle biopsy performed during laparotomy. A: From the head; B: From the body; C: From the tail of the pancreas. All specimens were diagnosed as poorly differentiated adenocarcinoma.

(GOT 416 IU/L, GPT 489 IU/L, ALP 2055 IU/L and γGTP 190 IU/L). Initial laboratory studies included a serum total bilirubin value of 2.6 mg/dL, serum glutamic-oxaloacetic transaminase (GOT) of 416 U/L, serum glutamic-pyruvic transaminase (GPT) of 489 IU/L, alkaline phosphatase (ALP) of 2055 IU/L, and γ-glutamyl transferase (γGTP) of 190 IU/L. Her CA19-9 was not elevated (27.8 U/mL). She was referred to this hospital. She had a past history of rhabdomyosarcoma of the urinary bladder at the age of 56. On admission, abdominal computed tomography (CT) revealed that the density of the whole pancreas had decreased, and showed dilatation of the common bile duct (CBD) and the main pancreatic duct (MPD). In addition, stricture of the lower CBD was revealed on magnetic resonance cholangio-pancreatography (MRCP) (Figures 1 and 2). The MPD in the pancreas head appeared irregular on endoscopic retrograde cholangio-pancreatography (ERCP). The presence of pancreatic cancer or bleary duct cancer was suspected, because a cytological examination of the pancreatic juice obtained by ERCP

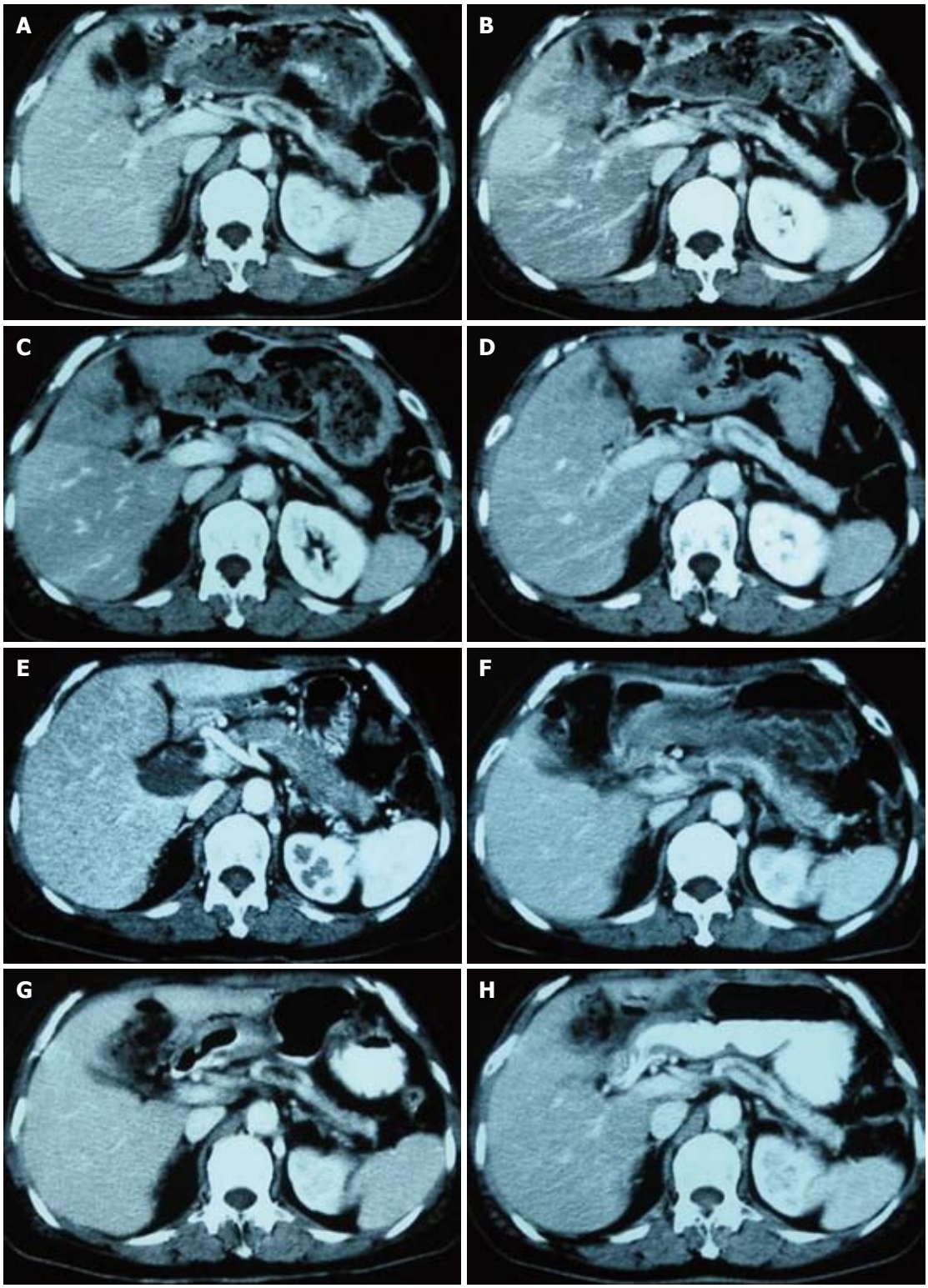

Figure 4 Abdominal CT. A: 12 mo after CTx; B: 16 mo after chemotherapy (CTx); C: 18 mo after CTx; D: 24 mo after CTx; E: preoperative; F: 1 mo after CTx; G: 2 mo after CTx; H: 6 mo after CTx; CT: Computed tomography; CTx: Chemotherapy.

detected adenocarcinoma cells. After amelioration of her jaundice by biliary drainage through percutaneous transhepatic gallbladder drainage (PTGBD), surgical treatment was selected based on a presumptive diagnosis of pancreatic cancer or biliary duct cancer. Laparotomy revealed no liver metastasis or peritoneal dissemination. The entire pancreas was hard, and a needle biopsy was obtained from the head, body, and tail of the pancreas. Examination of frozen sections revealed a poorly differentiated adenocarcinoma in each specimen (Figure 3). We therefore considered total pancreatectomy, however, a radical resection was considered excessive. Hepaticojejunostomy was performed. The postoperative course was uneventful. On postoperative day 14,

administration of chemotherapy with 1000 mg/body per week of gemcitabine was initiated. However, because the patient experienced adverse effects, such as eyelid swelling, larynx swelling, grade 1 fever and grade 3 leucopenia and neutropenia, the chemotherapy schedule was thus changed to biweekly, with the additional of granulocyte colony stimulating factor (G-CSF) (Figure 4).

After 3 courses of chemotherapy, an abdominal CT scan revealed a complete response (CR), in which the decrease in density of the entire pancreas had disappeared, and dilatation of the MPD had improved. Therefore, weekly chemotherapy was stopped after 3 courses, and changed to monthly chemotherapy with hospitalization. To date, 4 years after the initial chemotherapy, the tumor has not reappeared.

DISCUSSION

The incidence of pancreatic cancer has recently increased worldwide, and the prognosis of patients with this disease remains very poor[2,6]. Pancreatic cancer is a major cause of cancer-related mortality in Japan and remains one of the most aggressive diseases in the world. In fact, the National Registry of the Japan Pancreas Society reported that only 9.7% of these patients attained 5-year survival[7]. Early-stage diagnosis of pancreatic cancer is difficult because of the lack of specific early symptoms, and surgery with a curative intent can be performed in only 5%-20% of patients[2]. When the diagnosis is made, less than 10% of patients will survive for a year, and many require relief from jaundice and the symptoms of gastric outlet obstruction[2,5,8,9]. The prognosis for unresectable pancreatic cancer thus remains extremely poor.

Gemcitabine was recently developed for the treatment of advanced pancreatic cancer, and current studies have reported an improved survival as well as clinical benefit in patients treated with this agent[5,10]. Gemcitabine is a prodrug that requires initial intracellular phosphorylation by deoxycytidine kinase, ultimately undergoing phosphorylation to the active gemcitabine triphosphate, a cytotoxic agent that inhibits DNA synthesis.

Today an extensive pancreatic resection may not influence survival, although some studies have recommended an extensive resection for advanced pancreatic cancer[11,12]. Gemcitabine has been reported to be a strong factor in influencing the survival of patients after resection of pancreatic cancer, and the resection techniques used may not influence a patient's survival when gemcitabine is administered[9]. Although stage IVb pancreatic cancer without distant metastasis can technically be resected, it normally does not lead to a good recovery and a satisfactory QOL for the patient. As a result, gemcitabine may be the most effective clinically proven treatment for patients with stage IVb pancreatic cancer.

It is interesting that this case achieved a lengthy CR of 4 years after a gemcitabine dose of 800 mg/m^2 per month. The prognosis of pancreatic cancer is still poor, despite the findings in this patient who achieved long-term survival following the administration of gemcitabine for progressive pancreatic carcinoma which could not be excised. In addition, such a high success is surprising. Recently, the receptivity of anti-cancer drugs in cancer therapy has been shown to be remarkable, and there may be a specific mechanism which results in the high receptivity of gemcitabine in pancreatic cancer. The publication of further studies on the use of gemcitabine is therefore necessary. In several phase Ⅲ studies[5,13-23], objective responses were observed in 4.4%-17.3% of patients treated with gemcitabine alone. Progression-free survival in patients treated with gemcitabine alone was 2-4 mo and median survival was 5.4-7.3 mo for gemcitabine alone. In these studies, patients received either 1000 mg/m^2 per week of gemcitabine for 3 wk out of every 4 or for 7 wk out of every 8.

Total pancreatectomy results in complete ablation of the exocrine and endocrine pancreatic functions. Despite the administration of insulin and pancreatic enzyme replacement, total pancreatectomy often results in uncontrollable diabetes, as well as persistent diarrhea and steatorrhea, which compromises patients' nutritional status.

In this case, long-term CR and long-term survival were achieved without excision, however, this is a very rare case. The development of gemcitabine has brought about a change in the treatment of pancreatic cancer, but the effect is less than 20%, and the drug has yet to demonstrate satisfactory results. Gemcitabine may be used as second line treatment in patients who have failed first line therapy. Early detection of pancreatic cancer is important fin treatment selection, and gemcitabine has been shown to be effective in some pancreatic cancers, as it was in this case. When it is deemed that an aggressive operation would clearly be disadvantageous to a patient, then the patient may undergo conservative surgery followed by appropriate chemotherapy.

REFERENCES

1 **Tepper J**, Nardi G, Sutt H. Carcinoma of the pancreas: review of MGH experience from 1963 to 1973. Analysis of surgical failure and implications for radiation therapy. *Cancer* 1976; **37**: 1519-1524
2 **Warshaw AL**, Fernández-del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992; **326**: 455-465
3 **Matsuno S**, Egawa S, Fukuyama S, Motoi F, Sunamura M, Isaji S, Imaizumi T, Okada S, Kato H, Suda K, Nakao A, Hiraoka T, Hosotani R, Takeda K. Pancreatic Cancer Registry in Japan: 20 years of experience. *Pancreas* 2004; **28**: 219-230
4 **Fujino Y**, Ueda T, Kamigaki T, Takase S, Ajiki T, Kamoda Y, Matsumoto I, Yasuda T, Kuroda Y. Impact of gemcitabine on the survival of patients with stage IV pancreatic cancer. *Pancreas* 2007; **34**: 335-339
5 **Burris HA 3rd**, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403-2413
6 **Niederhuber JE**, Brennan MF, Menck HR. The National

Cancer Data Base report on pancreatic cancer. *Cancer* 1995; **76**: 1671-1677
7 **Matsuno M**. The report from the registration committee of pancreatic cancer. *J Jpn Pancr Soc* 2003; **18**: 97–169
8 Guidelines for the management of patients with pancreatic cancer periampullary and ampullary carcinomas. *Gut* 2005; **54** Suppl 5: v1-v16
9 **Fujino Y**, Suzuki Y, Kamigaki T, Mitsutsuji M, Kuroda Y. Evaluation of gastroenteric bypass for unresectable pancreatic cancer. *Hepatogastroenterology* 2001; **48**: 563-568
10 **Rothenberg ML**, Moore MJ, Cripps MC, Andersen JS, Portenoy RK, Burris HA 3rd, Green MR, Tarassoff PG, Brown TD, Casper ES, Storniolo AM, Von Hoff DD. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. *Ann Oncol* 1996; **7**: 347-353
11 **Nakao A**, Takeda S, Sakai M, Kaneko T, Inoue S, Sugimoto H, Kanazumi N. Extended radical resection versus standard resection for pancreatic cancer: the rationale for extended radical resection. *Pancreas* 2004; **28**: 289-292
12 **Nagakawa T**, Nagamori M, Futakami F, Tsukioka Y, Kayahara M, Ohta T, Ueno K, Miyazaki I. Results of extensive surgery for pancreatic carcinoma. *Cancer* 1996; **77**: 640-645
13 **Berlin JD**, Catalano P, Thomas JP, Kugler JW, Haller DG, Benson AB 3rd. Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. *J Clin Oncol* 2002; **20**: 3270-3275
14 **Herrmann R**, Bodoky G, Ruhstaller T, Glimelius B, Bajetta E, Schüller J, Saletti P, Bauer J, Figer A, Pestalozzi B, Köhne CH, Mingrone W, Stemmer SM, Tàmas K, Kornek GV, Koeberle D, Cina S, Bernhard J, Dietrich D, Scheithauer W. Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group. *J Clin Oncol* 2007; **25**: 2212-2217
15 **Oettle H**, Richards D, Ramanathan RK, van Laethem JL, Peeters M, Fuchs M, Zimmermann A, John W, Von Hoff D, Arning M, Kindler HL. A phase III trial of pemetrexed plus gemcitabine versus gemcitabine in patients with unresectable or metastatic pancreatic cancer. *Ann Oncol* 2005; **16**: 1639-1645
16 **Colucci G**, Giuliani F, Gebbia V, Biglietto M, Rabitti P, Uomo G, Cigolari S, Testa A, Maiello E, Lopez M. Gemcitabine alone or with cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic carcinoma: a prospective, randomized phase III study of the Gruppo Oncologia dell'Italia Meridionale. *Cancer* 2002; **94**: 902-910
17 **Heinemann V**, Quietzsch D, Gieseler F, Gonnermann M, Schönekäs H, Rost A, Neuhaus H, Haag C, Clemens M, Heinrich B, Vehling-Kaiser U, Fuchs M, Fleckenstein D, Gesierich W, Uthgenannt D, Einsele H, Holstege A, Hinke A, Schalhorn A, Wilkowski R. Randomized phase III trial of gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer. *J Clin Oncol* 2006; **24**: 3946-3952
18 **Louvet C**, Labianca R, Hammel P, Lledo G, Zampino MG, André T, Zaniboni A, Ducreux M, Aitini E, Taïeb J, Faroux R, Lepere C, de Gramont A. Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. *J Clin Oncol* 2005; **23**: 3509-3516
19 **Rocha Lima CM**, Green MR, Rotche R, Miller WH Jr, Jeffrey GM, Cisar LA, Morganti A, Orlando N, Gruia G, Miller LL. Irinotecan plus gemcitabine results in no survival advantage compared with gemcitabine monotherapy in patients with locally advanced or metastatic pancreatic cancer despite increased tumor response rate. *J Clin Oncol* 2004; **22**: 3776-3783
20 **Abou-Alfa GK**, Letourneau R, Harker G, Modiano M, Hurwitz H, Tchekmedyian NS, Feit K, Ackerman J, De Jager RL, Eckhardt SG, O'Reilly EM. Randomized phase III study of exatecan and gemcitabine compared with gemcitabine alone in untreated advanced pancreatic cancer. *J Clin Oncol* 2006; **24**: 4441-4447
21 **Moore MJ**, Hamm J, Dancey J, Eisenberg PD, Dagenais M, Fields A, Hagan K, Greenberg B, Colwell B, Zee B, Tu D, Ottaway J, Humphrey R, Seymour L. Comparison of gemcitabine versus the matrix metalloproteinase inhibitor BAY 12-9566 in patients with advanced or metastatic adenocarcinoma of the pancreas: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2003; **21**: 3296-3302
22 **Van Cutsem E**, van de Velde H, Karasek P, Oettle H, Vervenne WL, Szawlowski A, Schoffski P, Post S, Verslype C, Neumann H, Safran H, Humblet Y, Perez Ruixo J, Ma Y, Von Hoff D. Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. *J Clin Oncol* 2004; **22**: 1430-1438
23 **Moore MJ**, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960-1966

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CASE REPORT

Strangulated small bowel hernia through the port site: A case report

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Abstract

Port site hernia develops through a fascial or peritoneal layer that was inadequate or not repaired. It is a rare complication of laparoscopic surgery which may lead to serious problems. Here, we present a 77-year-old female, diagnosed with a small bowel hernia through a 10-mm port site. We had performed ten cases of laparoscopy-assisted distal gastrectomy before this case. The patient complained of left lower abdominal pain with a palpable mass. Abdominal CT showed an incarcerated small bowel hernia and the patient underwent segmental resection of the strangulated small bowel through a minimally extended port site incision.

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Key words: Port site hernia; Strangulated small bowel; Minimally extended port site incision

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INTRODUCTION

Recently, laparoscopy-assisted gastrectomy (LAG) for early gastric cancer has been accepted as technically and oncologically safe and feasible. During LAG, 10 mm or larger trocars are used for traction of the intra-abdominal organs, and fascial defects created by those trocars are usually closed because of concern over the potential for small bowel hernia. Port site hernia is a rare complication following laparoscopic surgery: however, occurrence of this may be dangerous and, hence, may have a fatal outcome. Therefore, we report the clinical course of our case and prepare a review for the management of port site hernia.

CASE REPORT

A 77-year-old woman was admitted to our hospital for gastric cancer treatment. She had complained of dyspepsia and epigastric discomfort for 20 d: following gastrofiberoscopy, early gastric cancer (type I + IIa) was found at the prepyloric antrum. She had no past medical history except well-controlled hypertension, and no abnormalities were found during preoperative evaluation. During laparoscopic surgery, a total of 5 trocars were applied to perform surgical procedures, and all the trocars used were the bladed type. A 10 mm trocar was inserted into the abdominal cavity via the umbilicus to prepare the pneumoperitoneum for electro-laparoscopy. After sufficient inflation of the abdominal cavity, 5 mm and 12 mm trocars were placed into the right side, and 5 mm and 10 mm trocars were inserted into the left side. She underwent LAG with D1 + β lymph node dissection and Billorth-II gastrojejunostomy. The fascial defects at the 10 mm and 12 mm port sites were repaired by 2-0 absorbable vicryl sutures. The patient's recovery was progressing favorably, so we put her on a diet on the 2nd day after surgery, and no abnormalities were detected. On the 7th day after the operation, the patient complained of vague abdominal pain and vomiting, but those symptoms were alleviated by conservative support. The next day, however, the patient developed a fever (38℃) and manifested left lower quadrant abdominal pain with a palpable mass. Emergency ultrasonography and abdominal CT were performed, and a structure that looked like an abscess

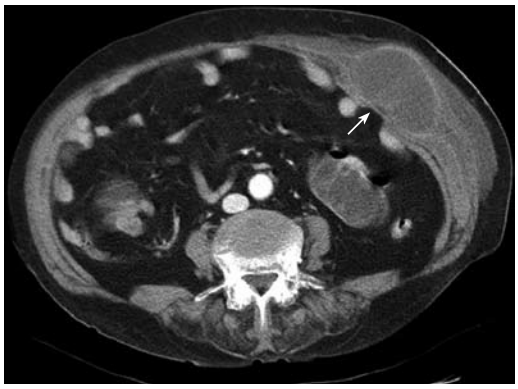

Figure 1 Port site hernia located at the left flank on computed tomography. Arrow indicates hernia.

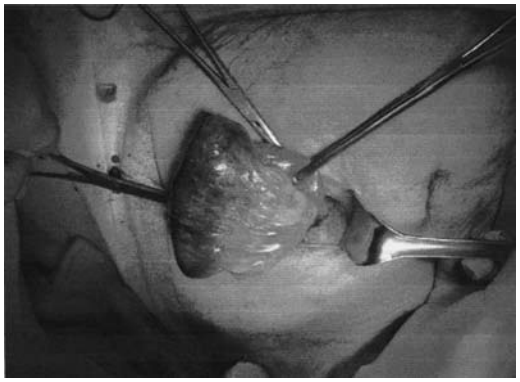

Figure 2 Strangulated small bowel was pulled through the extended port site.

or hernia of the small bowel was detected (Figure 1). We suspected an incarcerated port site or strangulated hernia of the small bowel and performed exploratory surgery. The port site incision that had been made on the left lower quadrant was transversely extended to about 4 cm long. After fascial layer sutures were untied, it was observed that the strangulated small bowel was pushing into a peritoneal defect in the abdominal wall (Figure 2). The length of incarcerated small bowel was about 10 cm long. After that was taken out through an extended incision, we carefully observed its viability and decided to remove a ischemic portion of the small bowel through an extended port site. Then we performed segmental resection of the strangulated small bowel and end-to-end anastomosis. After the operation, there were no specific events, and the patient recovered.

DISCUSSION

Port site hernia is a rare complication following laparoscopic surgery. Tonouchi *et al*[1] reported that the incidence of port site hernia was 0.65%-2.80% and classified port site hernia into three types. The early-onset type occurs immediately after the operation, the late-onset type develops after several months and the special-type presents as dehiscence of the whole abdominal wall. Relative to the size of the port site, Kadar *et al*[2] reported that incidence of port site hernia was 0.23% at a 10 mm port site and 3.1% at a 12 mm port site, and Mayol *et al*[3] reported umbilical port site hernia with an incidence of 1.6%. Port site hernia may occur at any time during the postoperative course, though our case occurred early during the postoperative course[4-6]. It is known that a port site of larger than 10 mm in diameter usually causes hernia while a 5 mm port site rarely causes hernia[5]. Hence, most surgeons routinely repair the fascia of 10 mm or larger port sites to prevent hernia.

In an experimental study of wounds relative to the type of trocar, Kolata *et al*[7] reported that a non-bladed trocar created smaller peritoneal port site wounds than a conventional pyramidal tip trocar and they hypothesized that a non-bladed type of trocar might reduce the chance of trocar wound hernia. Another study showed that a 10 mm or 12 mm port site created by a non-bladed trocar did not require fascial closure if it was placed in a non-midline position above the arcuate line[8]. Since the non-bladed trocar split the muscle rather than cutting it, it allowed the oblique muscle to reanneal together more readily. Moreover, this study suggested that misalignment of the fascial defect (created by the non-bladed trocar) aided inhibition of trocar site hernia. However, recently non-bladed trocars have mostly been used to access the port site; hence, many cases occur under this condition and some reports have suggested that herniation through the peritoneal defect might develop even though the fascial defect has been repaired. In our case, an intestinal hernia via peritoneal defect developed below the repaired fascial layer. Therefore, fascial and, if possible, peritoneal repair of port sites are necessary to prevent port site hernia.

Many methods and devices for closure of port sites have been introduced[9,10], and each of them has its merits and demerits. Generally, it is not difficult to repair port sites that are larger than 10 mm using a small retractor and hemostat unless a patient is severely obese. However, if the port site is less than 5 mm, it will be hard to close and extension of the port site may be necessary for repair. Some devices, such as a Deschamps needle, a suture carrier, and an Endoclose suture device (Tyco Auto Suture International, Inc. Norwalk, CT, USA) , may be helpful in repairing fascial and peritoneal defects[9]. In addition to direct repair of the port site, recently the port plug technique without suturing was introduced. Moreno-Sanz *et al*[10] reported that inserting a Bioabsorbable Hernia Plug (W.L Gore and Associates Inc, Flagstaff, AZ, USA) to the port site was safe and feasible as a way to prevent port site hernia. Before this unexpected event, trocar sites had been imprudently repaired. After that incident, we made every effort to repair the fascial and peritoneal defects of the port site using an extremely concaved needle and a hemostat as far as possible. After skin and subcutaneous tissue were retracted to opposite sites using small retractors, visualized fascia were clamped using a hemostat and the hemostat was then lifted to allow for detection of

the peritoneum. After the peritoneum was clamped by the hemostat, the two layers were closed separately or together. This technique was similar to the dual-hemostat technique introduced by Spalding *et al*[11] and was feasible and simple to perform unless a patient was severely obese.

Clinical courses of port site hernia are varied and depend on the degree of intestinal entry to the defect. Most patients present with vague abdominal pain, nausea, vomiting, a palpable or painful mass around port site, and fever if the bowel is incarcerated. If a patient complains of mild symptoms including nausea, vomiting and vague abdominal pain, early diagnosis may be delayed. Plain radiographs may be useful in some cases; however, plain radiographs only reveal the existence or nonexistence of the bowel obstruction. Therefore, they are limited in diagnosing port site hernia. In the case of Boughey *et al*[12], a definite diagnosis was achieved by explorative laparoscopic examination. Abdominal CT may be an effective diagnostic method if a patient complains of the symptoms described above without a palpable mass as it can differentiate adhesion from port site hernia and indicate the location of the port site hernia. Therefore, if atypical symptoms persist with obscure plain radiographs, abdominal CT is helpful in early diagnosis. Exploratory surgery with reduction of the incarcerated bowel is used to treat a port site hernia. According to previous reports, there are a few ways to access the abdominal cavity. One is a laparoscopic approach and another is an open approach through extension of the port site involved or other incision sites. Unless the incarcerated bowel is frankly ischemic, the laparoscopic approach is an acceptable method. In our case, we extended the port site and corrected the disorder because strangulation of the bowel was apparent.

In conclusion, it is necessary to repair the fascial and peritoneal layers to prevent port site hernia. Although port site hernia is rare, surgeons should keep this possibility in mind. Prompt intervention may reduce unfavorable events if port site hernia is suspected.

REFERENCES

1 **Tonouchi H**, Ohmori Y, Kobayashi M, Kusunoki M. Trocar site hernia. *Arch Surg* 2004; **139**: 1248-1256
2 **Kadar N**, Reich H, Liu CY, Manko GF, Gimpelson R. Incisional hernias after major laparoscopic gynecologic procedures. *Am J Obstet Gynecol* 1993; **168**: 1493-1495
3 **Mayol J**, Garcia-Aguilar J, Ortiz-Oshiro E, De-Diego Carmona JA, Fernandez-Represa JA. Risks of the minimal access approach for laparoscopic surgery: multivariate analysis of morbidity related to umbilical trocar insertion. *World J Surg* 1997; **21**: 529-533
4 **Itoh T**, Fuji N, Taniguchi H, Watanabe T, Kosuga T, Kashimoto K, Naito K. Port site herniation of the small bowel following laparoscopy-assisted distal gastrectomy: a case report. *J Med Case Reports* 2008; **2**: 48
5 **Reardon PR**, Preciado A, Scarborough T, Matthews B, Marti JL. Hernia at 5-mm laparoscopic port site presenting as early postoperative small bowel obstruction. *J Laparoendosc Adv Surg Tech A* 1999; **9**: 523-525
6 **Cadeddu MO**, Schlachta CM, Mamazza J, Seshadri PA, Poulin EC. Soft-tissue images. Trocar-site hernia after laparoscopic procedures. *Can J Surg* 2002; **45**: 9-10
7 **Kolata RJ**, Ransick M, Briggs L, Baum D. Comparison of wounds created by non-bladed trocars and pyramidal tip trocars in the pig. *J Laparoendosc Adv Surg Tech A* 1999; **9**: 455-461
8 **Liu CD**, McFadden DW. Laparoscopic port sites do not require fascial closure when nonbladed trocars are used. *Am Surg* 2000; **66**: 853-854
9 **Shaher Z**. Port closure techniques. *Surg Endosc* 2007; **21**: 1264-1274
10 **Moreno-Sanz C**, Picazo-Yeste JS, Manzanera-Díaz M, Herrero-Bogajo ML, Cortina-Oliva J, Tadeo-Ruiz G. Prevention of trocar site hernias: description of the safe port plug technique and preliminary results. *Surg Innov* 2008; **15**: 100-104
11 **Spalding SC**, Ponsky TA, Oristian E. A new Dual-hemostat technique to facilitate the closure of small laparoscopic trocar incisions. *Surg Endosc* 2003; **17**: 164-165
12 **Boughey JC**, Nottingham JM, Walls AC. Richter's hernia in the laparoscopic era: four case reports and review of the literature. *Surg Laparosc Endosc Percutan Tech* 2003; **13**: 55-58

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CASE REPORT

Gastric carcinoid tumor in a patient with a past history of gastrointestinal stromal tumor of the stomach

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Abstract

Gastrointestinal stromal tumor is the most common mesenchymal tumor in the gastrointestinal tract. It may coexist with other type of cancers, and if so, the tumors usually involve the stomach. The most common associated cancers are gastrointestinal carcinomas. We report a 65-year-old woman with a history of gastric gastrointestinal stromal tumor who had undergone subtotal segmental gastrectomy. New polypoid lesions were detected on a follow-up gastroscopy one year later. The lesions were biopsied and found to be carcinoid tumors. There was serum hypergastrinemia, and type 1 gastric carcinoid tumor was diagnosed. A total gastrectomy was performed. Pathologic examination revealed both carcinoid tumors and a recurrent gastrointestinal stromal tumor.

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Key words: Hypergastrinemia; Multiple primary neoplasms; Stomach; Gastrointestinal stromal tumor; Carcinoid tumor

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INTRODUCTION

Gastrointestinal stromal tumors (GIST) are the most frequent mesenchymal tumors in the gastrointestinal tract. They may coexist with other types of cancers with a reported frequency from 4.5% to 33%[1,2]. The most common location of a GIST associated with other cancers is the stomach[1], and the most frequently associated tumor type is gastrointestinal adenocarcinoma[1,2]. Infrequently, other primary cancers have been reported along with GIST, including lymphoma, leukemia, breast cancer, prostate cancer, pancreatic adenocarcinoma, or lung cancer[1-4]. GIST has been reported to occur simultaneously with ileal carcinoid tumor[5]. We report a patient with both GIST and carcinoid tumor in the stomach, an association that has not been previously reported.

CASE REPORT

A 65-year-old woman had undergone subtotal segmental gastrectomy for a 5.5 cm GIST in the lower gastric corpus. The diagnosis was based on immunohistochemical staining that was diffusely positive for CD-117 and focally positive for CD-34 and smooth muscle actin; it was negative for desmin. The patient received periodic follow-up gastroscopy. One year after surgery, she was found to have two distinct sessile polypoid lesions in the cardia and lower body of the stomach (Figure 1). The lesion in the cardia, about 2 cm distal to the gastroesophageal junction was a 1.2 cm polyp with a central ulceration. The other lesion was a 0.6 cm mass on the anterior wall near the earlier anastomosis. Immunohistochemical staining of a biopsy specimen of the larger polyp was strongly positive for

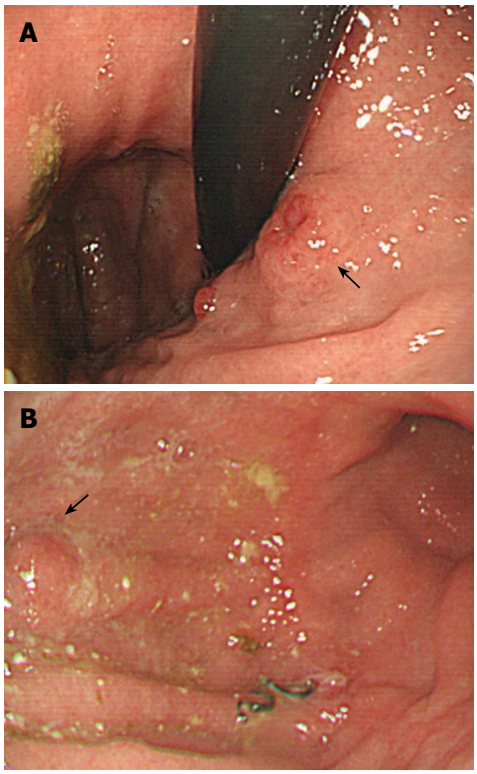

Figure 1 Gastroscopic findings. A: 1.2 cm ulcerated polyp in the cardia (arrow); B: 0.6 cm polyp on the anterior wall of the corpus near the previous anastomosis (arrow).

chromogranin-A, weakly positive for synaptophysin, and negative for CD56, neuron-specific enolase, and cytokeratin 7. Gastric carcinoid was therefore diagnosed. There was inflammation, but no *Helicobacter pylori* (*H pylori*) was found. The normal gastric body glands were atrophic and replaced by pyloric and intestinal glands. Abdominal ultrasonography, computerized tomography, barium small bowel series, and colonoscopy were performed to exclude other possible gastrointestinal tumors; there were no other abnormalities found. The patient denied any symptoms of diarrhea, palpitation, facial flushing, or weight loss, and there was no family history suggestive of similar disorders or multiple endocrine syndromes. Her abdominal examination was normal except for the previous surgical scar. The fasting serum gastrin level was 1920 pg/mL (normal 25-125 pg/mL). The 24-h urinary 5-HIAA level was normal. These findings were consistent with a type 1 hypergastrinemic gastric carcinoid tumor. Total gastrectomy was performed because the patient was concerned about further recurrences of gastric tumors. The pathologist reported two carcinoid tumors in the cardia and lower corpus near the previous anastomosis. There was no immunohistochemical evidence of GIST in the two carcinoid tumors (Figure 2). One dissected lymph node had metastatic carcinoid tumor. A 0.6 cm focal GIST was also found on the lesser curvature; and there was no carcinoid component on immunohistochemical staining (Figure 3). The pathologic slides from the first surgery were reviewed and immunohistochemical staining

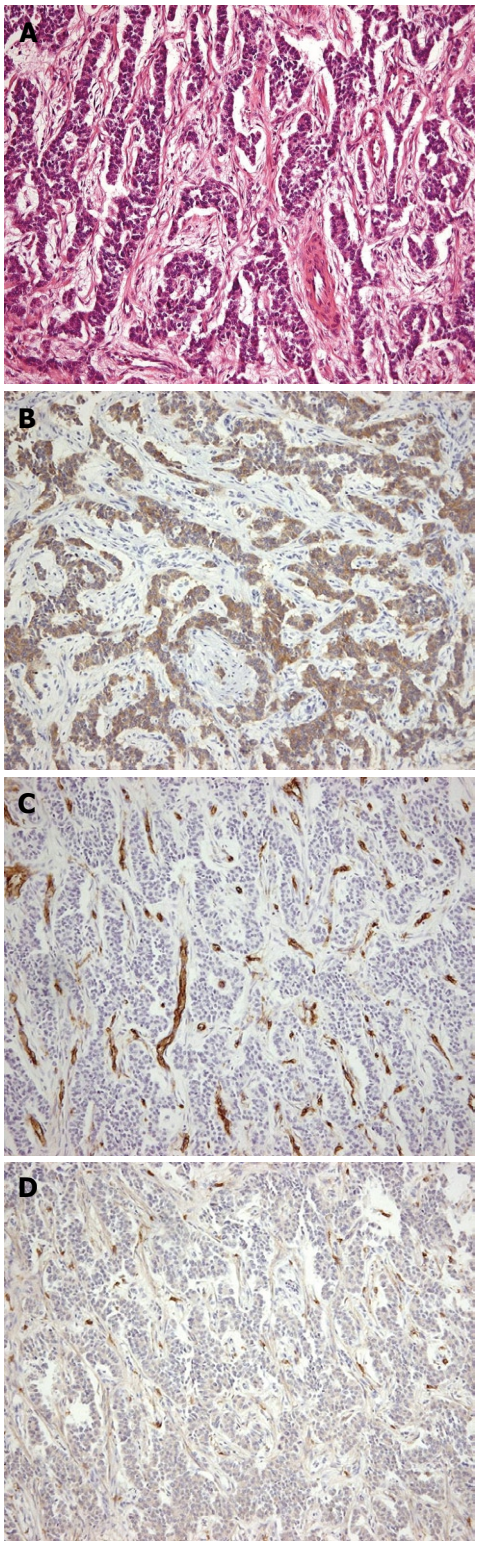

Figure 2 Carcinoid tumor. A: Uniform round-to-oval cells in trabeculae, nests, and gland-like structures (hematoxylin and eosin, × 100); B: Positive immunohistochemical staining for synaptophysin (× 100); C: Negative immunohistochemical staining for CD 34 (× 100); D: Negative immunohistochemical staining for CD 117 (× 100).

repeated, which showed no evidence of carcinoid tumor. Three weeks after the total gastrectomy, the patient's fasting gastrin level had returned to normal (72.40 pg/mL). She recovered uneventfully and has remained well for six months after surgery.

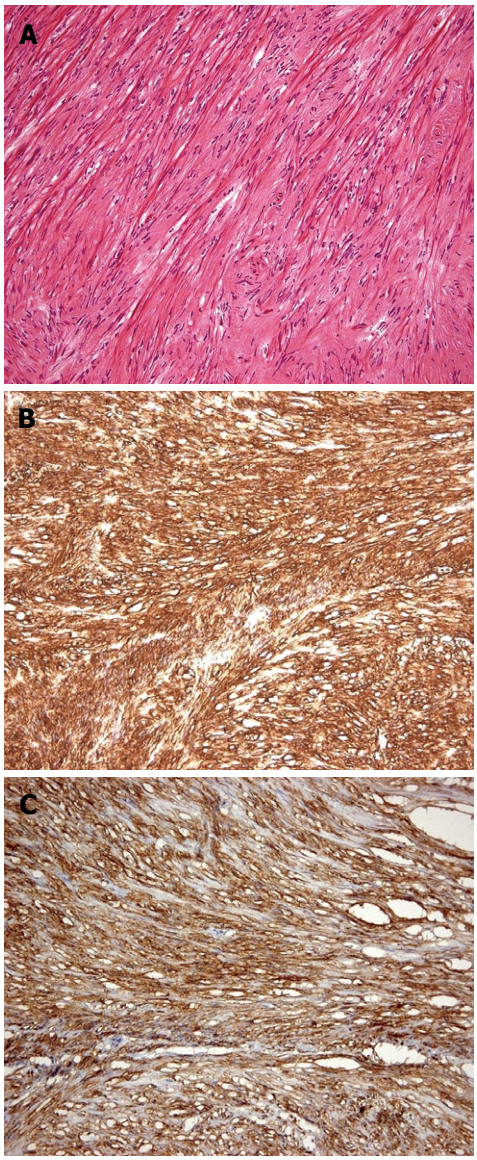

Figure 3 Recurrent gastrointestinal stromal tumor. A: Composed of spindle cells (hematoxylin and eosin × 100); B: Positive immunohistochemical staining for CD34 (× 100); C: Positive immunohistochemical staining for CD 117 (× 100).

DISCUSSION

This patient had metachronous gastric GIST and gastric carcinoid tumors, a previously unreported phenomenon. The tumors were confirmed by pathologic examination, including immunohistochemical studies, to be totally distinct neoplasms.

Gastrointestinal carcinoids comprised 73% of all carcinoid tumors in a large series, the most common sites being in the small bowel, appendix and rectum[6]. Gastric carcinoids are less common, reported as being only 8.7% of enteric carcinoids. Among all gastric cancers, carcinoids are quite rare, with a reported incidence of only 0.35%-1.77%[7]. Gastric carcinoid tumors may present with anemia caused by bleeding from an ulcerative mass, abdominal pain, or with a classical carcinoid syndrome. Histologically, the tumors have uniformly round cells growing in rosettes, cords, or trabecular patterns. Immunohistochemical stains are usually positive for chromogranin A or C and synaptophysin but negative for CD117. The latter marker distinguishes carcinoid from GIST, which is positive for CD117.

Gastric carcinoid tumors can be classified into four types: type 1, with enterochromaffin-like cells, chronic atrophic gastritis, achlorhydria, hypergastrinemia, and often pernicious anemia; type 2, with enterochromaffin-like cells, Zollinger-Ellison syndrome, multiple endocrine neoplasia type 1, and hypergastrinemia; type 3, with enterochromaffin-like cells but which is gastrin-independent and occurs sporadically; and type 4, with miscellaneous non-enterochromaffin-like endocrine cells[8-10]. The prognosis of types 1 and 2 gastric is better than that of types 3 or 4 gastric carcinoid tumors. The 5-year and 10-year survival rates were 96.1% and 73.9% for type 1 disease, compared with only 33.3% and 22.2% for type 4 gastric carcinoid tumors[9]. For type 1 gastric carcinoids, limited surgery, such as endoscopic mucosal resection[11] or partial gastrectomy is adequate, along with appropriate treatment for the hypergastrinemia[9,10,12]. Radical resection is recommended for types 3 and 4[9,10]. Our patient had type 1 gastric carcinoid with hypergastrinemia but no associated symptoms, such as Zollinger-Ellison syndrome or multiple endocrine neoplasia type 1. If she had had only the type 1 gastric carcinoid, more conservative surgery might have been considered. The history of the previous GIST, however, influenced the choice of total gastrectomy.

In a retrospective review of 200 patients with gastrointestinal GIST (with 78% or 39% in the stomach), DeMatteo *et al*[13] reported an overall 5-year survival rate of about 35%, but it depended on the extent of resection. For patients undergoing complete resection, the 5-year survival was 54%, with those patients surviving a median of 66 mo compared with 22 mo for those who had incomplete resection. The recurrence rate was 40% (median 24 mo' follow-up). The median disease-specific survival for pateints with local recurrence was 12 mo. Metastases usually occurred with tumors larger than 5 cm or with a mitotic index of more than 10 mitoses per 50 high power fields. The authors recommended complete surgical resection for GIST if possible.

In estimating our patient's prognosis, it is difficult to know if it should be predicted based on the locally recurrent GIST or the carcinoid metastatic to a lymph node. Carcinoid metastases to regional lymph nodes cannot be reliably predicted by tumor size or depth of invasion, their impact on survival is uncertain[14], and there is no recommended adjuvant treatment. However, type 1 carcinoid is fairly indolent and is less likely than the GIST to limit our patient's survival. We intend to monitor the patient at 6-12 mo intervals with computed tomography for GIST and using plasma chromogranin measurements and octreoscan for carcinoid. Adjuvant therapy with imatinib is an option for treating residual GIST, with evaluation of *c-Kit* and *PDGFRA* mutations a useful tool to predict efficacy[15,16]. There was also a clinical trial conducted by Yao *et al*[17], testing the imatinib in 27 carcinoid patients with a modest clinical

response of median overall survival of 36 mo. However, our patient's recurrent GIST was totally removed by gastrectomy, and the mitotic index was low. There would be no way of assessing treatment response and therefore no clear benefit, so imatinib is not indicated in this patient unless the GIST is found to recur on follow-up.

In conclusion, our patient had an apparently unique combination of two unusual gastric tumors. Accurate diagnosis of her GIST and carcinoid tumors, based on immunohistochemical studies, was important both for deciding on treatment and follow-up and in estimating prognosis. However, as this case clearly illustrates, a number of individual patient variables affect those decisions and estimates. Inevitably we must rely on clinical judgment which, while informed by what the evidence says about the diseases in general, must also take into account a patient's particular characteristics, needs, and preferences.

REFERENCES

1 **Agaimy A**, Wünsch PH, Sobin LH, Lasota J, Miettinen M. Occurrence of other malignancies in patients with gastrointestinal stromal tumors. *Semin Diagn Pathol* 2006; **23**: 120-129

2 **Liszka L**, Zielinska-Pajak E, Pajak J, Golka D, Huszno J. Coexistence of gastrointestinal stromal tumors with other neoplasms. *J Gastroenterol* 2007; **42**: 641-649

3 **Kövér E**, Faluhelyi Z, Bogner B, Kalmár K, Horváth G, Tornóczky T. [Dual tumours in the GI tract: synchronous and metachronous stromal (GIST) and epithelial/neuroendocrine neoplasms] *Magy Onkol* 2004; **48**: 315-321

4 **Melis M**, Choi EA, Anders R, Christiansen P, Fichera A. Synchronous colorectal adenocarcinoma and gastrointestinal stromal tumor (GIST). *Int J Colorectal Dis* 2007; **22**: 109-114

5 **Buragas M**, Kidd M, Modlin IM, Cha C. Multiple gastrointestinal stromal tumors and synchronous ileal carcinoids. *Nat Clin Pract Oncol* 2005; **2**: 166-170; quiz 1 p following 170

6 **Hu YQ**, Qian JM, Zhou XD. [Comparison and analysis of the clinical features of different types of gastrointestinal cancers] *Zhonghua Neike Zazhi* 2004; **43**: 900-902

7 **Modlin IM**, Lye KD, Kidd M. A 50-year analysis of 562 gastric carcinoids: small tumor or larger problem? *Am J Gastroenterol* 2004; **99**: 23-32

8 **Modlin IM**, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* 2003; **97**: 934-959

9 **Borch K**, Ahren B, Ahlman H, Falkmer S, Granerus G, Grimelius L. Gastric carcinoids: biologic behavior and prognosis after differentiated treatment in relation to type. *Ann Surg* 2005; **242**: 64-73

10 **Burkitt MD**, Pritchard DM. Review article: Pathogenesis and management of gastric carcinoid tumours. *Aliment Pharmacol Ther* 2006; **24**: 1305-1320

11 **Ichikawa J**, Tanabe S, Koizumi W, Kida Y, Imaizumi H, Kida M, Saigenji K, Mitomi H. Endoscopic mucosal resection in the management of gastric carcinoid tumors. *Endoscopy* 2003; **35**: 203-206

12 **Gough DB**, Thompson GB, Crotty TB, Donohue JH, Kvols LK, Carney JA, Grant CS, Nagorney DM. Diverse clinical and pathologic features of gastric carcinoid and the relevance of hypergastrinemia. *World J Surg* 1994; **18**: 473-479; discussion 479-480

13 **DeMatteo RP**, Lewis JJ, Leung D, Mudan SS, Woodruff JM, Brennan MF. Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. *Ann Surg* 2000; **231**: 51-58

14 **Mullen JT**, Wang H, Yao JC, Lee JH, Perrier ND, Pisters PW, Lee JE, Evans DB. Carcinoid tumors of the duodenum. *Surgery* 2005; **138**: 971-977; discussion 977-978

15 **Paul PC**, Chakraborty J, Kundu D, Sarkar R. Gastrointestinal stromal tumour--role of CD117 in diagnosis and management. *Indian J Pathol Microbiol* 2007; **50**: 279-283

16 **Joensuu H**. Gastrointestinal stromal tumor (GIST). *Ann Oncol* 2006; **17** Suppl 10: x280-x286

17 **Yao JC**, Zhang JX, Rashid A, Yeung SC, Szklaruk J, Hess K, Xie K, Ellis L, Abbruzzese JL, Ajani JA. Clinical and in vitro studies of imatinib in advanced carcinoid tumors. *Clin Cancer Res* 2007; **13**: 234-240

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
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18th World Congress of the International Association of Surgeons,
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59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
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Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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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

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[1]Passed away on October 20, 2007
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Contents

Contents

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Volume 14 **Number** 45 **December 7**, 2008

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Contents

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EDITORIAL

Function of the hemochromatosis protein HFE: Lessons from animal models

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Abstract

Hereditary hemochromatosis (HH) is caused by chronic hyperabsorption of dietary iron. Progressive accumulation of excess iron within tissue parenchymal cells may lead to severe organ damage. The most prevalent type of HH is linked to mutations in the *HFE* gene, encoding an atypical major histocompatibility complex class I molecule. Shortly after its discovery in 1996, the hemochromatosis protein HFE was shown to physically interact with transferrin receptor 1 (TfR1) and impair the uptake of transferrin-bound iron in cells. However, these findings provided no clue why *HFE* mutations associate with systemic iron overload. It was later established that all forms of HH result from misregulation of hepcidin expression. This liver-derived circulating peptide hormone controls iron efflux from duodenal enterocytes and reticuloendothelial macrophages by promoting the degradation of the iron exporter ferroportin. Recent studies with animal models of HH uncover a crucial role of HFE as a hepatocyte iron sensor and upstream regulator of hepcidin. Thus, hepatocyte HFE is indispensable for signaling to hepcidin, presumably as a constituent of a larger iron-sensing complex. A working model postulates that the signaling activity of HFE is silenced when the protein is bound to TfR1. An increase in the iron saturation of plasma transferrin leads to displacement of TfR1 from HFE and assembly of the putative iron-sensing complex. In this way, iron uptake by the hepatocyte is translated into upregulation of hepcidin, reinforcing the concept that the liver is the major regulatory site for systemic iron homeostasis, and not merely an iron storage depot.

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Key words: Hepcidin; Iron metabolism; Transferrin; Hemojuvelin; Bone morphogenetic proteins

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PHYSIOLOGY AND PATHOPHYSIOLOGY OF IRON HOMEOSTASIS

Iron is essential for various physiological and metabolic pathways. However, unshielded iron is toxic, as a catalyst of free radical generation[1,2]. The adult human body contains a pool of 3-5 g of iron (about 55 mg and 44 mg per kilogram body weight in males and females, respectively), the majority of which (> 70%) is utilized by erythroid cells for heme synthesis and integration into hemoglobin[3]. A daily requirement of about 20-30 mg iron for erythropoiesis is mainly covered by recycling of the metal from senescent erythrocytes *via* reticuloendothelial macrophages. These cells metabolize heme and release iron into the circulation, where it is scavenged by plasma transferrin and delivered to tissues. A considerable amount of iron (about 1 g) is stored in the liver. Dietary iron absorption by duodenal enterocytes compensates for losses through bleeding or desquamation; a physiological rate of 1-2 mg/d suffices to maintain the body iron pool. This is subjected to feedback regulation and may adjust to fluctuations in iron demands.

In hereditary hemochromatosis (HH), disruption of this homeostatic loop leads to unrestricted dietary iron absorption at a rate that may reach 8-10 mg/d[4,5]. This

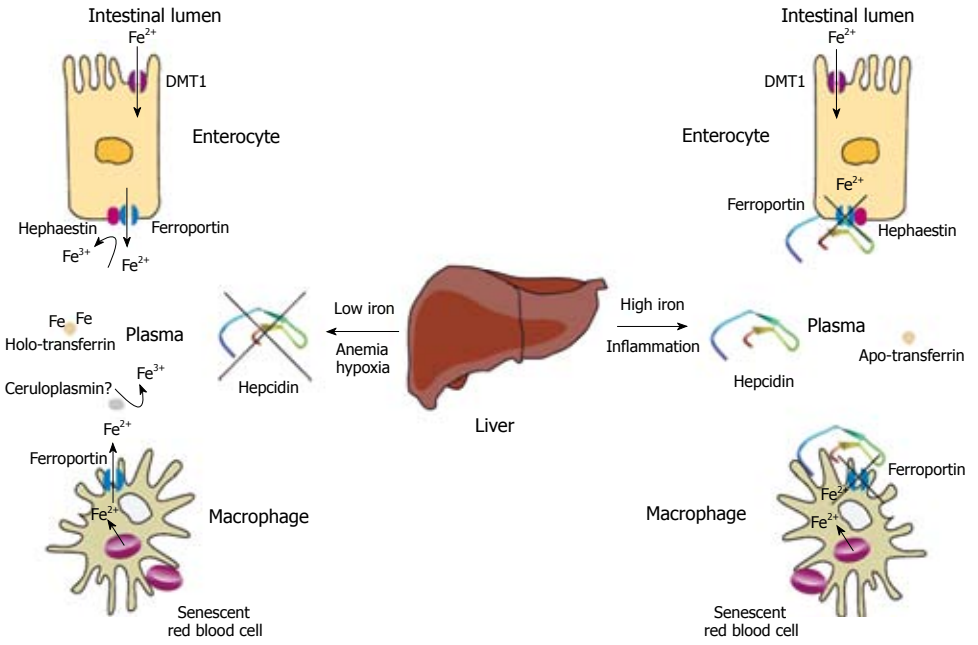

Figure 1 Regulation of iron efflux from enterocytes and macrophages by hepcidin. Duodenal enterocytes absorb dietary iron *via* DMT1 and reticuloendothelial macrophages phagocytose iron-loaded senescent red blood cells. Both cell types release ferrous iron (Fe^{2+}) into plasma via ferroportin, which is incorporated into transferrin following oxidation into the ferric form (Fe^{3+}) *via* hephaestin or ceruloplasmin. The secretion of the iron-regulatory hormone hepcidin from the liver in response to high body iron stores or inflammatory signals results in internalization and degradation of ferroportin, and retention of iron within enterocytes and macrophages. A decrease in body iron stores, a requirement of iron for erythropoiesis, or hypoxia, inhibit hepcidin expression, permitting dietary iron absorption by enterocytes and iron release from macrophages.

is accompanied by a gradual increase in the saturation of transferrin with iron (from physiological 30% up to 100%), a buildup of non-transferrin-bound iron and excessive accumulation of the metal in parenchymal cells of the liver, pancreas, pituitary, heart, joints and skin. Notably, macrophages and absorptive duodenal enterocytes are spared from iron loading and exhibit increased rates of iron release. Excessive iron deposition in the liver constitutes a risk factor for fibrosis, cirrhosis and hepatocellular cancer[6-8], and may exacerbate other types of liver disease[9,10]. Iron overload may also lead to cardiomyopathy, diabetes mellitus, hypogonadism, arthritis and skin pigmentation[3]. HH is efficiently treated by phlebotomy.

HORMONAL REGULATION OF IRON TRAFFIC BY HEPCIDIN

The discoveries of the divalent metal transporter (DMT1), the iron exporter ferroportin, and the iron regulatory hormone hepcidin provided a framework to understand the molecular mechanisms for systemic iron traffic and homeostasis[11,12]. DMT1 accounts for the absorption of ferrous ions across the apical membrane of duodenal enterocytes, but also for intracellular transport of transferrin-derived iron across the endosomal membrane in many cell types. Ferroportin mediates efflux of ferrous iron from enterocytes and macrophages to plasma transferrin. The transport of iron by DMT1 requires its reduction by ferric reductases (such as Dcytb or the Steap proteins), while its export by ferroportin is coupled by re-oxidation *via* ferroxidases (such as ceruloplasmin or hephaestin).

The ferroportin-mediated efflux of iron from enterocytes and macrophages defines a key regulatory checkpoint for iron homeostasis. This process is negatively controlled by hepcidin, a cysteine-rich peptide hormone that binds to ferroportin and promotes its internalization and lysosomal degradation[13]. Hepcidin is synthesized in hepatocytes as a pro-peptide, which undergoes proteolytic processing to form a bioactive molecule of 25 amino acids[14]. The mature peptide is secreted into plasma and orchestrates homeostatic responses to iron, erythropoiesis, hypoxia and inflammation. An increase in hepcidin levels, commonly encountered following dietary iron intake or in inflammation[15,16], impairs iron absorption by duodenal enterocytes and promotes retention of the metal within macrophages (Figure 1), limiting its availability for erythropoiesis. Excessive hepcidin expression, in response to prolonged inflammation, contributes to the anemia of chronic disease[17]. On the other hand, low hepcidin levels triggered by iron deficiency, hypoxia or phlebotomy[18] facilitate duodenal iron absorption and iron release from macrophages (Figure 1). Importantly, HH patients fail to mount an appropriate upregulation of hepcidin expression, despite high transferrin saturation and elevated body iron stores[19,20]. Thus, HH is largely based on the loss of feedback control in dietary iron absorption due to defects in the hepcidin pathway.

Juvenile hemochromatosis, a rare but severe form of hereditary iron overload results from genetic inactivation of the hepcidin gene[21] or mutations in hemojuvelin (HJV) associated with profound hepcidin deficiency[22]. The most prevalent form of HH is linked to mutations in HFE[23], while another less common but phenotypically indistinguishable HH subtype is caused by mutations in transferrin receptor 2 (TfR2)[24]. Iron overload patients with either HFE or TfR2 mutations exhibit inappropriately decreased hepcidin levels or blunted hepcidin responses[19,20,25,26]. Similar results were obtained with mouse models of iron overload, bearing targeted disruptions of the *HFE*[27-30], *HJV*[31,32] or *TfR2*[33] genes. These findings suggest that HFE, HJV and TfR2 are upstream regulators of hepcidin expression.

REGULATION OF HEPCIDIN EXPRESSION

Hepcidin is transcriptionally activated by distinct iron-

and cytokine-dependent pathways. The latter is mediated by IL-6 (and IL-1) *via* STAT3[34-36]. The iron-dependent pathway is less well characterized and involves proximal and distal promoter elements[37,38]. The lack of hepcidin expression, accompanied by iron overload, in mice carrying a hepatocyte-specific disruption of SMAD4[39] has linked iron-sensing with bone morphogenetic protein (BMP) signaling. In fact, BMP-2, -4 and -9 are potent inducers of hepcidin transcription, while hemojuvelin stimulates this pathway as a BMP co-receptor[40-42]. The CCAAT/enhancer-binding protein α (C/EBPα) appears necessary for basal hepcidin transcription[43].

Hepcidin expression is suppressed in anemia by a mechanism that requires erythropoietic activity[44,45]. At least in thalassemia patients, the silencing of hepcidin is mediated by overexpression of growth differentiation factor 15 (GDF15), a member of the transforming growth factor β (TGFβ) superfamily[46]. Erythropoietin (EPO) directly reduces the binding of C/EBPα to the hepcidin promoter *via* EPO receptor signaling[47]. Hepcidin is also negatively regulated by hypoxia[18]. Experiments in mice with hepatic disruption of HIF-1α provided evidence for the involvement of this transcription factor in the underlying pathway[48]. However, other reports suggested that the hypoxic downregulation of hepcidin is HIF-independent[49,50] and involves oxidative stress-mediated repression of C/EBPα and STAT3[49], or inhibition of 2-oxoglutarate dependent oxygenases[50]. Recent work revealed that the transmembrane serine protease TMPRSS6 negatively regulates signaling to hepcidin[51-55], by a yet unknown mechanism.

What is the role of HFE in hepcidin regulation?

DISCOVERY OF HFE AS THE HEMOCHROMATOSIS GENE

The *HFE* gene was elucidated by linkage disequilibrium and haplotype analysis from a large group of HH patients[23], culminating lengthy efforts to map the hemochromatosis locus. It encodes an atypical major histocompatibility complex (MHC) class I protein, which is processed *via* the Golgi network to the cell surface, following interaction with β2-microglobulin. Structural analysis revealed that in contrast to typical MHC class I homologues, HFE formed a smaller groove between the α1 and α2 subunits, which was predicted to preclude peptide antigen presentation[56]. The majority of HH patients carry an *HFE* C282Y substitution. This abrogates a disulphide bridge and prevents the association of HFE with β2-microglobulin, a necessary step for its processing and transport to the plasma membrane[57,58]. Unprocessed *HFE* C282Y undergoes proteasomal degradation following retention in the endoplasmic reticulum (ER), which promotes ER stress[59]. An *HFE* H63D mutation may also lead to HH, especially in the compound heterozygous state with C282Y. Homozygosity for the *HFE C282Y* genotype is highly prevalent (1:200) in populations of Northern European ancestry; however, the clinical penetrance is lower and remains a matter of debate[4-6,60,61]. It appears that HH is a multifactorial disease and the development of iron overload in individuals bearing disease-associated *HFE* mutations requires the contribution of additional, yet incompletely understood environmental, genetic and/or epigenetic factors[62]. Nevertheless, mice with either targeted disruption of the *HFE*[63,64] or β2-microglobulin[65,66] genes, or expressing orthologues of the *HFE* C282Y[67] or H63D[68] mutants, develop progressive iron overload, the degree of which depends on the genetic background of the animals[69-71]. Collectively, these findings underlie the significance of HFE in the control of body iron homeostasis.

EARLY MODELS FOR THE FUNCTION OF HFE

Biochemical[72,73] and crystallographic[74] studies revealed that HFE interacts with TfR1 (Kd about 60 nmol/L) and competes for the binding of transferrin to its receptor, which has a Kd of about 1 nmol/L[75]. However, considering that the physiological concentration of plasma diferric holotransferrin is about 5 μmol/L[76], HFE is unlikely to affect the rate of TfR1 endocytosis *in vivo*. In transfected cell lines, overexpressed HFE reduced the efficiency of the transferrin cycle[77] and promoted an iron-deficient phenotype[78-81], without or with co-expression of β2-microglobulin[82]. Notably, a similar phenotype was observed with an *HFE* W81A mutant that is unable to bind to TfR1, suggesting that the HFE-mediated decrease of intracellular iron levels is independent of the HFE/TfR1 interaction[83].

The above data did not shed much light on how HFE controls systemic iron homeostasis and rather created some confusion. The immunohistochemical detection of HFE in precursor enterocytes of the intestinal crypts[84] and its association with TfR1 in these cells[85] laid the foundation for the "crypt-programming model"[86]. This postulates that iron absorption is regulated by signals that are sensed by precursor enterocytes, which undergo maturation and migrate along the crypt-villus axis. An iron deficient status in the crypt cells would program mature enterocytes to absorb more dietary iron from the lumen. According to the crypt-programming model, HFE would serve to promote iron retention within crypt cells, possibly by increasing the uptake of plasma transferrin[87] and/or inhibiting iron efflux[88]. The model is supported by the iron deficient status manifested in duodenal biopsies from HH patients[89,90]. Experimental evidence has been provided that HFE may also facilitate iron accumulation[91] or retention[92] within macrophages, which are likewise iron-deficient in HH patients[93]. In the pre-hepcidin era, these findings have highlighted the enterocytes and macrophages as possible primary sites of the HFE regulatory function. Nonetheless, HFE is expressed in multiple cell types, including hepatocytes[94], the major producers of hepcidin.

LESSONS FROM ANIMAL MODELS I: THE SITE OF HFE REGULATORY FUNCTION

Definite clues as to the site of HFE regulatory function

in the context of systemic iron homeostasis were recently provided by experiments with genetically engineered mice, bearing a targeted, tissue-specific disruption of the *HFE* gene. The technology is based on the generation of animals carrying a "floxed" *HFE* allele, surrounded by loxP sites, which are specific targets of the Cre recombinase. Crossing of "floxed" *HFE* mice with a transgenic line expressing the Cre recombinase under the control of the villin promoter resulted in intestinal-specific disruption of HFE in the progeny[95]. Importantly, mice lacking HFE expression in the intestine did not show any signs of abnormal iron metabolism, at least with regard to liver iron content, serum iron parameters and serum ferritin levels. Moreover, they exhibited physiological expression of the mRNAs encoding liver hepcidin and the intestinal iron transporters DMT1 and ferroportin[95]. By showing that intestinal HFE expression is dispensable for the regulation of body iron homeostasis, these data challenge the validity of the "crypt-programming model" and raise the possibility for a critical role of HFE in the liver.

In a follow-up study, "floxed" *HFE* mice were crossed with transgenic animals expressing the Cre recombinase under the control of either the hepatocyte-specific albumin promoter, or the macrophage-specific lysozyme M promoter[96]. While HFE ablation in macrophages did not affect body iron metabolism, the disruption of HFE in hepatocytes recapitulated the hemochromatosis phenotype of null *HFE*-/-mice. Thus, mice lacking HFE expression in hepatocytes exhibited hyperabsorption of dietary iron, increased serum iron, transferrin saturation and iron deposition in the liver[96]. Taken together, the tissue-specific knock-out experiments demonstrate that hepatocyte HFE is necessary to promote appropriate hepcidin responses and thereby prevent iron overload.

These data also corroborate clinical findings, showing that the iron status of recipients of a liver transplant was largely dependent on the *HFE* genotype of the donors[97,98]. Nevertheless, a contribution of macrophage HFE to hepcidin regulation cannot be completely ruled out. While macrophages are dispensable for hepcidin expression in response to iron or inflammatory signals[99,100], bone marrow transplantation from wild type mice into irradiated *HFE*-/-counterparts corrected iron parameters and significantly increased hepcidin levels in the recipients[101]. Conceivably, this could be the result of intercellular communication and signaling to hepatocytes and/or HFE-mediated autocrine production of hepcidin in macrophages[102].

LESSONS FROM ANIMAL MODELS II: THE ROLE OF TFR1 IN THE CONTROL OF HFE ACTIVITY

How does HFE modulate signaling to hepcidin? Biochemical work showed that HFE not only interacts with TfR1, but also with TfR2[103]. Moreover, the HFE/TfR2 interaction leads to an increase in TfR2 levels[104]. TfR2 is primarily expressed in hepatocytes[105] and stabilized by diferric holo-transferrin[106,107]. While TfR1 mediates cellular iron uptake from circulating transferrin, TfR2 is thought to function as an upstream regulator of hepcidin, and possibly an iron sensor[14]. A testable prediction arising from the capacity of HFE to interact with both TfR1 and TfR2 would be that the choice of its binding partner is regulated by transferrin and, furthermore, this event is crucial for signaling to hepcidin.

This hypothesis was explored in a recent study, based on the idea to induce or abolish HFE/TfR1 interactions *in vivo*[108]. To this end, transgenic mice were engineered for expression of TfR1 mutants that prevent the binding of either transferrin (R654A) or HFE (L622A). In light of the early embryonic lethality of *TfR1*-/-mice[109], indicating an utmost importance for the interaction of TfR1 with transferrin, a TfR1 R654A cDNA was integrated by homologous recombination into the heterologous ROSA26 locus, maintaining endogenous wild type *TfR1* expression (thus, the transgenic product did not disrupt the transferrin cycle, excluding abnormalities of erythropoiesis). In contrast, the L622A mutation was introduced by homologous recombination within the TfR1 locus ("knock-in").

TfR1 R654A, that is unable to bind to transferrin, would be expected to constitutively associate with HFE. Transgenic mice expressing *TfR1* R654A developed iron overload, associated with decreased hepcidin mRNA levels, closely resembling the *HFE*-/-phenotype. On the other hand, TfR1 L622A, that is unable to bind to HFE, would be expected to be highly efficient in the uptake of transferrin-bound iron. Interestingly, transgenic mice expressing TfR1 L622A developed a mild hypochromic microcytic anemia, and exhibited decreased serum iron and elevated hepcidin mRNA levels. These results suggest that HFE stimulates hepcidin expression when it is free of TfR1. In support of this notion, the hepatocyte-specific transgenic overexpression of an HFE cDNA in *HFE*-/-mice substantially induced hepcidin mRNA expression to the extent that it not only corrected hepatic iron overload, but also promoted hypochromic microcytic anemia.

A MODEL FOR THE IRON REGULATORY FUNCTION OF HFE

A model accommodating the above findings postulates that under low serum iron conditions, hepatocyte HFE is predominantly bound to TfR1 (Figure 2A). An increase in transferrin saturation triggers the release of HFE from TfR1 and concomitantly stabilizes TfR2[106,107]. In that way, TfR1 becomes accessible for the binding and endocytosis of holo-transferrin, resulting in cellular iron uptake. At the same time, HFE associates with stabilized TfR2 and possibly other proteins, such as hemojuvelin and BMPs and their receptor (BMPR), to form a putative iron signaling complex that induces hepcidin transcription *via* Smad proteins (Figure 2B). Thus, an increase in the iron content of the hepatocyte is

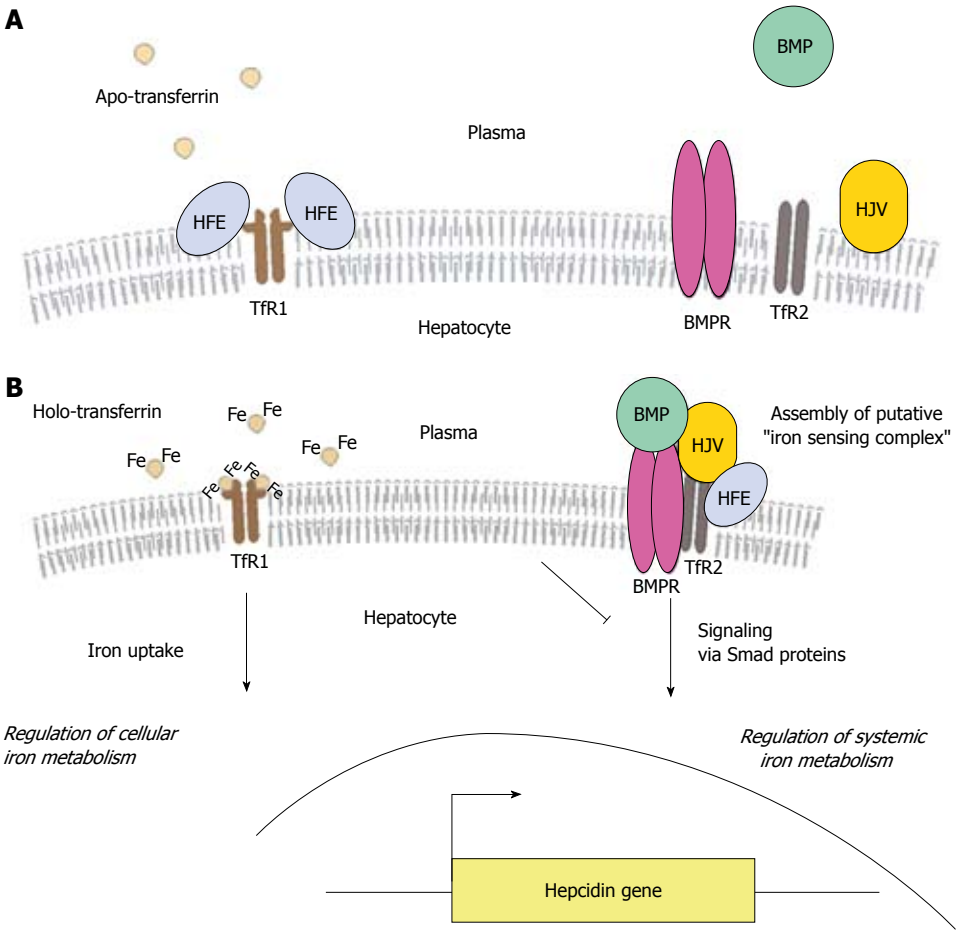

Figure 2 A model for HFE-mediated signaling to hepcidin in hepatocytes. A: At low plasma iron concentration, HFE is bound to TfR1 and other proteins involved in signaling to hepcidin remain silent; B: An increase in plasma iron levels results in displacement of HFE from TfR1, followed by iron uptake. This triggers the assembly of a putative "iron-sensing" complex, comprising of HFE, TfR2, BMPs (such as BMP-2, BMP-4 and BMP-9) and their receptor BMPR, and Hjv, which mediates signaling to activate hepcidin transcription via Smad proteins. Thus, the hepatocyte integrates signals for regulation of iron metabolism at the cellular and systemic level.

indirectly translated into a systemic regulatory response *via* hepcidin. Iron-dependent degradation of TfR1 mRNA by iron regulatory proteins[110] would terminate this process in a feedback loop. According to this model, HFE serves to sense alterations in transferrin saturation.

Considering that a number of HH patients with HFE C282Y mutations[25] and some *HFE*-/-mice[29] express normal (or close to normal) basal hepcidin mRNA levels but exhibit blunted hepcidin responses to dietary iron, it is conceivable that the role of HFE is somehow restricted to the fine-tuning of iron-dependent signaling to hepcidin. Along these lines, BMP-2, -4 and -9 can induce hepcidin mRNA transcription in *HFE*-/-and *TfR2*-/-hepatocytes[41]. Several reports have also shown that HFE is dispensable for signaling to hepcidin *via* the inflammatory pathway[29,41,111,112], even though opposing views exist[113].

Recent animal studies[95,96,108] have not entirely solved the mystery of HFE function, but have significantly advanced our understanding on how this protein regulates systemic iron homeostasis. First, they uncovered HFE as a hepatocyte iron sensor, necessary to prevent iron overload and sufficient to control hepcidin expression (at least at the mRNA level). And second, they demonstrated that HFE-dependent signaling to hepcidin is regulated by the interaction of HFE with TfR1.

OUTLOOK AND PERSPECTIVES

Several outstanding issues remain to be addressed. For example, the proposed function of HFE as a sensor of transferrin saturation requires experimental validation. The functional significance of the interaction between HFE and TfR2, as well as the role and composition of the putative iron-sensing complex await further investigation. It will be interesting to explore a potential functional redundancy between HFE and classical MHC class I molecules with regard to iron regulation, considering that mice lacking such molecules develop iron overload[114]. Conversely, the proposed capacity of HFE to engage into immune responses, following recognition by cytotoxic T lymphocytes[115], deserves additional attention, especially in light of immunological abnormalities of HH patients[116]. A possible connection between the unfolded protein response, caused by defective processing of HFE C282Y, and the hepcidin pathway would not be totally unexpected[117]. Finally, it will be important to examine whether HFE may also affect the maturation of hepcidin; this would necessitate analytical methods for direct measurement of the peptide in plasma[118,119] and in cell culture.

REFERENCES

1 **Hentze MW**, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. *Cell* 2004; **117**: 285-297
2 **Papanikolaou G**, Pantopoulos K. Iron metabolism and toxicity. *Toxicol Appl Pharmacol* 2005; **202**: 199-211
3 **Andrews NC**. Disorders of iron metabolism. *N Engl J Med* 1999; **341**: 1986-1995

4 **Pietrangelo A**. Hereditary hemochromatosis--a new look at an old disease. *N Engl J Med* 2004; **350**: 2383-2397
5 **Beutler E**. Hemochromatosis: genetics and pathophysiology. *Annu Rev Med* 2006; **57**: 331-347
6 **Adams PC**, Barton JC. Haemochromatosis. *Lancet* 2007; **370**: 1855-1860
7 **Ramm GA**, Ruddell RG. Hepatotoxicity of iron overload: mechanisms of iron-induced hepatic fibrogenesis. *Semin Liver Dis* 2005; **25**: 433-449
8 **Kowdley KV**. Iron, hemochromatosis, and hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S79-S86
9 **Pietrangelo A**. Hemochromatosis gene modifies course of hepatitis C viral infection. *Gastroenterology* 2003; **124**: 1509-1523
10 **Sebastiani G**, Walker AP. HFE gene in primary and secondary hepatic iron overload. *World J Gastroenterol* 2007; **13**: 4673-4689
11 **Andrews NC**, Schmidt PJ. Iron homeostasis. *Annu Rev Physiol* 2007; **69**: 69-85
12 **De Domenico I**, McVey Ward D, Kaplan J. Regulation of iron acquisition and storage: consequences for iron-linked disorders. *Nat Rev Mol Cell Biol* 2008; **9**: 72-81
13 **Nemeth E**, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004; **306**: 2090-2093
14 **Nemeth E**, Ganz T. Regulation of iron metabolism by hepcidin. *Annu Rev Nutr* 2006; **26**: 323-342
15 **Pigeon C**, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem* 2001; **276**: 7811-7819
16 **Nemeth E**, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004; **113**: 1271-1276
17 **Weiss G**, Goodnough LT. Anemia of chronic disease. *N Engl J Med* 2005; **352**: 1011-1023
18 **Nicolas G**, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest* 2002; **110**: 1037-1044
19 **Gehrke SG**, Kulaksiz H, Herrmann T, Riedel HD, Bents K, Veltkamp C, Stremmel W. Expression of hepcidin in hereditary hemochromatosis: evidence for a regulation in response to the serum transferrin saturation and to non-transferrin-bound iron. *Blood* 2003; **102**: 371-376
20 **Bridle KR**, Frazer DM, Wilkins SJ, Dixon JL, Purdie DM, Crawford DH, Subramaniam VN, Powell LW, Anderson GJ, Ramm GA. Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis. *Lancet* 2003; **361**: 669-673
21 **Roetto A**, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D, Camaschella C. Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nat Genet* 2003; **33**: 21-22
22 **Papanikolaou G**, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dube MP, Andres L, MacFarlane J, Sakellaropoulos N, Politou M, Nemeth E, Thompson J, Risler JK, Zaborowska C, Babakaiff R, Radomski CC, Pape TD, Davidas O, Christakis J, Brissot P, Lockitch G, Ganz T, Hayden MR, Goldberg YP. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. *Nat Genet* 2004; **36**: 77-82
23 **Feder JN**, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R Jr, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 1996; **13**: 399-408
24 **Camaschella C**, Roetto A, Cali A, De Gobbi M, Garozzo G, Carella M, Majorano N, Totaro A, Gasparini P. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. *Nat Genet* 2000; **25**: 14-15
25 **Piperno A**, Girelli D, Nemeth E, Trombini P, Bozzini C, Poggiali E, Phung Y, Ganz T, Camaschella C. Blunted hepcidin response to oral iron challenge in HFE-related hemochromatosis. *Blood* 2007; **110**: 4096-4100
26 **Nemeth E**, Roetto A, Garozzo G, Ganz T, Camaschella C. Hepcidin is decreased in TFR2 hemochromatosis. *Blood* 2005; **105**: 1803-1806
27 **Ahmad KA**, Ahmann JR, Migas MC, Waheed A, Britton RS, Bacon BR, Sly WS, Fleming RE. Decreased liver hepcidin expression in the Hfe knockout mouse. *Blood Cells Mol Dis* 2002; **29**: 361-366
28 **Muckenthaler M**, Roy CN, Custodio AO, Minana B, deGraaf J, Montross LK, Andrews NC, Hentze MW. Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis. *Nat Genet* 2003; **34**: 102-107
29 **Constante M**, Jiang W, Wang D, Raymond VA, Bilodeau M, Santos MM. Distinct requirements for Hfe in basal and induced hepcidin levels in iron overload and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2006; **291**: G229-G237
30 **Ludwiczek S**, Theurl I, Bahram S, Schumann K, Weiss G. Regulatory networks for the control of body iron homeostasis and their dysregulation in HFE mediated hemochromatosis. *J Cell Physiol* 2005; **204**: 489-499
31 **Huang FW**, Pinkus JL, Pinkus GS, Fleming MD, Andrews NC. A mouse model of juvenile hemochromatosis. *J Clin Invest* 2005; **115**: 2187-2191
32 **Niederkofler V**, Salie R, Arber S. Hemojuvelin is essential for dietary iron sensing, and its mutation leads to severe iron overload. *J Clin Invest* 2005; **115**: 2180-2186
33 **Kawabata H**, Fleming RE, Gui D, Moon SY, Saitoh T, O'Kelly J, Umehara Y, Wano Y, Said JW, Koeffler HP. Expression of hepcidin is down-regulated in TfR2 mutant mice manifesting a phenotype of hereditary hemochromatosis. *Blood* 2005; **105**: 376-381
34 **Wrighting DM**, Andrews NC. Interleukin-6 induces hepcidin expression through STAT3. *Blood* 2006; **108**: 3204-3209
35 **Pietrangelo A**, Dierssen U, Valli L, Garuti C, Rump A, Corradini E, Ernst M, Klein C, Trautwein C. STAT3 is required for IL-6-gp130-dependent activation of hepcidin in vivo. *Gastroenterology* 2007; **132**: 294-300
36 **Verga Falzacappa MV**, Vujic Spasic M, Kessler R, Stolte J, Hentze MW, Muckenthaler MU. STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. *Blood* 2007; **109**: 353-358
37 **Truksa J**, Peng H, Lee P, Beutler E. Different regulatory elements are required for response of hepcidin to interleukin-6 and bone morphogenetic proteins 4 and 9. *Br J Haematol* 2007; **139**: 138-147
38 **Truksa J**, Lee P, Peng H, Flanagan J, Beutler E. The distal location of the iron responsive region of the hepcidin promoter. *Blood* 2007; **110**: 3436-3437
39 **Wang RH**, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, Cooperman S, Eckhaus M, Rouault T, Mishra L, Deng CX. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. *Cell Metab* 2005; **2**: 399-409
40 **Babitt JL**, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, Andrews NC, Lin HY. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet* 2006; **38**: 531-539
41 **Truksa J**, Peng H, Lee P, Beutler E. Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6. *Proc Natl Acad Sci USA* 2006; **103**: 10289-10293
42 **Babitt JL**, Huang FW, Xia Y, Sidis Y, Andrews NC, Lin HY.

Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. *J Clin Invest* 2007; **117**: 1933-1939

43 **Courselaud B**, Pigeon C, Inoue Y, Inoue J, Gonzalez FJ, Leroyer P, Gilot D, Boudjema K, Guguen-Guillouzo C, Brissot P, Loreal O, Ilyin G. C/EBPalpha regulates hepatic transcription of hepcidin, an antimicrobial peptide and regulator of iron metabolism. Cross-talk between C/EBP pathway and iron metabolism. *J Biol Chem* 2002; **277**: 41163-41170

44 **Pak M**, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. *Blood* 2006; **108**: 3730-3735

45 **Vokurka M**, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. *Physiol Res* 2006; **55**: 667-674

46 **Tanno T**, Bhanu NV, Oneal PA, Goh SH, Staker P, Lee YT, Moroney JW, Reed CH, Luban NL, Wang RH, Eling TE, Childs R, Ganz T, Leitman SF, Fucharoen S, Miller JL. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nat Med* 2007; **13**: 1096-1101

47 **Pinto JP**, Ribeiro S, Pontes H, Thowfeequ S, Tosh D, Carvalho F, Porto G. Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPalpha. *Blood* 2008; **111**: 5727-5733

48 **Peyssonnaux C**, Zinkernagel AS, Schuepbach RA, Rankin E, Vaulont S, Haase VH, Nizet V, Johnson RS. Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). *J Clin Invest* 2007; **117**: 1926-1932

49 **Choi SO**, Cho YS, Kim HL, Park JW. ROS mediate the hypoxic repression of the hepcidin gene by inhibiting C/EBPalpha and STAT-3. *Biochem Biophys Res Commun* 2007; **356**: 312-317

50 **Braliou GG**, Verga Falzacappa MV, Chachami G, Casanovas G, Muckenthaler MU, Simos G. 2-Oxoglutarate-dependent oxygenases control hepcidin gene expression. *J Hepatol* 2008; **48**: 801-810

51 **Du X**, She E, Gelbart T, Truksa J, Lee P, Xia Y, Khovananth K, Mudd S, Mann N, Moresco EM, Beutler E, Beutler B. The serine protease TMPRSS6 is required to sense iron deficiency. *Science* 2008; **320**: 1088-1092

52 **Finberg KE**, Heeney MM, Campagna DR, Aydinok Y, Pearson HA, Hartman KR, Mayo MM, Samuel SM, Strouse JJ, Markianos K, Andrews NC, Fleming MD. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nat Genet* 2008; **40**: 569-571

53 **Folgueras AR**, de Lara FM, Pendas AM, Garabaya C, Rodriguez F, Astudillo A, Bernal T, Cabanillas R, Lopez-Otin C, Velasco G. Membrane-bound serine protease matriptase-2 (Tmprss6) is an essential regulator of iron homeostasis. *Blood* 2008; **112**: 2539-2545

54 **Guillem F**, Lawson S, Kannengiesser C, Westerman M, Beaumont C, Grandchamp B. Two nonsense mutations in the TMPRSS6 gene in a patient with microcytic anemia and iron deficiency. *Blood* 2008; **112**: 2089-2091

55 **Melis MA**, Cau M, Congiu R, Sole G, Barella S, Cao A, Westerman M, Cazzola M, Galanello R. A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. *Haematologica* 2008; **93**: 1473-1479

56 **Lebron JA**, Bennett MJ, Vaughn DE, Chirino AJ, Snow PM, Mintier GA, Feder JN, Bjorkman PJ. Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. *Cell* 1998; **93**: 111-123

57 **Feder JN**, Tsuchihashi Z, Irrinki A, Lee VK, Mapa FA, Morikang E, Prass CE, Starnes SM, Wolff RK, Parkkila S, Sly WS, Schatzman RC. The hemochromatosis founder mutation in HLA-H disrupts beta2-microglobulin interaction and cell surface expression. *J Biol Chem* 1997; **272**: 14025-14028

58 **Waheed A**, Parkkila S, Zhou XY, Tomatsu S, Tsuchihashi Z, Feder JN, Schatzman RC, Britton RS, Bacon BR, Sly WS. Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with beta2-microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells. *Proc Natl Acad Sci USA* 1997; **94**: 12384-12389

59 **de Almeida SF**, Fleming JV, Azevedo JE, Carmo-Fonseca M, de Sousa M. Stimulation of an unfolded protein response impairs MHC class I expression. *J Immunol* 2007; **178**: 3612-3619

60 **Allen KJ**, Gurrin LC, Constantine CC, Osborne NJ, Delatycki MB, Nicoll AJ, McLaren CE, Bahlo M, Nisselle AE, Vulpe CD, Anderson GJ, Southey MC, Giles GG, English DR, Hopper JL, Olynyk JK, Powell LW, Gertig DM. Iron-overload-related disease in HFE hereditary hemochromatosis. *N Engl J Med* 2008; **358**: 221-230

61 **Waalen J**, Beutler E. Iron-overload-related disease in HFE hereditary hemochromatosis. *N Engl J Med* 2008; **358**: 2293-2294; author reply 2294-2295

62 **Beutler E**. Iron storage disease: facts, fiction and progress. *Blood Cells Mol Dis* 2007; **39**: 140-147

63 **Zhou XY**, Tomatsu S, Fleming RE, Parkkila S, Waheed A, Jiang J, Fei Y, Brunt EM, Ruddy DA, Prass CE, Schatzman RC, O'Neill R, Britton RS, Bacon BR, Sly WS. HFE gene knockout produces mouse model of hereditary hemochromatosis. *Proc Natl Acad Sci USA* 1998; **95**: 2492-2497

64 **Bahram S**, Gilfillan S, Kuhn LC, Moret R, Schulze JB, Lebeau A, Schumann K. Experimental hemochromatosis due to MHC class I HFE deficiency: immune status and iron metabolism. *Proc Natl Acad Sci USA* 1999; **96**: 13312-13317

65 **de Sousa M**, Reimao R, Lacerda R, Hugo P, Kaufmann SH, Porto G. Iron overload in beta 2-microglobulin-deficient mice. *Immunol Lett* 1994; **39**: 105-111

66 **Rothenberg BE**, Voland JR. beta2 knockout mice develop parenchymal iron overload: A putative role for class I genes of the major histocompatibility complex in iron metabolism. *Proc Natl Acad Sci USA* 1996; **93**: 1529-1534

67 **Levy JE**, Montross LK, Cohen DE, Fleming MD, Andrews NC. The C282Y mutation causing hereditary hemochromatosis does not produce a null allele. *Blood* 1999; **94**: 9-11

68 **Tomatsu S**, Orii KO, Fleming RE, Holden CC, Waheed A, Britton RS, Gutierrez MA, Velez-Castrillon S, Bacon BR, Sly WS. Contribution of the H63D mutation in HFE to murine hereditary hemochromatosis. *Proc Natl Acad Sci USA* 2003; **100**: 15788-15793

69 **Fleming RE**, Holden CC, Tomatsu S, Waheed A, Brunt EM, Britton RS, Bacon BR, Roopenian DC, Sly WS. Mouse strain differences determine severity of iron accumulation in Hfe knockout model of hereditary hemochromatosis. *Proc Natl Acad Sci USA* 2001; **98**: 2707-2711

70 **Levy JE**, Montross LK, Andrews NC. Genes that modify the hemochromatosis phenotype in mice. *J Clin Invest* 2000; **105**: 1209-1216

71 **Sproule TJ**, Jazwinska EC, Britton RS, Bacon BR, Fleming RE, Sly WS, Roopenian DC. Naturally variant autosomal and sex-linked loci determine the severity of iron overload in beta 2-microglobulin-deficient mice. *Proc Natl Acad Sci USA* 2001; **98**: 5170-5174

72 **Feder JN**, Penny DM, Irrinki A, Lee VK, Lebron JA, Watson N, Tsuchihashi Z, Sigal E, Bjorkman PJ, Schatzman RC. The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc Natl Acad Sci USA* 1998; **95**: 1472-1477

73 **Lebron JA**, West AP Jr, Bjorkman PJ. The hemochromatosis protein HFE competes with transferrin for binding to the transferrin receptor. *J Mol Biol* 1999; **294**: 239-245

74 **Bennett MJ**, Lebron JA, Bjorkman PJ. Crystal structure of the hereditary haemochromatosis protein HFE complexed with transferrin receptor. *Nature* 2000; **403**: 46-53

75 **West AP Jr**, Bennett MJ, Sellers VM, Andrews NC, Enns CA, Bjorkman PJ. Comparison of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the

hereditary hemochromatosis protein HFE. *J Biol Chem* 2000; **275**: 38135-38138
76 **Ponka P**, Beaumont C, Richardson DR. Function and regulation of transferrin and ferritin. *Semin Hematol* 1998; **35**: 35-54
77 **Roy CN**, Penny DM, Feder JN, Enns CA. The hereditary hemochromatosis protein, HFE, specifically regulates transferrin-mediated iron uptake in HeLa cells. *J Biol Chem* 1999; **274**: 9022-9028
78 **Riedel HD**, Muckenthaler MU, Gehrke SG, Mohr I, Brennan K, Herrmann T, Fitscher BA, Hentze MW, Stremmel W. HFE downregulates iron uptake from transferrin and induces iron-regulatory protein activity in stably transfected cells. *Blood* 1999; **94**: 3915-3921
79 **Muckenthaler M**, Richter A, Gunkel N, Riedel D, Polycarpou-Schwarz M, Hentze S, Falkenhahn M, Stremmel W, Ansorge W, Hentze MW. Relationships and distinctions in iron-regulatory networks responding to interrelated signals. *Blood* 2003; **101**: 3690-3698
80 **Corsi B**, Levi S, Cozzi A, Corti A, Altimare D, Albertini A, Arosio P. Overexpression of the hereditary hemochromatosis protein, HFE, in HeLa cells induces and iron-deficient phenotype. *FEBS Lett* 1999; **460**: 149-152
81 **Gross CN**, Irrinki A, Feder JN, Enns CA. Co-trafficking of HFE, a nonclassical major histocompatibility complex class I protein, with the transferrin receptor implies a role in intracellular iron regulation. *J Biol Chem* 1998; **273**: 22068-22074
82 **Wang J**, Chen G, Pantopoulos K. The haemochromatosis protein HFE induces an apparent iron-deficient phenotype in H1299 cells that is not corrected by co-expression of beta 2-microglobulin. *Biochem J* 2003; **370**: 891-899
83 **Zhang AS**, Davies PS, Carlson HL, Enns CA. Mechanisms of HFE-induced regulation of iron homeostasis: Insights from the W81A HFE mutation. *Proc Natl Acad Sci USA* 2003; **100**: 9500-9505
84 **Parkkila S**, Waheed A, Britton RS, Feder JN, Tsuchihashi Z, Schatzman RC, Bacon BR, Sly WS. Immunohistochemistry of HLA-H, the protein defective in patients with hereditary hemochromatosis, reveals unique pattern of expression in gastrointestinal tract. *Proc Natl Acad Sci USA* 1997; **94**: 2534-2539
85 **Waheed A**, Parkkila S, Saarnio J, Fleming RE, Zhou XY, Tomatsu S, Britton RS, Bacon BR, Sly WS. Association of HFE protein with transferrin receptor in crypt enterocytes of human duodenum. *Proc Natl Acad Sci USA* 1999; **96**: 1579-1584
86 **Roy CN**, Enns CA. Iron homeostasis: new tales from the crypt. *Blood* 2000; **96**: 4020-4027
87 **Trinder D**, Olynyk JK, Sly WS, Morgan EH. Iron uptake from plasma transferrin by the duodenum is impaired in the Hfe knockout mouse. *Proc Natl Acad Sci USA* 2002; **99**: 5622-5626
88 **Davies PS**, Enns CA. Expression of the hereditary hemochromatosis protein HFE increases ferritin levels by inhibiting iron export in HT29 cells. *J Biol Chem* 2004; **279**: 25085-25092
89 **Pietrangelo A**, Rocchi E, Casalgrandi G, Rigo G, Ferrari A, Perini M, Ventura E, Cairo G. Regulation of transferrin, transferrin receptor, and ferritin genes in human duodenum. *Gastroenterology* 1992; **102**: 802-809
90 **Pietrangelo A**, Casalgrandi G, Quaglino D, Gualdi R, Conte D, Milani S, Montosi G, Cesarini L, Ventura E, Cairo G. Duodenal ferritin synthesis in genetic hemochromatosis. *Gastroenterology* 1995; **108**: 208-217
91 **Montosi G**, Paglia P, Garuti C, Guzman CA, Bastin JM, Colombo MP, Pietrangelo A. Wild-type HFE protein normalizes transferrin iron accumulation in macrophages from subjects with hereditary hemochromatosis. *Blood* 2000; **96**: 1125-1129
92 **Drakesmith H**, Sweetland E, Schimanski L, Edwards J, Cowley D, Ashraf M, Bastin J, Townsend AR. The hemochromatosis protein HFE inhibits iron export from macrophages. *Proc Natl Acad Sci USA* 2002; **99**: 15602-15607
93 **Moura E**, Noordermeer MA, Verhoeven N, Verheul AF, Marx JJ. Iron release from human monocytes after erythrophagocytosis in vitro: an investigation in normal subjects and hereditary hemochromatosis patients. *Blood* 1998; **92**: 2511-2519
94 **Zhang AS**, Xiong S, Tsukamoto H, Enns CA. Localization of iron metabolism-related mRNAs in rat liver indicate that HFE is expressed predominantly in hepatocytes. *Blood* 2004; **103**: 1509-1514
95 **Vujic Spasic M**, Kiss J, Herrmann T, Kessler R, Stolte J, Galy B, Rathkolb B, Wolf E, Stremmel W, Hentze MW, Muckenthaler MU. Physiologic systemic iron metabolism in mice deficient for duodenal Hfe. *Blood* 2007; **109**: 4511-4517
96 **Vujic Spasic M**, Kiss J, Herrmann T, Galy B, Martinache S, Stolte J, Grone HJ, Stremmel W, Hentze MW, Muckenthaler MU. Hfe acts in hepatocytes to prevent hemochromatosis. *Cell Metab* 2008; **7**: 173-178
97 **Wigg AJ**, Harley H, Casey G. Heterozygous recipient and donor HFE mutations associated with a hereditary haemochromatosis phenotype after liver transplantation. *Gut* 2003; **52**: 433-435
98 **Crawford DH**, Fletcher LM, Hubscher SG, Stuart KA, Gane E, Angus PW, Jeffrey GP, McCaughan GW, Kerlin P, Powell LW, Elias EE. Patient and graft survival after liver transplantation for hereditary hemochromatosis: Implications for pathogenesis. *Hepatology* 2004; **39**: 1655-1662
99 **Montosi G**, Corradini E, Garuti C, Barelli S, Recalcati S, Cairo G, Valli L, Pignatti E, Vecchi C, Ferrara F, Pietrangelo A. Kupffer cells and macrophages are not required for hepatic hepcidin activation during iron overload. *Hepatology* 2005; **41**: 545-552
100 **Lou DQ**, Lesbordes JC, Nicolas G, Viatte L, Bennoun M, Van Rooijen N, Kahn A, Renia L, Vaulont S. Iron- and inflammation-induced hepcidin gene expression in mice is not mediated by Kupffer cells in vivo. *Hepatology* 2005; **41**: 1056-1064
101 **Makui H**, Soares RJ, Jiang W, Constante M, Santos MM. Contribution of Hfe expression in macrophages to the regulation of hepatic hepcidin levels and iron loading. *Blood* 2005; **106**: 2189-2195
102 **Theurl I**, Theurl M, Seifert M, Mair S, Nairz M, Rumpold H, Zoller H, Bellmann-Weiler R, Niederegger H, Talasz H, Weiss G. Autocrine formation of hepcidin induces iron retention in human monocytes. *Blood* 2008; **111**: 2392-2399
103 **Goswami T**, Andrews NC. Hereditary hemochromatosis protein, HFE, interaction with transferrin receptor 2 suggests a molecular mechanism for mammalian iron sensing. *J Biol Chem* 2006; **281**: 28494-28498
104 **Chen J**, Chloupkova M, Gao J, Chapman-Arvedson TL, Enns CA. HFE modulates transferrin receptor 2 levels in hepatoma cells via interactions that differ from transferrin receptor 1-HFE interactions. *J Biol Chem* 2007; **282**: 36862-36870
105 **Kawabata H**, Yang R, Hirama T, Vuong PT, Kawano S, Gombart AF, Koeffler HP. Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. *J Biol Chem* 1999; **274**: 20826-20832
106 **Johnson MB**, Enns CA. Diferric transferrin regulates transferrin receptor 2 protein stability. *Blood* 2004; **104**: 4287-4293
107 **Robb A**, Wessling-Resnick M. Regulation of transferrin receptor 2 protein levels by transferrin. *Blood* 2004; **104**: 4294-4299
108 **Schmidt PJ**, Toran PT, Giannetti AM, Bjorkman PJ, Andrews NC. The transferrin receptor modulates Hfe-dependent regulation of hepcidin expression. *Cell Metab* 2008; **7**: 205-214
109 **Levy JE**, Jin O, Fujiwara Y, Kuo F, Andrews NC. Transferrin receptor is necessary for development of erythrocytes and the nervous system. *Nat Genet* 1999; **21**: 396-399
110 **Pantopoulos K**. Iron metabolism and the IRE/IRP regulatory

system: an update. *Ann N Y Acad Sci* 2004; **1012**: 1-13
111 **Lee P**, Peng H, Gelbart T, Beutler E. The IL-6- and lipopolysaccharide-induced transcription of hepcidin in HFE-, transferrin receptor 2-, and beta 2-microglobulin-deficient hepatocytes. *Proc Natl Acad Sci USA* 2004; **101**: 9263-9265
112 **Frazer DM**, Wilkins SJ, Millard KN, McKie AT, Vulpe CD, Anderson GJ. Increased hepcidin expression and hypoferraemia associated with an acute phase response are not affected by inactivation of HFE. *Br J Haematol* 2004; **126**: 434-436
113 **Roy CN**, Custodio AO, de Graaf J, Schneider S, Akpan I, Montross LK, Sanchez M, Gaudino A, Hentze MW, Andrews NC, Muckenthaler MU. An Hfe-dependent pathway mediates hyposideremia in response to lipopolysaccharide-induced inflammation in mice. *Nat Genet* 2004; **36**: 481-485
114 **Cardoso EM**, Macedo MG, Rohrlich P, Ribeiro E, Silva MT, Lemonnier FA, de Sousa M. Increased hepatic iron in mice lacking classical MHC class I molecules. *Blood* 2002; **100**: 4239-4241
115 **Rohrlich PS**, Fazilleau N, Ginhoux F, Firat H, Michel F, Cochet M, Laham N, Roth MP, Pascolo S, Nato F, Coppin H, Charneau P, Danos O, Acuto O, Ehrlich R, Kanellopoulos J, Lemonnier FA. Direct recognition by alphabeta cytolytic T cells of Hfe, a MHC class Ib molecule without antigen-presenting function. *Proc Natl Acad Sci USA* 2005; **102**: 12855-12860
116 **Porto G**, De Sousa M. Iron overload and immunity. *World J Gastroenterol* 2007; **13**: 4707-4715
117 **de Almeida SF**, de Sousa M. The unfolded protein response in hereditary haemochromatosis. *J Cell Mol Med* 2008; **12**: 421-434
118 **Tomosugi N**, Kawabata H, Wakatabe R, Higuchi M, Yamaya H, Umehara H, Ishikawa I. Detection of serum hepcidin in renal failure and inflammation by using ProteinChip System. *Blood* 2006; **108**: 1381-1387
119 **Swinkels DW**, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, Tjalsma H. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. *PLoS ONE* 2008; **3**: e2706

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EDITORIAL

Current treatment indications and strategies in chronic hepatitis B virus infection

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Abstract

The optimal approach to the management of several marginal cases with chronic hepatitis B virus (HBV) infection is controversial. Serum HBV DNA and aminotransferase levels, and the degree of necroinflammation and fibrosis determine the therapeutic decisions. All patients with elevated aminotransferase (> twice the upper limit of normal) and serum HBV DNA above 20 000 IU/mL should be treated. Liver biopsy is important for therapeutic decisions in cases with mild aminotransferase elevations and serum HBV DNA below 20 000 IU/mL. Chronic HBV patients who do not receive treatment should be followed for life. There are seven agents licensed for chronic hepatitis B: standard and pegylated interferon-alpha, lamivudine, adefovir, entecavir, telbivudine and tenofovir. One-year courses with pegylated interferon-alpha induce sustained off-therapy remission in 30%-32% of patients with HBeAg-positive chronic hepatitis B and in a smaller proportion of patients with HBeAg-negative chronic hepatitis B. Oral antivirals achieve initial on-therapy responses in the majority of patients, but are intended as long-term therapies. Viral suppression has favourable effects on patients' outcome and modifies the natural course of the disease. Viral resistance, however, is the major drawback of long-term oral antiviral therapy. Lamivudine monotherapy is associated with the highest and entecavir monotherapy with the lowest resistance rate so far. There has been no resistance to tenofovir, but after only 18 mo of treatment to date. The optimal first-line anti-HBV therapy with the best long-term cost/benefit ratio remains unclear. If oral antiviral agents are used, compliance should always be ascertained and HBV DNA levels should be regularly tested.

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Key words: Hepatitis B; Hepatitis B virus DNA; Interferon; Antivirals; Resistance

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INTRODUCTION

Despite the universal vaccination of neonates and infants during the last years and the subsequent reduction in the incidence of new infections with hepatitis B virus (HBV), chronic HBV infection remains a significant public health problem worldwide[1,2]. It is estimated that there are approximately 400 000 000 people with chronic HBV infection and that more than 500 000 people die every year due to complications of HBV related chronic liver disease[2]. Although considerable improvements in the evaluation and treatment of patients with chronic HBV infection have occurred during the last decade, several issues regarding the optimal management of such patients still remain controversial. This short review focuses on two such controversies: the most appropriate treatment indications and the optimal therapeutic strategy for patients with chronic hepatitis B (CHB).

TREATMENT INDICATIONS

Every patient with chronic HBV infection is potentially infectious and at risk for liver complications and is ide-

ally a candidate for therapy, if the virus can be eradicated[3,4]. However, current medications rarely achieve viral eradication in patients with chronic HBV infection and therefore only patients who are at risk for progression to advanced liver disease should be considered for treatment[3,5,6]. Thus, the knowledge of the natural history and the significance of the elements used in the evaluation of disease are necessary for decisions on treatment indications. Moreover, the efficacy and safety of current therapies may also affect the treatment indications, as there is no reason to recommend an ineffective therapy to any patient, even if they have progressive liver disease.

In general, the natural history of chronic HBV infection includes four phases of variable duration distinguished by the presence of hepatitis B e antigen (HBeAg) or its antibody (anti-HBe) in the serum and the serum HBV DNA and aminotransferases levels[7-9]. It starts with an HBeAg-positive, immune-tolerant phase, characterized by high viremia, normal serum aminotransferases and minimal histological changes. The phase of HBeAg-positive CHB follows at a variable rate. It may also be called the HBeAg seroconversion phase and is characterized by positive HBeAg, high serum HBV DNA levels, elevated aminotransferases and active necroinflammation and/or fibrosis. The annual probability of HBeAg seroconversion (disappearance of HBeAg and development of anti-HBe) depends on several factors, such as the age of acute infection and HBV genotype, and is lower in Asian patients infected at birth (lower in those with genotype C than B) and higher in Caucasian patients infected during childhood, adolescence or adulthood[7,10-12]. If HBeAg seroconversion occurs, patients progress to the HBeAg-negative phases, which can be separated into the inactive carrier state and the HBeAg-negative CHB phase. The inactive chronic HBV carrier state is characterized by low levels of viral replication, normal aminotransferases and minimal histological lesions, while HBeAg-negative CHB is characterized by higher viral replication, elevated aminotransferases and active liver necroinflammation and fibrosis[9,13]. HBeAg-negative CHB may develop immediately after the HBeAg seroconversion phase or after several years of an inactive chronic carrier state[9], but many inactive chronic HBV carriers never progress to the HBeAg-negative CHB phase.

General indications for treatment in chronic hepatitis B

Significant histological lesions and progression of liver disease are observed almost exclusively in patients with HBeAg-positive and HBeAg-negative CHB, therefore these patients are considered as cases with widely accepted treatment indications. In clinical practice, HBeAg-positive or HBeAg-negative CHB can be diagnosed in patients with compensated chronic HBV infection (positive or negative HBeAg respectively) by evidence of viral replication (high serum HBV DNA levels) and biochemical and histological evidence of hepatocellular injury [increased alanine aminotransferase (ALT) activity and liver histological lesions at liver biopsy]. On the other hand, treatment indications are based on specific criteria and require certain cut-off points, which may sometimes be arbitrary due to the lack of strong data to support them. Treatment indications currently focus on serum HBV DNA levels, ALT activity and severity of liver histological lesions.

According to the most recent guidelines, treatment is recommended to all patients with either HBeAg-positive or HBeAg-negative CHB who have serum HBV DNA > 20 000 IU/mL and ALT higher than two times the upper limit of normal (> 2 xULN) for at least 3 mo[5,6,14]. In such cases, liver biopsy is considered to be optional, as it may offer prognostic information but it is not expected to affect the decision to treat. On the other hand, treatment is also recommended in HBeAg-positive CHB patients with ALT between 1-2 x ULN or HBeAg-negative CHB patients with ALT between 1-2 xULN and serum HBV DNA between 2000-20 000 IU/mL who have at least moderate necroinflammatory activity and/or significant fibrosis[5,6]. So, liver biopsy is mandatory in the latter cases with mild ALT elevations or relatively low viremia levels.

Controversial issues-The role of serum HBV DNA

All patients with chronic HBV infection are at increased risk for hepatocellular carcinoma (HCC) compared with the general population; while the risk increases substantially in patients with prolonged high viremia and cirrhosis[15,16]. Recent data suggest that patients with chronic HBV infection and HBV DNA above 10^4 copies/mL (approximately 2000 IU/mL) are at increased risk for cirrhosis and HCC regardless of ALT activity and are therefore possible candidates for treatment[16,17]. Such data have created some debate on whether patients in the HBeAg-positive immune tolerant phase, who have very high serum HBV DNA levels, should be left untreated. However, because they have minimal to mild histological liver lesions and the currently available agents may reduce viremia but offer minimal chances to induce HBeAg seroconversion[3,5,6], there is currently no widely accepted indication for treatment in such cases.

Despite the recent advances in the treatment indications, several issues remain unanswered, most probably because of the fluctuating activity of chronic HBV infection and the existence of patients who do not fulfil all criteria. HBeAg-negative CHB is defined as increased ALT/AST, serum HBV-DNA > 2000 IU/mL and moderate/severe necroinflammation, while inactive HBV carrier state is defined as persistently normal ALT/AST on ≥ 3-4 3-monthly determinations (then every 6-12 mo) and HBV-DNA < 2000 IU/mL[5]. However, it is well known that viremia levels fluctuate substantially in patients with HBeAg-negative CHB, often being lower than 2000 IU/mL. In one study that included mostly Chinese patients, 42% of cases with HBeAg-negative CHB occasionally had serum HBV DNA levels < 10 000 copies/mL (or approximately < 2000 IU/mL)[18]. In one recently published, prospective study from our group, 5% of HBeAg-negative CHB patients and 16% of their

serum samples had HBV DNA levels < 2000 IU/mL, while HBV DNA was persistently ⩾ 2000 IU/mL in 82% or occasionally < 2000 IU/mL in 18% of our HBeAg-negative CHB patients[19]. Although some patients may eventually develop HBV DNA levels > 2000 IU/mL after repeated testing, it is not clear how often and how many times HBV DNA determinations should be repeated. Since the current treatment indications include increased ALT and HBV DNA > 2000 IU/mL[5], HBeAg-negative patients with increased ALT/AST and HBV DNA < 2000 IU/mL are not considered for liver biopsy and are excluded from treatment. However, they may represent a sizeable proportion of HBeAg-negative CHB patients in clinical practice. In a retrospective, multicenter Greek study including 399 HBeAg-negative chronic HBV patients with increased ALT, at least moderate necroinflammation and/or moderate fibrosis were detected in the majority (62%) of such cases, who had HBV DNA < 2000 IU/mL and represented 14% of the total study population[20]. Therefore, liver biopsy is an useful tool in the evaluation of patients with persistent or transient mild transaminase elevations, regardless of the levels of serum HBV DNA.

On the other hand, if viremia is considered as the main criterion for performing a liver biopsy in order to decide the treatment indications, then the 2000 IU/mL cut-off serum HBV DNA level may cause a substantial proportion of inactive carriers to undergo unnecessary liver biopsies. In our prospective cohort of 150 HBeAg-negative chronic HBV patients with close biochemical and virological follow-up, a substantial proportion (22% of cases or 28% of 228 serum samples tested) of 85 patients with persistently normal ALT have HBV-DNA > 2000 IU/mL (2000-5000 IU/mL: 15%, 5000-20 000 IU/mL: 7%)[19]. According to our data, in 35 such HBeAg-negative cases with persistently normal ALT and HBV DNA > 2000 IU/mL, liver biopsies showed minimal necroinflammation in all but one (mild in one case) and minimal to mild fibrosis in 29 (83%) of them [fibrosis score of 2 in Ishak's scoring system was detected in the remaining 6 (17%) cases][20]. Thus, such cases seem to represent true inactive chronic HBV carriers, who require only close follow-up but no therapeutic intervention. It should be noted, however, that all HBeAg-negative cases with persistently normal ALT had HBV DNA < 20 000 IU/mL and that HBeAg-negative cases with persistently normal ALT and HBV DNA ⩾ 20 000 IU/mL are extremely rare in Greece. Close follow-up still remains the cornerstone of diagnosis. HBeAg-negative cases with relatively high normal ALT values (> 30 IU/L for men and > 20 IU/L for women) and/or serum HBV DNA ⩾ 2000 IU/mL warrant closer follow-up, because they are at increased risk of developing HBeAg-negative CHB in the future[19].

Indications for treatment in specific settings

Current guidelines support the view that all patients with decompensated liver disease and detectable serum HBV DNA should receive antiviral treatment regardless of aminotransferases and viremia levels[5]. The main goal of treatment in this group of severely ill patients is to completely inhibit viral replication in order to improve liver function and survival and, in case of liver transplantation, to prevent graft re-infection[21-23].

Another very important indication of anti-HBV therapy is the prevention of HBV reactivation in chronic HBV patients who are treated with corticosteroids or any other immunosuppressive therapy or cancer chemotherapy. It is well known that reactivation occurs in 29%-56% of chronic HBV carriers treated with chemotherapy and even in a small proportion of HBsAg negative and anti-HBc positive subjects regardless of anti-HBs status[24,25]. HBV reactivation after immunosuppressive therapy may have deleterious effects, often resulting in acute liver failure and death, therefore all patients who are going to be treated with any type of immunosuppression should be tested for HBsAg and receive pre-emptive anti-HBV therapy in case they are positive for HBsAg[5,26].

THERAPEUTIC STRATEGIES

In CHB, the more realistic therapeutic targets are suppression of HBV replication, induction of biochemical remission and ultimately prevention of cirrhosis and HCC[3,5,6]. Studies in non-Asian patients have shown that long-term benefits on survival are strongly linked to the induction of sustained HBeAg seroconversion in HBeAg-positive CHB[27-30] and with sustained biochemical and virological remission in HBeAg-negative CHB[31,32]. HBeAg seroconversion, however, may not be a sufficient therapeutic end-point in all patients with HBeAg-positive CHB, since some of them may subsequently develop HBeAg-negative CHB. This might depend on the patients' origin and perhaps the HBV genotype. Asian studies suggested that interferon-alpha (IFNα) induced HBeAg seroconversion improves the long-term outcome[33,34] but others reported no long-term benefit from IFNα therapy[35]. Thus, the induction of persistent biochemical and virological remission appears to be the most important therapeutic target in CHB, as the risk of major complications is strongly related to the viremia levels being independent of the HBeAg status[16,17,36]. Effective long-term antiviral therapy has been shown to prevent or diminish the development of decompensation and major complications in patients with advanced fibrosis or cirrhosis and to improve patients' outcome and survival[32,37,38].

Currently, there are seven drugs licensed for treatment of CHB: standard IFNa, pegylated IFNα-2a (Peg-IFNa-2a) (Peg-IFNα-2b is also licensed in some countries), lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (TBV) and tenofovir disoproxil fumarate (TDF). These drugs may be broadly classified into (1) IFNalphas (standard or pegylated), which have both antiviral and immunomodulatory activities and are administered subcutaneously, and (2) the pure antiviral agents (LAM, ADV, ETV, TBV, TDF), which are analogs of natural nucleosides (LAM, ETV, TBV) or nucleotides (ADV, TDF) and are administered orally once daily.

Anti-HBV agents can be given as therapies of finite duration or as long-term therapies[4].

Therapies of finite duration

Therapies of finite duration are usually given for 12 mo and aim to induce sustained off-treatment response[3,4]. In current clinical practice, only IFNα/Peg-IFNα are used as therapy of finite duration in chronic hepatitis B, because sustained off-therapy responses after one-year courses with oral antivirals are rather limited.

In HBeAg-positive CHB, HBeAg seroconversion rates were reported to be 25%-33% with IFNα (5 MU daily or 10 MU thrice weekly for 4-6 mo) or Peg-IFNα (Peg-IFNα-2a: 180 μg/wk, Peg-IFNα-2b: 100 μg/wk for 12 mo)[39-41], 18%-22% after 12-mo courses with LAM (100 mg daily)[42-44], ETV (0.5 mg daily)[44], TBV (600 mg daily)[45] or TDF (300 mg daily) and only 12% with ADV (10 mg daily)[46]. The antiviral potency expressed by the reduction in serum HBV DNA levels differs among the anti-HBV agents (highest with TDF, ETV and TBV, intermediate with LAM and lowest with IFNα/Peg-IFNα and ADV), but these differences do not translate into different HBeAg seroconversion rates, at least within the first year of therapy. Patients with genotypes A and B respond better to IFNα/Peg-IFNα than those with genotypes D and C[34,40,41], while response rates to oral antivirals are not affected by the HBV genotype[5].

In HBeAg-negative CHB, cohort studies using insensitive virological assays showed that 12- or 24-mo courses of standard IFNα (3 or 5 MU thrice weekly) may achieve sustained long-term off-therapy biochemical and virological responses in 22%-30% of patients[47,48], who often (> 40%) clear HBsAg after some years[31,47]. More recently, Peg-IFNα-2α (180 μg/wk for 12 mo) was reported to induce biochemical and virological response rates of 35% at 24 wk post-treatment[49], which decreased with further follow-up to 25%-30% at 1-3 post-treatment years[49-51]. Again, 35% of sustained responders lost HBsAg over the 3-year post-treatment follow-up[51]. On the other hand, 12-mo courses with oral antivirals achieve high on-therapy response rates (> 75%), but sustained off-therapy responses are rare (< 8%-11%)[52-54] and therefore these agents are only given as long-term therapies.

Anti-HBV treatment is also given for finite duration in chronic inactive HBV carriers who receive immunosuppressive therapy[5,26]. In such cases, most of the data comes from pre-emptive LAM therapy, which has been shown to prevent or ameliorate the course of HBV reactivation[26,55,56]. However, it is anticipated that all oral anti-HBV agents will also be effective in the prevention of HBV reactivation, while the newer, more potent agents are expected to be more effective in patients who develop clinically apparent HBV reactivation after immunosuppressive therapy. Pre-emptive oral anti-HBV therapy should ideally start at least 2 wk before the onset of immunosuppressive therapy and should continue for at least 6-12 mo after the completion of immunosuppressive courses[5,26].

Long-term therapies

Long-term or "maintenance" therapy is the most commonly used treatment strategy in CHB, because IFNa/Peg-IFNa as a therapy of finite duration can achieve sustained off-therapy responses only in a minority of cases[3,4,57], while a proportion of patients do not wish to be treated with IFNα/Peg-IFNα because of the frequently anticipated side effects or cannot tolerate or have contraindications to IFNα/Peg-IFNα therapy[40,41,49]. Only oral antiviral agents are used as long-term therapies because of their good tolerability, safety profile and on-therapy efficacy[3,4,9]. In particular, for patients with HBV decompensated cirrhosis, life-long treatment with a potent oral anti-HBV agent is the only possible therapeutic option[5].

In HBeAg-positive CHB, prolongation of therapy with oral antiviral agents has been shown to increase the probability of HBeAg seroconversion, although the interpretation of most of such long-term data needs close scrutiny as protocols for treatment after one year have differed markedly. The duration of therapy after HBeAg seroconversion induced by oral antivirals seems to be rather important for the maintenance of response[5]. There are no good studies to define the optimal duration of antiviral therapy, but most experts and recommendations now agree that any oral antiviral agent should continue for at least 6 mo after HBeAg seroconversion in order to maximize the possibility of sustained off-therapy responses[5]. Thus, treatment may stop after a certain consolidation period in HBeAg-positive CHB patients who achieve HBeAg seroconversion.

In HBeAg-negative CHB, however, it is still unclear whether long-term courses with oral antivirals can induce sizeable sustained off-therapy response rates. In particular, there are some conflicting reports for long-term LAM courses[58,59] and one encouraging report for ADV[60]. In the latter study, 67% of 33 patients maintained sustained off-therapy biochemical and virological (HBV DNA < 50000 cp/mL) remission for a median of 17 mo after discontinuation of a 4-5-year effective ADV course[60]. Thus, sustained off-therapy responses may be achieved in a proportion for HBeAg-negative CHB patients treated successfully for some years with oral antiviral agents, but more studies are needed before firm conclusions can be drawn.

Viral resistance

The main limitation of long-term therapies with antiviral agents is the progressively increasing rates of viral resistance due to selection of treatment resistant HBV mutant strains[61-63]. The emergence of genotypic viral resistance is clinically expressed by the subsequent development of virological breakthrough or secondary antiviral treatment failure, which is usually defined as reappearance or $\geqslant 1$ $\log_{10}$ IU/mL increase after initial lack of detection or initial $\geqslant 1$ $\log_{10}$ IU/mL reduction of serum HBV DNA[5,64,65]. Genotypic resistance may be also detected in patients without virological response or with primary antiviral treatment failure (no or < 1

log_{10} IU/mL decrease of HBV DNA), while virological breakthroughs may also develop from non-compliance to therapy[5,61,65,66]. Virological breakthroughs are usually followed by biochemical breakthroughs[67,68], which eventually worsen liver histology[67,69] and may even result in decompensation and death, particularly in patients with pre-existing cirrhosis[21,22,32,66].

Viral resistance may develop under any anti-HBV oral agent, but the rate of resistance differs markedly among the different agents. Long-term LAM monotherapy results in rather high rates of resistance due to emergence of HBV strains with mutation within the YMDD motif (rtM204V/I with or without rtL180M)[61,63,64,70,71]. LAM resistance rates usually exceed 15%-20% at year-1 and 60%-65% at year-4[38,67,72,73]. Due to the high probability of resistance, LAM monotherapy is not currently considered as an optimal first-line long-term therapy for CHB[5,14]. However, LAM is still used in many countries because of its low cost. Resistance also increases progressively with prolongation of ADV monotherapy but at much slower rates compared to LAM[74]. There are no good long-term ADV data in naïve patients with HBeAg-positive CHB. In HBeAg-negative CHB, ADV resistant strains (rtN236T and/or rtA181V/T mutations)[63,71,75], first emerge during the second year of therapy reaching cumulative rates of 3% at week-96 and 29% at week-240[74]. Resistance to ETV in nucleoside naive CHB patients seems to be rather rare, since it has been detected in < 1.5% of naive patients treated with ETV monotherapy for up to five years[76]. ETV resistance requires selection of two LAM resistant mutations (rtM204V/I and rtL180M) and at least one additional substitution (rtI169, rtT184, rtS202, or rtM250)[77]. TBV also selects for mutations in the YMDD motif with only rtM204I resistant strains being detected to date[78]. TBV resistance has been observed in 4.4% and 21.6% of HBeAg-positive and 2.7% and 8.6% of HBeAg-negative CHB patients treated with TBV for 1 and 2 years, respectively[45,79]. No resistance to TDF has been reported to date after 18 mo of therapy in monoinfected CHB patients.

Resistance is the major limitation of long-term oral antiviral therapy, therefore its management is of great importance. First, there are several strategies to prevent the development of resistance. Such strategies include: (1) the use as first-line therapy of agents with high genetic barrier or low resistance profile, such as TDF and ETV; (2) the non-use of agents with high probability of resistance, such as LAM; and (3) the careful on-treatment monitoring and the prompt modification of therapy that does not result in complete suppression of HBV replication. Since residual viremia at 6 mo of TBV or LAM and at 12 mo of ADV monotherapy represents the strongest risk factor for subsequent resistance[74,80], some treatment algorithms suggest that antiviral therapy should be modified in CHB patients who remain viremic after the first 6 or 12 mo therapy with these agents.

Whatever strategy is adopted, resistance may eventually develop and therefore management of HBV strains with resistance against some agents is often required. The wide use of LAM monotherapy in CHB during the last 7-8 years, an anti-HBV therapeutic strategy with a poor resistance profile[63,65], has progressively increased the numbers of patients with LAM resistant HBV mutant strains[57,81]. ADV, ETV (1.0 mg daily) and TDF have been shown to be effective in patients with LAM resistance[65,82-85]. Pre-existing LAM resistant mutations favour the emergence of resistance to ETV[77,81], therefore the cumulative ETV resistance rates are substantially higher in LAM resistant than in naïve patients (6% at year-1 and 51% at year-5)[76]. These data make ETV a less attractive therapeutic option for the long-term treatment of patients with LAM resistance. The prompt addition of ADV to on-going LAM therapy was for years the treatment of choice for CHB patients with LAM resistance[5,14,57,65,86-89]. However, after the recent license of TDF, which is a more potent agent for both naïve and LAM resistant patients compared to ADV, TDF will probably be used for the treatment of patients with LAM resistance.

In vitro data and clinical studies reported that ADV resistant mutants are susceptible to LAM, ETV and TBV therapy[65,75,81,84,90]. Some recent *in vitro* data, however, suggested that LAM, ETV and TBV are effective against the N236T HBV mutant strain, which represents the most frequent ADV resistant mutant, while all three agents may have relatively reduced efficacy against the A181V HBV mutant strain, which seem to be more sensitive to TDF[81]. It should be noted that LAM should be avoided in patients with prior LAM resistance who develop ADV resistance under ADV monotherapy, as reintroduction of LAM was reported to be associated with rapid re-emergence of LAM resistant strains[66]. There are very few data regarding management of patients with ETV or TDV resistance. Since resistance against these two agents requires the presence of M204V/I (± L180M) mutations, LAM will be ineffective. However, according to in vitro resistance data and the type of resistant mutations, ADV or now, TDF, seem to be the most reasonable currently available therapeutic options for patients with ETV or TDV resistance due to lack of cross-resistance[14].

CONCLUSION

The decision to treat any patient with chronic HBV infection should be based on reasonable clinical judgment. Treatment should be given to all chronic HBV patients with HBV DNA > 20 000 IU/mL, elevated ALT > 2 xULN, regardless of HBeAg status. Liver biopsy does not affect the therapeutic decisions in this group of patients and therefore has only prognostic significance. In contrast to above, treatment indications remain debatable in the group of patients in the HBeAg-positive immunotolerant phase (high HBV DNA and normal ALT) as well as in marginal HBeAg-negative chronic HBV patients. Liver biopsy is extremely important for therapeutic decisions in patients with marginal ALT elevations (1-2 xULN) and/or relatively low HBV DNA levels (< 20 000 IU/mL). All existing data suggest that

there is no clear cut-off HBV DNA level to differentiate inactive chronic HBV carriers from HBeAg-negative CHB patients. Any such a cut-off level would not correctly diagnose a substantial proportion of inactive carriers perhaps leading to unnecessary liver biopsies as well as a proportion of HBeAg-negative CHB patients who may not receive appropriate treatment. In clinical practice, all HBeAg-negative patients with any persistent or transient mild ALT elevation may benefit from a liver biopsy regardless of viremia levels, while HBeAg-negative patients with persistently normal ALT and HBV DNA < 20 000 IU/mL should be followed for life.

Therapeutic strategies for CHB can be summarized as therapies of finite duration aiming to offer sustained off-therapy response and long-term therapies aiming to maintain remission under oral antiviral agents. Peg-IFNα, or even standard IFNa, therapy represents the only practical treatment given for finite duration, which offers a chance of sustained off-therapy response with a considerable probability of HBsAg loss. However, IFNα/Peg-IFNα therapy has relatively poor safety and tolerance profile, is contraindicated in specific patient populations and eventually achieves sustained off-therapy responses in the minority of CHB patients. Long-term treatment with oral anti-HBV agents represents the therapeutic option for the majority of chronic HBV patients who require treatment. They achieve high initial on-therapy biochemical and virological response rates, but viral resistance may develop with prolongation of therapy. Therefore, judicious use of oral anti-HBV agents is recommended, particularly in patients with mild liver disease. The optimal strategy for oral anti-HBV therapy with the best long-term cost/benefit ratio has not been completely clarified. LAM monotherapy is associated with the highest resistance rates and therefore is considered as a suboptimal first-line long-term monotherapy in CHB, However, despite the current availability of more potent agents with better resistance profile, such as ETV and TDF, which have become the treatment of choice in Western countries, LAM is still widely used in many parts of the world because of its low cost. Regardless of the anti-HBV agent used, compliance should always be ascertained and most current guidelines recommend HBV DNA testing at least every 6 mo for the prompt diagnosis of lack of response or virological breakthroughs and timely treatment modification. Whether the 6-monthly HBV DNA determinations are necessary during the long-term treatment with any anti-HBV agent, even those with very low resistance rates after 4-5 years of therapy, is currently unclear.

REFERENCES

1 **Lee WM**. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733-1745
2 **Maddrey WC**. Hepatitis B: an important public health issue. *J Med Virol* 2000; **61**: 362-366
3 **Papatheodoridis GV**, Hadziyannis SJ. Review article: current management of chronic hepatitis B. *Aliment Pharmacol Ther* 2004; **19**: 25-37
4 **Papatheodoridis GV**, Manolakopoulos S, Dusheiko G, Archimandritis AJ. Therapeutic strategies in the management of patients with chronic hepatitis B virus infection. *Lancet Infect Dis* 2008; **8**: 167-178
5 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539
6 **Liaw YF**, Leung N, Guan R, Lau GK, Merican I, McCaughan G, Gane E, Kao JH, Omata M. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2005 update. *Liver Int* 2005; **25**: 472-489
7 **Fattovich G**. Natural history and prognosis of hepatitis B. *Semin Liver Dis* 2003; **23**: 47-58
8 **McMahon BJ**. The natural history of chronic hepatitis B virus infection. *Semin Liver Dis* 2004; **24** Suppl 1: 17-21
9 **Hadziyannis SJ**, Papatheodoridis GV. Hepatitis B e antigen-negative chronic hepatitis B: natural history and treatment. *Semin Liver Dis* 2006; **26**: 130-141
10 **Chu CJ**, Hussain M, Lok AS. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. *Gastroenterology* 2002; **122**: 1756-1762
11 **Yuen MF**, Sablon E, Yuan HJ, Wong DK, Hui CK, Wong BC, Chan AO, Lai CL. Significance of hepatitis B genotype in acute exacerbation, HBeAg seroconversion, cirrhosis-related complications, and hepatocellular carcinoma. *Hepatology* 2003; **37**: 562-567
12 **Chu CJ**, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, Carey W, Brown RS Jr, Luketic VA, Terrault N, Lok AS. Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology* 2003; **125**: 444-451
13 **Papatheodoridis GV**, Hadziyannis SJ. Diagnosis and management of pre-core mutant chronic hepatitis B. *J Viral Hepat* 2001; **8**: 311-321
14 **Keeffe EB**, Dieterich DT, Han SH, Jacobson IM, Martin P, Schiff ER, Tobias H, Wright TL. A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: an update. *Clin Gastroenterol Hepatol* 2006; **4**: 936-962
15 **Beasley RP**. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer* 1988; **61**: 1942-1956
16 **Chen CJ**, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65-73
17 **Iloeje UH**, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006; **130**: 678-686
18 **Chu CJ**, Hussain M, Lok AS. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. *Hepatology* 2002; **36**: 1408-1415
19 **Papatheodoridis GV**, Chrysanthos N, Hadziyannis E, Cholongitas E, Manesis EK. Longitudinal changes in serum HBV DNA levels and predictors of progression during the natural course of HBeAg-negative chronic hepatitis B virus infection. *J Viral Hepat* 2008; **15**: 434-441
20 **Papatheodoridis GV**, Manesis EK, Manolakopoulos S, Elefsiniotis IS, Goulis J, Giannousis J, Bilalis A, Kafiri G, Tzourmakliotis D, Archimandritis AJ. Is there a meaningful serum hepatitis B virus DNA cutoff level for therapeutic decisions in hepatitis B e antigen-negative chronic hepatitis B virus infection? *Hepatology* 2008; **48**: 1451-1459
21 **Villeneuve JP**, Condreay LD, Willems B, Pomier-Layrargues G, Fenyves D, Bilodeau M, Leduc R, Peltekian K, Wong F, Margulies M, Heathcote EJ. Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. *Hepatology* 2000; **31**: 207-210
22 **Manolakopoulos S**, Karatapanis S, Elefsiniotis J, Mathou N, Vlachogiannakos J, Iliadou E, Kougioumtzan A, Economou M, Triantos C, Tzourmakliotis D, Avgerinos A. Clinical course of lamivudine monotherapy in patients with decompensated cirrhosis due to HBeAg negative chronic

HBV infection. *Am J Gastroenterol* 2004; **99**: 57-63
23 **Papatheodoridis GV**, Sevastianos V, Burroughs AK. Prevention of and treatment for hepatitis B virus infection after liver transplantation in the nucleoside analogues era. *Am J Transplant* 2003; **3**: 250-258
24 **Hui CK**, Lie A, Au WY, Leung YH, Ma SY, Cheung WW, Zhang HY, Chim CS, Kwong YL, Liang R, Lau GK. A long-term follow-up study on hepatitis B surface antigen-positive patients undergoing allogeneic hematopoietic stem cell transplantation. *Blood* 2005; **106**: 464-469
25 **Hui CK**, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, Leung N, Luk JM, Lie AK, Kwong YL, Liang R, Lau GK. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. *Gastroenterology* 2006; **131**: 59-68
26 **Kohrt HE**, Ouyang DL, Keeffe EB. Systematic review: lamivudine prophylaxis for chemotherapy-induced reactivation of chronic hepatitis B virus infection. *Aliment Pharmacol Ther* 2006; **24**: 1003-1016
27 **Niederau C**, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, Haussinger D. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. *N Engl J Med* 1996; **334**: 1422-1427
28 **Korenman J**, Baker B, Waggoner J, Everhart JE, Di Bisceglie AM, Hoofnagle JH. Long-term remission of chronic hepatitis B after alpha-interferon therapy. *Ann Intern Med* 1991; **114**: 629-634
29 **Fattovich G**, Giustina G, Realdi G, Corrocher R, Schalm SW. Long-term outcome of hepatitis B e antigen-positive patients with compensated cirrhosis treated with interferon alfa. European Concerted Action on Viral Hepatitis (EUROHEP). *Hepatology* 1997; **26**: 1338-1342
30 **van Zonneveld M**, Honkoop P, Hansen BE, Niesters HG, Murad SD, de Man RA, Schalm SW, Janssen HL. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. *Hepatology* 2004; **39**: 804-810
31 **Papatheodoridis GV**, Manesis E, Hadziyannis SJ. The long-term outcome of interferon-alpha treated and untreated patients with HBeAg-negative chronic hepatitis B. *J Hepatol* 2001; **34**: 306-313
32 **Papatheodoridis GV**, Dimou E, Dimakopoulos K, Manolakopoulos S, Rapti I, Kitis G, Tzourmakliotis D, Manesis E, Hadziyannis SJ. Outcome of hepatitis B e antigen-negative chronic hepatitis B on long-term nucleos(t)ide analog therapy starting with lamivudine. *Hepatology* 2005; **42**: 121-129
33 **Lin SM**, Sheen IS, Chien RN, Chu CM, Liaw YF. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection. *Hepatology* 1999; **29**: 971-975
34 **Lin SM**, Yu ML, Lee CM, Chien RN, Sheen IS, Chu CM, Liaw YF. Interferon therapy in HBeAg positive chronic hepatitis reduces progression to cirrhosis and hepatocellular carcinoma. *J Hepatol* 2007; **46**: 45-52
35 **Yuen MF**, Hui CK, Cheng CC, Wu CH, Lai YP, Lai CL. Long-term follow-up of interferon alfa treatment in Chinese patients with chronic hepatitis B infection: The effect on hepatitis B e antigen seroconversion and the development of cirrhosis-related complications. *Hepatology* 2001; **34**: 139-145
36 **Yuan HJ**, Yuen MF, Ka-Ho Wong D, Sablon E, Lai CL. The relationship between HBV-DNA levels and cirrhosis-related complications in Chinese with chronic hepatitis B. *J Viral Hepat* 2005; **12**: 373-379
37 **Liaw YF**, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J. Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351**: 1521-1531
38 **Di Marco V**, Marzano A, Lampertico P, Andreone P, Santantonio T, Almasio PL, Rizzetto M, Craxi A. Clinical outcome of HBeAg-negative chronic hepatitis B in relation to virological response to lamivudine. *Hepatology* 2004; **40**: 883-891
39 **Wong DK**, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med* 1993; **119**: 312-323
40 **Lau GK**, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, Gane E, Fried MW, Chow WC, Paik SW, Chang WY, Berg T, Flisiak R, McCloud P, Pluck N. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005; **352**: 2682-2695
41 **Janssen HL**, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, Simon C, So TM, Gerken G, de Man RA, Niesters HG, Zondervan P, Hansen B, Schalm SW. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. *Lancet* 2005; **365**: 123-129
42 **Lai CL**, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61-68
43 **Dienstag JL**, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256-1263
44 **Chang TT**, Gish RG, de Man R, Gadano A, Sollano J, Chao YC, Lok AS, Han KH, Goodman Z, Zhu J, Cross A, DeHertogh D, Wilber R, Colonno R, Apelian D. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001-1010
45 **Lai CL**, Gane E, Liaw YF, Hsu CW, Thongsawat S, Wang Y, Chen Y, Heathcote EJ, Rasenack J, Bzowej N, Naoumov NV, Di Bisceglie AM, Zeuzem S, Moon YM, Goodman Z, Chao G, Constance BF, Brown NA. Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med 2007*; **357**: 2576-2588
46 **Marcellin P**, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, Jeffers L, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; **348**: 808-816
47 **Manesis EK**, Hadziyannis SJ. Interferon alpha treatment and retreatment of hepatitis B e antigen-negative chronic hepatitis B. *Gastroenterology* 2001; **121**: 101-109
48 **Lampertico P**, Del Ninno E, Vigano M, Romeo R, Donato MF, Sablon E, Morabito A, Colombo M. Long-term suppression of hepatitis B e antigen-negative chronic hepatitis B by 24-month interferon therapy. *Hepatology* 2003; **37**: 756-763
49 **Marcellin P**, Lau GK, Bonino F, Farci P, Hadziyannis S, Jin R, Lu ZM, Piratvisuth T, Germanidis G, Yurdaydin C, Diago M, Gurel S, Lai MY, Button P, Pluck N. Peginterferon alfa-2a alone, lamivudine alone, and the two in combination in patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2004; **351**: 1206-1217
50 **Marcellin P**, Bonino F, Lau GK, Farci P, Yurdaydin C, Piratvisuth T, Jin R, Gurel S, Hadziyannis S, Lu ZM, Popescu M. Factors associated with sustained virologic response 1 year after treatment with peginterferon alfa-2a (40KD) (Pegasys) monotherapy for HBeAg-negative chronic hepatitis B. *Hepatology* 2005; **42** (Suppl 1): 580A
51 **Marcellin P**, Bonino F, Lau GK, Farci P, Yurdaydin C, Piratvisuth T, Gurel S, Hadziyannis S, Wang Y, Popescu M. Virological and biochemical response in patients with HBeAg-negative CHB treated with peginterferon alfa-2a (40KD) + lamivudine: 3-year follow-up results. *J Hepatol* 2007; **46** (Suppl 1): S25-S26
52 **Tassopoulos NC**, Volpes R, Pastore G, Heathcote J, Buti M, Gray DF, Barber J, Hawley S. Post lamivudine treatment follow up of patients with HBeAg negative chronic hepatitis

B. *J Hepatol* 1999; **30** (Suppl 1): 117
53 **Santantonio T**, Mazzola M, Iacovazzi T, Miglietta A, Guastadisegni A, Pastore G. Long-term follow-up of patients with anti-HBe/HBV DNA-positive chronic hepatitis B treated for 12 months with lamivudine. *J Hepatol* 2000; **32**: 300-306
54 **Hadziyannis SJ**, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, Marcellin P, Lim SG, Goodman Z, Ma J, Arterburn S, Xiong S, Currie G, Brosgart CL. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B. *N Engl J Med* 2005; **352**: 2673-2681
55 **Idilman R**, Arat M, Soydan E, Toruner M, Soykan I, Akbulut H, Arslan O, Ozcan M, Turkyilmaz AR, Bozdayi M, Karayalcin S, Van Thiel DH, Ozden A, Beksac M, Akan H. Lamivudine prophylaxis for prevention of chemotherapy-induced hepatitis B virus reactivation in hepatitis B virus carriers with malignancies. *J Viral Hepat* 2004; **11**: 141-147
56 **Hsu C**, Hsiung CA, Su IJ, Hwang WS, Wang MC, Lin SF, Lin TH, Hsiao HH, Young JH, Chang MC, Liao YM, Li CC, Wu HB, Tien HF, Chao TY, Liu TW, Cheng AL, Chen PJ. A revisit of prophylactic lamivudine for chemotherapy-associated hepatitis B reactivation in non-Hodgkin's lymphoma: a randomized trial. *Hepatology* 2008; **47**: 844-853
57 **Hadziyannis SJ**, Papatheodoridis GV. Emerging treatments in chronic hepatitis B. *Expert Opin Emerg Drugs* 2004; **9**: 207-221
58 **Fung SK**, Wong F, Hussain M, Lok AS. Sustained response after a 2-year course of lamivudine treatment of hepatitis B e antigen-negative chronic hepatitis B. *J Viral Hepat* 2004; **11**: 432-438
59 **Barbon V**, Gaia S, Marzano A, Lagget M, Rizzetto M. Prompt relapse of viremia after lamivudine discontinuation in e-minus chronic hepatitis B patients completely responders during 5 years of therapy. *J Hepatol* 2004; **41**: 500-501
60 **Hadziyannis SJ**, Sevastianos V, Rapti IN, Tassopoulos N. Sustained biochemical and virological remission after discontiuation of 4 to 5 years of adefovir dipivoxil (ADV) treatment in HBeAg-negative chronic hepatitis B. *Hepatology* 2006; **44** (Suppl 1): 231A-232A
61 **Papatheodoridis GV**, Dimou E, Papadimitropoulos V. Nucleoside analogues for chronic hepatitis B: antiviral efficacy and viral resistance. *Am J Gastroenterol* 2002; **97**: 1618-1628
62 **Zoulim F**. Mechanism of viral persistence and resistance to nucleoside and nucleotide analogs in chronic hepatitis B virus infection. *Antiviral Res* 2004; **64**: 1-15
63 **Shaw T**, Bartholomeusz A, Locarnini S. HBV drug resistance: mechanisms, detection and interpretation. *J Hepatol* 2006; **44**: 593-606
64 **Hunt CM**, McGill JM, Allen MI, Condreay LD. Clinical relevance of hepatitis B viral mutations. *Hepatology* 2000; **31**: 1037-1044
65 **Locarnini S**, Hatzakis A, Heathcote J, Keeffe EB, Liang TJ, Mutimer D, Pawlotsky JM, Zoulim F. Management of antiviral resistance in patients with chronic hepatitis B. *Antivir Ther* 2004; **9**: 679-693
66 **Fung SK**, Andreone P, Han SH, Rajender Reddy K, Regev A, Keeffe EB, Hussain M, Cursaro C, Richtmyer P, Marrero JA, Lok AS. Adefovir-resistant hepatitis B can be associated with viral rebound and hepatic decompensation. *J Hepatol* 2005; **43**: 937-943
67 **Papatheodoridis GV**, Dimou E, Laras A, Papadimitropoulos V, Hadziyannis SJ. Course of virologic breakthroughs under long-term lamivudine in HBeAg-negative precore mutant HBV liver disease. *Hepatology* 2002; **36**: 219-226
68 **Hadziyannis SJ**, Papatheodoridis GV, Dimou E, Laras A, Papaioannou C. Efficacy of long-term lamivudine monotherapy in patients with hepatitis B e antigen-negative chronic hepatitis B. *Hepatology* 2000; **32**: 847-851
69 **Dienstag JL**, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, Gardner S, Gray DF, Schiff ER. Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; **124**: 105-117
70 **Allen MI**, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* 1998; **27**: 1670-1677
71 **Bartholomeusz A**, Locarnini S. Hepatitis B virus mutations associated with antiviral therapy. *J Med Virol* 2006; **78** Suppl 1: S52-S55
72 **Liaw YF**, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *Gastroenterology* 2000; **119**: 172-180
73 **Lok AS**, Lai CL, Leung N, Yao GB, Cui ZY, Schiff ER, Dienstag JL, Heathcote EJ, Little NR, Griffiths DA, Gardner SD, Castiglia M. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003; **125**: 1714-1722
74 **Hadziyannis SJ**, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, Marcellin P, Lim SG, Goodman Z, Ma J, Brosgart CL, Borroto-Esoda K, Arterburn S, Chuck SL. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. *Gastroenterology* 2006; **131**: 1743-1751
75 **Angus P**, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, Brosgart C, Colledge D, Edwards R, Ayres A, Bartholomeusz A, Locarnini S. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology* 2003; **125**: 292-297
76 **Tenney D**, Pokorowski KA, Rose RE, Baldick CJ, Eggers BJ, Fang J, Yang JY, Xu D, Brett-Smith H, Colonno RJ. Entecavir at five years shows long-term maintenance of high genetic barrier to hepatitis B virus resistance. *Hepatol Int* 2008; **2**: S76-S77
77 **Tenney DJ**, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, Plym M, Pokornowski K, Yu CF, Angus P, Ayres A, Bartholomeusz A, Sievert W, Thompson G, Warner N, Locarnini S, Colonno RJ. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob Agents Chemother* 2004; **48**: 3498-3507
78 **Lai CL**, Leung N, Teo EK, Tong M, Wong F, Hann HW, Han S, Poynard T, Myers M, Chao G, Lloyd D, Brown NA. A 1-year trial of telbivudine, lamivudine, and the combination in patients with hepatitis B e antigen-positive chronic hepatitis B. *Gastroenterology* 2005; **129**: 528-536
79 **Lai CL**, Gane E, Hsu CW, Thongsawat S, Wang Y, Chen Y, Heathcote EJ, Rasenack J, Bzowej N, Naoumov N, Zeuzem S, Di Bisceglie A, Chao GC, Fielman BA, Brown NA. Two-years results from the GLOBE trial in patients with hepatitis B: greater clinical and antiviral efficacy for telbivudine (LdT) vs lamivudine. *Hepatology* 2006; **44** (Suppl 1): 222A
80 **Di Bisceglie A**, Lai CL, Gane E, Chen YC, Thongsawat S, Wang Y, Chen Y, Heathcote EJ, Zeuzem S, Rasenack J, Bzowej N, Han SH, Naoumov N, Hwang SG, Lim SG, Chao GC, Fielman BA, Brown NA. Telbivudine GLOBE trial: Maximal early HBV suppression is predictive of optimal two-year efficacy in nucleoside-treated hepatitis B patients. *Hepatology* 2006; **44** (Suppl 1): 230A-231A
81 **Locarnini S**, Mason WS. Cellular and virological mechanisms of HBV drug resistance. *J Hepatol* 2006; **44**: 422-431
82 **Hadziyannis SJ**, Papatheodoridis GV. Adefovir dipivoxil in the treatment of chronic hepatitis B virus infection. *Expert Rev Anti Infect Ther* 2004; **2**: 475-483
83 **Chang TT**, Gish RG, Hadziyannis SJ, Cianciara J, Rizzetto M, Schiff ER, Pastore G, Bacon BR, Poynard T, Joshi S, Klesczewski KS, Thiry A, Rose RE, Colonno RJ, Hindes RG. A dose-ranging study of the efficacy and tolerability

of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; **129**: 1198-1209

84 **Yang H**, Qi X, Sabogal A, Miller M, Xiong S, Delaney WE 4th. Cross-resistance testing of next-generation nucleoside and nucleotide analogues against lamivudine-resistant HBV. *Antivir Ther* 2005; **10**: 625-633

85 **Sherman M**, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, Boron-Kaczmarska A, Martin P, Goodman Z, Colonno R, Cross A, Denisky G, Kreter B, Hindes R. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. *Gastroenterology* 2006; **130**: 2039-2049

86 **Shaw T**, Bowden S, Locarnini S. Rescue therapy for drug resistant hepatitis B: another argument for combination chemotherapy? *Gastroenterology* 2004; **126**: 343-347

87 **Rapti I**, Dimou E, Mitsoula P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. *Hepatology* 2007; **45**: 307-313

88 **Lampertico P**, Vigano M, Manenti E, Iavarone M, Lunghi G, Colombo M. Adefovir rapidly suppresses hepatitis B in HBeAg-negative patients developing genotypic resistance to lamivudine. *Hepatology* 2005; **42**: 1414-1419

89 **Manolakopoulos S**, Bethanis S, Koutsounas S, Goulis J, Vlachogiannakos J, Christias E, Saveriadis A, Pavlidis C, Triantos C, Christidou A, Papatheodoridis G, Karamanolis D, Tzourmakliotis D. Long-term therapy with adefovir dipivoxil in hepatitis B e antigen-negative patients developing resistance to lamivudine. *Aliment Pharmacol Ther* 2008; **27**: 266-273

90 **Villeneuve JP**, Durantel D, Durantel S, Westland C, Xiong S, Brosgart CL, Gibbs CS, Parvaz P, Werle B, Trepo C, Zoulim F. Selection of a hepatitis B virus strain resistant to adefovir in a liver transplantation patient. *J Hepatol* 2003; **39**: 1085-1089

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Adult celiac disease in the elderly

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Abstract

There is an increased awareness that celiac disease may occur in the elderly although presentations with either diarrhea, weight loss or both may be less common causing delays in diagnosis for prolonged periods. Higher detection rates also seem evident owing to active case screening, largely through serodiagnostic measures. In some elderly patients who are genetically predisposed, it has been hypothesized that celiac disease might be precipitated late in life by an antigen, possibly from an infectious agent. As a result, peptide mimicry or other poorly-defined mechanisms may precipitate an autoimmune gluten-dependent clinical state. Although diarrhea and weight loss occur, only isolated iron deficiency anemia may be present at the time of initial diagnosis. In addition, the risk of other autoimmune disorders, particularly autoimmune thyroiditis, and bone disease, are increased. Osteopenia may also be associated with an increased risk of fractures. Finally, elderly celiacs have an increased risk of malignant intestinal disease, especially lymphoma.

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Key words: Celiac disease; Elderly; Lymphoma; Silent celiac disease

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INTRODUCTION

Celiac disease has been traditionally recognized in children and young adults, however, in recent years, detection in the elderly population has increased[1]. This is reflected historically in published clinical series from North America and Europe (e.g. about 7% in a Birmingham series were diagnosed after the age of 60 years, similar to a Mayo Clinic series of 4%[2]. Later, studies from the United Kingdom[3] recorded that about 25% were initially diagnosed in their seventh decade. Similar findings were also recorded in the United States[4], Sweden[5], Scotland[6], Ireland[7] and Canada[8]. In each, typical symptoms of impaired intestinal absorption, such as diarrhea or weight loss, while common, were less prominent compared to those with celiac disease diagnosed at a younger age, even though adult celiac disease was reported to be the most common cause of steatorrhea after the age of 50 years[9]. Others, however, have also recently emphasized the need to suspect celiac disease in the elderly, as even classical features of celiac disease may be ignored leading to prolonged delays in diagnosis[10-12].

CLINICAL PRESENTATION

A precise explanation for the apparent paucity of clinical findings in the elderly age group is still required. Firstly, this may be due, in part, to a limited mucosal extent of disease in the proximal duodenum and jejunum. As a result, symptoms and signs, such as diarrhea and weight loss, may be minimized. Instead, isolated deficiencies of specific nutrients, such as iron, may occur. Secondly, a low index of suspicion by the physician for celiac disease may lead to a delay in recognition or a distraction to other sinister disorders. For example, iron deficiency anemia may lead to studies to exclude colon cancer. Thirdly, some patients with elderly celiac disease may have cognitive impairment which makes the definition of some symptoms more difficult[13]. Occasionally, in those treated with a gluten-free diet, cognitive decline may be minimized or may resolve[11]. Finally, and most intriguing, recognition of celiac disease, in some cases, may not be delayed. Possibly, the disease may only be precipitated later in life by some environmental factor, such as a viral agent (e.g. adenovirus) or an as yet unidentified superantigen with a peptide structure that mimics, at a molecular level, the offending gluten peptide[14,15]. Especially intriguing is a recent report showing that in a biopsy-defined population,

celiac disease was more commonly found in the elderly than any other younger age group implying a critical need for active case finding, possibly through serological screening[16]. The reason for this late clinical definition of celiac disease needs to be further elucidated.

ASSOCIATED DISORDERS

A number of disorders have now been recognized in the elderly which might provide a clinical clue to underlying or occult celiac disease. In addition to isolated iron deficiency, folic acid or calcium deficiencies may also occur because the principal uptake site for these nutrients is also the proximal small intestine. Moreover, autoimmune disorders linked to adult celiac disease (and possibly with a similar etiopathogenesis) may be the predominant clinical manifestation of occult celiac disease, such as dermatitis herpetiformis[17,18] or autoimmune thyroiditis[19]. Celiac disease may also be associated with collagenous mucosal diseases (i.e. collagenous colitis)[20], as well as epithelial lymphocytosis of the stomach, colon and biliary tract[21-23]. In some patients, collagenous colitis may be a clue to underlying celiac disease[24], or may complicate celiac disease causing an apparently "refractory" diarrhea, despite a gluten-free diet. Finally, specific malignancies traditionally associated with celiac disease may occur, including lymphoma and small bowel adenocarcinoma[25,26].

Other disorders of the intestinal tract have been recorded. Gastric and duodenal ulcers may occur, but jejunal or ileal ulcers are especially worrying, since these may harbour a malignant small bowel lymphoma[25], especially if the presentation is associated with a free perforation of the small intestine[27]. Also, pancreatic calcification has been described, similar to the calcific pancreatitis noted in autoimmune pancreatitis, protein-energy malnutrition and long-term alcohol abuse[28].

LATENT CELIAC DISEASE

Latent celiac disease has also been documented in some elderly patients with dermatitis herpetiformis. In these patients, initial small bowel biopsies were architecturally normal (suggesting that celiac disease might not be present)[29]. Following a high gluten diet, however, histological changes of variable severity were induced, indicating that the small bowel mucosa was gluten-sensitive. In some biopsies, architectural changes were only mildly or moderately abnormal, but in others, changes were severely ("flat") abnormal. These small intestinal biopsy changes do not occur in normal volunteers fed high gluten-containing diets. Later, improvement in the small bowel biopsies occurred with gluten restriction. Similar changes were documented in serial small intestinal biopsies carried out in an elderly patient with intestinal lymphoma[30].

SPECIAL PROBLEMS IN ELDERLY CELIAC DISEASE

Anemia and abnormal laboratory tests

Up to 80% of elderly patients with celiac disease in a British series had anemia[31]. Iron deficiency was usually the cause but other nutrients may be deficient, such as folic acid, sometimes producing a dimorphic peripheral blood smear (if combined with iron deficiency). In these elderly patients, concomitant hypoalbuminemia with peripheral edema and ascites may also occur with hypocalcemia and hypomagnesemia[8]. Others may have liver chemistry test changes[23], initially believed, in some cases, to be related to alcohol overuse. In these patients, a hepatocellular injury profile may normalize with gluten restriction. In others with a cholestatic injury profile, lymphocytic sclerosing cholangitis or primary biliary cirrhosis may be present, but these do not improve with a gluten-free diet.

Bone disease and fractures

Bone disease may develop with few clinical features of malabsorption. Decreased bone mass is the most common form of metabolic bone disorder in celiac disease. Up to 70% of adult and elderly patients with celiac disease have a bone mineral density that is less than one standard deviation below normal controls (osteopenia)[32,33]. Men and post-menopausal women are affected more often than pre-menopausal women[33]. Reduced bone mineral density may also occur in treated patients with celiac disease[33,34].

The mechanism for osteopenia in celiac disease may be related, in part, to calcium malabsorption causing increased secretion of parathyroid hormone. Increased bone turnover leads to cortical bone loss. Impaired absorption of vitamin D may also occur. Pro-inflammatory and anti-inflammatory cytokines are believed to play an active role in the pathogenesis of osteopenia in celiac disease. Although celiacs may improve their bone mineral density with a gluten-free diet, the bone mass increment is limited in elderly patients and may be incomplete.

A higher prevalence of fractures also occurs in the peripheral skeleton of elderly celiacs. Most fractures occur before the diagnosis of celiac disease is initially defined and commonly occur in those with poor diet compliance. Most believe that a gluten-free diet is the most important factor providing protection from the risk of fracture. In a recent population-based study on long-term fracture risk[35], celiac disease was linked to an increased fracture risk before and after diagnosis. Appendicular and axial fractures were more common supporting a rationale for earlier detection of celiac disease and active management of bone disease before bone effects, specifically fractures, occur[35].

Recurrent or refractory disease

In well-defined celiac disease, recurrent diarrhea or malabsorption may occur. In an elderly celiac, this may be particularly disconcerting since the number of potentially serious disorders that may complicate the clinical course of celiac disease is significant[36]. In most, limited compliance to the gluten-free diet or inadvertent consumption of gluten remains the most common cause. Ubiquitous sources of gluten include the fillers in pill capsules, a particularly critical issue in this age group. In others, diarrhea or weight loss may be related

to another cause with no specific relationship to celiac disease (e.g. ischemic bowel disease, infectious diarrhea, colonic malignancy). Alternatively, other superimposed small intestinal histological changes may be present which could be related to impaired absorption of a specific nutrient (e.g. folic acid deficiency). In others, an associated cause, such as pancreatic exocrine insufficiency, may be present. Re-evaluation of the original diagnosis may be necessary to ensure that a separate diagnosis has not been overlooked. Sometimes, an associated complication may be responsible, e.g. collagenous or lymphocytic colitis, or a more serious complication may supervene, e.g. lymphoma[36].

In elderly patients with established celiac disease, a rare disorder, collagenous sprue, may develop[36]. In most of these patients, severe pan-malabsorption may occur with recurrent diarrhea, progressive weight loss and significant nutritional and electrolyte deficiencies. IgA-endomysial antibodies may also be detected in collagenous sprue, further evidence for an etiopathogenetic link with celiac disease[37]. In addition, collagenous sprue may be complicated by lymphoma[38]. Collagenous mucosal changes may also concomitantly occur in the colon, and rarely, these may completely disappear with treatment[39]. Most intriguing was the finding of complete histopathological resolution of collagenous small bowel and colonic involvement after resection of an unrelated colon cancer, suggesting the possibility of a paraneoplastic phenomenon[40].

Occasionally, no specific cause for persistent or refractory symptoms can be documented. Some have a poorly understood syndrome characterized by recurrent or persistent small bowel changes of variable severity, splenic hypofunction and cavitation of mesenteric lymph nodes. This disorder may also be complicated by lymphoma[41]. Others in this heterogeneous group remain severely symptomatic with malabsorption and profound wasting despite a gluten-free diet. Some may have a "clinically-resistant" form of celiac disease. Interestingly, a recent report documented persistent changes in elderly celiacs on a gluten-free diet and suggested that follow-up biopsies be done only after two years on a gluten-free diet to document histological recovery[42]. This suggests that the label of "refractory" may only be applied after an extended period of time. Others will be eventually proved to have a difficult-to-diagnose intestinal lymphoma[36].

CONCLUSION

Despite a paucity of symptoms, such as diarrhea and weight loss, celiac disease is becoming increasingly recognized in the elderly. Especially intriguing is a recent report which suggests that celiac disease occurs in the elderly more often than in any other age group, possibly related to serological screening. Other presentations in this elderly age group include iron deficiency anemia (often refractory to oral iron), other autoimmune disorders (dermatitis herpetiformis, thyroiditis), osteopenic bone disease, including fractures, and malignant intestinal disease, especially lymphoma. Diagnosis may be delayed due to limited symptoms, a low index of suspicion or diagnostic difficulties related to cognitive impairment. Conversely, celiac disease may not develop in the genetically predisposed until late in life, possibly being precipitated by an environmental factor. Finally, celiac disease diagnosed late in life is more often associated with recurrent or refractory disease and the appearance of lymphoma.

REFERENCES

1 **Freeman H**, Lemoyne M, Pare P. Coeliac disease. *Best Pract Res Clin Gastroenterol* 2002; **16**: 37-49
2 **Green PA**, Wollaeger EE. The clinical behavior of sprue in the United States. *Gastroenterology* 1960; **38**: 399-418
3 **O'Morain C**, Segal AW, Levi AJ. Elemental diets in treatment of acute Crohn's disease. *Br Med J* 1980; **281**: 1173-1175
4 **Mann JG**, Brown WR, Kern F Jr. The subtle and variable clinical expressions of gluten-induced enteropathy (adult celiac disease, nontropical sprue). An analysis of twenty-one consecutive cases. *Am J Med* 1970; **48**: 357-366
5 **Hallert C**, Gotthard R, Norrby K, Walan A. On the prevalence of adult coeliac disease in Sweden. *Scand J Gastroenterol* 1981; **16**: 257-261
6 **Rifkind EA**, Logan RF, Busuttil A, Gilmour H, Ferguson A. Coeliac disease in Edinburgh and the Lothians 1900-1980. *Scott Med J* 1982; **27**: 256
7 **Kirby J**, Fielding JF. Very adult coeliac disease! The need for jejunal biopsy in the middle aged and elderly. *Ir Med J* 1984; **77**: 35-36
8 **Freeman HJ**. Clinical spectrum of biopsy-defined celiac disease in the elderly. *Can J Gastroenterol* 1995; **9**: 42-46
9 **Price HL**, Gazzard BG, Dawson AM. Steatorrhoea in the elderly. *Br Med J* 1977; **1**: 1582-1584
10 **Gasbarrini G**, Ciccocioppo R, De Vitis I, Corazza GR. Coeliac Disease in the Elderly. A multicentre Italian study. *Gerontology* 2001; **47**: 306-310
11 **Patel D**, Kalkat P, Baisch D, Zipser R. Celiac disease in the elderly. *Gerontology* 2005; **51**: 213-214
12 **Lurie Y**, Landau DA, Pfeffer J, Oren R. Celiac disease diagnosed in the elderly. *J Clin Gastroenterol* 2008; **42**: 59-61
13 **Freeman HJ**. Neurological disorders in adult celiac disease. *Can J Gastroenterol* 2008; **22**: 909-911
14 **Kagnoff MF**, Austin RK, Hubert JJ, Bernardin JE, Kasarda DD. Possible role for a human adenovirus in the pathogenesis of celiac disease. *J Exp Med* 1984; **160**: 1544-1557
15 **Barbeau WE**, Novascone MA, Elgert KD. Is celiac disease due to molecular mimicry between gliadin peptide-HLA class II molecule-T cell interactions and those of some unidentified superantigen? *Mol Immunol* 1997; **34**: 535-541
16 **Vilppula A**, Collin P, Maki M, Valve R, Luostarinen M, Krekela I, Patrikainen H, Kaukinen K, Luostarinen L. Undetected coeliac disease in the elderly: a biopsy-proven population-based study. *Dig Liver Dis* 2008; **40**: 809-813
17 **Brow JR**, Parker F, Weinstein WM, Rubin CE. The small intestinal mucosa in dermatitis herpetiformis. I. Severity and distribution of the small intestinal lesion and associated malabsorption. *Gastroenterology* 1971; **60**: 355-361
18 **Weinstein WM**, Brow JR, Parker F, Rubin CE. The small intestinal mucosa in dermatitis herpetiformis. II. Relationship of the small intestinal lesion to gluten. *Gastroenterology* 1971; **60**: 362-369
19 **Freeman HJ**. Celiac associated autoimmune thyroid disease. A study of 16 patients with overt hypothyroidism. *Can J Gastroenterol* 1995; **9**: 242-246
20 **Freeman HJ**. Collagenous mucosal inflammatory diseases of the gastrointestinal tract. *Gastroenterology* 2005; **129**: 338-350
21 **Wolber R**, Owen D, DelBuono L, Appelman H, Freeman H. Lymphocytic gastritis in patients with celiac sprue or spruelike intestinal disease. *Gastroenterology* 1990; **98**: 310-315

22 **Wolber R**, Owen D, Freeman H. Colonic lymphocytosis in patients with celiac sprue. *Hum Pathol* 1990; **21**: 1092-1096
23 **Freeman HJ**. Hepatobiliary and pancreatic disorders in celiac disease. *World J Gastroenterol* 2006; **12**: 1503-1508
24 **Freeman HJ**. Collagenous colitis as the presenting feature of biopsy-defined celiac disease. *J Clin Gastroenterol* 2004; **38**: 664-668
25 **Freeman HJ**, Weinstein WM, Shnitka TK, Piercey JR, Wensel RH. Primary abdominal lymphoma. Presenting manifestation of celiac sprue or complicating dermatitis herpetiformis. *Am J Med* 1977; **63**: 585-594
26 **Freeman HJ**. Lymphoproliferative and intestinal malignancies in 214 patients with biopsy-defined celiac disease. *J Clin Gastroenterol* 2004; **38**: 429-434
27 **Freeman HJ**. Free perforation due to intestinal lymphoma in biopsy-defined or suspected celiac disease. *J Clin Gastroenterol* 2003; **37**: 299-302
28 **Freeman HJ**. Pancreatic endocrine and exocrine changes in celiac disease. *World J Gastroenterol* 2007; **13**: 6344-6346
29 **Weinstein WM**. Latent celiac sprue. *Gastroenterology* 1974; **66**: 489-493
30 **Freeman HJ**, Chiu BK. Multifocal small bowel lymphoma and latent celiac sprue. *Gastroenterology* 1986; **90**: 1992-1997
31 **Hankey GL**, Holmes GK. Coeliac disease in the elderly. *Gut* 1994; **35**: 65-67
32 **Corazza GR**, Di Sario A, Cecchetti L, Tarozzi C, Corrao G, Bernardi M, Gasbarrini G. Bone mass and metabolism in patients with celiac disease. *Gastroenterology* 1995; **109**: 122-128
33 **Meyer D**, Stavropolous S, Diamond B, Shane E, Green PH. Osteoporosis in a north american adult population with celiac disease. *Am J Gastroenterol* 2001; **96**: 112-119
34 **McFarlane XA**, Bhalla AK, Reeves DE, Morgan LM, Robertson DA. Osteoporosis in treated adult coeliac disease. *Gut* 1995; **36**: 710-714
35 **Jafri MR**, Nordstrom CW, Murray JA, Van Dyke CT, Dierkhising RA, Zinsmeister AR, Melton LJ 3rd. Long-term fracture risk in patients with celiac disease: a population-based study in Olmsted County, Minnesota. *Dig Dis Sci* 2008; **53**: 964-971
36 **Freeman HJ**. Refractory celiac disease and sprue-like intestinal disease. *World J Gastroenterol* 2008; **14**: 828-830
37 **Freeman HJ**. Hyposplenism, antiendomysial antibodies and lymphocytic colitis in collagenous sprue. *Can J Gastroenterol* 1999; **13**: 347-350
38 **Freeman HJ**. Collagenous sprue associated with an extensive T-cell lymphoma. *J Clin Gastroenterol* 2003; **36**: 144-146
39 **Freeman HJ**, Davis JE, Myers DM. Complete histological resolution of collagenous sprue. *Can J Gastroenterol* 2004; **18**: 333-336
40 **Freeman HJ**, Berean KW. Resolution of paraneoplastic collagenous enterocolitis after resection of colon cancer. *Can J Gastroenterol* 2006; **20**: 357-360
41 **Freeman HJ**, Chiu BK. Small bowel malignant lymphoma complicating celiac sprue and the mesenteric lymph node cavitation syndrome. *Gastroenterology* 1986; **90**: 2008-2012
42 **Tursi A**, Brandimarte G, Giorgetti GM, Elisei W, Inchingolo CD, Monardo E, Aiello F. Endoscopic and histological findings in the duodenum of adults with celiac disease before and after changing to a gluten-free diet: a 2-year prospective study. *Endoscopy* 2006; **38**: 702-707

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, *Series Editor*

Mouse models in liver cancer research: A review of current literature

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Abstract

Primary liver cancer remains one of the most lethal malignancies worldwide. Due to differences in prevalence of etiological factors the incidence of primary liver cancer varies among the world, with a peak in East-Asia. As this disease is still lethal in most of the cases, research has to be done to improve our understanding of the disease, offering insights for possible treatment options. For this purpose, animal models are widely used, especially mouse models. In this review, we describe the different types of mouse models used in liver cancer research, with emphasis on genetically engineered mice used in this field. We focus on hepatocellular carcinoma (HCC), as this is by far the most common type of primary liver cancer, accounting for 70%-85% of cases.

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Key words: Primary liver cancer; Hepatocellular carcinoma; Mouse model; Genetically engineered mice

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INTRODUCTION

The American Cancer Society has estimated that, in 2007, there were over 700 000 new cases of primary liver cancer worldwide. It is the fifth most common malignancy in men and the eighth in women. Liver cancer is among the most lethal cancers (five-year survival rates under 11%), which makes it the third most frequent cause of cancer death in men and the sixth in women[1]. Liver cancer consists of several histologically different primary hepatic malignancies, such as cholangiocarcinoma, hepatoblastoma and haemangiosarcoma, but hepatocellular carcinoma (HCC) is by far the most common type, accounting for 70%-85% of cases[1,2].

Cirrhosis (due to for instance hemochromatosis), chronic hepatitis B and C viral infections, chronic alcohol consumption, aflatoxin-B1 intake (from contaminated food) are the most important of the well-defined risk factors for HCC. Variations in the prevalence of these etiological factors mirror the geographical distribution of the incidence of HCC. The worldwide (age-adjusted) incidence per 100 000 persons is 14.9/5.5 (men/women), varying from 2.6/1.3 in Northern Europe to 35.4/12.6 in East Asia[2,3].

Animal models for human HCC can be helpful to our understanding of the (molecular) mechanisms underlying the pathogenesis of HCC. The laboratory mouse remains one of the best models to study cancer *in vivo* due to various features, such as the small size, the similarities to humans and the entirely sequenced genome and the similarities to humans[4].

For instance, the risk of HCC in males is approximately 2-5 times greater than in females[5,6]. This gender difference is seen in mice as well. Recently, Naugler *et al*[5] attributed this disparity to a higher serum interleukin-6 (IL-6) concentration in male than in female mice after administration of the chemical hepatocarcinogen diethylnitrosamine. IL-6 is secreted by Kupffer cells in response to, for example, necrotic hepatocytes. They demonstrated that estrogen inhibits the secretion of IL-6.

Since the spontaneous incidence of liver tumors in

the most frequently used strains of mice is < 4%, mouse models have been developed to induce HCC-formation[7,8].

None of the currently available mouse models meet all criteria of the ideal animal model, which include biologic, genetic, etiologic and therapeutic criteria[9]. Therefore, the most appropriate model for a particular experimental question should be used to answer the specific research question.

This review highlights the currently used mouse models for HCC, with emphasis on genetically engineered models.

CARCINOGEN-INDUCED MOUSE MODELS OF HCC

An etiological role for external agents in contributing to human HCC, also referred to as hepatocarcinogens, has been established by primarily epidemiological studies[9]. Examples of known human hepatocarcinogens include aflatoxins, ethanol and combined oral contraceptives[10]. Despite the fact that some of these chemicals are carcinogenic to the mouse liver as well, this is not true for all human hepatocarcinogens. For example, cirrhosis and liver cancer have not been observed in mice subjected to ethanol solely[10-12]. At the same time, hardly any of the mouse hepatocarcinogens have shown to be carcinogenic for humans (aflatoxin B1 and oral contraceptives belong to the exceptions). This discrepancy in response to carcinogens can probably be explained by species differences[9-11]. Nonetheless, carcinogen-induced mouse models are still frequently used for HCC research.

The (hepato) carcinogens are subdivided into two classes, namely genotoxic and non-genotoxic (or epigenetic) carcinogens. The genotoxic carcinogens can presumably cause cancer by forming DNA adducts, which lead to genetic changes of the target cell. These changes can direct normal cells to a preneoplastic state (initiation). The non-genotoxic carcinogens do not modify DNA structure, but generally stimulate the preneoplastic or initiated cells to evolve into a malignant neoplasm by controlling cell proliferation, apoptosis and cell differentiation[10,11,13]. Because of the high incidence of altered (or preneoplastic) hepatocytes in certain mouse strains (especially C3H and B6C3F1 mice, the latter corresponding to the progeny of C3H mice coupled to C57BL/6 mice), epigenetic carcinogens alone can be used to induce HCC-formation in these mice[8,11].

Many chemicals have been shown to induce HCCs in the mouse liver[10,14]. To date, the most frequently used hepatocarcinogens in mice are diethylnitrosamine (DEN) and phenobarbital (PB), although the carcinogenic effect of PB is controversial[8]. Furthermore, attention has been paid to the hepatocarcinogenic effect of peroxisome proliferators such as clofibrate and the experimental Wy-14.643[13].

The single most frequently used chemical for induction of HCCs in mice is DEN, a genotoxic carcinogen. DEN is typically administered to mice between 12 and 15 d of age by a single intraperitoneal injection (5 μg/g body weight). Using this protocol, originally described by Vesselinovitch and Mihailovich, 100% of B6C3F1 male mice developed HCCs, on average, 44 wk after intraperitoneal injection of DEN[15-17]. Less frequently used protocols for DEN-administration include intraperitoneal injection of a higher dose of DEN (for example 80-90 μg/g body weight) to older mice (4-5 wk of age) and intraperitoneal DEN-injection 36 h after a partial hepatectomy (mice 5-6 wk of age). These protocols are less efficient in producing HCCs than those previously mentioned[8]. DEN-induced mouse models of HCC are predominantly used to study the molecular mechanisms of (chemical) hepatocarcinogenesis. Furthermore, the influence of (trans) genes and chemicals that might prevent HCC development can be evaluated by means of this model.

In the background of carcinogen-induced mouse models for HCC, identifying chemicals that might be carcinogenic to humans is the major application of laboratory mice. For this purpose, chemicals are either administered to newborn mice in order to determine genotoxicity, or compounds are administered for longer periods (usually 2 years) to assess epigenetic carcinogenity[6,14]. However, because of the significant inconsistencies between mouse and human carcinogens, extrapolating the results of these mouse studies to the human situation remains difficult[18].

Carcinogen-induced mouse models for HCC are useful for establishing a relationship between carcinogen exposure and specific genetic changes[19]. However, the influences of sex, age and genetic background of the mice on the predictability of HCC-development remain disadvantages of these models.

IMPLANTATION MODELS OF HCC

Implantation models are among the most widely used models to accomplish HCC formation in mice, because of their suitability in studies for preclinical evaluation of anticancer agents. In implantation models that are currently used to induce HCC formation in mice, HCC cell lines or tumor tissue fragments are implanted in recipient mice. Here, we will give an impression of the approaches that can be used to obtain HCCs in these mice.

The earliest implantation model is the syngeneic transplantable tumor model, in which a HCC cell line or tissue fragment is implanted in mice of the strain from which the implant originates. To date, this model is less frequently used, because of the discovery that human tumor cell lines and tissue fragments can be implanted into immunodeficient mice. Nevertheless, this model is still required when anticancer agents are tested that work by activating the immune system (immune therapy)[20-22]. The unpredictability of the effectiveness of studied anticancer agents in human HCC and the small number of available murine cell lines are the most important disadvantages of this implantation model[20].

As mentioned, nowadays, primary human HCC cell lines or tissue fragments are implanted into immunocompromised mice (xenograft models).

The most widely used mouse for this approach is the nude mouse (*nu* -/-). These mice are athymic, hairless and have a deficiency of T lymphocytes as well as an impaired T and B cell function[23]. Next to nude mice, severe combined immunodeficient (SCID) mice are frequently used in xenograft models of HCC. These mice have a deficiency in number and function of both T and B lymphocytes[24]. Since the establishment of the first human HCC cell line in 1963[25], many human HCC cell lines have been described, of which the HepG2, the Hep3B, the SMMC-7721 and the HuH7 cell lines are the most commonly used.

In allograft models, murine HCC cell lines or tumor fragments are implanted in (not necessarily syngeneic) mice. The HCC grafts have been derived from spontaneously occurring HCCs in mice, from carcinogen-induced tumors or from genetically engineered mice (GEM), which is discussed in the last part of this review.

The above mentioned xenograft or allograft HCC cell suspension or tumor tissue fragments can be implanted into recipient mice, either at ectopic or at orthotopic sites. Ectopic implantation of tumor cells or fragments usually occurs subcutaneously. Orthotopic implantation can be accomplished by subserosal injection of HCC cells or by surgical orthotopical implantation (SOI) of tumor fragments. These fragments, approximately 1 mm^3 in size, are derived from surgical specimens of human HCCs or from pieces of subcutaneously grown HCC cells, either from human or mouse origin.

Xenografts of human HCCs growing subcutaneously in mice are predominantly used in the preclinical evaluation of anticancer agents. The rapid formation of tumors, the minimal labor that is required, the relative inexpensiveness and the ability to measure tumor size non-invasively are the main advantages of the ectopic, subcutaneous, application of grafts from human HCCs[21,22]. However, many research groups have described the importance of the microenvironment on the biological behaviour of malignant cells. For example, many tumor cell lines do not spontaneously metastasize when they are subcutaneously implanted, while they do metastasize when they are orthotopically implanted. Hence, the interaction of organ-specific factors (such as fibroblasts, endothelial cells and inflammatory cells) with tumor cells, is important for HCC development[21,26]. For this reason, therapeutic results obtained by an ectopic implantation model of a HCC graft must always be verified in orthotopic models.

These orthotopic implantation models mimic human HCCs in a better way with respect to tumor morphology, microenvironment, metastatic potential and the response to anticancer agents[27,28]. Furthermore, processes involved in local invasion, like angiogenesis, can be examined in their normal microenvironment[20]. Nonetheless, disadvantages of orthotopic implants of HCC xenografts include a more difficult surgical implantation procedure and more expensive procedures. Furthermore, tumor growth and response cannot be determined as easily as in ectopic transplantation models.

Despite the fact that (ectopic and orthotopic) xenograft implantation models are among the most widely used models for preclinical evaluation of anticancer agents, it has been demonstrated excessively that these models have a poor predictive value for the anti-tumor effects in patients. This can possibly be explained by the fact that the injected tumor cells are often cultured for a long period. Due to selection pressures in culture, these tumor cells have no longer maintained the original molecular characteristics and the heterogeneity of the patients' tumor[4,20,28,29]. By implanting a tumor fragment of a patient, the morphological and molecular characteristics of a patient's tumor are preserved better. However, establishing a parallel in vitro cell line from a patient's tumor is often very difficult.

The abovementioned features of implantation models are but a few arguments why implantation models are not ideal for the preclinical evaluation of anticancer agents. However, because of the suitability of these models, implantation models are still frequently used for this purpose.

GENETICALLY ENGINEERED MOUSE MODELS FOR HCC

The introduction of transgenic mouse models in the early 1980's made it possible to study the molecular features of human malignancies *in vivo*[30,31]. Since then, much progress has been made in techniques of producing GEM[4].

In studying the molecular mechanisms involved in hepatocarcinogenesis, GEM are particularly used to explore the role of a specific gene and to explore the interaction of different genes (e.g. oncogenes and tumor-suppressor genes) in the development of HCC. GEM is also suitable to investigate the role of specific genes in combination with a liver-specific carcinogen.

As is the case in other types of cancer, genetic alterations in various cellular pathways (including pathways involved in growth, apoptosis, proliferation and angiogenesis) are needed for the development of HCC. Although the exact genetic events in hepatocarcinogenesis are not entirely clear, there is evidence that the p53, Rb and Wnt/β-catenin pathways are involved[32,33]. Several transgenic mouse lineages that are nowadays used to induce formation of HCCs are transgenic in one of these pathways (Table 1). The most commonly used models will be discussed here.

Since the late 1980's, transgenic SV40 T-Ag (Simian Virus 40 T-antigen) mice have been studied extensively. The genome of the simian virus 40 (a DNA tumor virus) encodes two oncogenic proteins, the large and small T antigen (T-Ag and tAg, respectively, herein together referred to as T-Ag). After infection, large T-Ag can cause malignant transformation of the host cell primarily by inactivating the tumor-suppressor genes *p53* and *Rb*[34,35].

Research groups have reported the production of transgenic mice expressing SV40 T-Ag directed to the liver by the promoter/enhancer antithrombin-Ⅲ (AT Ⅲ)[36], albumin (Alb)[37] and α-1-antitrypsin (AAT)[38]. For

Table 1 Transgenic mouse models for HCC

Transgene	Promoter	Mouse strain	Percentage HCCs	Reference
c-myc	Alb	C57BL/6 × CBA/J	65% in males at 20 mo	40
TGF-α	MT	CD1	50% in males > 12 mo	77, 78
c-myc/TGF-α	Alb, MT	C57BL/6 × CBA/J × CD1	100% in males at 8 mo	40
SV40 T-Ag	ATⅢ	C57BL/6 × DBA2	100% at 8 mo	36
E2F-1	Alb	C57BL/6 × CBA/J	33%-60% at 12 mo	79, 80
c-myc/E2F-1	Alb	C57BL/6 × CBA/J	100% at 9 mo	80

example, Dubois *et al*[36] produced transgenic mice by putting the SV40 T-Ag under the control of the human ATⅢ promoter. In mouse lineages that expressed the highest level of the transgene, by the age of 8 mo, 100% of mice had developed HCCs and 10% had developed lung metastases.

Another commonly used transgenic mouse model was described by Murakami *et al*[39]. They generated double transgenic mice overexpressing c-myc and TGF-α in the liver (Alb-c-myc/MT-TGF-α mice) by crossing Alb/c-myc mice (transgenic mice overexpressing c-myc, directed by the albumin promoter) with MT/TGF-α mice (transgenic mice overexpressing TGF-α, directed by the metallothionein 1 promoter). Santoni-Rugiu *et al*[40] demonstrated that these mice developed HCCs substantially earlier and at a higher rate than single transgenic mice, overexpressing either c-myc or TGF-α. Within 8 mo after birth, 100% of male and 30% of female Alb-c-myc/MT-TGF-α mice had developed HCCs.

Although these conventional transgenic mouse models have been very useful to study the role of particular genes in hepatocarcinogenesis and to study the multistep nature of HCC development, one limitation of these models is the fact that the transgene is expressed in all hepatocytes, including the tumor microenvironment. Furthermore, the mutations are already present during embryogenesis and thus, might activate compensatory (molecular) pathways[26]. To overcome these limitations, mouse models have recently been developed in which the genetic alterations are induced in a tissue-specific and time-controlled fashion (conditional mouse models).

For instance, Lewis *et al*[41] used a retroviral transduction strategy to deliver oncogenes to hepatocytes *in situ*. They made use of the fact that mice do not express the TVA receptor, which is the receptor for the avian leukosis sarcoma virus subgroup A (ALSV-A). Lewis *et al*[41] generated TVA transgenic mice, in which TVA was specifically expressed within the liver. Delivery of ALSV-A viruses encoding PyMT (mouse polyoma virus middle T antigen, an oncogene) to these mice at the age of 2-3 d, subsequently led to tumor formation by the age of 4-6 mo (in 17 of 26 mice). They also exposed TVA transgenic mice that were deficient for p53 to PyMT-bearing ALSV-A viruses. Interestingly, the tumor incidence in these mice was not increased, but 6 of 16 p53 null mice that had developed HCCs, showed lung metastases (in contrast with 1 of 17 p53 wild-type mice). Consequently, this mouse model might be of value as a metastatic HCC model. Moreover, this model can be easily used to study the effect of other oncogenes in hepatocarcinogenesis, through the delivery of other oncogene-bearing ALSV-A viruses to TVA transgenic mice.

In addition, Lou *et al*[42] created mice with a regulated expression of liver-specific SV40 T-Ag. The SV40 T-Ag in these mice is preceded by a stop signal flanked by *loxP sites*. Hence, the SV40 T-Ag is expressed upon Cre-mediated excision, either by adenoviral expression of Cre recombinase or by administration of tamoxifen to mice that are transgenic for a liver-specific tamoxifen-inducible Cre. HCCs were observed in mice 5 mo after administration of adenoviral Cre recombinase or tamoxifen.

Several research groups employed alternative recombinase-mediated conditional gene-mutation strategies[32,33,43]. Colnot *et al* generated a mouse strain in which exon 14 of both *Apc* (adenomatous polyposis coli) alleles were flanked by *loxP* sites. The *Apc* alleles become invalidated (leading to β-catenin signaling) upon liver-targeted expression of Cre recombinase. Of these mice, 67% develop HCCs 8-9 mo after Cre recombinase administration[32,33].

Promising results have been published with these and other conditional mouse models to induce HCC-formation. Nonetheless, to date, these models are mainly used to study the effect of genetic alterations (mutation, deletion, or overexpression of a certain gene) on hepatocarcinogenesis and not to induce HCCs.

VIRAL HEPATOCARCINOGENESIS

More than 80% of HCCs in humans are attributable to infection with either hepatitis B virus (HBV) or hepatitis C virus (HCV) or both[44]. HBV- and HCV-related HCC are characteristically preceded by liver cirrhosis, though this is not always the case[45]. It may take more than 20 years for HCC to develop in HBV or HCV infected persons. For this reason, hepatocarcinogenesis due to viral hepatitis probably requires multiple steps of genetic alterations.

Finding the molecular mechanisms that drive these multiple steps by using cell-culture and non-genetic animal models is difficult. Therefore, in the past decades various animal models for investigation of viral hepatitis were developed. One problem in establishing such a model is that HBV and HCV require human hepatocytes to induce hepatitis, due to the stringent human tropism of these viruses[46,47].

In HBV research the finding of HBV-related viruses, e.g. the woodchuck hepatitis virus (WHV) and the ground squirrel hepatitis virus (GSHV), has provided opportunities for *in vivo* studies[46,48]. Another approach

for studying hepatitis B and C infection is the use of immunocompromised mice or rats. Recently, several animal models have been developed in which human hepatocytes or human liver tissue are transplanted into these animals. The transplanted hepatocytes in these animals can be infected with HBV or HCV *in vivo* or *ex vivo*. Alternatively, an already intrinsically infected specimen is transplanted[46,47]. These models are promising for the evaluation of therapeutics and prophylactics against hepatitis due to HBV or HCV, but are not useful to study HBV- or HCV-associated HCC. For that purpose, transgenic mice expressing HBV or HCV proteins represent a better model. In this section, the most frequently used transgenic mouse models for studying HBV- and HCV-associated HCC will be discussed.

By means of these models, two pathways have been proposed that might participate in the hepatocarcinogenic effect of chronic viral hepatitis. First, it is considered that chronic inflammation of the liver, continuous cell death and subsequent chronic hepatocyte regeneration due to viral hepatitis might increase the incidence of genetic alterations[48-53]. The second pathway encompasses a direct oncogenic effect of HBV or HCV on the infected hepatocyte. In the case of HBV (a DNA virus), this carcinogenic effect is believed to be accomplished through cis-activation or trans-activation of cellular genes. In cis-activation, genomic instability is a result of integration of HBV DNA into the host genome. In trans-activation, HBV proteins activate transcription of the HBV genome and host genes by binding to cellular sequences[48,50,51]. For HCV, a direct cytopathic effect has also been reported. As HCV is a RNA virus, it cannot integrate into the host genome. Therefore, other pathways must be of importance[52,53].

As a consequence of the chronic inflammatory state of the infected liver and the direct oncogenic effects of the hepatic viruses (as mentioned before), genetic alterations occur in various cellular pathways, which might eventually lead to the development of HCC. HBV proteins have been shown to manipulate the *p53*-, *Rb*-, *cyclinD1*- and *p21*-genes[51]. HCV is frequently associated with mutations of p53 and β-catenin[52,53].

HEPATITIS B VIRUS-ASSOCIATED HCC

Approximately 380 million people are chronically infected with HBV. These chronic HBV infected people have a 100-fold greater lifetime risk of developing HCC in comparison with non-carriers[3]. For this reason, HBV infection is the leading risk factor for the development of HCC. Worldwide, over 50% of HCC cases are associated with chronic HBV infection and the highest incidence of HCC is in South East Asia and sub-Saharan Africa, regions with a high prevalence of HBV infection[44,54].

As early as 1985, the first transgenic mouse models for investigating HBV infection were developed[55,56]. HBV transgenic mice have been created with the full HBV genome and with every single HBV gene, namely those encoding for the surface envelope proteins (large, middle and small), X protein (HBx), core and precore proteins.

It did not take long before the first transgenic mouse models for evaluation of HBV-associated hepatocarcinogenesis appeared. Until now, merely the large envelope protein and the HBx protein have displayed a carcinogenic role[48,57].

Chisari *et al*[58] described a mouse model in which transgenic mice were generated that carried an integrated HBV DNA fragment coding for the HBV large envelope polypeptides on a C57BL/6 genetic background. As a result, non-secretable hepatitis B surface antigen (HBsAg) particles formed that accumulated in the endoplasmic reticulum of the hepatocyte. In mice with 100% of the hepatocytes expressing HBsAg (lineage 50-4), liver injury begins at 2-3 mo of age; at 6 mo regenerative nodules appear and from the age of 15 mo HCCs develop.

Another HBV gene that has been extensively studied is the HBx gene. Though several research groups could not find evidence for a hepatocarcinogenic role of HBx in HBx transgenic mice[54,59], Kim *et al*[60] did report such a role in 1991. They produced HBx transgenic mice by injection of HBV DNA containing the HBx gene into single-cell embryos from CD1-mice. In these mice, liver tumors began to emerge after 8-10 mo. Both male and female transgenic mice died early in comparison with control CD1 mice, at the age of 11-15 mo *vs* 17-21 mo, respectively. On autopsy, 80%-91% of male transgenic mice and 60%-67% of female transgenic mice showed one or multiple HCCs. Yu *et al*[61] generated transgenic HBx mice using the same technique as Kim *et al*[60], but in a C57BL/6 genetic background and with a much weaker HBx expression in the liver. They reported an incidence of grossly identified HCCs and small neoplastic nodules, without signs of cirrhosis or inflammation, in 86% of 11-18 mo old HBx transgenic mice.

Possible explanations for the different outcome in transgenic mouse models for the hepatocarcinogenic role of HBx, may include a difference in mouse strains that were used. Male mice of the CD-1 strain develop spontaneous HCC in 5.7%[62], an incidence that is somewhat higher than the rate in for instance C57BL/6J mice (< 4.0%)[61]. In addition, the expression level of HBx-mRNA in the livers of transgenic mice and the type of HBx used may be different in the various studies. Finally, the integration site of HBx in the genome of the mice might influence the hepatocarcinogenic effect of HBx[57,61,63].

Efforts have been made to accomplish a model in which complete HBV genome transgenic mice demonstrate HCCs. Thus far, this has not been successful[63,64].

Nowadays, models based on the HBsAg transgenic mouse model of Chisari *et al* and the HBx transgenic mouse model of Kim *et al*[60] are commonly used to study mechanisms involved in hepatocarcinogenesis. These models are also applied to study possible synergistic relations between chemical carcinogens (such as aflatoxin B1 or diethyl nitrosamine) and HBV-infection[65-67]. Another application is the use of bitransgenic mouse models, in which mice are produced that are transgenic for a gene

of interest (such as *c-myc* or *TGF*-α) in conjunction with HBsAg or HBx[48,68,69]. For instance, Jakubczak *et al*[68] produced bitransgenic mice by pairing HBsAg transgenic mice described by Chisari *et al* to TGF-α transgenic mice. At 8 mo of age, 76% (13 of 17) of male bitransgenic mice developed HCCs, while TGF-α transgenic control mice showed HCC in only 6% (1 of 17) and HBsAg transgenic control mice in 0%. These bitransgenic mouse models can be used to investigate the effect of a particular gene on HBV-induced hepatocarcinogenesis.

HEPATITIS C VIRUS-ASSOCIATED HCC

Worldwide, approximately 30% of HCC cases are related to chronic HCV infection, making HCV the second most frequent cause of HCC[44]. In some areas, like Southern Europe and Japan, HCV infection is the strongest predisposing factor for HCC[70]. Patients infected with HCV have a risk of up to 35% for developing liver cirrhosis[47,70]. Thereafter, the cumulative risk of developing HCC in these cirrhotic patients is 1%-7% per year. HCC is the most frequent cause of death in HCV infected persons[47,70,71].

Various HCV proteins have been expressed in transgenic mice to study the pathogenesis of HCV-associated HCC, particularly the HCV polyprotein, the core protein and the core protein in combination with E1 and E2 envelope proteins. Interestingly, the expression of the core protein of HCV seems to be the major factor contributing to the hepatocarcinogenic effect of HCV infection, as transgenic mice that do not express this protein, no HCCs arise[47,52].

Moriya *et al*[72] were the first to describe such a transgenic mouse model. They generated transgenic mice that carried the HCV core gene. These mice showed histological features of steatosis in the liver, without inflammation, from the age of 3 mo and showed HCCs with close histological resemblance of HCCs in human chronic HCV infection, by the time they were 16 mo old. The incidence of HCC in 16-19 mo old male transgenic mice was 26% to 31%, in contrast to a low incidence in the female transgenic mice, which is in accordance with the human situation[73]. By means of such transgenic mouse models numerous molecular and pathogenetic pathways have been investigated that have led to a better understanding of HCV-associated hepatocarcinogenesis.

To study the role of HCV proteins other than the HCV core protein in hepatocarcinogenesis, Lerat *et al*[74] developed full-length HCV polyprotein transgenic mice and compared them with transgenic mice encoding merely the structural HCV proteins (including the core and the E1 and E2 envelope proteins). HCCs occurred (exclusively in males) in 5 of 38 transgenic mice expressing the full HCV polyprotein and in 1 of 43 transgenic mice expressing the structural HCV proteins. These findings suggest that HCV proteins, other than the HCV core protein, may endorse development of HCC as well, because in these mice the HCV protein levels are much lower in the first group[74].

The HCCs that develop in the mouse models described by Moriya *et al* and Lerat *et al* show proper (histological) resemblance to the corresponding lesions in patients with HCV-associated HCC. In the model described by Lerat *et al*[74], tumors develop regardless of the absence of detectable levels of the expression of HCV proteins, mimicking the situation in HCV infected patients. Furthermore, constitutive HCV gene expression results in immunological tolerance to the HCV genes, allowing the study of the direct hepatocarcinogenic effect of HCV proteins in the absence of an immune response to the viral proteins[52,74]. Disadvantages of these models are the possible significance of the genetic background of the mice and the relative unpredictability of HCC formation.

As described above for HBV, the HCV transgenic mouse models of Moriya *et al*, Lerat *et al* and comparable models, are presently used to study carcinogenic mechanisms in HCV-related HCC. In addition, these models are applied to study relations between carcinogens (like DEN) and HCV-infection in inducing HCC[75,76]. Furthermore, bitransgenic mouse models have been developed to investigate the effect of a particular gene on HCV-induced hepatocarcinogenesis.

CONCLUSION AND FUTURE PERSPECTIVES

Mouse models in cancer research are developed to imitate human carcinogenesis. Although the ideal animal model does not (yet) exist, mouse models can imitate parts of human carcinogenesis. To date, different types of mouse models are available to induce HCC, varying in complexity. The most appropriate model for a particular research question should be chosen to answer that specific question. Each approach has its own advantages and disadvantages, which are discussed in this review.

First, carcinogen-induced models are used to identify chemicals that might be carcinogenic to humans. Furthermore, these models are used for establishing a relationship between carcinogen exposure and specific genetic changes. In HCC, DEN is especially used to induce HCC. Major disadvantages remain the influence of sex, age and genetic background of the mice on the predictability of HCC-development. Moreover, there is a species difference in the response to hepatocarcinogens between humans and mice.

Next, because of their suitability, implantation models are still frequently used for the screening of different types of anticancer drugs. Nonetheless, these models have a poor predictive value for the anti-tumor effects in patients. This is probably the consequence of culturing the tumor cells for a long period, which alters the molecular characteristics and the heterogeneity of the original tumor.

Agents tested in mice with subcutaneously implanted tumors, should always be tested in orthotopic models as well, because of the importance of the microenvironment on the biological behaviour of malignant cells.

HCC is a result of different genetic mutations. Con-

ventional transgenic mouse models have been developed to study the role of different genes in HCC formation and to study molecular features involved in hepatocarcinogenesis. Limitations of these models include the expression of the transgene in all hepatocytes (hence, the tumor microenvironment as well) and the presence of the genetic alterations during embryogenesis (which might activate compensatory pathways). To overcome these problems, conditional mouse models have been developed recently. To date, these models are mainly used to study the effect of genetic alterations and, unfortunately, not to induce HCCs. Genetically engineered mouse models are also used in studying the role of viral hepatitis in HCC formation, as HBV and HCV require human hepatocytes to induce hepatitis and, consequently, hepatitis-induced hepatocarcinogenesis.

Highly sophisticated genetically engineered mouse models will become increasingly available and will help to answer a variety of research questions. Nevertheless, significant differences between mice and humans have to be taken into account when interpreting the (molecular) mechanisms of hepatocarcinogenesis. The most familiar of these (interspecies) differences are the much longer telomeres in mice, due to persistent telomerase expression in mice (as opposed to limited or absent telomerase expression in humans). Humans also differ from mice in respect to, for instance, their metabolism and immune system. To extrapolate the results from cancer studies in mice to humans, humanized mice should be generated and used in (genetically engineered) mouse models in the future.

This will bring us one step closer to the ideal animal model for cancer research.

As mentioned above, the adequate mouse model will be used depending on the research question. However, whether the impact of a possible carcinogen is investigated, the development of anticancer drugs or the genetic background of HCC formation is studied, all experiments have the goal to reduce the prevalence of HCC.

REFERENCES

1 **American Cancer Society**. Global Cancer Facts & Figures 2007. Available from: URL: http://www cancer org/docroot/STT/stt_0_2007 asp?sitearea=STT&level=1
2 **Farazi PA**, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer* 2006; **6**: 674-687
3 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917
4 **Frese KK**, Tuveson DA. Maximizing mouse cancer models. *Nat Rev Cancer* 2007; **7**: 645-658
5 **Naugler WE**, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, Karin M. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* 2007; **317**: 121-124
6 **Chen CJ**, Yu MW, Liaw YF. Epidemiological characteristics and risk factors of hepatocellular carcinoma. *J Gastroenterol Hepatol* 1997; **12**: S294-S308
7 **Buchmann A**, Bauer-Hofmann R, Mahr J, Drinkwater NR, Luz A, Schwarz M. Mutational activation of the c-Ha-ras gene in liver tumors of different rodent strains: correlation with susceptibility to hepatocarcinogenesis. *Proc Natl Acad Sci USA* 1991; **88**: 911-915
8 **Lee GH**. Paradoxical effects of phenobarbital on mouse hepatocarcinogenesis. *Toxicol Pathol* 2000; **28**: 215-225
9 **Hann B**, Balmain A. Building 'validated' mouse models of human cancer. *Curr Opin Cell Biol* 2001; **13**: 778-784
10 **Wogan GN**. Impacts of chemicals on liver cancer risk. *Semin Cancer Biol* 2000; **10**: 201-210
11 **Williams GM**. Chemicals with carcinogenic activity in the rodent liver; mechanistic evaluation of human risk. *Cancer Lett* 1997; **117**: 175-188
12 **Seitz HK**, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer* 2007; **7**: 599-612
13 **Gonzalez FJ**. The peroxisome proliferator-activated receptor alpha (PPARalpha): role in hepatocarcinogenesis. *Mol Cell Endocrinol* 2002; **193**: 71-79
14 **Huff J**, Cirvello J, Haseman J, Bucher J. Chemicals associated with site-specific neoplasia in 1394 long-term carcinogenesis experiments in laboratory rodents. *Environ Health Perspect* 1991; **93**: 247-270
15 **Vesselinovitch SD**, Mihailovich N. Kinetics of diethylnitrosamine hepatocarcinogenesis in the infant mouse. *Cancer Res* 1983; **43**: 4253-4259
16 **Vesselinovitch SD**, Koka M, Mihailovich N, Rao KV. Carcinogenicity of diethylnitrosamine in newborn, infant, and adult mice. *J Cancer Res Clin Oncol* 1984; **108**: 60-65
17 **Vesselinovitch SD**. Infant mouse as a sensitive bioassay system for carcinogenicity of N-nitroso compounds. *IARC Sci Publ* 1980; 645-655
18 **Anisimov VN**, Ukraintseva SV, Yashin AI. Cancer in rodents: does it tell us about cancer in humans? *Nat Rev Cancer* 2005; **5**: 807-819
19 **Hirst GL**, Balmain A. Forty years of cancer modelling in the mouse. *Eur J Cancer* 2004; **40**: 1974-1980
20 **Troiani T**, Schettino C, Martinelli E, Morgillo F, Tortora G, Ciardiello F. The use of xenograft models for the selection of cancer treatments with the EGFR as an example. *Crit Rev Oncol Hematol* 2008; **65**: 200-211
21 **Killion JJ**, Radinsky R, Fidler IJ. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer Metastasis Rev* 1998; **17**: 279-284
22 **Heijstek MW**, Kranenburg O, Borel Rinkes IH. Mouse models of colorectal cancer and liver metastases. *Dig Surg* 2005; **22**: 16-25
23 **Clarke R**. Human breast cancer cell line xenografts as models of breast cancer. The immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. *Breast Cancer Res Treat* 1996; **39**: 69-86
24 **Bankert RB**, Egilmez NK, Hess SD. Human-SCID mouse chimeric models for the evaluation of anti-cancer therapies. *Trends Immunol* 2001; **22**: 386-393
25 **Tang ZY**, Sun FX, Tian J, Ye SL, Liu YK, Liu KD, Xue Q, Chen J, Xia JL, Qin LX, Sun SL, Wang L, Zhou J, Li Y, Ma ZC, Zhou XD, Wu ZQ, Lin ZY, Yang BH. Metastatic human hepatocellular carcinoma models in nude mice and cell line with metastatic potential. *World J Gastroenterol* 2001; **7**: 597-601
26 **Jonkers J**, Berns A. Conditional mouse models of sporadic cancer. *Nat Rev Cancer* 2002; **2**: 251-265
27 **Khanna C**, Hunter K. Modeling metastasis in vivo. *Carcinogenesis* 2005; **26**: 513-523
28 **Bibby MC**. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. *Eur J Cancer* 2004; **40**: 852-857
29 **Becher OJ**, Holland EC. Genetically engineered models have advantages over xenografts for preclinical studies. *Cancer Res* 2006; **66**: 3355-8335, discussion 3358-3359
30 **Shuldiner AR**. Transgenic animals. *N Engl J Med* 1996; **334**: 653-655
31 **Palmiter RD**, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* 1982;

300: 611-615
32 **Buendia MA**. Genetics of hepatocellular carcinoma. *Semin Cancer Biol* 2000; **10**: 185-200
33 **Colnot S**, Decaens T, Niwa-Kawakita M, Godard C, Hamard G, Kahn A, Giovannini M, Perret C. Liver-targeted disruption of Apc in mice activates beta-catenin signaling and leads to hepatocellular carcinomas. *Proc Natl Acad Sci USA* 2004; **101**: 17216-17221
34 **Ali SH**, DeCaprio JA. Cellular transformation by SV40 large T antigen: interaction with host proteins. *Semin Cancer Biol* 2001; **11**: 15-23
35 **Ahuja D**, Saenz-Robles MT, Pipas JM. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. *Oncogene* 2005; **24**: 7729-7745
36 **Dubois N**, Bennoun M, Allemand I, Molina T, Grimber G, Daudet-Monsac M, Abelanet R, Briand P. Time-course development of differentiated hepatocarcinoma and lung metastasis in transgenic mice. *J Hepatol* 1991; **13**: 227-239
37 **Kitagawa T**, Hino O, Lee GH, Li H, Liu J, Nomura K, Ohtake K, Furuta Y, Aizawa S. Multistep hepatocarcinogenesis in transgenic mice harboring SV40 T-antigen gene. *Princess Takamatsu Symp* 1991; **22**: 349-360
38 **Sepulveda AR**, Finegold MJ, Smith B, Slagle BL, DeMayo JL, Shen RF, Woo SL, Butel JS. Development of a transgenic mouse system for the analysis of stages in liver carcinogenesis using tissue-specific expression of SV40 large T-antigen controlled by regulatory elements of the human alpha-1-antitrypsin gene. *Cancer Res* 1989; **49**: 6108-6117
39 **Murakami H**, Sanderson ND, Nagy P, Marino PA, Merlino G, Thorgeirsson SS. Transgenic mouse model for synergistic effects of nuclear oncogenes and growth factors in tumorigenesis: interaction of c-myc and transforming growth factor alpha in hepatic oncogenesis. *Cancer Res* 1993; **53**: 1719-1723
40 **Santoni-Rugiu E**, Nagy P, Jensen MR, Factor VM, Thorgeirsson SS. Evolution of neoplastic development in the liver of transgenic mice co-expressing c-myc and transforming growth factor-alpha. *Am J Pathol* 1996; **149**: 407-428
41 **Lewis BC**, Klimstra DS, Socci ND, Xu S, Koutcher JA, Varmus HE. The absence of p53 promotes metastasis in a novel somatic mouse model for hepatocellular carcinoma. *Mol Cell Biol* 2005; **25**: 1228-1237
42 **Lou DQ**, Molina T, Bennoun M, Porteu A, Briand P, Joulin V, Vasseur-Cognet M, Cavard C. Conditional hepatocarcinogenesis in mice expressing SV 40 early sequences. *Cancer Lett* 2005; **229**: 107-114
43 **Harada N**, Oshima H, Katoh M, Tamai Y, Oshima M, Taketo MM. Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations. *Cancer Res* 2004; **64**: 48-54
44 **Parkin DM**. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 2006; **118**: 3030-3044
45 **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35-S50
46 **Dandri M**, Volz TK, Lutgehetmann M, Petersen J. Animal models for the study of HBV replication and its variants. *J Clin Virol* 2005; **34** Suppl 1: S54-S62
47 **Kremsdorf D**, Brezillon N. New animal models for hepatitis C viral infection and pathogenesis studies. *World J Gastroenterol* 2007; **13**: 2427-2435
48 **Singh M**, Kumar V. Transgenic mouse models of hepatitis B virus-associated hepatocellular carcinoma. *Rev Med Virol* 2003; **13**: 243-253
49 **Cougot D**, Neuveut C, Buendia MA. HBV induced carcinogenesis. *J Clin Virol* 2005; **34** Suppl 1: S75-S78
50 **Brechot C**. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. *Gastroenterology* 2004; **127**: S56-S61
51 **Park NH**, Song IH, Chung YH. Chronic hepatitis B in hepatocarcinogenesis. *Postgrad Med J* 2006; **82**: 507-515
52 **Liang TJ**, Heller T. Pathogenesis of hepatitis C-associated hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S62-S71
53 **Guidotti LG**, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006; **1**: 23-61
54 **Lee TH**, Finegold MJ, Shen RF, DeMayo JL, Woo SL, Butel JS. Hepatitis B virus transactivator X protein is not tumorigenic in transgenic mice. *J Virol* 1990; **64**: 5939-5947
55 **Chisari FV**, Pinkert CA, Milich DR, Filippi P, McLachlan A, Palmiter RD, Brinster RL. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. *Science* 1985; **230**: 1157-1160
56 **Babinet C**, Farza H, Morello D, Hadchouel M, Pourcel C. Specific expression of hepatitis B surface antigen (HBsAg) in transgenic mice. *Science* 1985; **230**: 1160-1163
57 **Akbar SK**, Onji M. Hepatitis B virus (HBV)-transgenic mice as an investigative tool to study immunopathology during HBV infection. *Int J Exp Pathol* 1998; **79**: 279-291
58 **Chisari FV**, Klopchin K, Moriyama T, Pasquinelli C, Dunsford HA, Sell S, Pinkert CA, Brinster RL, Palmiter RD. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. *Cell* 1989; **59**: 1145-1156
59 **Reifenberg K**, Lohler J, Pudollek HP, Schmitteckert E, Spindler G, Kock J, Schlicht HJ. Long-term expression of the hepatitis B virus core-e- and X-proteins does not cause pathologic changes in transgenic mice. *J Hepatol* 1997; **26**: 119-130
60 **Kim CM**, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature* 1991; **351**: 317-320
61 **Yu DY**, Moon HB, Son JK, Jeong S, Yu SL, Yoon H, Han YM, Lee CS, Park JS, Lee CH, Hyun BH, Murakami S, Lee KK. Incidence of hepatocellular carcinoma in transgenic mice expressing the hepatitis B virus X-protein. *J Hepatol* 1999; **31**: 123-132
62 **Chandra M**, Frith CH. Spontaneous neoplasms in aged CD-1 mice. *Toxicol Lett* 1992; **61**: 67-74
63 **Bagis H**, Arat S, Mercan HO, Aktoprakligil D, Caner M, Turanli ET, Baysal K, Turgut G, Sekmen S, Cirakoglu B. Stable transmission and expression of the hepatitis B virus total genome in hybrid transgenic mice until F10 generation. *J Exp Zoolog A Comp Exp Biol* 2006; **305**: 420-427
64 **Guidotti LG**, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus replication in transgenic mice. *J Virol* 1995; **69**: 6158-6169
65 **Ghebranious N**, Sell S. Hepatitis B injury, male gender, aflatoxin, and p53 expression each contribute to hepatocarcinogenesis in transgenic mice. *Hepatology* 1998; **27**: 383-391
66 **Sell S**, Hunt JM, Dunsford HA, Chisari FV. Synergy between hepatitis B virus expression and chemical hepatocarcinogens in transgenic mice. *Cancer Res* 1991; **51**: 1278-1285
67 **Kaplanski C**, Chisari FV, Wild CP. Minisatellite rearrangements are increased in liver tumours induced by transplacental aflatoxin B1 treatment of hepatitis B virus transgenic mice, but not in spontaneously arising tumours. *Carcinogenesis* 1997; **18**: 633-639
68 **Jakubczak JL**, Chisari FV, Merlino G. Synergy between transforming growth factor alpha and hepatitis B virus surface antigen in hepatocellular proliferation and carcinogenesis. *Cancer Res* 1997; **57**: 3606-3611
69 **Terradillos O**, Billet O, Renard CA, Levy R, Molina T, Briand P, Buendia MA. The hepatitis B virus X gene potentiates c-myc-induced liver oncogenesis in transgenic mice. *Oncogene* 1997; **14**: 395-404
70 **Di Bisceglie AM**. Hepatitis C and hepatocellular carcinoma. *Hepatology* 1997; **26**: 34S-38S
71 **Koike K**, Tsutsumi T, Fujie H, Shintani Y, Kyoji M. Molecular mechanism of viral hepatocarcinogenesis. *Oncology* 2002; **62** Suppl 1: 29-37
72 **Moriya K**, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997; **78** (Pt 7): 1527-1531

73 **Moriya K**, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065-1067
74 **Lerat H**, Honda M, Beard MR, Loesch K, Sun J, Yang Y, Okuda M, Gosert R, Xiao SY, Weinman SA, Lemon SM. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; **122**: 352-365
75 **Kamegaya Y**, Hiasa Y, Zukerberg L, Fowler N, Blackard JT, Lin W, Choe WH, Schmidt EV, Chung RT. Hepatitis C virus acts as a tumor accelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis. *Hepatology* 2005; **41**: 660-667
76 **Kato T**, Miyamoto M, Date T, Yasui K, Taya C, Yonekawa H, Ohue C, Yagi S, Seki E, Hirano T, Fujimoto J, Shirai T, Wakita T. Repeated hepatocyte injury promotes hepatic tumorigenesis in hepatitis C virus transgenic mice. *Cancer Sci* 2003; **94**: 679-685
77 **Jhappan C**, Stahle C, Harkins RN, Fausto N, Smith GH, Merlino GT. TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell* 1990; **61**: 1137-1146
78 **Lee GH**, Merlino G, Fausto N. Development of liver tumors in transforming growth factor alpha transgenic mice. *Cancer Res* 1992; **52**: 5162-5170
79 **Conner EA**, Lemmer ER, Omori M, Wirth PJ, Factor VM, Thorgeirsson SS. Dual functions of E2F-1 in a transgenic mouse model of liver carcinogenesis. *Oncogene* 2000; **19**: 5054-5062
80 **Calvisi DF**, Conner EA, Ladu S, Lemmer ER, Factor VM, Thorgeirsson SS. Activation of the canonical Wnt/beta-catenin pathway confers growth advantages in c-Myc/E2F1 transgenic mouse model of liver cancer. *J Hepatol* 2005; **42**: 842-849

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Helicobacter pylori

Helicobacter pylori damages human gallbladder epithelial cells *in vitro*

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Abstract

AIM: To study the mechanism by which *Helicobacter pylori* (*H pylori*) damages human gallbladder epithelial cells (HGBEC).

METHODS: *H pylori* isolated from gallbladder were cultured in a liquid medium. Different concentration supernatants and sonicated extracts of *H pylori* cells were then added to HGBEC in a primary culture. The morphological changes in HGBEC as well as changes in the levels of alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and glutamyltransferase (GGT) were measured.

RESULTS: According to the culture curve of HGBEC, it was convenient to study the changes in HGBEC by adding *H pylori* sonicated extracts and *H pylori* culture supernatants. Both *H pylori* sonicated extracts and *H pylori* culture supernatants had a significant influence on HGBEC morphology, i.e. HGBEC grew more slowly, their viability decreased and their detachment increased. Furthermore, HGBEC ruptured and died. The levels of ALP (33.84 ± 6.00 *vs* 27.01 ± 4.67, $P < 0.05$), LDH (168.37 ± 20.84 *vs* 55.51 ± 17.17, $P < 0.01$) and GGT (42.01 ± 6.18 *vs* 25.34 ± 4.33, $P < 0.01$) significantly increased in the HGBEC culture supernatant in a time- and concentration-dependent. The damage to HGBEC in *H pylori* culture liquid was more significant than that in *H pylori* sonicated extracts.

CONCLUSION: *H pylori* induces no obvious damage to HGBEC.

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Key words: Alkaline phosphatase; Glutamyltransferase; *Helicobacter pylori*; Human gallbladder epithelial cells; Lactate dehydrogenase

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INTRODUCTION

Cholecystitis can be caused by many factors[1-6], of which *Helicobacter pylori* (*H pylori*) has been reported to cause gallbladder mucosa injury in the colonial region and inflammatory reaction[7-9]. In this study, in order to ascertain if the damage to gallbladder mucosa relates to *H pylori*, we explored the damaging effect of *H pylori* on human gallbladder epithelial cells (HGBEC) using an enriched liquid and a primary culture of HGBEC to show that *H pylori* is an important clinical etiological factor leading to cholecystitis.

MATERIALS AND METHODS

Materials

H pylori colonies extracted from the gallbladder were selected and transferred to a modified Brucella broth liquid medium, with a density of up to 10^7/L, and placed into a triangular filter which was then sealed with a vacuum lipid and connected to a rubber tube for air exchange. The rubber tube was clipped with hemostatic forceps so that the oxygen inside the filter was maintained at 50 mL/L. The rubber tube was then immobilized in a type THI-82 homeothermia oscillator (Shanghai Yuejin Medical Instrument Corporation) at 37℃, 150 r/min, and observed with regard to bacterial opacity at 24, 48 and 72 h. The extracted bacteria were used to determine the A660 value using a 721

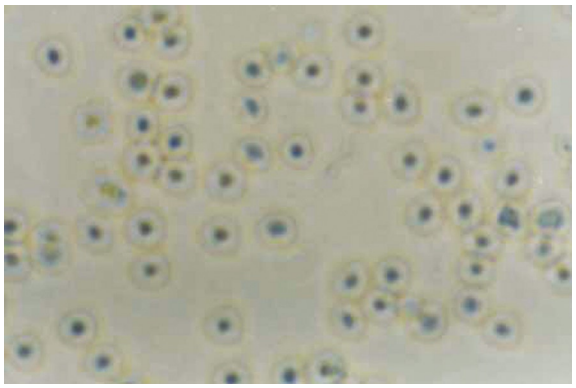

Figure 1 Culture of normal human gallbladder epithelial cells (HGBEC) (x 400).

spectrophotometer (1.0 A660 was approximately equal to 1×10^{11}/L) and the density of *H pylori* calculated. The bacterial status was observed *via* smeared Gram staining. The optimal growth period of *H pylori* was determined by the density and morphology of the bacteria. Thereafter, the liquid culture was terminated to allow the bacteria to adjust to 10^{11}/L for centrifugation, after which supernatants and precipitates were collected and frozen at -30℃ until use. *H pylori* grew well in the liquid culture medium and reached a live bacterial density of 10^{12}-10^{13}/L after shaking for 24-48 h, when the bacterial morphology was typical, but was atypical with less live bacteria and many spheroids after 72 h. Therefore, *H pylori* cultivated for 48 h was considered suitable for this study. HGBEC during their optimal growth period (6 d after primary culture) taken from the HGBEC growth curve was considered suitable for this study[10] (Figure 1). HGBEC cultured and supplemented with Brucella broth were used as controls.

Methods

An enriched liquid containing 10^{12}/L of *H pylori* was diluted to 10^{11}/L of *H pylori* using ultrapure water. The collected supernatants, following centrifugation, were diluted to a final density of 1/10 and 1/100 using ultrapure water, filtered using a 0.22 μm filter coat and frozen at -70℃ until use. The supernatants and ultrapure water were used to dilute the bacteria to a density of 10^{11}/L, where an ultrasound cell breaker, with an output of 80 W for 1 min/time was employed, until Gram staining showed that all *H pylori* were broken. *H pylori* were diluted to 1/10 and 1/100, filtered using a 0.22 μm filter coat and frozen at -70℃ until use. At the bacterial logarithmic growth period, different concentrations of supernatants from enriched *H pylori*, *H pylori* break liquid as well as Brucella broth, each 150 μL, were added and the morphological and anchoring changes in the cells were observed on days 1, 2, 3 and 4. Supernatants of the cell culture were kept at -30℃, and the cells were collected at the most typical stage and immobilized with 30 g/L glutaral for electron microscopic observation. Finally, assays to determine the levels of alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and glutamyltransferase (GGT) in the cell culture supernatants were performed. The results were expressed as nKat/L.

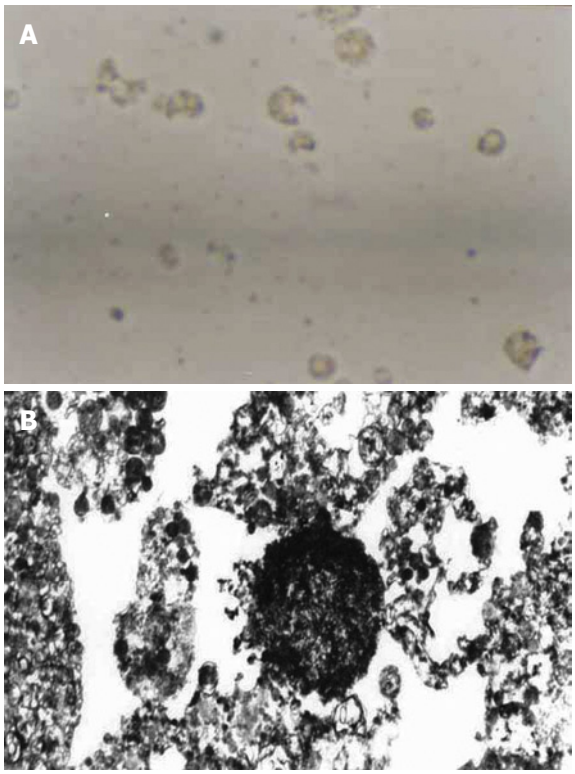

Figure 2 Four d after addition of enriched *H pylori*. A: dead HGBEC (x 400); B: HGBEC characterized by karyopyknosis, vacuoles and incomplete cell structure (x 6000).

Statistical analysis

SSAP 10.0 software was used for statistical analyses. Data were processed using the Student's *t* test and expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Morphological changes in HGBEC

From the second day after addition of *H pylori* supernatants, electron microscopy showed that cell division decreased and the anchored HGBEC loosened leading to dissociation. On day 3, the cell number significantly decreased, with rarefaction of the cytoplasm, reduced cell division, significant vacuoles, slow motion and deepened nuclei chromatin. On day 4, the cells ruptured into fragments and shrank, showing karyopyknosis, light color and different sizes, loss of adhesiveness and increased cell death (Figure 2A). The addition of diluted supernatants of *H pylori* to HGBEC alleviated the cell damage and prolonged survival time and anchoring time compared with HGBEC with added primary *H pylori*. The addition of 1/100 *H pylori* supernatants did not significantly change cell morphology, suggesting that the addition of 1/100 *H pylori* supernatants induced no obvious damage to HGBEC. The cell changes in the controls (Brucella broth added) were in accordance with the growth of

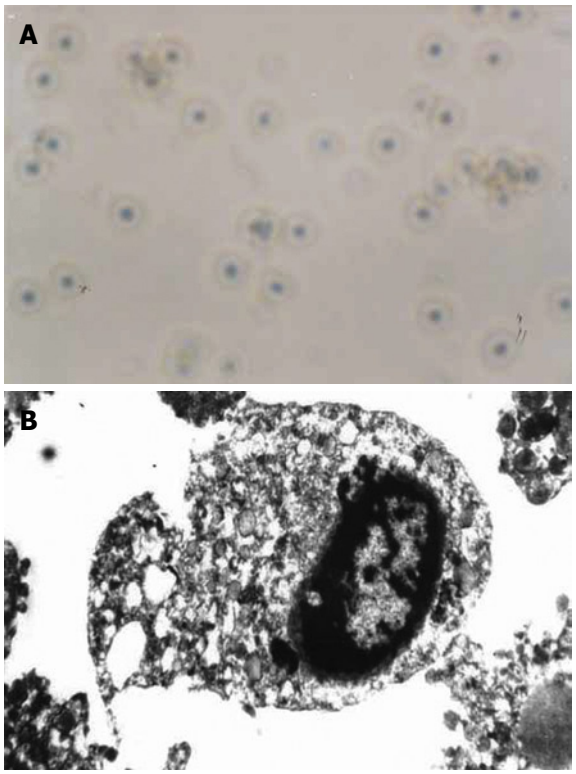

Figure 3 Four days after addition of *H pylori* sonicated extracts. A: Most HGBEC were normal, however, a few HGBEC died (x 400); B: Basically normal HGBEC and some cellular plasm under the vacuole (x 6000).

HGBEC receiving no other substance, i.e. at day 7, cell vitality was reduced, cell number decreased, and on day 14, all cultured HGBEC died. Electron microscopy showed the following characteristics in HGBEC supplemented with primary *H pylori* supernatants for 3 and 4 d. Cells were scattered and reduced in size, with rarefaction and swelling of the cytoplasm, mitochondrion swelling, myelin sheath changes and obvious vacuoles as well as karyopyknosis, assembled chromatin scattered to blocks, incomplete structure of cell nuclei and nuclear membrane in some regions, and even a dense body formed by the whole cell nuclei and hence the cell nuclei disappeared, leading to broken cells (Figure 2B). It was shown that *H pylori* supernatants had a significant damaging effect on HGBEC in a time- and dose-dependent manner, resulting in obvious vacuoles.

From the second day after the addition of sonicated extracts of *H pylori*, electron microscopy showed that the primary HGBEC had decreased cell division and had insignificant morphological changes. At day 3, the anchored cells were dissociated, with slow motion of free cells, morphological changes, decreased numbers, increased vacuoles, loss of HGBEC adhesiveness, cell malformation, karyopyknosis and even cell rupture or death, which was less than HGBEC exposed to *H pylori* supernatants with regard of injury severity. Sonicated extracts of *H pylori* at 1/10 exerted a slight damaging effect on HGBEC, but at 1/100 showed an insignificant damaging effect (Figure 3A). Under the electron microscope, the cells had irregular morphology and were reduced in size, with cytoplasm rarefaction, vacuoles, dilatation of endoplasmic reticulum and mitochondria, reduced nuclei, aggregation of chromatin and even cell rupture and death, which resulted in a slightly more overall damage compared with *H pylori* supernatants (Figure 3B).

Enzymological changes in HGBEC culture supernatants

In comparison with the controls, the three enzymological indices were significantly higher following the addition of different concentrations of enriched liquid into the HGBEC culture supernatants ($P < 0.01$) and still higher on days 2 and 3 following the addition of 1/10 enriched *H pylori* ($P < 0.01$). However, only the values for LDH and GGT were significantly higher on day 4 than those for the controls ($P < 0.01$). There were no significant differences with respect to the enzymological indices in the supernatants after the addition of 1/100 enriched *H pylori*, compared with the controls ($P > 0.05$). With the addition of the stock solution of enriched *H pylori* and 1/10 diluted fluid, the enzymological indices in the supernatants gradually increased with time ($P < 0.01$) with exception on days 3 and 4 (Figure 4).

ALP, LDH and GGT in the supernatants of the HGBEC culture significantly increased 2, 3 and 4 d after the addition of sonicated extracts of *H pylori*, compared with the controls ($P < 0.01$). The addition of 1/10 and 1/100 sonicated extracts of *H pylori* produced an insignificant effect on the enzymological indices in the supernatants ($P > 0.05$). From the second day after the addition of the stock solution of *H pylori*, the levels of ALP, LDH and GGT increased each day ($P < 0.01$) with the exception on days 3 and 4 ($P > 0.05$) (Figure 5).

The above-mentioned results showed that the supernatants of enriched *H pylori* significantly affected the enzymological indices of cultured HGBEC supernatants. The effect of *H pylori* on cell damage and permeability weakened with the gradual decrease in concentration of *H pylori* supernatants. The sonicated extracts of *H pylori* had similar effects, but were far weaker than those of the enriched *H pylori* supernatants.

DISCUSSION

The addition of enriched *H pylori* supernatants resulted in a rapid decrease in cell division, motion and adhesiveness of HGBEC, with obvious vacuoles, cell rupture and necrosis, and even cell death. The sonicated extracts of *H pylori* had similar effects, but were weaker. The reasons for the above changes may be that *H pylori* poisonous substances which permeate the liquid medium so that the supernatants of enriched *H pylori* contain many poisonous substances or metabolites when *H pylori* are under continual oscillation. When the supernatants enter the cultured HGBEC, the poisonous substances exert effects on the HGBEC which damage the cell membrane, increase permeability, induce rarefaction of cytoplasm, vacuoles, dilatation of mitochondria and endoplasmic reticulum, nuclei membrane and nuclei, leading to a decrease in cell division, disordered metabolism and even cell disruption and death[11-12]. The rarefaction of cytoplasm and

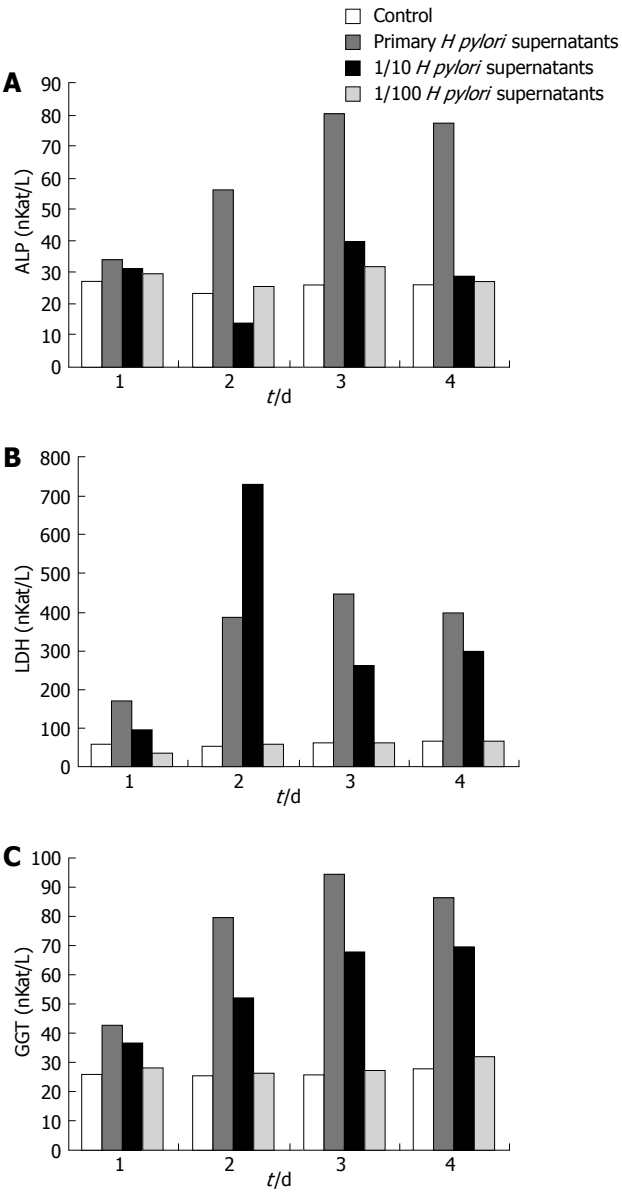

Figure 4 Effect of different concentrations of *H pylori* supernatants on ALP, LDH and GGT in the supernatants of HGBEC culture. A: ALP; B: LDH; C: GGT.

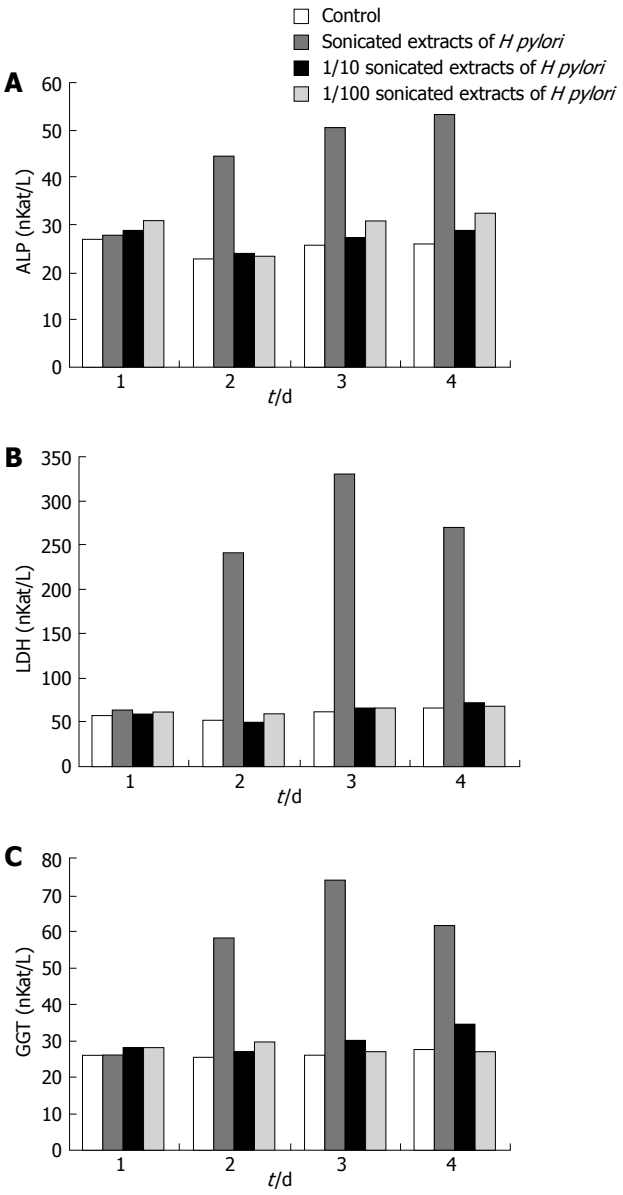

Figure 5 Effect of different concentrations of sonicated extracts of *H pylori* on HGBEC supernatant ALP, LDH and GGT. A: ALP; B: LDH; C: GGT.

obvious vacuoles seen under the optic microscope and electron microscope are due to the actions of *H pylori* and other cells such as HeLa cells and hepatocytes, which contain vacuolating cytotoxin A (VacA)[13-14]. After VacA disrupts the function of the ion transport protein, which in turn affects the motion of the ion transport protein in HGBEC. The new vacuoles mainly consist of cell endoplasmic reticulum fused with lysosome. The formation of vacuoles in cells is a result of self-phagocytosis. Another *H pylori* toxin, cytotoxin-associated protein (CagA) has a high homogeneity with sodium passage and can affect the sodium pump of HGBEC which results in cell swelling, disruption and even death. We have hypothesized that the damaging effect of *H pylori*, isolated from gallbladder, on HEBEC and epithelial cells of gastric mucosa, is similar with respect to the method of action, i.e. by way of enzymes, toxins like VacA and CagA, urease, lipase, phospholipase A, hemolysin and lipopolysaccharide of *H pylori*[15-19]. In addition, *H pylori* may damage the epithelial cells of gallbladder mucosa and eventually result in cholecystitis by activating inflammatory cells and secreting inflammatory medium as well as by cellular immunity, humoral immunity and autoimmunity[20-22]. The toxic factors in *H pylori* can activate factors inhibiting cell proliferation, promote the expression of regulatory genes like *bcl-2*, *bax* and *fas* and finally induce cell apoptosis[11], which is consistent with the apoptosis we observed in HGBEC. The reason why the sonicated extracts of *H pylori* had a weaker damaging effect on HGBEC than the enriched *H pylori*, is probably because the main toxic factor within the sonicated extracts (lipopolysaccharide) is not a major toxic factor compared to VacA and CagA[23].

Determination of the ALP, LDH and GGT levels in HEBEC culture supernatants showed the

functional status of HGBEC and the integrity of cell membranous structures, as gallbladder epithelial cells containing ALP, LDH and GGT. Our study has clearly demonstrated that the addition of sonicated extracts or supernatants of *H pylori* to cultured HGBEC can, to a certain degree, raise the activity of these three enzymes in cell culture supernatants. With the decrease in cell vitality and enhanced disruption, the increased level of the three enzymes in cell culture supernatants was not due to cell function enhancement and an increase in enzyme synthesis, but to cell damage which resulted in the destruction of membranous structures and enzyme leaking into the supernatants, suggesting that the supernatants and sonicated extracts of *H pylori* can damage cell membranous structures, cause cell disruption and raise the levels of ALP, LDH and GGT in cell culture supernatants. The assayed enzyme levels in cell culture supernatants increased with time, with the exception on days 3 and 4, which reflected the increased enzyme leakage from the cells with aggravated HGBEC injury. Following cell disruption and death, the enzymes in the cells leaked and the content showed no increase in the cell culture supernatants, suggesting that *H pylori* can damage gallbladder epithelial cells and may be one of the factors leading to clinical cholecystitis.

REFERENCES

1 **Sun XF**, Chi BE, Luo L. Expression of p53, bcl-2 and EGFR in cholecyst polyp and gallbladder carcinoma and its clinical significance. *Shijie Huaren Xiaohua Zazhi* 1998; **6** (SE7): 491
2 **Chen SH**. Clinical exploration on cholelithiasis and infection of biliary tract. *Shijie Huaren Xiaohua Zazhi* 1998; **6** (SE7): 510-511
3 **Zhou YJ**. Cholecyst schistosomiasis complicated by cholelithiasis: a report of 21 cases. *Shijie Huaren Xiaohua Zazhi* 1998; **6** (SE7): 458
4 **Wu YD**, Yang WY. Posthepatitic cirrhosis and cholecyst lesion: a report of 50 cases. *Shijie Huaren Xiaohua Zazhi* 1998; **6** (SE7): 337
5 **Wei Y**, Zheng H, Shen AD, Huangfu G. Correlative study of changes of sonic permeability and viscosity of the bile. *Shijie Huaren Xiaohua Zazhi* 1998; **6** (SE7): 474
6 **Shi DL**, Cong WG, Sun DM. Comprehensive treatment of biliary calculus. *Shijie Huaren Xiaohua Zazhi* 1998; **6** (SE7): 443
7 **Yu JP**, Li L, Luo XY. Significance of gallbladder mucosa and bile Hp and anti-Hp CagA antibody in patients with cholelithiasis. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 826-827
8 **Fang CH**, Yang JZ, Kang HG. A PCR study on Hp DNA of bile, mucosa and stone in gallstones patients and its relation to stone nuclear formation. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 233-235
9 **Shi JS**, Han WS, Zhuo JS, Lu Y, Jiao XY, Yang YJ. An exploration on the role of L form bacteria in formation of cholelithiasis. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 170
10 **Chen DF**, Liu WW, Fang DC, Liu P, Yi P. Isolation, culture and identification of human gallbladder epithelial cells. *Disan Junyi Daxue Xuebao* 2001; **23**: 1109-1111
11 **Neu B**, Randlkofer P, Neuhofer M, Voland P, Mayerhofer A, Gerhard M, Schepp W, Prinz C. Helicobacter pylori induces apoptosis of rat gastric parietal cells. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G309-G318
12 **Bebb JR**, Letley DP, Rhead JL, Atherton JC. Helicobacter pylori supernatants cause epithelial cytoskeletal disruption that is bacterial strain and epithelial cell line dependent but not toxin VacA dependent. *Infect Immun* 2003; **71**: 3623-3627
13 **Supajatura V**, Ushio H, Wada A, Yahiro K, Okumura K, Ogawa H, Hirayama T, Ra C. Cutting edge: VacA, a vacuolating cytotoxin of Helicobacter pylori, directly activates mast cells for migration and production of proinflammatory cytokines. *J Immunol* 2002; **168**: 2603-2607
14 **McClain MS**, Schraw W, Ricci V, Boquet P, Cover TL. Acid activation of Helicobacter pylori vacuolating cytotoxin (VacA) results in toxin internalization by eukaryotic cells. *Mol Microbiol* 2000; **37**: 433-442
15 **Silva CP**, Pereira-Lima JC, Oliveira AG, Guerra JB, Marques DL, Sarmanho L, Cabral MM, Queiroz DM. Association of the presence of Helicobacter in gallbladder tissue with cholelithiasis and cholecystitis. *J Clin Microbiol* 2003; **41**: 5615-5618
16 **Dohmen K**, Shigematsu H, Miyamoto Y, Yamasaki F, Irie K, Ishibashi H. Atrophic corpus gastritis and Helicobacter pylori infection in primary biliary cirrhosis. *Dig Dis Sci* 2002; **47**: 162-169
17 **Banic M**, Buljevac M, Kujundzic M, Jelic D, Dominis M, Colic-Cvrlje V, Kardum D, Katicic M. [Extra-gastrointestinal tract diseases and Helicobacter pylori infection] *Lijec Vjesn* 2002; **124** Suppl 1: 63-68
18 **Kuroki T**, Fukuda K, Yamanouchi K, Kitajima T, Matsuzaki S, Tajima Y, Furui J, Kanematsu T. Helicobacter pylori accelerates the biliary epithelial cell proliferation activity in hepatolithiasis. *Hepatogastroenterology* 2002; **49**: 648-651
19 **Leong RW**, Sung JJ. Helicobacter species and hepatobiliary diseases. *Aliment Pharmacol Ther* 2002 ;**16**: 1037-1045
20 **Backhed F**, Torstensson E, Seguin D, Richter-Dahlfors A, Rokbi B. Helicobacter pylori infection induces interleukin-8 receptor expression in the human gastric epithelium. *Infect Immun* 2003; **71**: 3357-3360
21 **Straubinger RK**, Greiter A, McDonough SP, Gerold A, Scanziani E, Soldati S, Dailidiene D, Dailide G, Berg DE, Simpson KW. Quantitative evaluation of inflammatory and immune responses in the early stages of chronic Helicobacter pylori infection. *Infect Immun* 2003; **71**: 2693-2703
22 **Nedrud JG**, Blanchard SS, Czinn SJ. Helicobacter pylori inflammation and immunity. *Helicobacter* 2002; **7** Suppl 1: 24-29
23 **Hansen PS**, Petersen SB, Varning K, Nielsen H. Additive effects of Helicobacter pylori lipopolysaccharide and proteins in monocyte inflammatory responses. *Scand J Gastroenterol* 2002; **37**: 765-771

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CLINICAL RESEARCH

A feasibility trial of computer-aided diagnosis for enteric lesions in capsule endoscopy

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Abstract

AIM: To investigate and evaluate the feasibility of the computer-aided screening diagnosis for enteric lesions in the capsule endoscopy (CE).

METHODS: After developing a series of algorithms for the screening diagnosis of the enteric lesions in CE based on their characteristic colors and contours, the normal and abnormal images obtained from 289 patients were respectively scanned and diagnosed by the CE readers and by the computer-aided screening for the enteric lesions with the image-processed software (IPS). The enteric lesions shown by the images included esoenteritis, mucosal ulcer and erosion, bleeding, space-occupying lesions, angioectasia, diverticula, parasites, etc. The images for the lesions or the suspected lesions confirmed by the CE readers and the computers were collected, and the effectiveness rate of the screening and the number of the scanned images were evaluated, respectively.

RESULTS: Compared with the diagnostic results obtained by the CE readers, the total effectiveness rate (sensitivity) in the screening of the commonly-encountered enteric lesions by IPS varied from 42.9% to 91.2%, with a median of 74.2%, though the specificity and the accuracy rates were still low, and the images for the rarely-encountered lesions were difficult to differentiate from the normal images. However, the number of the images screened by IPS was 5000 on average, and only 10%-15% of the original images were left behind. As a result, a large number of normal images were excluded, and the reading time decreased from 5 h to 1 h on average.

CONCLUSION: Though the total accuracy and specificity rates by the computer-aided screening for the enteric lesions with IPS are much lower than those by the CE readers, the computer-aided screening diagnosis can exclude a large number of the normal images and confine the enteric lesions to 5000 images on average, which can reduce the workload of the readers in the scanning of the images. This computer-aided screening technique can make a correct diagnosis as efficiently as possible in most of the patients.

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Key words: Enteric lesions; Image processing; Capsule endoscopy; Diagnosis

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INTRODUCTION

Depiction of the small intestines has still been a challenge because of their length and tortuosity. In the past time when some lesions occurred in an intestinal segment, they were usually difficult to identify because of a shortage of the efficiently used tools[1,2]. When the capsule endoscopy (CE) was introduced to the clinical practice in 2000, it became a revolutionary diagnostic tool in diagnosing small intestinal diseases[3]. Besides, there is growing evidence that CE may be used in diagnosing some diseases occurring in the colon and the esophagus[4,5]. Because of the incapability of its use in the insufflation, affusion, biopsy and therapy, when

compared with the balloon or push enteroscope[6-8], CE still has some limitations in diagnosing and treating the enteric lesions[9]; however, the patients are willing to accept it because it is painless and noninvasive. It is also much superior to the small intestinal radiology in management of some diseases[10].

CE is virtually a microcamera, which can create 2 images/s in the human gastrointestinal (GI) tract, obtain 40-60 thousand images of the GI lumen from the mouth to the anus before its batteries are exhausted[5].

The principal task of the CE readers is to scan all the images and find out what diseases occur in the GI tract, especially those occurring in the small intestines, because they cannot be detected by the routine gastroscopy or colonoscopy. So, the screening task is a heavy burden on the readers because there is a large number of images for them to screen. Though the number of the images for the lesions is smaller than 500 in most of the patients, the CE reader still has to scan ten thousands of the images one by one because the reader cannot make sure which images the lesions are in. As a rule, it would take 4-6 h at least for two readers (2-3 h for each reader) to finish this tiring work in our trial, and the 4-6 h was only the time for the two readers to finish scanning the images once. If there were some doubts and suspicions, much more time would be spent in repeating the scanning, which might do a great harm to the readers' eyes. In order to solve this problem, the high-speed reading (HSR) technique is the commonly-used method at the present time[11], and the reading time can be decreased to 0.5-1 h for one reader[12], but the work intensity is much greater, and some lesions may be missed, too[12,13]. So, if the computer-aided screening diagnosis with image-processed software (IPS) can be used to identify the images for the enteric lesions from a large number of the images that contain the normal ones, it will greatly decrease the workload of the readers for doing this kind of work and make a correct diagnosis as soon as possible.

However, the previous studies showed that the computer-aided diagnosis could not be as accurate as the one made by the reader because of the complexity and multiformity of the lesions in the GI tract[14]. So, our research focused on the feasibility of "screening" the normal images. After the exclusion of a large number of the normal images with the help of IPS, the remaining images can efficiently be evaluated; whether the images indicate the confirmed lesions or indicate only the suspected lesions, they can efficiently be preserved for a further diagnosis.

MATERIALS AND METHODS

Image-sample collection and pretreatment

All the images, derived from the CE produced by OMOM Capsule Endoscopic Company (OCEC), Chongqing City, China, were obtained from the Endoscopic Center of West China Hospital of Sichuan University or provided by OCEC directly. Two hundred and eighty-nine patients, with a median age of 54.2 years (range, 17-83 years), were included. All the patients finished the CE examination without any capsule retention. No lesions were found in 86 of the patients, but 1 lesion was found in 182 patients, 2 in 12 patients, 3 in 6 patients, and 4 in 3 patients. The enteric lesions included esoenteritis, mucosal ulcer and erosion, bleeding, space-occupying lesions, angioectasia, diverticula, parasites, *etc* (Table 1). All the images would be converted to the bitmap (BMP) format directly before they were analyzed by IPS[15].

Table 1 Proportions of enteric lesions and non-lesions in 289 patients

Enteric lesion	No. of lesions and non-lesions	Proportion (%)
Non-lesion	86	26.7
Common lesion		
Esoenteritis	42	13.0
Mucosal ulcer and erosion	47	14.6
Bleeding	61	18.9
Space-occupying lesion	28	8.7
Angioectasia		
Telangiectasis	5	1.6
Angioectasia	32	9.9
Angioma	7	2.2
Rare lesion		
Diverticulum	3	0.9
Parasite	5	1.7
Enteric stenosis	2	0.6
Intussusception	2	0.6
Enteric lymphangiectasis	2	0.6
Total	322	100.0

Algorithm analysis

In every enteric image obtained by CE, all the pictorial contents could be grouped as one of the following three categories: enteric mucosa, enteric lesions, and enteric contents. Each of them had its own characteristic colors, coloring distribution or borderlines of the body to the surroundings, which might be distinguished from one another by the computer-aided IPS in most of the images. According to the Tricolor Theory, the color of every dot in the image consisted of three types of the primary pels: red (R), green (G) and blue (B), whose chromatic values varied from 0 to 255. The characteristic color (primary hue of the enteric mucosa was R, with a common value greater than 100 (average value, 150-200), which was different from that of the lesions or that of the contents in the small intestines in most of the images. Besides esoenteritis and bleeding, there was a series of algorithms developed to simulate the scanning of the human eyes to "screen" a large number of the images for the normal mucosa.

Algorithm principle

The first step was to decide the analyzed region in the image. The dots with an R value (Rv) less than 100 would be excluded because they formed the "dark region", which was hard to be discerned even by the human eyes (Figure 1A and B), and the remaining dots would be contained for the analysis.

The second step was to exclude the region of the

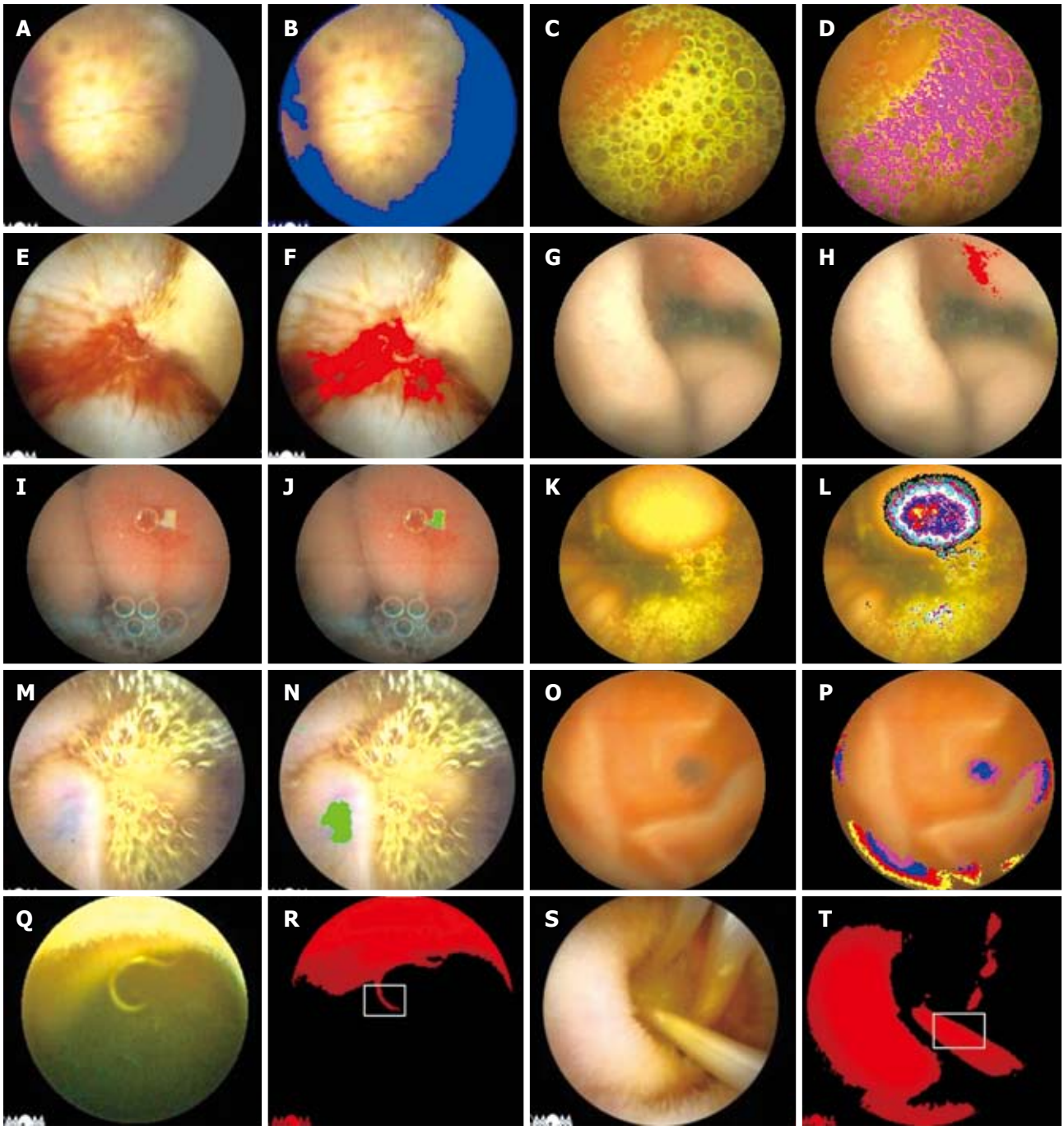

Figure 1 The screened results of enteric lesions by the IPS. A: Space-occupying lesions; B: The blue area in the image for the "dark region"; C: The enteric bile; D: The purple area in the image for the enteric bile; E: The enteric blood (bleeding); F: The red area in the image for the enteric blood; G: Esoenteritis; H: The red area in the image for esoenteritis; I: Mucosal ulcer; J: The green area in the image for mucosal ulcer; K: Space-occupying lesions; L: Different coloring circle in the image for space-occupying lesions; M: Venous angioma in the bright background; N: The green area in the image for venous angioma; O: Venous angioma in the dark background; P: The loop-closed distribution of Gv of the venous angiomain image; Q: Parasites (hook worms); R: The image of red components (Rv > 150) and the curvature of the double limits in the pane calculated; S: Parasites (ascarides); T: The image of red components (Rv > 150) and the linearity of the double limits in the pane calculated.

enteric contents, such as bile, spume *etc*., by their own characteristic colors.

In the bile region, Rv was less than 230, and the ratio of Rv to Gv was less than 1.1, which led to the characteristic color of yellow or green (Figure 1C and D); in the blood region, the usual Rv of the dots was less than 180 but greater than 120, the ratio of Rv to Gv was greater than 2, and the B value (Bv) was greater than 30 at least (Figure 1E and F).

The third step was to extract the characteristic colors and the coloring distribution of the common lesions in the small intestines.

In the region of esoenteritis, the Rv (> 180) of the dots was greater than Gv, and the remaining value subtracted Gv from Rv was greater than 60-70, and Bv varied from 60 to 90 (Figure 1G and H); furthermore, the difference (60-70) between Rv and Gv in the inflammatory region was greater than that (30-40) between

the background regions (mucosal regions) on average.

In the region of mucosal ulcer and erosion, Rv (> 170, < 200) was greater than Gv (> 150), but the remaining value subtracted Gv from Rv was less than 40, and Bv was greater than 100, so the mucosal ulcer and erosion in the image looked yellow and white appreciably, which was different from the color of the enteric bile. Rv in the region of mucosal ulcer and erosion was greater than that of the background region (mucosal region) by 30-40 on average (Figure 1I and J).

Generally speaking, in the region of space-occupying lesions, there was no characteristic color due to the lesions derived from the mucosal or submucosal tissues, but the space-occupying lesions had a characteristic protrusion into the enteric lumen, which led to a circular distribution of the degressive Gv, and Rv in the center of the circle was usually greater than 245 (Figure 1K and L).

Venous angioma was a special type of space-occupying lesions without any enteric protrusion, but it looked blue. In the bright background, the remaining value subtracted Gv from Rv (> 160, < 200) was less than 30, and Bv from Gv (> 150) less than 10 (Figure 1M and N). In the dark background, the remaining value subtracted Gv (> 70) from Rv (> 100, < 170) was less than 50. Bv was greater than 45, but the most important characteristic was that the lesion had the loop-closed distribution of Rv and Gv, which was different from that of the fringe of the image (Figure 1O and P).

Though the common parasites in the small intestines (Figure 1Q and S), mainly hook worms and ascarides, had no special body colors, but the double borderlines of the body to the surroundings (contour) usually existed clearly. Thus, a kind of the algorithm was developed to distinguish the parasites from the background mucosa or the enteric contents. Firstly, the chromatic pels of the parasites (Rv > 150, Gv > 120) in the bright visual field would be extracted. Secondly, as the double limits of the parasites in the extracted region had a similar curvature (hook worms) or linearity (ascarides), it could be calculated in some width and be judged by the software whether the parasites existed or not (Figure 1R and T). Finally, whether the screened results were accurate or not, the images about the esophagus, stomach and large intestine would be excluded if they were in the remaining images.

The rarely-encountered lesions, including diverticulum (Figure 2A), intussusception (Figure 2B), enteric stenosis (Figure 2C), enteric lymphangiectasis (Figure 2D), angioectasia (Figure 2E and F) *etc.*, because of the absence of characteristic colors or contours compared with the background mucosa, could not be picked out by the algorithm principles mentioned above, which will be explained in Discussion.

Supplementary algorithms

If there were several lesions in the image, the image processing would be terminated immediately for the saving of time if only one lesion or parts of the lesions were confirmed by any of the algorithms mentioned above.

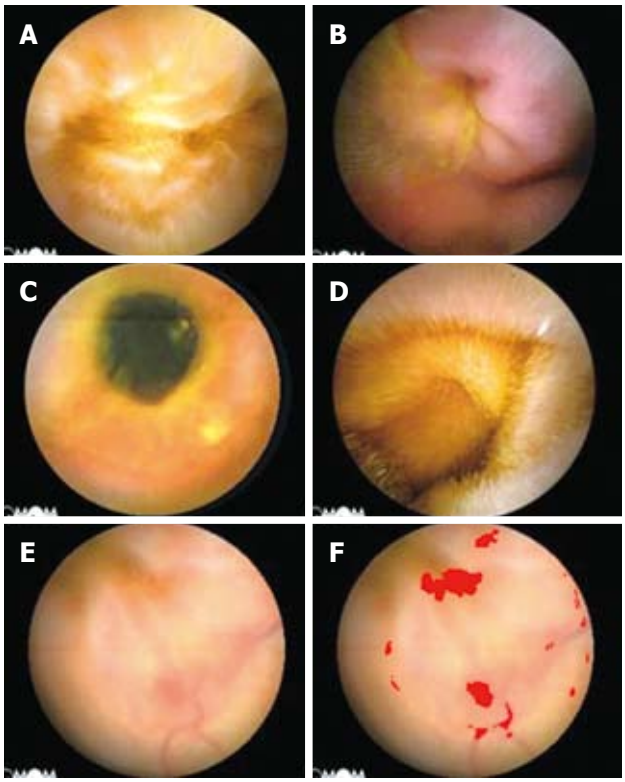

Figure 2 The enteric lesions not being recognised by the IPS. A: Enteric diverticulum; B: Intussusception; C: Enteric stenosis; D: Enteric lymphangiectasis; E: Angioectasia; F: The red area in the image for suspected bleeding, not angioectasia itself.

Software development and operation

IPS was developed and operated in the common personal computer (memory 1G, Intel® Pentium® Core 2 CPU 1.73GHz, Hard Disk 60GB at least), with Windows Operation System Xp or Vista® home basic.

Screening standard and evaluation

The gold standard for enteric lesions was the diagnostic conclusion drawn by the CE reader (the physician who reviews the CE study). In order to decrease the rate of misdiagnosis to a lowest extent, the primary images and the screened images by IPS from the same patient would respectively be scanned by two readers[13] with a speed of 20 000 images/h, and the marked images by IPS would be extracted out and ranked by the time-index in the images from the jejunum to the ileum, which would be scanned again by two readers and assessed whether those images of the lesions were preserved in the remaining images (screened results) and whether the lesions accorded with those of the readers' findings. If there was a series of continuous images of the confirmed lesions or the suspected lesions, only the first image and the last image would be marked out by IPS as shown by the index.

Inclusion standards for the screened results were: (1) A series of images for the lesions could be confirmed by IPS if one image was confirmed; (2) Only parts of the lesions in the images could be marked out by IPS.

Exclusion standards for the screened results were: None of the images for the lesions was picked out or

Table 2 Number of marked images in 289 patients

Group (*n*)	Total No. of images	No. of marked images	*P*
With lesion (236)	52 374 ± 4 865	4 156 ± 478[b]	< 0.001
Without lesion (86)	54 210 ± 3 739	4 219 ± 376[d]	< 0.001
P	> 0.10	> 0.10	

[b]*P* < 0.001, total No. of the images *vs* No. of the marked images in the group with lesions; [d]*P* < 0.001, total No. of the images *vs* No. of the marked images in the group without any lesions.

Table 3 Scanning time (ST) spent in each patient on marked images in 289 patients

Group (*n*)	ST for total images in each patient (h)	ST for marked images in each patient (h)	*P*
With lesion (236)	5.4 ± 1.2	1.2 ± 0.2[b]	< 0.001
Without lesion (86)	5.6 ± 1.3	1.3 ± 0.1[d]	< 0.001
P	> 0.10	> 0.10	

[b]*P* < 0.001, ST for the total images *vs* ST for the marked images by ISP in the group with lesions; [d]*P* < 0.001, ST for the total images *vs* ST for the marked images by ISP in the group without any lesions.

none of the marked parts in the images was the lesion itself.

Statistical analysis

Statistical analysis was performed using the SPSS software (ver.11.5). The correlation between two variables was evaluated using Pearson's χ^2, Fisher's exact test, and *u* test. Statistical significance was defined as $P < 0.05$.

RESULTS

Compared with the primary images in the left, the images in the right showed the suspected enteric lesions by different colors, such as red, blue, green, *etc.* (Figure 1F, H, J, L, N, P, R and T, and Figure 2F).

Screened results

In the 289 patients, the total number of the images varied from 41 358 to 62 874, with a median of 52 374 ± 4865 in the group with the confirmed lesions and 54 210 ± 3739 in the group without any lesions. Parts of the images in each patient were marked by at least one kind of color, and the number varied from 2353 to 18 732, with a median of 4156 ± 478 and 4219 ± 376, respectively, in the group with the lesions and the group without any lesions (Table 2). The mean time needed to read a CE study was 5.4 ± 1.2 h in the group with the lesions and 5.6 ± 1.3 h in the group without any lesions (Table 3). The mean time needed to read a CE study by a reader was 2.8 ± 0.7 h compared with the scanning time of 3.2 ± 0.6 h by IPS (most in 2.5-3.5 h, $P > 0.05$), and the number of the images for the lesions varied from 1 to 262 in the 236 patients.

For the common enteric lesions detected by CE, the effectiveness rate of the screened results (SRs) varied from 42.9% to 91.2%, with a median of 74.2%, but for the angioectasia and rare lesions, the effectiveness rate varied from 20% to 57.1%, with a median of only 22.9% (Table 4).

Table 4 Screened results (SR) of IPS in 289 patients

Enteric lesions	Effectiveness rate of SR (%)	False positive rate of SR (%)	*P*
Common lesion			
Esoenteritis	33/42 (78.6)	188/280 (67.1)	> 0.05
Mucosal ulcer/erosion	31/47 (66.0)	191/275 (69.5)	> 0.05
Bleeding	56/61 (91.8)	121/261 (46.4)	> 0.05
Space-occupying lesion	12/28 (42.9)	96/294 (32.7)	> 0.05
Angioectasia			
Telangiectasis	1/5 (20.0)	63/317 (19.9)	> 0.05
Angioectasia	3/32 (9.4)	37/290 (12.8)	> 0.05
Angioma	4/7 (57.1)	25/315 (7.9)	> 0.05
Rare lesion[1]	3/14 (21.4)	53/308 (17.2)	> 0.05

[1]The samples of the rare lesions were not enough, so they were pooled together for the research.

Table 5 Sensitivity, specificity, accuracy, positive/negative predictive value of SR for special lesions in 289 patients (%)

Enteric lesions	Sensitivity	Specificity	Accuracy	Positive pv	Negative pv
Common lesion					
Esoenteritis	78.6	32.9	38.8	14.9	91.1
Mucosal ulcer/erosion	66.0	30.5	35.7	14.0	84.0
Bleeding	91.8	53.6	60.9	31.6	96.6
Space-occupying lesion	42.9	67.3	65.2	11.1	92.5
Angioectasia					
Telangiectasis	20.0	80.1	79.2	1.6	98.4
Angioectasia	9.4	87.2	79.5	7.5	89.7
Angioma	57.1	92.1	91.3	13.8	98.9
Rare lesion[1]	21.4	82.8	80.1	5.4	5.9

pv: Predictive value. [1]Because the samples of the rare lesions were not enough, so they were pooled together for the research.

The screened results showed that the sensitivity rate (effectiveness rate of SRs) was higher than the specificity rate and the accuracy rate in the commonly-encountered lesions but lower in angioectasia and rarely-encountered lesions; the positive predictive value was low and the negative predictive value was high in all the lesions (Tables 4 and 5).

DISCUSSION

CE plays an important role in diagnosis of the small bowel diseases[16-18], such as bleeding[19-21], Crohn's disease[21-23], tumors[20], angioectasia[20], *etc.* With the bowel peristalsis, CE passes through the small intestines and takes pictures of the intestinal cavity, though the total number of the images in each patient can vary from 40 000 to 60 000, with a median of 50 000, and the definite

number of the images for the lesions is not greater than several hundreds in the patients. After the screening by the computer-aided diagnosis with the help of IPS, the number of the remaining images is only 10%-15% of the original ones (Table 2). Most of the lesions, especially the commonly-encountered enteric lesions, have been included and preserved, which can greatly reduce the workload on the readers and make an efficient diagnosis in most of the patients. However, one thing should be emphasized that the screened results provided by IPS are not the diseases themselves, but the lesions, because it was very difficult for IPS to make a diagnosis according to the lesions themselves just as the CE reader does; therefore, all the data in Table 4 only indicate the screening rates, not the diagnosis rates (Table 4).

The initial objective of this research was to make the diagnosis of the small bowel diseases directly, but the results were disappointing because of the complexity and the multiformity of the lesions. The complicated algorithm (Hidded Markov models, HMM[24,25] or artificial neural network, ANN[26-28]) was of a tryout. Though the diagnosis accuracy could be improved to some extent, the result was not satisfactory to the physicians, and the scanning time of IPS was much longer than that of the readers' performance; therefore, it could not be accepted in the clinical practice. So, we had to turn our steps just to "screening" the images for the lesions and making the normal images left behind. The results showed that about 75% of the images for the common lesions could be screened out and the scanning-time of IPS was limited to 2.5-3.5 h (average, 3.2 h) in most of the patients after the simple algorithms were used based on the characteristic colors, coloring distribution or contours, which proved its feasibility and availability in the clinical application in our trial.

Though a large number of the normal images were excluded and the reading time was decreased from 5 h to 1 h on average (Table 3), the total time (scanning time of IPS plus reading time of CE reader) spent on average in each CE study did not decrease significantly when compared with that of HSR. But with the development of the CE technology, when the IPS is installed in the image-receiver and the images can be analyzed directly just as the Personal Digital Assistant (PDA) does[29-31], the total time can be decreased.

As the images were taken by CE at random, CE could not control the azimuth angles and the luminosity as efficiently as the gastroscope or the colonoscope. As a result, the contrast between the diseased mucosa and the normal mucosa in some images was incorrectly changed. In the screened images, the great majority of the normal images (> 90%) were misdiagnosed (false positive) and some of the images for the lesions were missed out by IPS; therefore, the specificity, accuracy, and predictive value for the positivity were very low for most of the lesions (Table 5). Another problem of IPS was the deficiency of self-adjustment of the borderline between the diseased mucosa and the normal mucosa, which resulted in the fact that only part of the lesions in the image could be marked out (Figure 1F) or part of the normal mucosa was mistakenly marked out (Figure 2F). These were the reasons why the readers would scan the excluded images if they thought that the results obtained from IPS did not accord with the clinical manifestations of the patients given by the CE examination.

For the rarely-encountered lesions by CE, there was still a lack of an ideal discriminating algorithm. These lesions were not the main indications for the use of CE if much more time was consumed, the screening procession was prolonged and the clinical significance was lost. So, we did not develop any special algorithm aimed at those lesions. Another kind of the lesions, i.e., angioectasia, was difficult to be directly recognized because of the deficiency of characteristic colors and contours unless the lesions were accompanied by bleeding (Figure 2F). The proper algorithms have still been under development.

In conclusion, the result of this pilot study has indicated that the computer-aided screening diagnosis can be used as an efficient auxiliary measurement for the commonly-encountered enteric lesions when the images are taken by CE. We suggest that a further research should focus on improvement of the specificity rate and the screening rate for the rarely-encountered enteric lesions. If the robotistic and controllable CE[32] is used and more images with a better contrast are provided, the screening rate can be significantly enhanced.

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COMMENTS

Background

Capsule endoscopy (CE), which is virtually a microcamera, is a revolutionary diagnostic tool in diagnosing small bowel diseases, and CE can obtain 40-60 thousand images of the GI tract, though the number of the images for the lesions is smaller than 500 in most of the patients. The CE reader still has to scan ten thousands of the images one by one because the reader cannot make sure which images the lesions are in. So, it may be a big burden on the CE reader's eyes and energy.

Research frontiers

In order to decrease the reading time, the high-speed reading (HSR) is the commonly-used method at present, the reading time can be decreased to 0.5-1 h for one reader, but the work intensity is much greater and some lesions may be missed.

Innovations and breakthroughs

In this pilot study, a kind of the image-processed software (IPS) aided by a computer was introduced to screen the large number of normal images, and only 10%-15% of the original images were left behind, of which most of the commonly-encountered lesions in the small intestines were preserved and diagnosed; therefore, the workload and the working time of the CE reader could be decreased significantly.

Applications

Though a large number of the normal images were excluded, and the reading time decreased significantly, the total time (scanning time of IPS plus reading time of a CE reader) spent on average in each CE study did not significantly decrease compared with that of HSR at present. But with the development of the CE technology and improvement of algorithms, when IPS is installed in the image-receiver and the images can be analyzed directly just as the Personal Digital Assistant (PDA) does, the total time can be decreased significantly.

Terminology

IPS is a kind of software installed in the computer workstation, which can identify the common lesions in the small intestines based on the characteristic colors and contours of the lesions. HSR system is a kind of software support system installed in the computer workstation, which can speed up the reading of the CE images.

Peer review

This article raises an interesting topic. Authors investigate and evaluate the feasibility of the computer-aided screening diagnosis for enteric lesions in the CE.

REFERENCES

1 **Meron GD**. The development of the swallowable video capsule (M2A). *Gastrointest Endosc* 2000; **52**: 817-819
2 **Swain P**. Wireless capsule endoscopy. *Gut* 2003; **52** Suppl 4: iv48-iv50
3 **Mazzarolo S**, Brady P. Small bowel capsule endoscopy: a systematic review. *South Med J* 2007; **100**: 274-280
4 **Seibel EJ**, Carroll RE, Dominitz JA, Johnston RS, Melville CD, Lee CM, Seitz SM, Kimmey MB. Tethered capsule endoscopy, a low-cost and high-performance alternative technology for the screening of esophageal cancer and Barrett's esophagus. *IEEE Trans Biomed Eng* 2008; **55**: 1032-1042
5 **Iddan G**, Meron G, Glukhovsky A, Swain P. Wireless capsule endoscopy. *Nature* 2000; **405**: 417
6 **Jensen TM**, Vilmann P, Hendel JW. [Double-balloon endoscopy for diagnosis and treatment of small bowel diseases. The first Danish experiences with 31 patients] *Ugeskr Laeger* 2008; **170**: 433-437
7 **Sidhu R**, McAlindon ME, Kapur K, Hurlstone DP, Wheeldon MC, Sanders DS. Push enteroscopy in the era of capsule endoscopy. *J Clin Gastroenterol* 2008; **42**: 54-58
8 **Rondonotti E**, Villa F, Mulder CJ, Jacobs MA, de Franchis R. Small bowel capsule endoscopy in 2007: indications, risks and limitations. *World J Gastroenterol* 2007; **13**: 6140-6149
9 **Rondonotti E**, Herrerias JM, Pennazio M, Caunedo A, Mascarenhas-Saraiva M, de Franchis R. Complications, limitations, and failures of capsule endoscopy: a review of 733 cases. *Gastrointest Endosc* 2005; **62**: 712-716; quiz 752, 754
10 **Marmo R**, Rotondano G, Piscopo R, Bianco MA, Cipolletta L. Meta-analysis: capsule enteroscopy vs. conventional modalities in diagnosis of small bowel diseases. *Aliment Pharmacol Ther* 2005; **22**: 595-604
11 **Yagi Y**, Vu H, Echigo T, Sagawa R, Yagi K, Shiba M, Higuchi K, Arakawa T. A diagnosis support system for capsule endoscopy. *Inflammopharmacology* 2007; **15**: 78-83
12 **Levinthal GN**, Burke CA, Santisi JM. The accuracy of an endoscopy nurse in interpreting capsule endoscopy. *Am J Gastroenterol* 2003; **98**: 2669-2671
13 **Lai LH**, Wong GL, Chow DK, Lau JY, Sung JJ, Leung WK. Inter-observer variations on interpretation of capsule endoscopies. *Eur J Gastroenterol Hepatol* 2006; **18**: 283-286
14 **Gurudu SR**, Vargas HE, Leighton JA. New frontiers in small-bowel imaging: the expanding technology of capsule endoscopy and its impact in clinical gastroenterology. *Rev Gastroenterol Disord* 2008; **8**: 1-14
15 **Gan T**, Rao N. [Study on the computer-assisted real-time diagnosis for micro-focus of esophagus based on the change of region-gradation] *Shengwu Yixue Gongchengxue Zazhi* 2007; **24**: 756-759
16 **Pennazio M**. Diagnosis of small-bowel diseases in the era of capsule endoscopy. *Expert Rev Med Devices* 2005; **2**: 587-598
17 **Ersoy O**, Sivri B, Arslan S, Batman F, Bayraktar Y. How much helpful is the capsule endoscopy for the diagnosis of small bowel lesions? *World J Gastroenterol* 2006; **12**: 3906-3910
18 **Fireman Z**, Kopelman Y. New frontiers in capsule endoscopy. *J Gastroenterol Hepatol* 2007; **22**: 1174-1177
19 **Leighton JA**, Sharma VK, Hentz JG, Musil D, Malikowski MJ, McWane TL, Fleischer DE. Capsule endoscopy versus push enteroscopy for evaluation of obscure gastrointestinal bleeding with 1-year outcomes. *Dig Dis Sci* 2006; **51**: 891-899
20 **Ohmiya N**, Yano T, Yamamoto H, Arakawa D, Nakamura M, Honda W, Itoh A, Hirooka Y, Niwa Y, Maeda O, Ando T, Yao T, Matsui T, Iida M, Tanaka S, Chiba T, Sakamoto C, Sugano K, Goto H. Diagnosis and treatment of obscure GI bleeding at double balloon endoscopy. *Gastrointest Endosc* 2007; **66**: S72-S77
21 **Leighton JA**, Triester SL, Sharma VK. Capsule endoscopy: a meta-analysis for use with obscure gastrointestinal bleeding and Crohn's disease. *Gastrointest Endosc Clin N Am* 2006; **16**: 229-250
22 **Saurin JC**. Capsule endoscopy. *Endoscopy* 2007; **39**: 986-991
23 **Pennazio M**. Crohn's disease: diagnostic and therapeutic potential of modern small-bowel endoscopy. *Gastrointest Endosc* 2007; **66**: S91-S93
24 **Pruteanu-Malinici I**, Carin L. Infinite hidden Markov models for unusual-event detection in video. *IEEE Trans Image Process* 2008; **17**: 811-822
25 **Bali N**, Mohammad-Djafari A. Bayesian approach with hidden Markov modeling and mean field approximation for hyperspectral data analysis. *IEEE Trans Image Process* 2008; **17**: 217-225
26 **La Cara GE**, Ursino M. A model of contour extraction including multiple scales, flexible inhibition and attention. *Neural Netw* 2008; **21**: 759-773
27 **Plagianakos VP**, Magoulas GD, Vrahatis MN. Distributed computing methodology for training neural networks in an image-guided diagnostic application. *Comput Methods Programs Biomed* 2006; **81**: 228-235
28 **Das A**, Ben-Menachem T, Farooq FT, Cooper GS, Chak A, Sivak MV Jr, Wong RC. Artificial neural network as a predictive instrument in patients with acute nonvariceal upper gastrointestinal hemorrhage. *Gastroenterology* 2008; **134**: 65-74
29 **De Ville K**. "The cure is in hand"? The brave new world of handheld computers in medicine. *Camb Q Healthc Ethics* 2008; **17**: 385-400
30 **Blaya JA**, Gomez W, Rodriguez P, Fraser H. Cost and implementation analysis of a personal digital assistant system for laboratory data collection. *Int J Tuberc Lung Dis* 2008; **12**: 921-927
31 **Forjuoh SN**, Reis MD, Couchman GR, Ory MG. Improving diabetes self-care with a PDA in ambulatory care. *Telemed J E Health* 2008; **14**: 273-279
32 **Park S**, Park H, Park S, Jee C, Kim J, Kim B. Capsular locomotive microrobot for gastrointestinal tract. *Conf Proc IEEE Eng Med Biol Soc* 2006; **1**: 2211-2214

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BASIC RESEARCH

Effects of Wy14643 on hepatic ischemia reperfusion injury in rats

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Abstract

AIM: To investigate the effects and possible mechanisms of Wy14643 on hepatic ischemia-reperfusion (I/R) injury in rats.

METHODS: Thirty male Sprague-Dawley rats weighing 220-280 g were randomly divided into five experimental groups: sham group (G1, *n* = 6): a sham operation was performed (except for liver I/R); I/R-untreated group (G2, *n* = 6): rats underwent liver ischemia for 90 min followed by reperfusion for 4 h; and I/R + Wy14643 groups (G3, G4, G5; *n* = 6): after the same surgical procedure as in group 2, animals were pretreated with Wy14643 at the dose of 1, 5 and 10 mg/kg 1 h before ischemia, respectively. Hepatic ischemia-reperfusion (I/R) was induced by clamping blood supply to the left lateral and median lobes of the liver for 90 min, and atraumatic clamp was removed for 4 h reperfusion. Blood samples and liver tissues were obtained at the end of reperfusion to assess serum and hepatic tissue homogenate aminotransferase (ALT), aspartate aminotransferase (AST), myeloperoxidase (MPO), serum interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α), as well as activity of superoxide dismutase (SOD) and content of malondialdehyde (MDA) in the hepatic tissue homogenate.

RESULTS: Hepatic I/R induced a significant increase in the serum levels of ALT, AST, TNF-α, IL-1β and MPO, as well as the levels of ALT, AST and MDA in the liver tissue homogenate, which were reduced by pretreatment with Wy14643 at the dose of 1, 5 and 10 mg/kg, respectively. The activity of SOD in the liver tissue homogenate was decreased after hepatic I/R, which was enhanced by Wy14643 pretreatment. In addition, serum and liver tissue homogenate ALT and AST in the Wy14643 10 mg/kg group were lower than in the Wy14643 1 mg/kg and 5 mg/kg groups, respectively.

CONCLUSION: Wy14643 pretreatment exerts significant protection against hepatic I/R injury in rats. The protective effects are possibly associated with enhancement of anti-oxidant and inhibition inflammation response.

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Key words: Wy14643; Liver; Ischemia-reperfusion; Effects

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INTRODUCTION

Interruption of hepatic inflow is a common procedure during trauma surgery, liver transplantation, and resectional surgery. However, the resulting period of hepatic ischemia and subsequent reperfusion can lead to liver injury and dysfunction through the initiation of a biphasic inflammatory response[1]. An excessive inflammation response is considered as a key mechanism of ischemia-reperfusion injury[2]. The acute phase of this response is characterized by activation of Kupffer cells and their subsequent production and release of reactive oxygen species, which may contribute to liver dysfunction and cell injury during reperfusion[3]. Proinflammatory cytokines, chemokines, and activated complement factors are responsible for neutrophil recruitment and the subsequent neutrophil-induced oxidant stress during the later reperfusion phase[4]. In addition, accumulated neutrophils release oxidants and

proteases that directly injure hepatocytes and vascular endothelial cells and may also obstruct hepatic sinusoids resulting in hepatic hypoperfusion[5].

Peroxisome proliferator-activated receptor-α (PPAR-α) is one of the three subtypes of the nuclear receptor PPAR family[6]. Activation of PPAR-α, by either natural ligands, such as polyunsaturated fatty acids and eicosanoids, or synthetic ligands, such as fibrates, Wy14643, stimulates target-gene transcription *via* the formation of heterodimeric transcription factor complexes with the retinoid X receptor[7]. PPAR-α has a wide range of effects on metabolism, cellular proliferation and the immune response[8]. Beyond metabolic effects, PPARα activation also induces anti-inflammatory and antioxidant effects in different organs. Several studies indicated that Wy14643 protected organs such as heart, kidney and brain against ischemia-reperfusion injury[9-12]. PPAR-α represses the expression of inflammatory-response genes *via* a mechanism termed ligand-dependent transrepression[13]. Direct binding of PPAR-α to NF-κB p65 was demonstrated by *in vitro* assays, suggesting that transrepression might be involved in direct interference with transcriptional activation by NF-κB, thus preventing the synthesis and release of cytokines (interleukin-1 and tumor necrosis factor α)[14,15]. Furthermore, PPAR-α activation induces the expression and activation of antioxidant enzymes such as superoxide dismutase(SOD), catalase and glutathione peroxidase[16,17]. PPAR-α is highly expressed in the liver, particularly in hepatic parenchymal cells, which can protect hepatocytes in the mice hepatic ischemia-reperfusion injury model[1]. But the effect of Wy14643 on hepatic ischemia-reperfusion injury was not clear. In the present study, we determined whether PPAR-α activation by the selective agonist Wy14643 may reduce hepatic I/R injury in rats through modulation of oxidative stress and inflammatory response.

MATERIALS AND METHODS

Chemicals

Wy14643, selective PPARα agonist, was purchased from Cayman Chemical (USA) and used as 1, 5 and 10 mg/kg homogenized in 20 mg/L ethanol *via* intraperitoneal (ip) route.

Ethanol was used to form a homogenized drug. Each dose was homogenized in 1ml ethanol and injected *via* ip. A previous study has found that 20 mg/L ethanol was not harmful to the liver[18].

Ethics and animals

Male Sprague-Dawley rats (weighing 220-280 g) were used in these experiments. Temperature and relative humidity were kept at 22 ± 2℃ and 50% ± 5%, respectively. All rats were obtained from the Center of Experimental Animals in Anhui Medical University. They were allowed free access to a commercial standard chow and water ad libitum before the experimental procedure began. All rats were acclimatized to our animal facility for at least 1 wk before experiment. Stressful stimuli were avoided. This project was approved by the Committee for Research and Animal Ethics of Anhui Medical University.

Experimental design

Rats were randomly divided into five experimental groups each containing six rats: (1) Sham (except for hepatic I/R), (2) Ischemia-reperfusion (I/R), (3) I/R + Wy14643 (1 mg/kg), (4) I/R + Wy14643 (5 mg/kg), and (5) I/R + Wy14643 (10 mg/kg). Partial hepatic ischemia was induced as described previously[1]. Briefly, each rat was weighed and anesthetized by intraperitoneal administration of 1.0 g/kg ethylurethanm. Anesthetized rats were placed onto a thermostatically controlled heating pad, a rectal temperature probe was inserted, and body temperature was monitored and maintained at 37℃. The abdomen was shaved and disinfected with 75% ethanol. A midline incision was performed; the first porta hepatis was exposed. An atraumatic clip was used to prevent blood supply to the left lateral and median lobes of the liver. After 90 min of partial hepatic ischemia, the clip was removed to recover hepatic reperfusion for 4 h. Sham control rats underwent the same protocol without vascular occlusion. Abdominal incision was closed in layers with 4-0 dexon and 2-0 nylon during reperfusion stage in order to prevent the loss of body fluid and quantity of heat.

Collection of blood and tissue samples

After 4 h of reperfusion, blood samples were drawn from aorta ventralis. The liver was carefully dissected out from its attachment, and totally excised. All rats were then sacrificed by hemorrhage. Blood samples were centrifuged with 4000 r/min for 15 min at 4℃, following collection of supernatant liquid, and were kept at -20℃ for biochemical analyses which were duplicated. Ischemia liver tissue was also divided into two parts, one part was fixed in 10 g/L glutaraldehyde to observe hepatic ultrastructure, the other part was snap frozen in liquid nitrogen. The samples were stored at -80℃ until assayed.

Ultrastructural assessment of liver tissue

Ischemia liver samples fixed in 10 g/L Glutaral, dehydrated, drying and surface gilding according to standard procedures. Electron microscope was used to assess the degree of hepatic damage.

Serum and hepatic tissue homogenate ALT and AST assay

Serum and hepatic tissue homogenate ALT and AST, a marker of hepatocellular injury, were measured using commercial available kit and the result was expressed as U/L as well as U/mg protein, respectively.

Total protein concentrations in liver homogenate samples were determined by the Coomassie blue method.

Serum and liver homogenate MPO assay

MPO activity in serum and hepatic tissue was detected by a spectrophotometric method, reflecting the number of polymorphonuclear neutrophils (PMN) in the liver.

This method uses 3, 3', 5, 5'-tetramethyl benzidine (TMB) as an oxidizable dye, and the reaction was started by adding hydrogen peroxide (H_2O_2) in the medium.

The assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Serum TNF-α and IL-1β assay

Serum TNF-α and IL-1β levels were determined with double antibody sandwich ABC-ELISA using a rat TNF-α and IL-1β kit method according to the manufacturer's instructions. The samples were compared with the standard curve and expressed as pg/mL. All assay kits were purchased from Shanghai Senxiong Technologyindustry Co. Ltd, China.

Hepatic tissue homogenate SOD and MDA assay

SOD activity was measured through the inhibition of nitroblue tetrazolium (NBT) reduction by O_2-generated by the xanthine/xanthine oxidase system. One SOD activity unit was defined as the enzyme amount causing 50% inhibition in 1 mL reaction solution per milligram tissue protein and the result was expressed as U/mg protein.

Liver homogenate malondialdehyde (MDA) concentration was measured using the thiobarbituric acid (TBA) method. The amount of lipid peroxides (LPO) was measured as the production of MDA, which in combination with TBA forms a pink chromogen compound whose absorbance at 532 nm was measured. The result was expressed as nmol/mg protein.

All assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Statistical analyses

All data were expressed as mean ± SD. The statistical significance of differences between groups was analyzed using the one-way analysis of variance (ANOVA) and methods of LSD with the SPSS11.5 for Windows XP statistical software package. The *P* values less than 0.05 were considered statistically significant.

RESULTS

Levels of serum and liver tissue homogenate ALT and AST

Liver function was examined by measuring serum and liver tissue levels of ALT and AST. Hepatic I/R caused a marked elevation of serum and liver tissue ALT as well as AST activity (serum ALT: 20 ± 4 U/L *vs* 485 ± 69 U/L, liver tissue homogenate ALT: 50 ± 10 U/mg protein *vs* 181 ± 16 U/mg protein, $P = 0.000$, $P = 0.000$, serum AST: 56 ± 5 U/L *vs* 252 ± 28 U/L, liver tissue homogenate AST: 20 ± 3 U/mg protein *vs* 46 ± 8 U/mg protein, $P = 0.000$, $P = 0.000$). The increase in serum and liver tissue homogenate ALT as well as AST activity induced by hepatic I/R was significantly attenuated by administration of Wy14643 at dose of 1, 5 and 10 mg/kg (serum ALT: 485 ± 69 U/L *vs* 386 ± 49 U/L, 259 ± 56 U/L, 139 ± 29 U/L, $P = 0.001$, $P = 0.000$, $P = 0.000$, liver tissue homogenate ALT: 181 ± 16 U/mg protein *vs* 164 ± 17 U/mg protein, 75 ± 13 U/mg protein, 47 ± 6 U/mg protein, $P = 0.031$, $P = 0.000$, $P = 0.000$, serum AST: 252 ± 28 U/L, 227 ± 30 U/L, 124 ± 19 U/L, 70 ± 8 U/L, $P = 0.048$, $P = 0.000$, $P = 0.000$, liver tissue homogenate AST: 46 ± 8 U/mg protein, 31 ± 7 U/mg protein, 22 ± 5 U/mg protein, 17 ± 4 U/mg protein, $P = 0.000$, $P = 0.000$, $P = 0.000$). The results demonstrated that Wy14643 has the dose-dependent protective effects on liver injury, and that alteration of ALT and AST activity in serum and liver tissue was coincident (Figure 1).

Ultrastructural alterations of liver tissue

The ultrastructural structure of cells was normal in the sham group. After 90 min of hepatic ischemia followed by reperfusion for 4 h, compared with the sham group, the occluded liver tissue from the I/R group was markedly damaged, with mitochondrion swollen, vacuolar degeneration and, mitochondrial crista destruction, marked decrease of rough endoplasmic reticulum and nucleus structure destruction under the electron microscope. Pretreatment of rats with 1, 5 and 10 mg/kg Wy14643 resulted in a significant amelioration of hepatic injury (Figure 2).

Levels of serum and liver tissue homogenate MPO

MPO activity in serum and liver tissue was detected, reflecting the number of polymorphonuclear neutrophils (PMN). The serum and liver tissue homogenate MPO activity was significantly increased after hepatic I/R compared with the sham group (serum MPO: 313 ± 55 U/L *vs* 574 ± 70 U/L, $P = 0.000$; liver tissue homogenate MPO: 0.23 ± 0.04 U/g *vs* 0.42 ± 0.06 U/g, $P = 0.000$). Pretreatment with Wy14643 at dose of 1, 5 and 10 mg/kg led to the marked reduction of MPO content compared with I/R group (serum MPO: 574 ± 70 U/L *vs* 479 ± 63 U/L, 334 ± 33 U/L, 365 ± 72 U/L, $P = 0.012$, $P = 0.000$, $P = 0.000$; liver tissue homogenate MPO: 0.42 ± 0.06 U/g *vs* 0.35 ± 0.05 U/g, 0.26 ± 0.02 U/g, 0.27 ± 0.03 U/g, $P = 0.010$, $P = 0.000$, $P = 0.000$). All these showed that Wy14643 reduced infiltration of leukocytes into the inflammatory sites (Figure 3).

Levels of TNF-α and IL-1β in serum

The TNF-α and IL-1β content was significantly higher after reperfusion in I/R group than in sham group (TNF-α: 1.37 ± 0.21 pg/mL *vs* 11.63 ± 1.11 pg/mL, $P = 0.000$; IL-1β: 78 ± 16 pg/mL *vs* 220 ± 30 pg/mL, $P = 0.000$), Pretreatment with Wy14643 1, 5 and 10 mg/kg resulted in the reduction in dependent-dose manner compared with the I/R group (TNF-α: 11.63 ± 1.11 pg/mL *vs* 10.83 ± 0.94 pg/mL, 9.89 ± 0.60 pg/mL, 9.29 ± 1.20 pg/mL $P = 0.132$, $P = 0.002$, $P = 0.000$; IL-1β: 220 ± 30 pg/mL *vs* 195 ± 27 pg/mL, 112 ± 17 pg/mL, 95 ± 20 pg/mL, $P = 0.070$, $P = 0.000$, $P = 0.000$) (Figure 3).

SOD activity in liver tissue homogenate

The levels of liver SOD lowered significantly after hepatic I/R compared with the sham group (SOD: 114.81 ± 1.13 U/mg protein *vs* 99.52 ± 1.68 U/mg protein, $P = 0.000$), after administration of Wy14643 1, 5 and 10

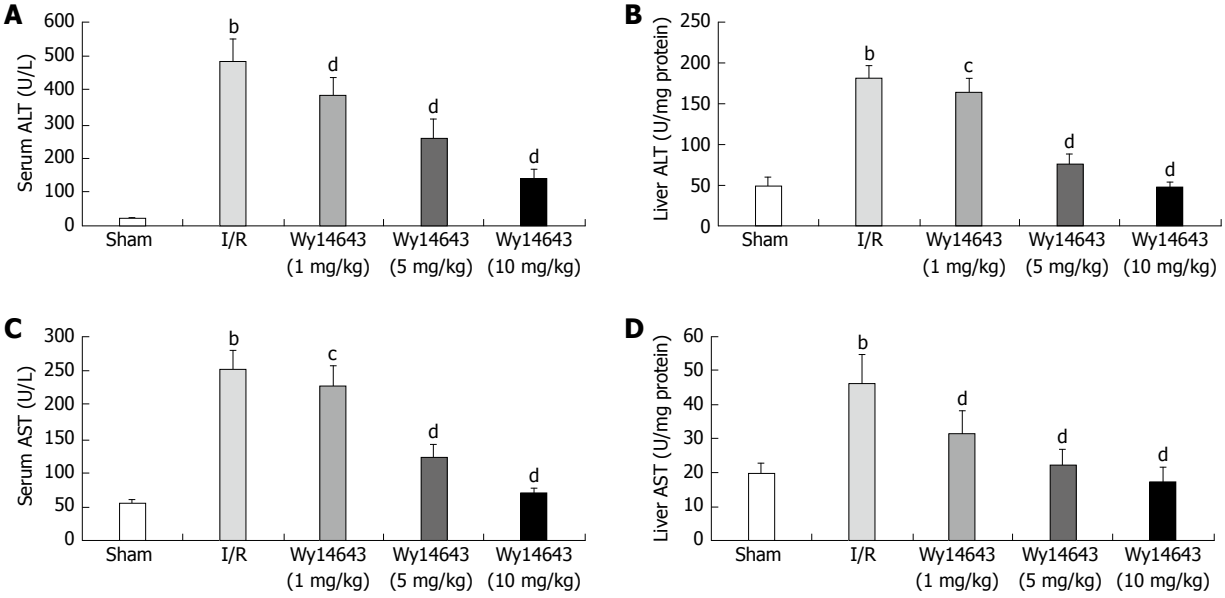

Figure 1 Levels of serum and liver tissue homogenate ALT and AST. Serum ALT (A), liver ALT (B), serum AST (C) and liver AST (D) levels in different groups (mean ± SD, $n = 6$). After 90 min of hepatic ischemia and 4 h of reperfusion, ALT and AST were determined with an ALT and AST assay kit. $^{b}P < 0.01$ *vs* sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* I/R group.

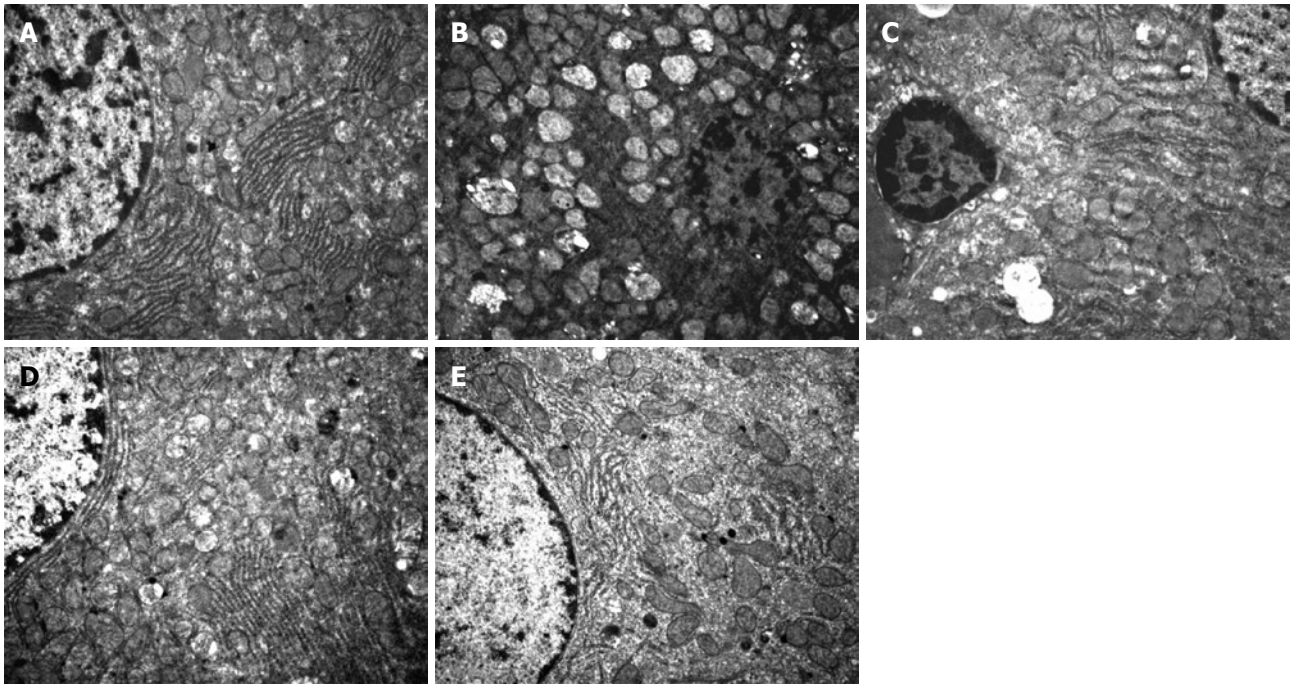

Figure 2 Ultrastructural alterations of liver tissue. A: Sham group. Normal appearance of mitochondrion, rough endoplasmic reticulum and nucleus structure; B: I/R group. Mitochondrion swelled significantly with vacuolar degeneration and mitochondrial crista destruction, marked decrease of rough endoplasmic reticulum and nucleus structure destruction; C: Wy14643 group (1 mg/kg). Mitochondrion swelled moderately, with mitochondrial crista interruption and vacuolar degeneration, increase of rough endoplasmic reticulum and nucleus structure destruction; D: Wy14643 group (5 mg/kg). Mitochondrion swelled mildly, with rough endoplasmic reticulu and normal nucleus structure; E: Wy14643 group (10 mg/kg). Normal appearance of mitochondrion, rough endoplasmic reticulum and nucleus structure.

mg/kg, SOD activity in liver was elevated (99.52 ± 1.68 U/mg protein *vs* 100.09 ± 3.75 U/mg protein, 103.45 ± 3.08 U/mg protein, 103.45 ± 1.73 U/mg protein, $P = 0.706$, $P = 0.014$, $P = 0.014$) (Figure 3).

MDA content in liver tissue homogenate

The MDA content as an index of lipid peroxidation became significantly higher after reperfusion in I/R group than in sham group (MDA: 6.48 ± 0.64 nmol/mg protein *vs* 11.36 ± 1.10 nmol/mg protein, $P = 0.000$). Pretreatment with Wy14643 1, 5 and 10 mg/kg caused marked reduction compared with the I/R group (MDA: 11.36 ± 1.10 nmol/mg protein *vs* 10.37 ± 0.99 nmol/mg protein, 6.59 ± 0.97 nmol/mg protein,7.50 ± 0.66 nmol/mg protein, $P = 0.066$, $P = 0.000$, $P = 0.000$) (Figure 3).

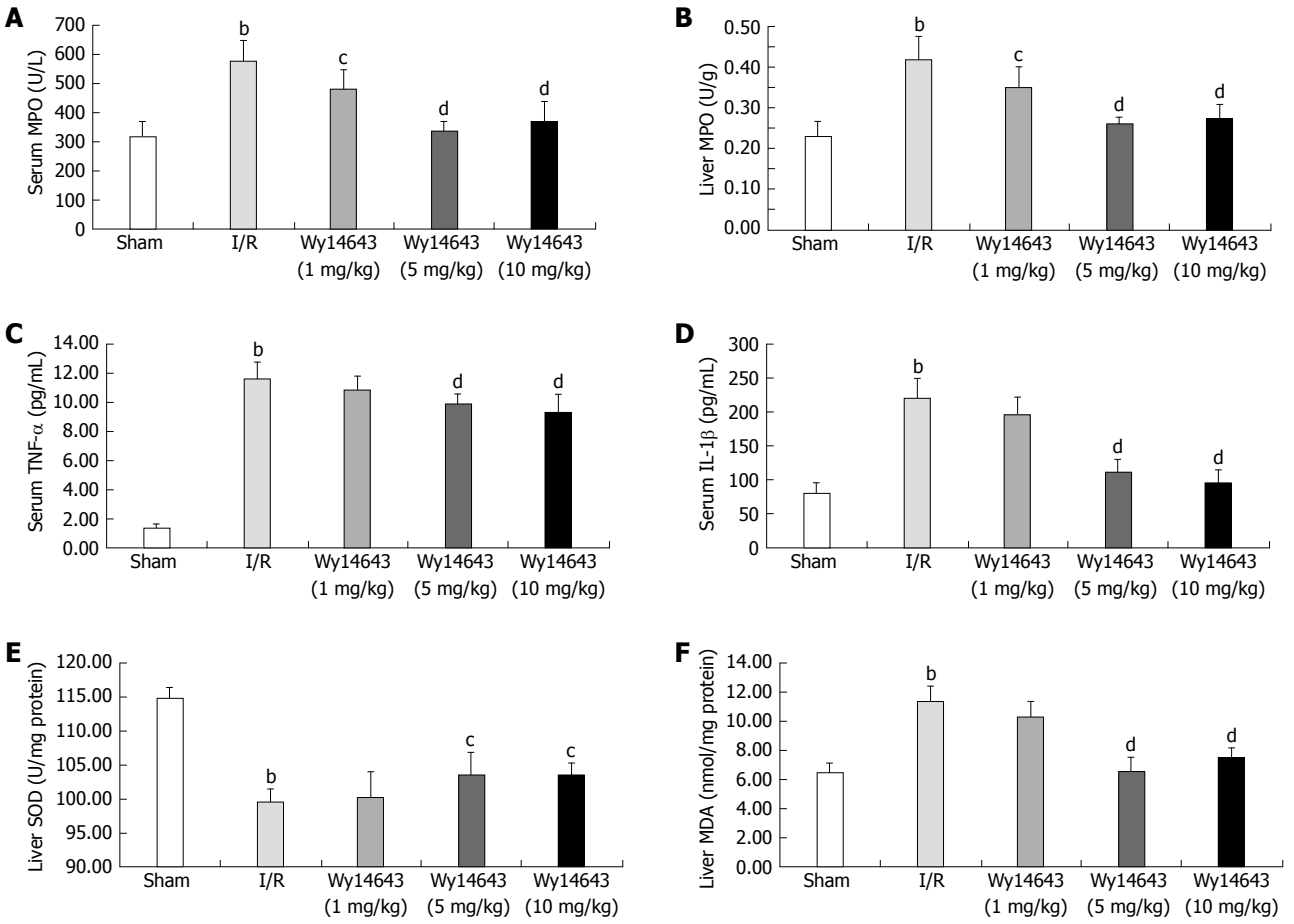

Figure 3 Activities of MPO in the serum (A), activities of MPO in the liver (B), serum TNF-α levels (C), serum IL-1β levels (D), activity of SOD in the liver (E) and content of MDA in the liver (F) in different groups (mean ± SD, *n* = 6). After 90 min of hepatic ischemia and 4 h of reperfusion, MPO, TNF-α, IL-1β, SOD and MDA were analyzed with an MPO, TNF-α, IL-1β, SOD and MDA assay kit. $^{b}P < 0.01$ *vs* sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* I/R group.

DISCUSSION

The present study provides noticeable evidence that the selective PPAR-α agonist Wy14643 protects the rat liver from hepatic I/R injury. This protective effect is demonstrated by reducing ALT and AST levels and is associated with an inhibition of oxidative stress and inflammatory response. An increase in serum and liver homogenate ALT and AST levels has been suggested to be an effective indicator of impaired liver parenchymal cells with hepatic I/R. Our results indicate that Wy14643 results in a marked reduction of ALT and AST levels with dose-dependent manner in the rat hepatic tissue homogenate compared with the sham group.

Hepatic ischemia-reperfusion is known to induce formation of reactive oxygen, as well as excessive inflammatory response, which is clearly recognized as a key mechanism of injury during reperfusion[2,19].

Hepatic ischemia activates Kupffer cells, which are the main sources of vascular reactive oxygen formation during the initial reperfusion period[20]. In addition to Kupffer cell-induced oxidant stress, with increasing length of the ischemic episode, components of NADPH oxidase are recruited to the cell surface leading to the priming for enhanced reactive oxygen formation[21]. Furthermore, ROS can activate diverse downstream signaling pathways, such as the transcription factor nuclear factor-κB (NF-κB), thus regulating expression of genes encoding a variety of proinflammatory proteins[22]. SOD, an oxygen radical scavenger which converts superoxide anion radicals present in the upper stream of reactive oxygen metabolism cascade, protects cells against damage. Lipid peroxidation, mediated by free oxygen radicals, is believed to be an important cause of destruction and damage to cell membranes. Membrane peroxidation can lead to changes in membrane fluidity and permeability and also to enhanced rates of protein degradation, eventually resulting in cell lysis[23]. MDA levels have been extensively used as markers of lipid peroxidation and lipid peroxidation damage in tissues, including cells and body fluids in both clinical and experimental studies[24,25]. In our study, we confirm that hepatic I/R caused significant increase of MDA, accompanied by SOD decrease. Administration of Wy14643 ip 1 h before I/R decreased lipid peroxidation in rats subjected to I/R and, at the same time, offered protection against SOD decrease. This finding is in agreement with other reports showing that Wy14643 enhances expression of antioxidant enzymes such as SOD and catalase in the rat liver[26].

TNF-α and IL-1β derived from activated Kupffer cells play an important role in the pathogenesis of hepatic ischemia-reperfusion injury. These cytokines

are capable of up-regulating adhesion molecules and causing polymorphonuclear neutrophils to adhere to endothelial cells. This causes microcirculation disturbance, which is thought to be a major mechanism of ischemia-reperfusion injury, including the "no-flow phenomenon"[27]. NF-κB is activated during I/R of the liver, and plays an important and complex role in the gene expression of proinflammatory cytokines (TNF-α and IL-1), which will lead to the tissue injury[28]. PPAR-α agonists may have the anti-inflammatory action, which are thought to be mediated through negative regulation of the transcription factors NF-κB and activator protein-1, resulting in decreased expression of their target genes[29,30]. Cuzzocrea *et al*[31] found that administration of Wy14643 before the onset of gut ischemia significantly reduced intestinal I/R injury in rats. The improved outcome was accompanied by reductions in neutrophil infiltration, proinflammatory cytokine(TNF-α and IL-1β) expression. Similarly, renal and liver I/R injury was reduced after Wy14643 pretreatment, partially due to its anti-inflammatory effects[1,32]. In mice, PPAR-α ligands attenuate cisplatin-induced ARF by repressing inflammation *via* inhibition of NF-κB binding activity, which attenuate neutrophil inflation and cytokine release(TNF-α and IL-1β)[33]. It is reported that Wy14643 lowers levels of TNF-α and IL-1β in plasma[34,35]. It is observed that pretreatment with PPAR-α agonists Wy14643 significantly decreased TNF-α and IL-1β in serum. Thus, the results showed that its reduction represents a further mechanism for hepatic protection by PPAR-α agonists.

MPO is an enzyme restricted mainly to polymorphonuclear neutrophils (PMNs), reflecting the number of PMNs in the serum and liver. The evidence indicated that Wy14643 reduced infiltration of the reperfused intestine with polymorphonuclear neutrophils[31]. Our result shows that Wy14643 significantly decreased the serum and liver MPO activity compared with the I/R group. That is to say, Wy14643 reduced infiltration of leukocytes into the inflammatory sites to decrease hepatic I/R injury. In the present study, the significant increase of MPO activity in the hepatic tissue homogenate after hepatic I/R is consistent with a previous study[36].

In summary, our results show that Wy14643 pretreatment protects liver injury induced by hepatic I/R. The protection effects are probably associated with enhancement of antioxidant capacities and inhibition of inflammation responses. However, the precise mechanisms of PPAR-α agonists need to be further investigated in the oxidative stress and inflammatory response implicated in the pathogenesis of hepatic I/R injury.

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COMMENTS

Background

The peroxisome proliferator-activated receptor-alpha (PPAR-α) is a member of the nuclear receptor family of ligand-dependent transcription factors. Several studies indicated that PPARα protected organs such as heart, kidney and brain against ischemia-reperfusion injury. PPARα activation also induces anti-inflammatory and antioxidant effects. In addition, PPAR-α is highly expressed in the liver, particularly in hepatic parenchymal cells, which can protect hepatocytes in the mouse model of hepatic ischemia-reperfusion injury.

Research frontiers

In recent years, more attention has been paid to the effects of PPAR-α on the important organs such as heart, brain, liver and kidney. PPAR-α is a transcription factor that in some *in vitro* systems has been linked with down-regulation of proinflammatory mediators, thus implicating a potential role for PPAR-α in the regulation of inflammatory processes. Meanwhile, PPAR-α enhances expression of antioxidant enzymes such as SOD and catalase.

Innovations and breakthroughs

Wy14643 was shown to enhance antioxidant capacities and inhibit inflammation responses in some studies. The present study analyzed serum and hepatic tissue homogenate indexes such as ALT, AST, MPO, SOD, MDA,TNF-α and TNF-α. Ultrastructural alterations of liver tissue were also observed. These results all indicated that Wy14643 significantly mitigated hepatic I/R injury in a dose-dependent manner.

Applications

This study has indicated that PPAR-α agonist Wy14643 pretreatment protects liver injury induced by hepatic I/R. The protective effects may be associated with enhancement of antioxidant capacities and inhibition of inflammation responses. This may present a novel and attractive approach to prevent hepatic I/R injury.

Peer review

The research is important because authors demonstrated that Wy14643 pretreatment exerts significant protection against hepatic I/R injury in rats. The protective effects are possibly associated with enhancement of anti-oxidant and inhibition inflammation response.

REFERENCES

1 **Okaya T**, Lentsch AB. Peroxisome proliferator-activated receptor-alpha regulates postischemic liver injury. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G606-G612
2 **Jaeschke H**. Mechanisms of reperfusion injury after warm ischemia of the liver. *J Hepatobiliary Pancreat Surg* 1998; **5**: 402-408
3 **Jaeschke H**, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991; **260**: G355-G362
4 **Jaeschke H**, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by neutrophils and Kupffer cells during in vivo reperfusion after hepatic ischemia in rats. *J Leukoc Biol* 1992; **52**: 377-382
5 **Jaeschke H**, Smith CW. Mechanisms of neutrophil-induced parenchymal cell injury. *J Leukoc Biol* 1997; **61**: 647-653
6 **Fruchart JC**, Duriez P, Staels B. Peroxisome proliferator-activated receptor-alpha activators regulate genes governing lipoprotein metabolism, vascular inflammation and atherosclerosis. *Curr Opin Lipidol* 1999; **10**: 245-257
7 **Berger J**, Moller DE. The mechanisms of action of PPARs. *Annu Rev Med* 2002; **53**: 409-435
8 **Moraes LA**, Piqueras L, Bishop-Bailey D. Peroxisome proliferator-activated receptors and inflammation. *Pharmacol Ther* 2006; **110**: 371-385
9 **Tabernero A**, Schoonjans K, Jesel L, Carpusca I, Auwerx J, Andriantsitohaina R. Activation of the peroxisome proliferator-activated receptor alpha protects against myocardial ischaemic injury and improves endothelial vasodilatation. *BMC Pharmacol* 2002; **2**: 10
10 **Yue TL**, Bao W, Jucker BM, Gu JL, Romanic AM, Brown PJ, Cui J, Thudium DT, Boyce R, Burns-Kurtis CL, Mirabile

RC, Aravindhan K, Ohlstein EH. Activation of peroxisome proliferator-activated receptor-alpha protects the heart from ischemia/reperfusion injury. *Circulation* 2003; **108**: 2393-2399
11 **Li S**, Gokden N, Okusa MD, Bhatt R, Portilla D. Anti-inflammatory effect of fibrate protects from cisplatin-induced ARF. *Am J Physiol Renal Physiol* 2005; **289**: F469-F480
12 **Collino M**, Aragno M, Mastrocola R, Benetti E, Gallicchio M, Dianzani C, Danni O, Thiemermann C, Fantozzi R. Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR-alpha agonist WY14643. *Free Radic Biol Med* 2006; **41**: 579-589
13 **Kielian T**, Drew PD. Effects of peroxisome proliferator-activated receptor-gamma agonists on central nervous system inflammation. *J Neurosci Res* 2003; **71**: 315-325
14 **Delerive P**, Martin-Nizard F, Chinetti G, Trottein F, Fruchart JC, Najib J, Duriez P, Staels B. Peroxisome proliferator-activated receptor activators inhibit thrombin-induced endothelin-1 production in human vascular endothelial cells by inhibiting the activator protein-1 signaling pathway. *Circ Res* 1999; **85**: 394-402
15 **Delerive P**, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, Gonzalez FJ, Fruchart JC, Tedgui A, Haegeman G, Staels B. Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1. *J Biol Chem* 1999; **274**: 32048-32054
16 **Marx N**, Sukhova GK, Collins T, Libby P, Plutzky J. PPARalpha activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells. *Circulation* 1999; **99**: 3125-3131
17 **Blanquart C**, Barbier O, Fruchart JC, Staels B, Glineur C. Peroxisome proliferator-activated receptors: regulation of transcriptional activities and roles in inflammation. *J Steroid Biochem Mol Biol* 2003; **85**: 267-273
18 **Daglar GO**, Kama NA, Atli M, Yuksek YN, Reis E, Doganay M, Dolapci M, Kologlu M. Effect of 5-lipoxygenase inhibition on Kupffer cell clearance capacity in obstructive jaundiced rats. *J Surg Res* 2001; **96**: 158-162
19 **Jaeschke H**. Preservation injury: mechanisms, prevention and consequences. *J Hepatol* 1996; **25**: 774-780
20 **Jaeschke H**. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G15-G26
21 **El-Benna J**, Dang PM, Gougerot-Pocidalo MA, Elbim C. Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. *Arch Immunol Ther Exp* (Warsz) 2005; **53**: 199-206
22 **Crack PJ**, Taylor JM. Reactive oxygen species and the modulation of stroke. *Free Radic Biol Med* 2005; **38**: 1433-1444
23 **Garcia JJ**, Reiter RJ, Guerrero JM, Escames G, Yu BP, Oh CS, Munoz-Hoyos A. Melatonin prevents changes in microsomal membrane fluidity during induced lipid peroxidation. *FEBS Lett* 1997; **408**: 297-300
24 **Trimarchi H**, Mongitore MR, Baglioni P, Forrester M, Freixas EA, Schropp M, Pereyra H, Alonso M. N-acetylcysteine reduces malondialdehyde levels in chronic hemodialysis patients--a pilot study. *Clin Nephrol* 2003; **59**: 441-446
25 **Freudenthaler SM**, Schreeb KH, Wiese A, Pilz J, Gleiter CH. Influence of controlled hypoxia and radical scavenging agents on erythropoietin and malondialdehyde concentrations in humans. *Acta Physiol Scand* 2002; **174**: 231-235
26 **Toyama T**, Nakamura H, Harano Y, Yamauchi N, Morita A, Kirishima T, Minami M, Itoh Y, Okanoue T. PPARalpha ligands activate antioxidant enzymes and suppress hepatic fibrosis in rats. *Biochem Biophys Res Commun* 2004; **324**: 697-704
27 **Andus T**, Bauer J, Gerok W. Effects of cytokines on the liver. *Hepatology* 1991; **13**: 364-375
28 **Ali S**, Mann DA. Signal transduction via the NF-kappaB pathway: a targeted treatment modality for infection, inflammation and repair. *Cell Biochem Funct* 2004; **22**: 67-79
29 **Reiterer G**, Toborek M, Hennig B. Peroxisome proliferator activated receptors alpha and gamma require zinc for their anti-inflammatory properties in porcine vascular endothelial cells. *J Nutr* 2004; **134**: 1711-1715
30 **Staels B**, Koenig W, Habib A, Merval R, Lebret M, Torra IP, Delerive P, Fadel A, Chinetti G, Fruchart JC, Najib J, Maclouf J, Tedgui A. Activation of human aortic smooth-muscle cells is inhibited by PPARalpha but not by PPARgamma activators. *Nature* 1998; **393**: 790-793
31 **Cuzzocrea S**, Di Paola R, Mazzon E, Genovese T, Muia C, Caputi AP. WY 14643, a potent exogenous PPAR-alpha ligand, reduces intestinal injury associated with splanchnic artery occlusion shock. *Shock* 2004; **22**: 340-346
32 **Sivarajah A**, Chatterjee PK, Hattori Y, Brown PA, Stewart KN, Todorovic Z, Mota-Filipe H, Thiemermann C. Agonists of peroxisome-proliferator activated receptor-alpha (clofibrate and WY14643) reduce renal ischemia/reperfusion injury in the rat. *Med Sci Monit* 2002; **8**: BR532-BR539
33 **Li S**, Basnakian A, Bhatt R, Megyesi J, Gokden N, Shah SV, Portilla D. PPAR-alpha ligand ameliorates acute renal failure by reducing cisplatin-induced increased expression of renal endonuclease G. *Am J Physiol Renal Physiol* 2004; **287**: F990-F998
34 **Muia C**, Mazzon E, Crisafulli C, Di Paola R, Genovese T, Caputi AP, Cuzzocrea S. ROLE of endogenous peroxisome proliferator-activated receptor-alpha (PPAR-alpha) ligands in the development of gut ischemia and reperfusion in mice. *Shock* 2006; **25**: 17-22
35 **Genovese T**, Mazzon E, Di Paola R, Muia C, Crisafulli C, Caputi AP, Cuzzocrea S. Role of endogenous and exogenous ligands for the peroxisome proliferator-activated receptor-alpha in the development of bleomycin-induce lung injury. *Shock* 2005; **24**: 547-555
36 **Yuan GJ**, Ma JC, Gong ZJ, Sun XM, Zheng SH, Li X. Modulation of liver oxidant-antioxidant system by ischemic preconditioning during ischemia/reperfusion injury in rats. *World J Gastroenterol* 2005; **11**: 1825-1828

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RAPID COMMUNICATION

Restrictive model of compensated carbon tetrachloride-induced cirrhosis in rats

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Abstract

AIM: To develop a simplified and quick protocol to induce cirrhosis and standardize models of partial liver resection in rats.

METHODS: In Fischer F344 rats two modified protocols of phenobarbital-carbon tetrachloride (CCl_4) (dilution 50%) gavage to induce cirrhosis (frequency adjusted according to weight, but each subsequent dose was systematically administered) were tested, i.e. the rapid and slow protocols. Prothrombin time (PT) and total bilirubin (TB) were also evaluated. Animals from the rapid group underwent 15% hepatectomy and animals from the slow group underwent 70% hepatectomy.

RESULTS: Rapid protocol: This corresponded to 1 gavage/4 d over 6 wk (mortality 30%). Mean PT was 35.2 ± 2.8 s (normal: 14.5 s), and mean TB was 1.8 ± 0.2 mg/dL (normal: 0.1 mg/dL). Slow protocol: This corresponded to 1 gavage/6 d over 9 wk (mortality 10%). Mean PT was 11.8 ± 0.2 s (normal: 14.5 s), and mean TB was 0.4 ± 0.04 mg/dL (normal: 0.1 mg/dL). Pathological analyses were performed in both protocols which showed persistent cirrhosis at 3 mo. Rat mortality in the rapid gavage group who underwent 15% hepatectomy and in the slow gavage group who underwent 70% hepatectomy was 50% and 70%, respectively.

CONCLUSION: Our modified model is a simplified method to induce cirrhosis which is rapid (6 to 9 wk), efficient and stable up to 3 mo. Using this method, "Child Pugh A" or "Child Pugh BC" cirrhotic rats were obtained. Our models of cirrhosis and hepatectomy can be used in various situations focusing on postoperative survival.

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Key words: Carbon tetrachloride; Cell therapy; Hepatectomy; Liver cirrhosis; Liver failure acute; Mortality; Surgery

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INTRODUCTION

There is currently a worldwide epidemic of chronic liver disease (chronic viral hepatitis B and C, and hepatopathy associated with obesity *via* the non alcoholic steatohepatitis (NASH) syndrome. At the same time, the number of patients presenting with hepatocellular carcinoma and/or persistent end-stage hepatic function alteration have increased. These two complications have led to more frequent discussions on hepatic resection and transplantation. However, the scope for both these solutions is limited by postoperative mortality and the constantly growing shortage of donors.

This situation led physicians and surgeons to propose new therapeutic solutions[1]. These solutions include: (1) Reduced-sized orthotopic liver graft[2,3], hepatic transplantation with living donor[4] and xenogenic transplantation[5,6]; (2) Cellular therapy by transplantation of hepatocytes into the liver, spleen and peritoneum (isolated or encapsulated)[7,8] (autologous, allogeneic, xenogenic, free or encapsulated, genetically modified *ex vivo*)[9].

It is thus necessary to have animal models of cirrhosis, which make it possible to obtain compensated (equivalent to Child Pugh A) or decompensated cirrhosis (equivalent

to Child Pugh BC). At best, liver cirrhosis must be rapidly obtainable, and durable. It is also necessary to have standardized models of partial hepatic resection in these cirrhotic animals with reproducible mortality.

The most validated cirrhosis model in the rat is the carbon tetrachloride (CCl_4) (induced by phenobarbital) cirrhosis model[10]. Many protocols exist, which differ in the route of administration (gavage, intraperitoneal or subcutaneous injection), the dilution of CCl_4 (1/20 to 1/10), the frequency (1 to 4 wk) and the duration (8 to 28 wk) of CCl_4 administration. The efficiency of these protocols (70% to 100%), as well as the inherent toxic-mortality (20% to 90%) is variable in the literature[11]. The study in which the CCl_4 protocol for cirrhosis is best described is that of Kobayashi *et al*[12,13]. Here CCl_4 was given by gavage twice weekly and diluted to 10%. The amount of CCl_4 was decreased if body weight remained constant, and not administered if the weight decreased. The study was carried out over a 28-wk period. The animals were studied only when evidence of liver failure did not improve after CCl_4 was withheld for 4 wk. However, this reference protocol is too restrictive particularly for pilot studies[14].

The aim of this study was to establish a restrictive CCl_4 cirrhosis model which resulted in "Child Pugh A" or "Child Pugh BC" cirrhotic rats, which could be carried out easily, quickly and in a reproducible and durable way by modulating the dilution of CCl_4 and the duration and frequency of gavage. In addition, we tested different types of hepatectomy in terms of the resected volume ("minor or major") to obtain reproducible postoperative mortality.

MATERIALS AND METHODS

Animals

Male Fischer F344 rats (Iffa Credo, France) weighing 150-180 g were used. The rats were given free access to standard laboratory food and water throughout the experiments. Rats were acclimatized to our laboratory conditions for 7 d. All the procedures performed on the animals were in accordance with the European Committee on the Use and Care of Animals.

Induction of phenobarbital-carbon tetrachloride-induced liver cirrhosis

Rats were given phenobarbital (lyophilized 200 mg, Rhône-Poulenc Rorer, France) in their drinking water at a concentration of 0.5 g/L throughout the establishment of liver cirrhosis. Two weeks later, CCl_4 (diluted 50% in olive oil) was given intragastrically using a gavage needle at different intervals determined during preliminary experiments.

Rat weight was approximately 200 g at the time of first gavage. The initial dose was 0.08 mL (0.20 mL/kg of CCl_4). Each subsequent dose was systematically administered (even if weight remained stable or decreased) but adjusted based on changes in body weight: from 150 to 220 g, 0.06 to 0.09 mL (0.20 mL/kg of CCl_4); from 230 to 270 g, 0.12 to 0.14 mL (0.25 mL/kg); from 280 to 300 g, 0.17 to 0.18 mL (0.30 mL/kg); from 310 to 330 g, 0.22 to 0.23 mL (0.35 mL/kg); from 340 to 360 g, 0.27 to 0.29 mL (0.40 mL/kg); from 370 to 390 g, 0.33 to 0.35 mL (0.45 mL/kg). Various gavage protocols (frequency, duration of gavage, dose adjustment, mortality during gavage) were tested leading to the selection of two protocols (rapid protocol, slow protocol).

Once these two protocols (rapid and slow) were selected, 3 groups of 10 animals for each protocol were used as test groups to affirm that these protocols were reproducible in terms of mortality related to gavage, gravity of cirrhosis (ascites, splenomegaly, portal hypertension, biology, pathology) and persistence of the histological lesions with time.

Biology

Prothrombin time (PT) and total bilirubin (TB) were assessed in 5 rats in the rapid and slow protocol groups 3 wk after gavage interruption.

Histological examination

Representative liver blocks, obtained when the rats were sacrificed, were prepared to establish the gavage protocols or after partial hepatectomy. After routine paraffin wax processing, hematoxylin eosin and Masson's trichromie stained sections were obtained and the presence of cirrhosis was assessed under light microscopy. Pathological analyses were performed in rats 3 wk after gavage interruption (T0) and at regular intervals (2 wk, 5, 9, 13, 18 s).

Anesthesia

Partial hepatectomy was performed under ether inhalation anesthesia. The day before intervention, the animals were fasted for 12 h prior to surgery. Postoperatively, animals received glucose supplementation (immediate postoperative subcutaneous glucose 30%, and glucose 10% added to drinking water during the first 24 h) and animals were heated during the first 24 h.

Hepatectomy

Hepatectomy was performed in cirrhotic rats 3 wk after gavage interruption. The extent of hepatectomy was adjusted by excising various combinations of liver lobes that are known to have a predictable volume[15] according to the nature of the protocol and the observed postoperative mortality: 15% hepatectomy (epiploïc lobes, i.e. minor hepatectomy) and 70% hepatectomy (left lateral and median lobes, i.e. major hepatectomy) were chosen after preliminary experiments.

Two groups of 10 animals from the rapid group underwent 15% hepatectomy and two groups of 10 animals from the slow group underwent 70% hepatectomy. The postoperative day 1 mortality was noted. Pathologic examination of the resected specimen was carried out systematically.

RESULTS

Rapid and slow protocol gavage mortality

Rapid protocol: This corresponded to 1 gavage/4 d over

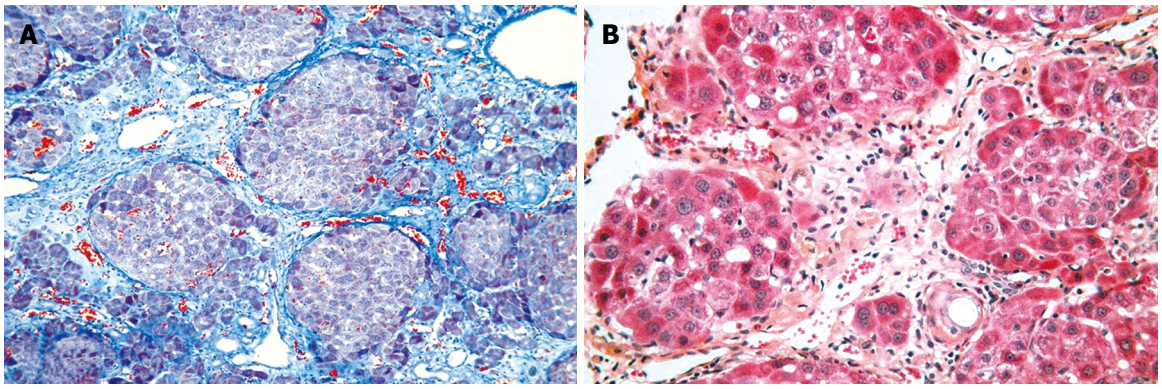

Figure 1 Rat liver after CCl_4 gavage. A: Rat liver [9 wk after T0 (12 wk after CCl4 gavage interruption)] extensive mutilating fibrosis, with nodules (cirrhosis). In the nodules separated by fibrous septa, hepatocytes with a basophil cytoplasm and a round nucleus are organized in fine spans (Masson's trichromie, × 10); B: Rat liver [6 wk after T0 (9 wk after CCl4 gavage interruption)] extensive mutilating fibrosis, with nodules (cirrhosis) (HE, × 10).

42 d (6 wk). Mortality in the rapid protocol was 30%, 30% and 40% (mean: 30%), from the first gavage to 3 wk after gavage interruption, respectively. In the surviving rats, 3 wk after gavage interruption, at the time of sacrifice, there were macroscopic micro nodular cirrhosis aspects in all animals, and ascites, splenomegaly and portal hypertension signs (at the level of the stomach) in 85%, 65% and 60% of animals, respectively.

Slow protocol: This corresponded to 1 gavage/6 d over 63 d (9 wk). Mortality in the rapid protocol was 10%, 20% and 10% (mean: 10%), from the first gavage to 3 wk after gavage interruption, respectively. In the surviving rats, 3 wk after gavage interruption, at the time of sacrifice, there were macroscopic micro nodular cirrhosis aspects in all animals, and ascites, splenomegaly and portal hypertension signs (at the level of the stomach) in 5%, 5% and 0% of animals, respectively.

Biology

Rapid protocol: In the surviving rats, 3 wk after gavage interruption, mean PT was 35.2 ± 2.8 s (normal: 14.5 s), and mean bilirubin was 1.8 ± 0.2 mg/dL (normal: 0.1 mg/dL) (n = 5).

Slow protocol: In the surviving rats, 3 wk after gavage interruption, mean PT was 11.8 ± 0.2 s (normal: 14.5 s), and mean bilirubin was 0.4 ± 0.04 mg/dL (normal: 0.1 mg/dL) (n = 5).

Pathological assessment

After both the rapid and slow protocols, all surviving rats presented with cirrhosis (T0 = 3 wk after gavage interruption) with extensive mutilating fibrosis and nodules (Figure 1). The cirrhosis aspects persisted on analyses performed at regular intervals (2 wk, 5, 9, 13, 18 s).

Mortality after partial hepatectomy

Mortality in the two groups of 10 animals from the rapid gavage group, who underwent 15% hepatectomy, was 50% at postoperative day 1. Mortality in the two groups of 10 animals from the slow gavage group, who underwent 70% hepatectomy, was 70% at postoperative day 1.

DISCUSSION

CCl_4 is widely used to induce experimental liver damage. The data from this study showed that our modified model is a simplified and improved method for inducing cirrhosis which was rapid (6 to 9 wk), efficient, stable up to 3 mo and which resulted in "Child Pugh A" or "Child Pugh BC" cirrhotic rats. Fifteen percent hepatectomy ("minor hepatectomy") in decompensated cirrhotic animals and 70% hepatectomy ("major hepatectomy") in compensated cirrhotic animals resulted in a postoperative mortality of 50% and 70%, respectively. These reproducible and high postoperative mortality rates can be used in protocols devoted to cellular therapy in chronic liver diseases[1,8]. Moreover, different series have studied the biological[16,17] and hemodynamic[18-20] variations induced by liver cirrhosis. Other authors have investigated various treatments in rats with liver cirrhosis[21-24].

In the Kobayashi *et al*[12,13], CCl_4 reference induced cirrhosis model, animals were studied only when evidence of liver failure did not improve after CCl_4 was withheld for 4 wk, following 28 wk of gavage. Overall, the length of the protocol was 8 mo. However, this protocol ensured the induction of cirrhosis which was slow, stable and very close to cirrhosis found in clinical practice. The main pitfall of this protocol was its length which is not compatible with small "pilot" or "preliminary" studies. In our study, serum albumin level, serum ammonia level and degree of encephalopathy (coma scale), are lacking, however, persistent histological alteration at 3 mo is, in our view, a major argument for the use of this method. To reinforce the pertinence of modified protocols for inducing cirrhosis in rats, at a recent congress, Quadrelli *et al*[25] presented a new approach to improve the model of cirrhotic liver induced by CCl_4, which associated gavage and subcutaneous injection of CCl_4. This new approach, associated with 45% mortality allowed cirrhosis induction in 60% of rats in a period of 9 wk.

We have found only 5 papers in the literature which reported mortality after hepatectomy (rational for the study of cirrhotic rats focusing on postoperative mortality) in various models of compensated "Child Pugh A" CCl_4 induced cirrhosis in rats. One of these studies reported a

postoperative mortality of 70% after 30% hepatectomy[26]. In the remaining four studies after 70% hepatectomy, a postoperative mortality of 15%, 20%, 25% and 40%, respectively was reported[27-30]. In this series, rat mortality after 70% hepatectomy in compensated "Child Pugh A" rats was higher, about 70% and could be explained by the more intensive gavage protocol used. However, the extent of the resection, i.e. 15% in decompensated cirrhotic animals, and 70% in compensated cirrhotic animals chosen in this series are compatible with minor and major hepatic resections performed in clinical practice in cirrhotic patients.

In conclusion, our modified model is a simplified method for inducing cirrhosis that is rapid (6 to 9 wk), efficient and stable up to 3 mo. It resulted in "Child Pugh A" or "Child Pugh BC" cirrhotic rats. Our models of cirrhosis and hepatectomy can be used in various situations focusing on postoperative survival.

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COMMENTS

Background

It is necessary to have animal models of compensated (equivalent to Child Pugh A) and decompensated cirrhosis (equivalent to Child Pugh BC) for various studies. At best, cirrhosis must be rapidly obtainable and durable. The most validated cirrhosis model in the rat is the carbon tetrachloride (CCl_4) (induced by phenobarbital) cirrhosis model. Many protocols exist, which differ in the route of administration (gavage, intraperitoneal, subcutaneous), the dilution of CCl_4, and in the frequency and the duration of CCl_4 administration. The efficiency of these protocols (70% to 100%), as well as the inherent toxic-mortality (20% to 90%) is variable in the literature.

Research frontiers

The establishment of an efficient and quick model of cirrhosis in rat with an acceptable rate of mortality has been the topic of several investigations in recent years.

Innovations and breakthroughs

This paper has reported a reproducible, rapid (6 to 9 wk), efficient and stable up to 3 mo model of liver cirrhosis, which resulted in "Child Pugh A" or "Child Pugh BC" cirrhotic rats.

Applications

Based on the results of this work, it is possible to standardize models of partial hepatic resection in cirrhotic rats with reproducible mortality. This rapid model is compatible with small "pilot" or "preliminary" studies. It can be used in protocols devoted to cellular therapy in chronic liver diseases, and in biological and hemodynamic variations induced by liver cirrhosis.

Terminology

The extent of hepatectomy was adjusted by excising various combinations of liver lobes that are known to have a predictable volume according to the nature of the protocol and the observed postoperative mortality: 15% hepatectomy (epiploïc lobes, i.e. minor hepatectomy) and 70% hepatectomy (left lateral and median lobes, i.e. major hepatectomy) were chosen after preliminary experiments.

Peer review

The data presented by the authors supports the possibility of a simplified method for inducing cirrhosis that is rapid, efficient and stable. The topic is very interesting because the model of "Child Pugh A" or "Child Pugh BC" cirrhosis and hepatectomy can be used in various situations focusing on postoperative survival.

REFERENCES

1 **Regimbeau JM**, Mallet VO, Bralet MP, Gilgenkrantz H, Houssin D, Soubrane O. [Transplantation of isolated hepatocytes. Principles, mechanisms, animal models, clinical results] *Gastroenterol Clin Biol* 2002; **26**: 591-601
2 **Bismuth H**, Houssin D. Reduced-sized orthotopic liver graft in hepatic transplantation in children. *Surgery* 1984; **95**: 367-370
3 **Soubrane O**, Houssin D. [All out search of graft for liver transplantation] *Gastroenterol Clin Biol* 1993; **17**: 845-850
4 **Raia S**, Nery JR, Mies S. Liver transplantation from live donors. *Lancet* 1989; **2**: 497
5 **White SA**, Nicholson ML. Xenotransplantation. *Br J Surg* 1999; **86**: 1499-1514
6 **Malassagne B**, Regimbeau JM, Taboit F, Troalen F, Chereau C, Moire N, Attal J, Batteux F, Conti F, Calmus Y, Houssin D, Boulard C, Houdebine LM, Weill B. Hypodermin A, a new inhibitor of human complement for the prevention of xenogeneic hyperacute rejection. *Xenotransplantation* 2003; **10**: 267-277
7 **Gupta S**, Hodgson HJ. Transplantation of isolated hepatocytes. *Indian J Gastroenterol* 1985; **4**: 97-100
8 **Ochenashko OV**, Volkova NA, Mazur SP, Somov AY, Fuller BJ, Petrenko AY. Cryopreserved fetal liver cell transplants support the chronic failing liver in rats with CCl4-induced cirrhosis. *Cell Transplant* 2006; **15**: 23-33
9 **Panis Y**, Cardoso J, Houssin D. [Gene therapy in Hepatology. Experimental results and clinical perspectives] *Gastroenterol Clin Biol* 1994; **18**: 262-276
10 **Proctor E**, Chatamra K. Standardized micronodular cirrhosis in the rat. *Eur Surg Res* 1984; **16**: 182-186
11 **Mullen KD**, McCullough AJ. Problems with animal models of chronic liver disease: suggestions for improvement in standardization. *Hepatology* 1989; **9**: 500-503
12 **Kobayashi N**, Ito M, Nakamura J, Cai J, Hammel JM, Fox IJ. Treatment of carbon tetrachloride and phenobarbital-induced chronic liver failure with intrasplenic hepatocyte transplantation. *Cell Transplant* 2000; **9**: 671-673
13 **Kobayashi N**, Ito M, Nakamura J, Cai J, Gao C, Hammel JM, Fox IJ. Hepatocyte transplantation in rats with decompensated cirrhosis. *Hepatology* 2000; **31**: 851-857
14 **Rivera-Huizar S**, Rincon-Sanchez AR, Covarrubias-Pinedo A, Islas-Carbajal MC, Gabriel-Ortiz G, Pedraza-Chaverri J, Alvarez-Rodriguez A, Meza-Garcia E, Armendariz-Borunda J. Renal dysfunction as a consequence of acute liver damage by bile duct ligation in cirrhotic rats. *Exp Toxicol Pathol* 2006; **58**: 185-195
15 **Madrahimov N**, Dirsch O, Broelsch C, Dahmen U. Marginal hepatectomy in the rat: from anatomy to surgery. *Ann Surg* 2006; **244**: 89-98
16 **Borkham-Kamphorst E**, Kovalenko E, van Roeyen CR, Gassler N, Bomble M, Ostendorf T, Floege J, Gressner AM, Weiskirchen R. Platelet-derived growth factor isoform expression in carbon tetrachloride-induced chronic liver injury. *Lab Invest* 2008; **88**: 1090-1100
17 **Lavina B**, Gracia-Sancho J, Rodriguez-Vilarrupla A, Chu Y, Heistad DD, Bosch J, Garcia-Pagan JC. Superoxide dismutase gene transfer reduces portal pressure in ccl4 cirrhotic rats with portal hypertension. *Gut* 2009; **58:** 118-125
18 **Cardenas A**, Lowe R, Oh S, Bodkin S, Kenney T, Lamorte WW, Afdhal NH. Hemodynamic effects of substance P and its receptor antagonist RP67580 in anesthetized rats with carbon tetrachloride-induced cirrhosis. *Scand J Gastroenterol* 2008; **43**: 328-333
19 **Maksan SM**, Ryschich E, Ulger Z, Gebhard MM, Schmidt J. Disturbance of hepatic and intestinal microcirculation in experimental liver cirrhosis. *World J Gastroenterol* 2005; **11**: 846-849
20 **Tsugawa K**, Hashizume M, Migou S, Kishihara F, Kawanaka H, Tomikawa M, Tanoue K, Sugimachi K. Role of nitric oxide and endothelin-1 in a portal hypertensive rat model. *Scand J Gastroenterol* 2000; **35**: 1097-1105
21 **Jang JH**, Kang KJ, Kim YH, Kang YN, Lee IS. Reevaluation of experimental model of hepatic fibrosis induced by hepatotoxic drugs: an easy, applicable, and reproducible

model. *Transplant Proc* 2008; **40**: 2700-2703
22 **Fang HL**, Lai JT, Lin WC. Inhibitory effect of olive oil on fibrosis induced by carbon tetrachloride in rat liver. *Clin Nutr* 2008; **27:** 900-907
23 **Perez R**, Garcia-Fernandez M, Diaz-Sanchez M, Puche JE, Delgado G, Conchillo M, Muntane J, Castilla-Cortazar I. Mitochondrial protection by low doses of insulin-like growth factor- I in experimental cirrhosis. *World J Gastroenterol* 2008; **14**: 2731-2739
24 **Yuan LP**, Chen FH, Ling L, Bo H, Chen ZW, Li F, Zhong MM, Xia LJ. Protective effects of total flavonoids of Bidens bipinnata L. against carbon tetrachloride-induced liver fibrosis in rats. *J Pharm Pharmacol* 2008; **60**: 1393-1402
25 **Quadrelli L**, Secchi MA, Consagra MF, Rossi L, Peralta E, Muniagurria C, Gabriele M, Figallo G. New approach to improved model of cirrhotic liver induced by carbon tetrachloride. *HPB* 2005; **7**: 35
26 **Kaido T**, Seto S, Yamaoka S, Yoshikawa A, Imamura M. Perioperative continuous hepatocyte growth factor supply prevents postoperative liver failure in rats with liver cirrhosis. *J Surg Res* 1998; **74**: 173-178
27 **MacIntosh E**, Gauthier T, Pettigrew N, Minuk G. Liver regeneration and the effect of exogenous putrescine on regenerative activity after partial hepatectomy in cirrhotic rats. *Hepatology* 1992; **16**: 1428-1433
28 **Hwang TL**, Yu HC, Chen PC, Chen MF. Liver regeneration following partial hepatectomy and stimulation by hepatic stimulatory substance in cirrhotic and non-cirrhotic rats. *Res Exp Med* (Berl) 1995; **195**: 201-208
29 **Moser M**, Zhang M, Gong Y, Johnson J, Kneteman N, Minuk GY. Effect of preoperative interventions on outcome following liver resection in a rat model of cirrhosis. *J Hepatol* 2000; **32**: 287-292
30 **Andiran F**, Ayhan A, Tanyel FC, Abbasoglu O, Sayek I. Regenerative capacities of normal and cirrhotic livers following 70% hepatectomy in rats and the effect of alpha-tocopherol on cirrhotic regeneration. *J Surg Res* 2000; **89**: 184-188

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RAPID COMMUNICATION

Prevalence of celiac disease in adult patients with refractory functional dyspepsia: Value of routine duodenal biopsy

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Abstract

AIM: To investigate the prevalence of celiac disease (CD) in adult patients referred to an open access gastroenterology clinic in the south of Italy and submitted to esophago-gastro-duodenoscopy (EGD) for evaluation of refractory functional dyspepsia.

METHODS: Seven hundred and twenty six consecutive dyspeptic patients (282 male, 444 female; mean age 39.6 years, range 18-75 years) with unexplained prolonged dyspepsia were prospectively enrolled. Duodenal biopsies were taken and processed by standard staining. Histological evaluation was carried out according to the Marsh-Oberhuber criteria.

RESULTS: The endoscopic findings were: normal in 61.2%, peptic lesions in 20.5%, malignancies in 0.5%, miscellaneous in 16.7%. CD was endoscopically diagnosed in 8 patients (1.1%), histologically in 15 patients (2%). The endoscopic features alone showed a sensitivity of 34.8% and specificity of 100%, with a positive predictive value (PPV) of 100% and a negative predictive value (NPP) of 97.9%.

CONCLUSION: This prospective study showed that CD has a high prevalence (1:48) in adult dyspeptic patients and suggests the routine use of duodenal biopsy in this type of patient undergoing EGD.

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Key words: Celiac disease; Endoscopy; Biopsy; Functional; Dyspepsia

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INTRODUCTION

Celiac disease (CD) has been considered for years to be a rare pathology that affects, in particular, pediatric patients who present with a clinical picture of malabsorption[1]. The development of sensitive and specific serological tests and their administration to subjects who are apparently healthy has shown that: CD is still under diagnosed in all age groups; the form with obvious symptoms is found in only a limited number of cases; in most patients, particularly adults, the disease has an atypical symptomatology or is completely silent[2-5]. The latter characteristics are responsible for a the length of time needed to have a correct diagnosis and expose patients to the possible development of severe pathologies. With the aim of discovering the hidden proportion of subjects with CD[6] two different diagnostic approaches have been proposed: to carry out a screening on the apparently healthy population, or to apply case-finding in subjects that are believed to be at high risk for the disease[7,8].

As regards mass screening, at the moment there is no evidence that supports this approach, since in the apparently healthy population the prevalence of

CD varies in relationship with geographical areas[9]. Furthermore, a cost-effectiveness analysis in support of a mass screening program has not been performed.

Case-finding is believed to be the most appropriate diagnostic approach to adopt for asymptomatic patients or for patients with subtle clinical features. This approach is particularly effective and becomes more so if in the selection of subjects to be investigated their family doctors are involved[10-12]. The activation of a celiac awareness program in the primary-care setting focusing on selective serological screening of high risk groups has doubled the number of cases diagnosed from among the adult asymptomatic population[13].

It was recently observed that CD had a greater prevalence, with respect to the general population, in dyspeptic patients[14,15] and that 30%-40% of CD patients have dyspeptic symptoms[2]. These findings suggested that it would be useful to carry out, in subjects undergoing esophago-gastro-duodenoscopy (EGD), biopsies of the descending duodenum independently of the endoscopic aspect of the mucosa[16-20]. The aim of our study was to determine, by means of duodenal biopsies, the prevalence of CD in dyspeptic patients submitted to EGD in an open access Gastroenterology Outpatient Clinic of a University Hospital in the south of Italy.

MATERIALS AND METHODS

From January 2005 to June 2007, 5413 patients underwent EGD at the Gastroenterology Unit of the University Hospital Policlinic of Catania.

The study was approved by the Bioethical Committee of the Polyclinic and carried out on 726 patients (282 male, 444 female; mean age 39.6 years, range 18-75 years) prospectively enrolled.

All patients gave their written informed consent before being enrolled in the study. During the entire period of the study, the first two dyspeptic patients admitted in our unit to undergo an EGD for the first time were included.

Patients with a positive family history for CD, those affected by pathologies known to be associated with CD[21] and patients with gastroesophageal reflux disease were excluded. Dyspeptic symptomatology was classified according to Roma Ⅱ criteria as: Ulcer-like Dyspepsia, Dysmotility-like Dyspepsia, and Indeterminate Dyspepsia.

During EGD, other than the observation of the esophageal and gastric mucosa, a precise evaluation of the duodenal mucosa up to the distal duodenum was carried out and any anomalies were classified as: micro nodular pattern; mosaic pattern; scalloped folds and loss or decrease of duodenal folds[22].

The endoscopic observation was completed with a rapid urease test to detect the presence of *H pylori*, 5 biopsies of the gastric mucosa (2 antrum, 1 angulus and 2 body) consistent with the Sydney system recommendations, and 4 biopsies of the descending duodenum. The bioptic duodenal samples were orientated and mounted villous side up before being immersed in formalin for standard staining (HE). The histological examination was carried out by a pathologist who did not know the clinical details and endoscopic reports of the patients. The pathologist gave a diagnosis of CD based on standard coloration and classified the entity of mucosal damage according to Marsh-Oberhuber criteria[23,24]. In patients with histological diagnosis of CD the study was completed with a specific antibody test, anti-Tissue Transglutaminase antibodies (tTG) and anti-Endomysial antibodies (EMA), and the determination of the HLA haplotypes (DQ2- DQ8).

Prevalence, the relative risk and 95% CI were calculated using the Biostat Program.

Statistical analysis

The difference between mean and the difference between proportions were evaluated by the *t*-test and the χ^2 test respectively. In the case of abnormal distribution an appropriate non-parametric test was performed. We also estimated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPP) and their 95% CI of the endoscopic examinations, considering the histological evaluation of duodenal biopsies as the gold standard.

RESULTS

Over a 30 mo period 726 dyspeptic patients were enrolled: 102 (14%) ulcer-like dyspepsia, 344 (47.4%) dysmotility-like dyspepsia and 280 (38.6%) indeterminate dyspepsia. No adverse events were reported during the endoscopic procedures and bioptic sampling. The endoscopic findings were normal in 444 (61.2%) patients, peptic lesions (esophagitis, erosive gastritis, peptic ulcer) were present in 149 (20.5%), endoscopic findings suggestive for CD in 8 (1.1%), malignancies in 4 (0.5%) and miscellaneous (chemical gastropathy, lymphocytic gastritis, submucosal mass lesions, hyperplastic polyps, cystic fundal hyperplasia) in 121 (16.7%) (Table 1). Endoscopic markers of CD consisted of a decrease in the number of folds in 5 cases, an association with a mosaic pattern in 2 cases, and in the remaining 3 cases the endoscopic aspect of the mucosa was respectively micro nodular, mosaic and scalloped. These alterations of the mucosa were localized in 3 cases (37.5%) to DI and in 5 (62.5%) both to DI and DⅡ.

The histological diagnosis of CD was made in 15 patients (5 male, 10 female; mean age 39.9 years, range 20-61 years), 8 were already suspected of being affected by CD on endoscopic evidence and 7 had an apparently normal duodenal endoscopic picture. Histological damage was classified as ⅢC category of Marsh (Total Villous Atrophy) in 5 cases, ⅢB (Subtotal Villous Atrophy) in 8 and ⅢA (Partial Villous Atrophy) in 2 cases. None of the patients had histological alterations of Marsh I or Ⅱ. The general prevalence of CD in dyspeptic patients that we examined was 2% (1/48). As regards *H pylori,* 7/15 (46.6%) CD patients were positive and 322/721

Table 1 Clinical characteristics and endoscopic findings of enrolled patients (n = 726)

Dyspepsia	Non CD (%) n = 711	CD (%) n = 15	OR	95% CI	P
Sex (female; male)	434 (61.0) 277 (39)	10 (66.7) 5 (33.3)	1.28	0.45-3.60	0.6
Mean age (yr)	39.5 ± 14.5	39.9 ± 11.2	1.86	0.71-4.65	0.3
Type of dyspepsia					
Dysmotility-like dyspepsia (DD)	336 (47.2)	8 (53.3)	1.27	0.47-3.42	0.6
Indeterminate dyspepsia (ID)	273 (38.4)	7 (46.7)	2.21	0.82-5.97	0.1
Ulcer-like dyspepsia (UD)	102 (14)	0			
Endoscopic findings					
Normal	437 (61.2)	7 (46.7)			/
Peptic lesion	149 (20.5)	/			
Malignancy	4 (0.5)	/			
Miscellaneous	121 (16.7)	/			
CD		8 (53.3)			

Overall prevalence: 2.01% (95% CI 1.2-3.3). Endoscopic evaluation: Sensitivity: 34.8% (95% CI 17.2-57.2); Specifity: 100% (95% CI 17.2-57.2); PPV: 100% (95% CI 59.8-100); NPV: 97.9% (95% CI 96.5-98.8).

Table 2 Demographic, clinical, endoscopic and histological data of celiac patients

Pts	Gender M/F	Age (yr)	Clinical findings	Endoscopic markers	Histological findings	Marsh
1	F	20	ID	RF/NM	TVA	ⅢC
2	F	27	DD	NM	TVA	ⅢC
3	F	54	ID	Normal	PVA	ⅢB
4	M	44	DD	Normal	STA	ⅢB
5	F	26	DD	RF	STA	ⅢB
6	F	36	ID	Normal	STA	ⅢB
7	F	41	DD	Normal	STA	ⅢB
8	M	34	ID	RF + Sll	TVA	ⅢC
9	F	29	DD	Normal	APV	ⅢA
10	M	54	DD	Normal	STA	ⅢB
11	F	52	ID	Normal	PVA	ⅢA
12	M	56	DD	MM	TVA	ⅢC
13	F	39	DD	RF	STA	ⅢB
14	M	41	ID	RF	STA	ⅢC
15	F	45	ID	Sll	STA	ⅢB

PVA: Partial villous atrophy; STA: Subtotal villous atrophy; TVA: Total villous atrophy; DD: Dysmotility-like dispepsia; ID: Indeterminate dispepsia; UD: Ulcer-like dispepsia; RF: Reduced folds; Sll: Scalloped folds; NM: Nodular mucosa; MM: Mosaic mucosa.

(45.3%) patients with normal duodenal mucosa histology were positive. We did not find differences in clinical features and in mean intraepithelial leucocyte (IEL) count in *H pylori* -negative and *H pylori* -positive patients.

Of the 15 patients diagnosed as celiac, 8 reported dysmotility-like and 7 indeterminate dyspepsia. The type of dyspepsia, endoscopic findings and histological diagnoses are shown in Table 2.

The EMA and tTG antibodies were both present in all but one case, in which only EMA was positive; the HLA associated haplotypes were, respectively, DQ2 in 12 patients, DQ2-DQ8 in 2 patients and DQ8 in one patient.

DISCUSSION

Over the last thirty years it has been established that CD is not a rare disease, rather it should be considered as a global health problem. It is estimated that CD currently affects 2.5/3 million in both American and European populations[25]. This observation confirms that the awareness for this under-diagnosed disease in clinical practice should be increased.

Recent investigations have shown that most patients affected by CD, in particular adults, do not have the typical symptoms of the disease, thus they remain misdiagnosed, delaying the diagnosis until an older age. In a study conducted on paucisymptomatic patients over 65 years old that had seen both family doctors and specialists, it was documented that the correct diagnosis was made with an average delay of 28 years[26].

The misdiagnosis of CD for such a long period exposes patients to the risk of developing severe gluten-related complications such as intestinal lymphoma, autoimmune disorders or neurological diseases[27-29].

To identify the sub-clinical or silent forms of CD, the suggested algorithm consists of the search for specific antibodies in categories of patients known to be at risk. The definitive confirmation of the disease will, however, come from the histological evaluation of the duodenal mucosa.

In recent publications[11,30,31] a high prevalence of CD has also been found in adult patients classified as functional dyspeptic who did not respond to an adequate pharmacological therapy. To identify in this particular population the subjects whose symptoms are really due to CD, three alternate approaches have been proposed: (1) Carry out biopsies from the descending duodenum[16,17] in all functional dyspeptic patients undergoing EGDS even if endoscopy does not reveal any lesions typical of CD[22]; (2) Use magnification tools or immersion techniques to better characterize the duodenal mucosa[32]; (3) Test for specific antibodies and, if positive, carry out EGD with biopsies of the descending duodenum[33].

The first approach has been criticized due to its cost for the limited number of CD cases that could be identified and for the amount of work for the pathology services[34,35]. The second approach, a modified version of the so-called immersion technique (MIT), which based on recent data has a sensitivity and specificity of 100%, is considered impractical though further studies are needed to assess its efficacy in routine practice as a screening or case-finding tool[36]. The third approach has diagnostic limitations, since the test for anti-tTG and anti-EMA antibodies[37] are relatively poor for adult smoker patients[38,39] or in the presence of a slight or medium (Marsh I to Ⅱ) histological damage.

The above cited observations suggest that for both asymptomatic risk groups and symptomatic risk groups in the general population the threshold for biopsies must become lower[38]. In Spain duodenal biopsy performed during upper GI endoscopy has been incorporated in daily practice in digestive endoscopic services[40]. This choice should be adopted especially in geographical areas

where there is a high prevalence of CD in an apparently healthy population.

In our study the biopsies were carried out in patients where EGD was used to clarify a functional dyspeptic symptomatology that did not resolve after an adequate pharmacological treatment. It should be noted that as regards *H pylori* positivity the percentage of *H pylori*-positive celiac patients is similar to non-celiac patients and, as recently shown[41], clinical features of CD patients are unrelated to simultaneous presence of *H pylori* gastritis.

In this population the prevalence of CD was 2% (1/48 patients) two/four times more than that found using serological tests in the general population[42-45] and more than that reported in two studies conducted in Italy and Brazil on dyspeptic patients[14,30]. The data is, however, similar to that obtained in a study conducted on patients who reported chronic abdominal pain[46].

Concerning the demographic characteristics of the 15 celiac patients (mean age 39.9 years; male/female ratio 2:1) our data are in agreement with what has already been observed in a multicenter retrospective study[3] and in a screening study[47], both carried out in Italy. A higher prevalence of females among celiac patients (3% *vs* 1%) has also been reported in a retrospective evaluation[40] of adult patients referred to an endoscopy unit with mild digestive symptoms (dyspepsia, abdominal discomfort) or analytical alterations (anemia, iron deficiency or hypertransaminasemia).

The greater prevalence of CD among patients who reported dysmotility or indeterminate dyspesia may be related to autoimmune damage of the extrinsic autonomic system[48] and/or to an increase in neurotensin and enteroglucagon plasma levels which inhibit the motility of the upper gastrointestinal tract[49,50]. Moreover a delayed oro-cecal transit time[51] and a post-prandial decrease in gallbladder emptying rate[52] have been found in untreated CD patients. Normalization of oro-cecal transit time was observed after gluten withdrawal using a hydrogen lactulose breath test[50]. The diagnostic precision of the endoscopic observations [8/15 (53%)] was similar to that observed in other samples of dyspeptic patients[14] but lower than that found in patients at high risk of CD[23,33,53]. The discrepancy of our study compared with the latter could be explained by the fact that the operator paid more attention to the observation of the duodenal mucosa in the patients affected by pathologies already recognized as being at risk for CD.

In conclusion, based on the results that we have obtained it can be hypothesized that in patients who have been diagnosed as having refractory functional dyspepsia and for whom an EGD has been prescribed, endoscopic observation should be routinely completed with a biopsy of the descending duodenum as suggested by the guidelines of the working group on CD[24]. Particular attention should be given to females who report dysmotility or indeterminate dyspepsia. Such an approach could reveal another submerged part of the "Celiac Iceberg" but it must be validated as regards the cost effectiveness, bearing in mind the variable prevalence of the disease in different geographical areas. The development of new and more precise serologic and/or immunohistochemical tests[54] would allow correct selection of subjects who need a bioptic examination of duodenal mucosa.

COMMENTS

Background

Celiac disease (CD) has been considered for years to be a rare pathology that affects, above all, pediatric patients presenting with a clinical picture of malabsorption. The development of sensitive and specific serological tests and their administration to subjects who are apparently healthy has shown that: CD is still under diagnosed in all age groups; the form with obvious symptoms is found in only a limited number of cases; in most patients, particularly adults, the disease has an atypical symptomatology or is completely silent. It was recently observed that CD had a greater prevalence, with respect to the general population, in dyspeptic patients and that 30%-40% of CD patients have dyspeptic symptoms. These findings suggested that it would be useful to carry out, in subjects undergoing esophago-gastro-duodenoscopy (EGD), biopsies of the descending duodenum independently of the endoscopic aspect of the mucosa.

Research frontiers

Based on the results that authors have obtained it can be hypothesized that in patients who have been diagnosed as having refractory functional dyspepsia and for whom an EGD has been prescribed, endoscopic observation should be routinely completed with a biopsy of the descending duodenum as suggested by the guidelines of the working group on CD. Particular attention should be given to females who report dysmotility or indeterminate dyspepsia.

Innovations and breakthroughs

This is a prospective study undertaken in the Mediterranean area. All the other related or similar articles are mainly retrospective and from different geographical areas.

Applications

Such an approach could reveal another submerged part of the "Celiac Iceberg" but it must be validated as regards the cost effectiveness, bearing in mind the variable prevalence of the disease in various geographical areas.

Peer review

This is an interesting study, looking into the prevalence of histological and serological proof of CD in consecutive patients undergoing upper GI endoscopy because of dyspepsia. Because of the high CD prevalence found, the authors conclude that duodenal biopsies should routinely be taken at this indication.

REFERENCES

1 **Marsh MN**. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992; **102**: 330-354

2 **Fasano A**, Catassi C. Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. *Gastroenterology* 2001; **120**: 636-651

3 **Bottaro G**, Cataldo F, Rotolo N, Spina M, Corazza GR. The clinical pattern of subclinical/silent celiac disease: an analysis on 1026 consecutive cases. *Am J Gastroenterol* 1999; **94**: 691-696

4 **Green PH**. The many faces of celiac disease: clinical presentation of celiac disease in the adult population. *Gastroenterology* 2005; **128**: S74-S78

5 **Craig D**, Robins G, Howdle PD. Advances in celiac disease. *Curr Opin Gastroenterol* 2007; **23**: 142-148

6 **Gelfond D**, Fasano A. Celiac disease in the pediatric population. *Pediatr Ann* 2006; **35**: 275-279

7 **Mulder CJ**, Bartelsman JF. Case-finding in coeliac disease should be intensified. *Best Pract Res Clin Gastroenterol* 2005; **19**: 479-486

8 **Mearin ML**, Ivarsson A, Dickey W. Coeliac disease: is it time for mass screening? *Best Pract Res Clin Gastroenterol* 2005; **19**: 441-452

9 **Collin P**. Should adults be screened for celiac disease? What

are the benefits and harms of screening? *Gastroenterology* 2005; **128**: S104-S108
10 **Berti I**, Della Vedova R, Paduano R, Devetta M, Caradonna M, Villanacci V, Not T, Martelossi S, Tamburlini G, Ventura A. Coeliac disease in primary care: evaluation of a case-finding strategy. *Dig Liver Dis* 2006; **38**: 461-467
11 **Catassi C**, Kryszak D, Louis-Jacques O, Duerksen DR, Hill I, Crowe SE, Brown AR, Procaccini NJ, Wonderly BA, Hartley P, Moreci J, Bennett N, Horvath K, Burk M, Fasano A. Detection of Celiac disease in primary care: a multicenter case-finding study in North America. *Am J Gastroenterol* 2007; **102**: 1454-1460
12 **Jones R**. Coeliac disease in primary care. *BMJ* 2007; **334**: 704-705
13 **Lanzini A**, Villanacci V, Apillan N, Lanzarotto F, Pirali F, Amato M, Indelicato A, Scarcella C, Donato F. Epidemiological, clinical and histopathologic characteristics of celiac disease: results of a case-finding population-based program in an Italian community. *Scand J Gastroenterol* 2005; **40**: 950-957
14 **Bardella MT**, Minoli G, Ravizza D, Radaelli F, Velio P, Quatrini M, Bianchi PA, Conte D. Increased prevalence of celiac disease in patients with dyspepsia. *Arch Intern Med* 2000; **160**: 1489-1491
15 **Keshavaraz AA**, Bashiri H, Izadi B. Celiac disease and refractory functional dyspepsia. Available from: URL: http://www.uegw.org/publications/uegw07/UEGW07_FinalProgramme_complete.pdf
16 **Green PH**, Murray JA. Routine duodenal biopsies to exclude celiac disease? *Gastrointest Endosc* 2003; **58**: 92-95
17 **Brocchi E**, Bonora M, Epifanio G, Tomassetti P, Biasco G, Corinaldesi R. Routine duodenal biopsies: is it time to change our minds? *Gastrointest Endosc* 2004; **59**: 331-332
18 **Emami M**, Karimi S. Is it or not? To do routine duodenal biopsy for every patient undergoing endoscopy. Available from: URL: http://www.uegw.org/publications/uegw07/UEGW07_FinalProgramme_complete.pdf
19 **Riestra S**, Dominguez F, Fernandez-Ruiz E, Garcia-Riesco E, Nieto R, Fernandez E, Rodrigo L. Usefulness of duodenal biopsy during routine upper gastrointestinal endoscopy for diagnosis of celiac disease. *World J Gastroenterol* 2006; **12**: 5028-5032
20 **Lecleire S**, Di Fiore F, Antonietti M, Savoye G, Lemoine F, Le Pessot F, Lerebours E, Ducrotte P. Endoscopic markers of villous atrophy are not useful for the detection of celiac disease in patients with dyspeptic symptoms. *Endoscopy* 2006; **38**: 696-701
21 **Talley NJ**, Vakil N. Guidelines for the management of dyspepsia. *Am J Gastroenterol* 2005; **100**: 2324-2337
22 **Brocchi E**, Tomassetti P, Misitano B, Epifanio G, Corinaldesi R, Bonvicini F, Gasbarrini G, Corazza G. Endoscopic markers in adult coeliac disease. *Dig Liver Dis* 2002; **34**: 177-182
23 **Oberhuber G**, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999; **11**: 1185-1194
24 **When is a coeliac a coeliac?** Report of a working group of the United European Gastroenterology Week in Amsterdam, 2001. *Eur J Gastroenterol Hepatol* 2001; **13**: 1123-1128
25 **Kolho KL**, Farkkila MA, Savilahti E. Undiagnosed coeliac disease is common in Finnish adults. *Scand J Gastroenterol* 1998; **33**: 1280-1283
26 **Hankey GL**, Holmes GK. Coeliac disease in the elderly. *Gut* 1994; **35**: 65-67
27 **Ventura A**, Magazzu G, Greco L. Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. SIGEP Study Group for Autoimmune Disorders in Celiac Disease. *Gastroenterology* 1999; **117**: 297-303
28 **Howdle PD**, Jalal PK, Holmes GK, Houlston RS. Primary small-bowel malignancy in the UK and its association with coeliac disease. *QJM* 2003; **96**: 345-353
29 **Logan RF**, Tucker G, Rifkind EA, Heading RC, Ferguson A. Changes in clinical features of coeliac disease in adults in Edinburgh and the Lothians 1960-79. *Br Med J* (Clin Res Ed) 1983; **286**: 95-97
30 **Lima VM**, Gandolfi L, Pires JA, Pratesi R. Prevalence of celiac disease in dyspeptic patients. *Arq Gastroenterol* 2005; **42**: 153-156
31 **Vivas S**, Ruiz de Morales JM, Martinez J, Gonzalez MC, Martin S, Martin J, Cechini C, Olcoz JL. Human recombinant anti-transglutaminase antibody testing is useful in the diagnosis of silent coeliac disease in a selected group of at-risk patients. *Eur J Gastroenterol Hepatol* 2003; **15**: 479-483
32 **Cammarota G**, Gasbarrini A, Gasbarrini G. No more biopsy in the diagnostic work-up of celiac disease. *Gastrointest Endosc* 2005; **62**: 119-121
33 **Bardella MT**, Minoli G, Radaelli F, Quatrini M, Bianchi PA, Conte D. Reevaluation of duodenal endoscopic markers in the diagnosis of celiac disease. *Gastrointest Endosc* 2000; **51**: 714-716
34 **Smith AD**, Ramesar K, Dunk AA. Routine duodenal biopsies to exclude celiac disease? Not yet. *Gastrointest Endosc* 2004; **60**: 164-165; author reply 165
35 **Dickey W**. Letters to the editor: Response to Dr. Radaelli et al. *Am J Gastroenterol* 2000; **95**: 1090
36 **Badreldin R**, Barrett P, Wooff DA, Mansfield J, Yiannakou Y. How good is zoom endoscopy for assessment of villous atrophy in coeliac disease? *Endoscopy* 2005; **37**: 994-998
37 **Tursi A**, Brandimarte G, Giorgetti GM. Prevalence of antitissue transglutaminase antibodies in different degrees of intestinal damage in celiac disease. *J Clin Gastroenterol* 2003; **36**: 219-221
38 **Mulder CJ**, Bartelsman JF. Case-finding in coeliac disease should be intensified. *Best Pract Res Clin Gastroenterol* 2005; **19**: 479-486
39 **Sanders DS**, Hurlstone DP, McAlindon ME, Hadjivassiliou M, Cross SS, Wild G, Atkins CJ. Antibody negative coeliac disease presenting in elderly people--an easily missed diagnosis. *BMJ* 2005; **330**: 775-776
40 **Riestra S**, Dominguez F, Fernandez-Ruiz E, Garcia-Riesco E, Nieto R, Fernandez E, Rodrigo L. Usefulness of duodenal biopsy during routine upper gastrointestinal endoscopy for diagnosis of celiac disease. *World J Gastroenterol* 2006; **12**: 5028-5032
41 **Villanacci V**, Bassotti G, Liserre B, Lanzini A, Lanzarotto F, Genta RM. Helicobacter pylori infection in patients with celiac disease. *Am J Gastroenterol* 2006; **101**: 1880-1885
42 **Mitka M**. Higher profile needed for celiac disease: underdiagnosis fosters treatment delays, says panel. *JAMA* 2004; **292**: 913-914
43 **NIH Consensus development conference on celiac disease, June 28-30, 2004**. http://consensus.nih.gov/2004/2004CeliacDisease118main.htm
44 **Catassi C**, Ratsch IM, Fabiani E, Rossini M, Bordicchia F, Candela F, Coppa GV, Giorgi PL. Coeliac disease in the year 2000: exploring the iceberg. *Lancet* 1994; **343**: 200-203
45 **Maki M**, Mustalahti K, Kokkonen J, Kulmala P, Haapalahti M, Karttunen T, Ilonen J, Laurila K, Dahlbom I, Hansson T, Hopfl P, Knip M. Prevalence of Celiac disease among children in Finland. *N Engl J Med* 2003; **348**: 2517-2524
46 **Yassin K**, Lachter J, Suissa A, Chermesh I, Eliakim R. [Undiagnosed celiac disease in adults unmasked by endoscopy] *Harefuah* 2003; **142**: 14-16, 79
47 **Catassi C**, Fabiani E, Ratsch IM, Coppa GV, Giorgi PL, Pierdomenico R, Alessandrini S, Iwanejko G, Domenici R, Mei E, Miano A, Marani M, Bottaro G, Spina M, Dotti M, Montanelli A, Barbato M, Viola F, Lazzari R, Vallini M, Guariso G, Plebani M, Cataldo F, Traverso G, Ventura A. The coeliac iceberg in Italy. A multicentre antigliadin antibodies screening for coeliac disease in school-age subjects. *Acta Paediatr Suppl* 1996; **412**: 29-35

48 **Usai P**, Usai Satta P, Savarino V, Boy MF. Autonomic neuropathy in adult celiac disease. *Am J Gastroenterol* 1996; **91**: 1676-1677
49 **Kilander AF**, Dotevall G, Lindstedt G, Lundberg PA. Plasma enteroglucagon related to malabsorption in coeliac disease. *Gut* 1984; **25**: 629-635
50 **Elli L**, Bardella MT. Motility disorders in patients with celiac disease. *Scand J Gastroenterol* 2005; **40**: 743-749
51 **Spiller RC**, Frost PF, Stewart JS, Bloom SR, Silk DB. Delayed postprandial plasma bile acid response in coeliac patients with slow mouth-caecum transit. *Clin Sci* (Lond) 1987; **72**: 217-223
52 **Maton PN**, Selden AC, Fitzpatrick ML, Chadwick VS. Defective gallbladder emptying and cholecystokinin release in celiac disease. Reversal by gluten-free diet. *Gastroenterology* 1985; **88**: 391-396
53 **Hayat M**, Cairns A, Dixon MF, O'Mahony S. Quantitation of intraepithelial lymphocytes in human duodenum: what is normal? *J Clin Pathol* 2002; **55**: 393-394
54 **Murdock AM**, Johnston SD. Diagnostic criteria for coeliac disease: time for change? *Eur J Gastroenterol Hepatol* 2005; **17**: 41-43

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RAPID COMMUNICATION

First attempt to produce experimental *Campylobacter concisus* infection in mice

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Abstract

AIM: To infect mice with atypical *Campylobacter concisus* (*C. concisus*) for the first time.
METHODS: Three separate experiments were conducted in order to screen the ability of five clinical *C. concisus* isolates of intestinal origin and the ATCC 33237 type strain of oral origin to colonize and produce infection in immunocompetent BALB/cA mice. The majority of the BALB/cA mice were treated with cyclophosphamide prior to *C. concisus* inoculation to suppress immune functions. Inoculation of *C. concisus* was performed by the gastric route.
RESULTS: *C. concisus* was isolated from the liver, ileum and jejunum of cyclophosphamide-treated mice in the first experiment. No *C. concisus* strains were isolated in the two subsequent experiments. Mice infected with *C. concisus* showed a significant loss of body weight from day two through to day five of infection but this decreased at the end of the first week. Histopathological examination did not consistently find signs of inflammation in the gut, but occasionally microabscesses were found in the liver of infected animals.
CONCLUSION: Transient colonization with *C. concisus* was observed in mice with loss of body weight. Future studies should concentrate on the first few days after inoculation and in other strains of mice.

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Key words: Animal model; BALB/cA mice; *Campylobacter concisus*; Colonization; Infection

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INTRODUCTION

The importance of *Campylobacter jejuni* (*C. jejuni*) and *C. coli* in causing diarrhoeal disease worldwide is well established[1]. However, a possible enteric pathogenic role for *Campylobacter* spp. other than *C. jejuni/coli* has not been established as yet, however, the potential pathogen *C. concisus* is at present the subject of investigation concerning its role in human gastrointestinal disease[2]. *Campylobacter concisus* (*C. concisus*) has been found in high proportions in patients with diarrhoeal disease, especially in tertiary hospital settings[3-6]. Other studies have shown *C. concisus* to possess virulence factors including a haemolytic phospholipase and cytotoxic activity caused by the Cytolethal Distending Toxin (CDT) as described in *C. jejuni*[7,8].

The presence of *C. concisus* in the gastrointestinal tract has been described by some investigators as that of a commensal as no statistical differences were found between symptomatic and asymptomatic carriers[9]. However, the fact that *C. concisus* is a heterogeneous

species with several subtypes and at least two genomospecies could in part account for the conflicting results[10-12]. To our knowledge, no reports of *C. concisus* in animal models have been presented.

Attempts to establish an animal model mimicking human campylobacteriosis using various animals have suffered from difficulties due to handling, lack of reproducibility, high costs and inadequate clinical pathology[13-17]. A major shortcoming of murine models is the inability to consistently reproduce the most common symptoms of enteritis following gastric inoculation, although sporadic colonization of the gastrointestinal tract has been achieved[18-20]. Colonization and development of gastrointestinal symptoms is augmented in immunodeficient or immune dysregulated mice and following pre-treatment with oral antibiotics or iron[21-24]. Consequently, no single animal model is widely accepted and applied in the study of *Campylobacter* infection. The objective of this study was to determine if five clinical *C. concisus* strains of intestinal origin and the type ATCC 33237 strain of oral origin could infect immunocompetent and immunodeficient mice and mimic human *Campylobacter* infections.

MATERIALS AND METHODS

Bacterial strains

The *C. concisus* reference and type ATCC 33237 strain of oral origin and five clinical isolates of *C. concisus* (RH10776.98, RH4204.98, RH4482.98, RH5097.98, RH15690.98) all from humans with diarrhoeal disease, were used. Strain details are given in Table 1. The isolates for inoculation were recovered from frozen storage, plated on 5% blood agar plates containing yeast extract (1%) and incubated in a microaerobic atmosphere (50 mL/L O_2, 100 mL/L H_2, 850 mL/L N_2) at 37℃ for 48 h as described previously[25]. The bacteria were harvested directly from plates into sterile phosphate buffered saline (PBS), washed and resuspended in PBS to the required cell density (10^9 CFU/mL) determined by optical density.

Test animals

Immunocompetent BALB/cA mice of both sexes (from the breeding colony of the University of Lund, Sweden), 6-7 wk old and with a mean weight of 16.7 g were used in the study. The mice were not defined as flora or specific pathogen free. The mice were housed in groups of a maximum of ten mice of equal gender per cage. All mice received vancomycin by intragastric intubation (0.3 mL, 80 μg/mL) three days prior to the *C. concisus* challenge. The mice had access, ad libitum, to water and standard food. Three days before the start of the experiments, faecal pellets were analysed for the presence of *Campylobacter* spp., and prior to all experiments 2-4 mice were sacrificed for baseline values with complete sampling including culture, PCR and histopathology of the stomach, liver, ileum, jejunum and colon tissue. All experiments were performed according to the recommendations of the Swedish Board of Animal Research and were approved by the Committee of Animal Ethics of the University of Lund.

Table 1 *C. concisus* **strains and patient data**

Strain	Origin	Diagnosis	No. of experiment	Protein group	AFLP group
ATCC 33237	Oral	Periodontitis	1 and 3	1	1
RH10776.98	Intestinal	Healthy traveller	1 and 3	2	2
RH4204.98	Intestinal	IBD	2 and 3	2	2
RH4482.98	Intestinal	NHL and IBD	2	2	1
RH5097.98	Intestinal	ALL (IR)	2	2	2
RH15690.98	Intestinal	ALL (IR)	2	2	-

NHL: Non Hogdkin Lymphoma; IBD: Inflammatory Bowel Disease; ALL: Acute Lymphoid Leukaemia; IR: In Remission; AFLP: Amplified Fragment Length Polymorphisms.

Experimental design

The study consisted of three experiments involving a total of 132 BALB/cA mice. Experiment 1 involved 22 animals, divided into 10 groups and the animals were inoculated with the type ATCC 33237 strain and the clinical isolate 10776.98. Half the mice received an intraperitoneal injection of cyclophosphamide (100 μL/10 g) three days before inoculation with the *C. concisus* strains in order to suppress normal immune systems by disrupting the T-cell population. The cyclophosphamide treated and untreated mice were kept in separate cages. Two mice were used for baseline values. Experiment 2 involved 54 animals, divided into 10 groups and the animals were inoculated with four clinical strains (RH4204.98; RH15690.98; RH4482.98; RH5097.98) or sham dosed with PBS. Half the mice received an intraperitoneal injection of cyclophosphamide (100 μL/10 g) three days before inoculation with the *C. concisus* strains. Four mice were used for baseline values. Experiment 3 involved 56 animals, divided into 13 groups and the animals were inoculated with the type ATCC 33237 strain, two clinical strains (RH4204.98; RH10776.98) or sham dosed with PBS. All mice received an intraperitoneal injection of cyclophosphamide (100 μL/10 g) three days before inoculation with *C. concisus* strains. Four mice were used for baseline values.

Inoculation of mice was performed by direct intragastric administration of 0.3 mL 10^9 CFU/mL or 0.3 mL of PBS by gastro-oesophageal tube (outer diameter 0.1 cm). Mice were challenged with a total of three equal doses of *C. concisus* on three consecutive days.

Evaluation of clinical status of the mice and necropsy

All animals were examined daily for signs of distress. The consistency of faecal pellets was also noted. Body weights of all mice in the third experiment were measured daily. Mice were euthanized by inhalation of carbon dioxide on day 7 (study 1: 3 mice; study 2: 5 mice; study 3: 5 mice), day 13 (study 1: 2 mice; study 2: 3 mice; study 3: 5 mice) and day 35 (study 1: 2 mice; study 2: 2 mice; study 3: 3 mice), and sampling from the stomach, liver, jejunum, ileum and colon was performed.

Identification of *C. concisus*

Microbiological culture: Faecal and tissue samples

Table 2 *P* values based in nominal weight changes from each *C. concisus* inoculated group compared to controls (n = 12), Weight (g) (*P*-value)

Day	Control group	Clinical strains		Type strain
		RH10776.98[1]	RH4204.98	ATCC 33237
0	16.0	17.2 (0.60)	16.9 (0.54)	16.6 (0.50)
1	16.4	17.5 (0.57)	16.9 (0.30)	16.6 (0.42)
2	16.7	17.2 (0.20)	16.4 (0.03)	16.6 (0.27)
3	16.8	17.0 (0.13)	16.6 (0.06)	15.4 (0.08)
4	17.3	17.5 (0.17)	16.7 (0.09)	16.5 (0.20)
5	17.5	17.3 (0.04)	16.8 (0.11)	16.6 (0.15)
6	18.1	18.1 (0.12)	17.6 (0.20)	17.4 (0.21)

[1]n = 12 from day 5. Weights are expressed as group mean. *P*-values are calculated using the Mann Whitney *U* test.

Table 3 Anatomical localization of *C. concisus* isolation (Experiment 1, day 7, n = 3)

	Stomach	Liver	Ileum	Jejunum	Colon	Stools
Culture	0	3	3	1	0	0
PCR	0	1	1	1	0	0

All isolations were from mice inoculated with the clinical strain RH10776.98.

were homogenized in PBS, plated onto blood agar containing yeast extract using the filter technique and incubated in a microaerobic environment for 48 h as previously described[25]. Identification was performed on phenotypic data according to On[26].

Detection by PCR: Specimens were stored at -18℃ until examination. DNA was extracted from faecal and tissue samples using DNeasy tissue minikit (Qiagen, Ballerup, Denmark) using a clean room procedure. Organ tissues were vortexed and manually degraded to homogenize the solutions before DNA extraction. Primers and amplification cycles were prepared according to Bastyns *et al*[27]. The mixture consisting of one forward primer MUC1 (5'-ATGAGTAGCGATAAATTGGG-3'), and two reverse primers CON1 (5'-CAGTATCGGCAATT CGCT-3') and CON2 (5'-GACAGTATCAAGGATTTA CG-3'). All *C. concisus* strains tested were PCR detectable when tested directly from agar plates prior to inoculation.

Histopathological examination

Specimens for histological examination were prepared throughout the study. Stomach, liver, small and large intestine were removed immediately after death, fixed in neutral buffered 4% formaldehyde, paraffin embedded and processed for histopathological evaluation. Sections were stained with haematoxylin and eosin and examined under a light microscope by an experienced pathologist. All examinations were performed blindly.

Statistical analysis

The loss of body weight in the various infected groups compared to the control group was evaluated using the Mann-Whitney *U*-test, with normalized weight ratios from day 1 of the experiment. Thus, for example, in Table 2 on day 2, the control group 0.7 (weight increase) is compared to ATCC 33237 0.0 (unchanged weight). Results were considered statistically significant when $P < 0.05$.

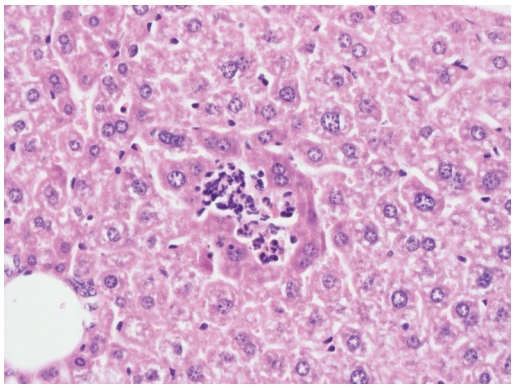

Figure 1 High power view of histological section of liver from experiment 1. Mouse inoculated with strain RH10776.98 in the non-treated group. A microabscess is seen in the centre. Haematoxylin-eosin (x 40).

RESULTS

Isolation of C. concisus

Culture: On day 7 of experiment 1, *C. concisus* was isolated from all three mice sacrificed in the cyclophosphamide-treated group, inoculated with the clinical isolate RH10776.98. All mice had positive liver and ileum cultures, whereas only one mouse had a positive jejunum culture (Table 3). Faecal pellets examined throughout the study were consistently negative. Tissue samples obtained on day 21 and day 56 were negative for *C. concisus*. Isolation of *C. concisus* was not obtained during the two subsequent experiments (2 and 3).

PCR: PCR testing of the samples yielded comparable results on day 7 of experiment 1, as all three mice sacrificed in the cyclophosphamide-treated group, inoculated with the clinical isolate RH10776.98, were PCR positive when analysing ileum tissue (Tables 2 and 3). Using PCR, *C. concisus* was not detected from liver or jejunum tissue. Tissue samples obtained on day 21 and day 56 were not positive for *C. concisus*. PCR results were consistently negative in the two subsequent experiments (2 and 3).

Histological examinations

Stomach: No evidence of inflammation or infection was noted throughout the study.

Liver: One mouse inoculated with the clinical isolate RH10776.98 and one mouse inoculated with the type ATCC 33237 strain, both from non-treated groups in experiment 1, harboured microabscesses in their livers (Figure 1). Megakaryocytes were occasionally noted in the livers of cyclophosphamide-treated groups, including the controls, but not in untreated controls. The three *C. concisus*-positive livers all showed evidence of megakaryocytes and haematopoiesis.

Jejunum: No evidence of inflammation or infection

was found, although oedema of intestinal villi was detected in the cyclophosphamide-treated control group. One mouse inoculated with the clinical *C. concisus* isolate RH15690.98, showed infiltration of lymphocytes as evidence of inflammation.

Ileum: Villi oedema was occasionally noted in the various untreated and treated groups, but not in controls. The three *C. concisus*-positive mice in experiment 1 had no signs of inflammation of the ileum.

Colon: Two mice in the untreated group infected with the type ATCC 33237 strain, showed evidence of inflammation and lymphocyte infiltration.

Clinical parameters

Loss of body weight: Compared to controls, the mice infected with the *C. concisus* strains RH4204.98 and RH 10776.98 showed a significant weight loss ($P < 0.05$), while ATCC 33237-infected mice showed a similar trend, however, this was only borderline significant ($P = 0.08$) (experiment 3). The effect was clear from day 2 but wore off on day 5. At the end of the first week, all three groups of *C. concisus*-infected mice showed a net gain less than one g of bodyweight. The controls had a slowly progressive net weight gain of two g. These results are summarized in Table 2. After the first week no differences in weight were observed between the groups.

Loose stools: On day 2 and 3 faecal pellets in *C. concisus*-inoculated groups were loose and slimy compared to control groups (experiment 3).

Mortality: Two mice in experiment 2 died after the second *C. concisus* inoculation with the clinical strains RH4204.98 and RH5097.98. One mouse inoculated with the clinical isolate RH10776.98 died on day 5 (experiment 3).

DISCUSSION

The present model mimics a relevant intragastric exposure to *C. concisus* infection in immunocompetent BALB/cA mice upon cyclophosphamide and vancomycin pretreatment. Taken together, the results indicate a transient colonization of liver and ileum, with clinical signs of illness seen as loss of body weight and loose stools, however, the lack of consistent *C. concisus* isolation is troublesome in the present model.

The reason for these discrepancies is not clear, but may predominately arise from (1) difficulties in isolation of *C. concisus* and (2) lack of tissue samples from sick animals. The problem encountered in the isolation of *C. concisus* from faecal pellets collected in the cages may have been due to swarming contamination of Proteus spp. on many of the blood agar plates, which obstructed identification of other bacteria including *C. concisus*. Using the filter technique reduced this problem but did not alleviate this swarming. A method to separate faecal pellets from urine might increase the isolation rate of *C. concisus* in future studies. In addition, the inoculation dose (0.3×10^9 CFU) may have limited the chance of persistent colonization and development of full scale illness, as many researchers report doses above 10^{10} CFU before the successful recovery of bacteria is achieved[20,21]. PCR detection was poor and refined techniques must be used, which could also solve the problem of swarming. In the clinical parameters assessed, loss of body weight and loose stools were most prominent on the first three days of infection; after that time the effects of colonization seemed to wear off. As the first mice were sacrificed on day 7, no symptomatic animals were sacrificed, leaving the cultivation of *C. concisus* and histopathological studies to determine the presence of inflammation only in asymptomatic mice.

The fact that *C. concisus* was isolated from the liver of infected animals is consistent with previous studies of *C. jejuni* extraintestinal manifestations[28-30], where histopathological lesions have been described, as opposed to the present study. There have been occasional reports of humans developing hepatitis due to *C. jejuni* infections[31,32]. In our study of 98 patients with *C. concisus* infection[5] none had hepatitis, although several had disturbed liver biochemistry. The presence of *Campylobacter* spp. in liver is an intriguing finding that merits further investigation.

Overall inflammatory changes in the present study were scarce and limited to a total of three mice. The absence of substantial histopathological findings could be due to localised patches of colonization in the GI-tract, or that the damaging effects of colonization were small and resolved quickly before the mice were sacrificed seven days post-inoculation. Some studies have reported similar findings of discrepancies between colonization and the absence of lesions on histological examination[23], whilst studies of infections on limited-flora SCID mice present with severe histopathological lesions when symptomatically sick mice are sacrificed[24]. Similar results were found in human studies, as histopathological evidence of intestinal damage is limited to sick patients and not found in asymptomatically colonized patients[33].

The present panel of six *C. concisus* strains (Table 1) showed only minor differences in clinical outcome, but the only isolated strain after inoculation was the clinical strain RH10776.98, originally from an otherwise healthy traveller with diarrhoea. We have previously shown clinical differences when applying protein profiles as well as DNA-based typing systems to differentiate clinical *C. concisus* strains[5,11,25]. The clinical strain RH10776.98 belonged to the protein profile group 2 and genomospecies 2, both of which consisted of predominately immunocompetent patients complaining of diarrhoeal disease. However, clinical differences were not apparent as the type ATCC strain of protein group 1 and genomospecies 1 was also able to induce a near significant and comparable weight loss. The fact that genetic differences exist and that many *Campylobacter* spp. exhibit interstrain differences in colonization has been shown previously[34,35]. However, more strains need to be tested to clarify the question of strain variability in

C. concisus infection.

Recently, a membrane bound haemolytic phospholipase has been identified in clinical *C. concisus* strains[7]. Also cytotoxic activity mimicking the Cytolethal Distending Toxin (CDT) has been found in the majority of tested *C. concisus* strains[8]. Taken together these findings suggest that a pathogenic role exists for at least certain subtypes of *C. concisus* strains. It is conceivable that *C. concisus* strains may differ in their pathogenicity due to the heterogeneity of the species[10-12,36].

Intragastric exposure to *C. concisus* infection in immunocompetent BALB/cA mice upon cyclophosphamide and vancomycin pretreatment resulted in transient colonization of liver, jejunum and ileum in a few mice. The symptoms were loss of body weight and loose stools. As most mice were not diseased, other strains of mice and increased dose levels of inoculum should be studied with a focus on the first days after inoculation. Future studies should concentrate on the first three days of infection and symptomatic mice should be sacrificed for optimal detection of histopathological changes, as the organism is rapidly cleared from the GI-tract and symptoms resolve.

COMMENTS

Background

Our knowledge of the etiological agent in infectious diarrhoea can at present only account for some 50% of the cases presenting with gastroenteritis. Lately, *Campylobacter concisus* (*C. concisus*) has been associated with gastroenteritis in humans, however clear evidence of a pathological role is lacking. At present only few clinical laboratories worldwide perform an active search for *C. concisus* strains in routine sampling from diarrhoeal patients. The present study aims to determine if *C. concisus* can cause diarrhoeal illness in mice.

Research frontiers

No study has addressed a pathogenic role of *C. concisus* in animal models. The demonstration of a pathogenic role of *C. concisus* in animals will fulfill Koch´s postulates and establish *C. concisus* as a pathogen to be considered in diarrhoeal disease. This study reports that transient colonization occurs and clinical symptoms are present in mice. However, the described model is not suitable for further research, as colonization is inconsistent and symptoms rapidly resolve.

Innovations and breakthroughs

This is the first study to report on *C. concisus* infection in mice.

Applications

This study proves that the colonization of mice GI tract and liver is possible and histopathological findings including microabscesses are present. Further studies using immunodeficient mice and focussing on the first days of infection are needed to justfully answer the question of pathogenicity. The liver pathology is an intriguing finding that merits attention in a human setting.

Peer review

The experimental study is focused on establishment of experimental infection with Campylobacter concisus in mice. The experiments are in the field of intestinal microbiology. Experimental cohorts are well clustered; important Campylobacter strains are used. The manuscript is well written.

REFERENCES

1 **Skirrow MB**. Diseases due to Campylobacter, Helicobacter and related bacteria. *J Comp Pathol* 1994; **111**: 113-149
2 **Newell DG**. Campylobacter concisus: an emerging pathogen? *Eur J Gastroenterol Hepatol* 2005; **17**: 1013-1014
3 **Lastovica AJ**. Emerging Campylobacter spp: The tip of the iceberg. *Clin Microbiol New* 2006; **28**: 49-55
4 **Engberg J**, On SL, Harrington CS, Gerner-Smidt P. Prevalence of Campylobacter, Arcobacter, Helicobacter, and Sutterella spp. in human fecal samples as estimated by a reevaluation of isolation methods for Campylobacters. *J Clin Microbiol* 2000; **38**: 286-291
5 **Aabenhus R**, Permin H, On SL, Andersen LP. Prevalence of Campylobacter concisus in diarrhoea of immunocompromised patients. *Scand J Infect Dis* 2002; **34**: 248-252
6 **Maher M**, Finnegan C, Collins E, Ward B, Carroll C, Cormican M. Evaluation of culture methods and a DNA probe-based PCR assay for detection of Campylobacter species in clinical specimens of feces. *J Clin Microbiol* 2003; **41**: 2980-2986
7 **Istivan TS**, Coloe PJ, Fry BN, Ward P, Smith SC. Characterization of a haemolytic phospholipase A(2) activity in clinical isolates of Campylobacter concisus. *J Med Microbiol* 2004; **53**: 483-493
8 **Engberg J**, Bang DD, Aabenhus R, Aarestrup FM, Fussing V, Gerner-Smidt P. Campylobacter concisus: an evaluation of certain phenotypic and genotypic characteristics. *Clin Microbiol Infect* 2005; **11**: 288-295
9 **Van Etterijck R**, Breynaert J, Revets H, Devreker T, Vandenplas Y, Vandamme P, Lauwers S. Isolation of Campylobacter concisus from feces of children with and without diarrhea. *J Clin Microbiol* 1996; **34**: 2304-2306
10 **Matsheka MI**, Elisha BG, Lastovica AL, On SL. Genetic heterogeneity of Campylobacter concisus determined by pulsed field gel electrophoresis-based macrorestriction profiling. *FEMS Microbiol Lett* 2002; **211**: 17-22
11 **Aabenhus R**, On SL, Siemer BL, Permin H, Andersen LP. Delineation of Campylobacter concisus genomospecies by amplified fragment length polymorphism analysis and correlation of results with clinical data. *J Clin Microbiol* 2005; **43**: 5091-5096
12 **Vandamme P**, Falsen E, Pot B, Hoste B, Kersters K, De Ley J. Identification of EF group 22 campylobacters from gastroenteritis cases as Campylobacter concisus. *J Clin Microbiol* 1989; **27**: 1775-1781
13 **Fox JG**, Ackerman JI, Taylor N, Claps M, Murphy JC. Campylobacter jejuni infection in the ferret: an animal model of human campylobacteriosis. *Am J Vet Res* 1987; **48**: 85-90
14 **Babakhani FK**, Bradley GA, Joens LA. Newborn piglet model for campylobacteriosis. *Infect Immun* 1993; **61**: 3466-3475
15 **Beery JT**, Hugdahl MB, Doyle MP. Colonization of gastrointestinal tracts of chicks by Campylobacter jejuni. *Appl Environ Microbiol* 1988; **54**: 2365-2370
16 **Russell RG**, Blaser MJ, Sarmiento JI, Fox J. Experimental Campylobacter jejuni infection in Macaca nemestrina. *Infect Immun* 1989; **57**: 1438-1444
17 **Newell DG**. Animal models of Campylobacter jejuni colonization and disease and the lessons to be learned from similar Helicobacter pylori models. *Symp Ser Soc Appl Microbiol* 2001; 57S-67S
18 **Berndtson E**, Danielsson-Tham ML, Engvall A. Experimental colonization of mice with Campylobacter jejuni. *Vet Microbiol* 1994; **41**: 183-188
19 **Stanfield JT**, McCardell BA, Madden JM. Campylobacter diarrhea in an adult mouse model. *Microb Pathog* 1987; **3**: 155-165
20 **Jesudason MV**, Hentges DJ, Pongpech P. Colonization of mice by Campylobacter jejuni. *Infect Immun* 1989; **57**: 2279-2282
21 **Hodgson AE**, McBride BW, Hudson MJ, Hall G, Leach SA. Experimental campylobacter infection and diarrhoea in immunodeficient mice. *J Med Microbiol* 1998; **47**: 799-809
22 **Fox JG**, Rogers AB, Whary MT, Ge Z, Taylor NS, Xu S, Horwitz BH, Erdman SE. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type Camplyobacter jejuni but not with C. jejuni lacking cytolethal distending toxin despite persistent colonization with both strains. *Infect Immun* 2004; **72**: 1116-1125
23 **Young VB**, Dangler CA, Fox JG, Schauer DB. Chronic

atrophic gastritis in SCID mice experimentally infected with Campylobacter fetus. *Infect Immun* 2000; **68**: 2110-2118
24 **Chang C**, Miller JF. Campylobacter jejuni colonization of mice with limited enteric flora. *Infect Immun* 2006; **74**: 5261-5271
25 **Aabenhus R**, Permin H, Andersen LP. Characterization and subgrouping of Campylobacter concisus strains using protein profiles, conventional biochemical testing and antibiotic susceptibility. *Eur J Gastroenterol Hepatol* 2005; **17**: 1019-1024
26 **On SL**. Identification methods for campylobacters, helicobacters, and related organisms. *Clin Microbiol Rev* 1996; **9**: 405-422
27 **Bastyns K**, Chapelle S, Vandamme P, Goossens H, De Wachter R. Specific detection of Campylobacter concisus by PCR amplification of 23S rDNA areas. *Mol Cell Probes* 1995; **9**: 247-250
28 **Kita E**, Oku D, Hamuro A, Nishikawa F, Emoto M, Yagyu Y, Katsui N, Kashiba S. Hepatotoxic activity of Campylobacter jejuni. *J Med Microbiol* 1990; **33**: 171-182
29 **Kita E**, Nishikawa F, Kamikaidou N, Nakano A, Katsui N, Kashiba S. Mononuclear cell response in the liver of mice infected with hepatotoxigenic Campylobacter jejuni. *J Med Microbiol* 1992; **37**: 326-231
30 **Vuckovic D**, Abram M, Doric M. Primary Campylobacter jejuni infection in different mice strains. *Microb Pathog* 1998; **24**: 263-268
31 **Reddy KR**, Farnum JB, Thomas E. Acute hepatitis associated with campylobacter colitis. *J Clin Gastroenterol* 1983; **5**: 259-262
32 **Humphrey KS**. Campylobacter infection and hepatocellular injury. *Lancet* 1993; **341**: 49
33 **Black RE**, Perlman D, Clements ML, Levine MM, Blaser MJ. Human Volunteer Studies with Campylobacter jejuni. In: Nachamkin I, Blaser MJ, Tompkins LS, eds. Campylobacter jejuni: Current status and future trends. Washington DC: American Society for Microbiology, 1992: 207-215
34 **Carvalho AC**, Ruiz-Palacios GM, Ramos-Cervantes P, Cervantes LE, Jiang X, Pickering LK. Molecular characterization of invasive and noninvasive Campylobacter jejuni and Campylobacter coli isolates. *J Clin Microbiol* 2001; **39**: 1353-1359
35 **Ahmed IH**, Manning G, Wassenaar TM, Cawthraw S, Newell DG. Identification of genetic differences between two Campylobacter jejuni strains with different colonization potentials. *Microbiology* 2002; **148**: 1203-1212
36 **On SL**, Harrington CS. Identification of taxonomic and epidemiological relationships among Campylobacter species by numerical analysis of AFLP profiles. *FEMS Microbiol Lett* 2000; **193**: 161-169

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RAPID COMMUNICATION

Antioxidant enriched enteral nutrition and oxidative stress after major gastrointestinal tract surgery

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Abstract

AIM: To investigate the effects of an enteral supplement containing antioxidants on circulating levels of antioxidants and indicators of oxidative stress after major gastrointestinal surgery.

METHODS: Twenty-one patients undergoing major upper gastrointestinal tract surgery were randomised in a single centre, open label study on the effect of postoperative enteral nutrition supplemented with antioxidants. The effect on circulating levels of antioxidants and indicators of oxidative stress, such as F2-isoprostane, was studied.

RESULTS: The antioxidant enteral supplement showed no adverse effects and was well tolerated. After surgery a decrease in the circulating levels of antioxidant parameters was observed. Only selenium and glutamine levels were restored to pre-operative values one week after surgery. F2-isoprostane increased in the first three postoperative days only in the antioxidant supplemented group. Lipopolysaccharide binding protein (LBP) levels decreased faster in the antioxidant group after surgery.

CONCLUSION: Despite lower antioxidant levels there was no increase in the circulating markers of oxidative stress on the first day after major abdominal surgery. The rise in F2-isoprostane in patients receiving the antioxidant supplement may be related to the conversion of antioxidants to oxidants which raises questions on antioxidant supplementation. Module AOX restored the postoperative decrease in selenium levels. The rapid decrease in LBP levels in the antioxidant group suggests a possible protective effect on gut wall integrity. Further studies are needed on the role of oxidative stress on outcome and the use of antioxidants in patients undergoing major abdominal surgery.

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Key words: Antioxidants; Critical illness; Enteral nutrition; Oxidative stress; Surgery; Upper gastrointestinal tract

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INTRODUCTION

Major surgery and critical illness induce an immuno-

inflammatory response, which is accompanied by the production of reactive oxygen species (ROS) at the site of injury[1-5]. Oxidative stress is defined as a state in which the level of ROS exceeds the endogenous antioxidant defences of the host. Reactive oxygen species can cause direct cellular injury by damaging lipids, proteins and DNA. This might result in tissue injury and organ dysfunction. Therefore, oxidative stress probably plays a key role in the development of organ failure[6-12]. In situations of major surgery and critical illness, a redistribution of antioxidants occurs to tissues or organs in need. This results in a depletion of antioxidant stores that may be deleterious when oxidative stress is prolonged[13]. In these situations the supplementation of certain antioxidant amino acids (glutamine, cysteine) and antioxidant micronutrients (zinc, Vitamin C, Vitamin E, β-carotene, selenium) may improve outcome. There is little information on the effect of major abdominal surgery and antioxidant supplementation on the blood levels of parameters of antioxidant capacity and oxidative stress.

To protect the host from oxidative stress, humans have an extensive antioxidant defence system consisting of enzymatic and non-enzymatic factors. Enzymes which are involved in antioxidant function are superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Superoxide dismutase catalyzes dismutation of the superoxide anion into hydrogen peroxide[14]. Glutathione peroxidase reduces hydrogen peroxide and organic hydroperoxides into water or alcohol, and reverts two glutathione (GSH) molecules to glutathione disulfide (GSSG)[14,15]. For GSH-Px and SOD function, the trace elements selenium and zinc are important, respectively[16].

Among the non-enzymatic factors, alpha tocopherol (Vitamin E), Vitamin C, β-carotene, and GSH function as antioxidants. Vitamin E is the main fat-soluble antioxidant in humans. Vitamin E scavenges peroxyl radicals, produced during lipid peroxidation, which leads to a tocopherol radical[17,18]. Vitamin C, a potent intra- and extracellular antioxidant[19], scavenges superoxide, hydroxyl and peroxyl radicals, and reacts with hypochlorite and singlet oxygen[15]. β-carotene is a precursor of Vitamin A. The conjugated double-bonds of β-carotene are able to open and scavenge singlet molecular oxygen and peroxyl radicals[20]. Glutathione is an intracellular antioxidant and a co-enzyme of GSH-Px. Glutamine and cysteine are precursors of the antioxidant GSH[15]. Furthermore, selenium, GSH, Vitamin E and Vitamin C function synergistically to regenerate both water and fat-soluble antioxidants[10]. The extent of oxidative stress can be measured by determining the end-products of lipid peroxidation; malondialdehyde (MDA) and F_2-isoprostane[15,21,22].

The present study investigates the effect of antioxidant-supplemented enteral feeding on circulating factors of the antioxidant defence system and markers of oxidative stress in patients after major upper gastrointestinal tract surgery.

MATERIALS AND METHODS

Patients

From February 2002 until May 2003, twenty-one patients undergoing elective surgery of the oesophagus, stomach or pancreas, in the VU University Medical Center (Amsterdam, The Netherlands), were included in the study. Inclusion criteria were: eligible for jejunostomy feeding, between 18 and 75 years old, body mass index (BMI) below 35, written informed consent, and a surgical procedure of at least three hours. Exclusion criteria were: history of cardiovascular or kidney disease, weight loss of > 10% in last six months, steroids or investigational drug used in the last six weeks and human immunodeficiency virus (HIV) infection. The study was approved by the Ethics Committee of the VU University Medical Centre and conducted according to the Declaration of Helsinki.

Methods

The study was a prospective, open label, randomised clinical trial of two balanced groups in parallel design in one medical centre. On the first day after surgery, patients were randomly assigned to the control group and were started on standard tube feeding (Sondalis ISO®, Nestlé, Switzerland), or the treatment group, and were started on the same tube feeding in combination with Module AOX (Nestlé, Switzerland). Sondalis ISO® is a nutritionally balanced, complete liquid diet. Module AOX is a plastic unit containing powder which contains per unit 37 kcal, 7.4 g protein, 0.04 g lipid and 1.9 g carbohydrate. The contents of Sondalis ISO® and Module AOX are listed in Table 1. The dosages of the compounds in Module AOX were established with respect to safety aspects. One Module AOX unit was added to one pouch of Sondalis ISO® (500 mL), with a maximum intake of two devices of Module AOX per day. Module AOX was upstream connected to the enteral feeding pouch. After mixing with enteral feeding, the module was connected to the administration set and was ready for immediate consumption by the patient. Feeding pouches were weighed before the start of feeding and after being discarded. This made it possible to calculate exactly the daily intake of kcal and nutritional compounds.

Feeding was administered by a jejunostomy feeding tube and started on the first day after surgery. In the treatment group, Module AOX was added to the enteral feeding from the first day after surgery. Patients received two Modules AOX per day when feeding could be increased beyond 500 mL per day. Module AOX was administered for a minimum of five and a maximum of seven days. Patients were fed continuously. The intention was to give the patient 500 mL of feeding on the first day after surgery, 1000-1500 mL on the second day and 1500-2000 mL from the third day. The feeding schedule was adjusted according to the energy requirements of the patient, which was established using indirect calorimetry. Oral food intake was allowed from day five after the

Table 1 Contents of Sondalis ISO® and Module AOX

	Sondalis ISO® per 100 mL	Module AOX per unit
kcal	100	37
Protein (g)	3.8	7.4
Glutamine (g)	0.34	6
Cysteine (g)	0.03	2.5
Vitamin C (mg)	5.4	140
Vitamin E (mg)	1	30
B-carotene (mg)	0	6
Zinc (mg)	1	6
Selenium (μg)	4.4	50

start of tube feeding. Patients were not allowed to receive additional Vitamins, amino acids or lipid solutions during the study period.

Endpoints of the study

The present study was part of a larger study testing the safety and tolerance of the supplemented ingredients. The effect of the intake of antioxidants (Module AOX, Nestlé, Switzerland) on indicators of oxidative stress was studied in a group of patients undergoing major elective surgery.

Levels of the administered nutritional compounds, indicators of oxidative stress after surgery and indicators of the inflammatory response after surgery were measured in plasma, serum and urine. F2-isoprostane (in urine) and malondialdehyde (MDA, in serum) were measured as parameters of oxidative stress. Total cysteine, Vitamin C, Vitamin E, β-carotene, zinc, selenium and GSH-Px were measured as parameters of antioxidant/oxidant status. Peripheral white blood cell count (WBC), Interleukin 6 (IL-6) and lipopolysaccharide-binding protein (LBP) were measured as parameters of surgery-induced inflammatory response.

Blood and urine samples were taken on the day before surgery (-1) and on day one (1), three (3), five (5) and seven (7) after surgery. The samples on day one (1) were taken before the start of enteral nutrition. All samples were taken between eight and ten a.m.

Preparation, storage and analysis of samples

Amino acids: Blood was collected on heparin. Plasma was separated immediately from the blood by centrifugation (2000 g) at 4℃ for ten minutes. Five hundreds μL of plasma was added to tubes containing 20 mg of solid sulfosalicyclic acid for deproteinizing, vortex mixed and subsequently stored at -80℃ until analysis. The concentration of glutamine was determined by reversed-phase high-performance liquid chromatography as previously described[23].

Cysteine: Blood was collected on heparin. Plasma was separated immediately from the blood by centrifugation (2000 g) at 4℃ for four minutes. Aliquots containing 500 μL of plasma were stored at -80℃ until analysis. Total cysteine concentrations were measured according to Malloy *et al*[24].

Vitamin E and β-carotene: Blood was collected in a serum separation tube. Blood was centrifuged (2000 g) at 20℃ for ten minutes and serum was stored at -80℃ until analysis. Serum vitamin E and β-carotene were determined as essentially described by Miller and Yang[25].

Vitamin C: Blood was collected in tubes containing EDTA (ethylenediaminetetraacetic acid). Plasma was separated immediately from the blood by centrifugation (1400 g) at 4℃ for ten minutes. Plasma was further purified by centrifugation (2700 g) at 4℃ for ten minutes. For the determination of total vitamin C (the sum of ascorbic acid and dehydroascorbic acid) plasma was stabilized by the addition of metaphosphoric acid[26] and stored at -80℃ until analysis. After deproteinization and enzymatic oxidation of ascorbic acid to dehydroascorbic acid, the latter was condensed with ortho-phenylenediamine to its derivative. This derivative was separated by reversed-phase high-performance liquid chromatography with fluorescence detection[27]. The between-assay coefficient of variation was < 4%.

Zinc and selenium: Serum was separated from the blood by centrifugation (2000 g) at 20℃ for ten minutes. Tubes cleaned with mineral-free water, were used to store serum at -80℃ until analysis. Zinc and selenium were determined by Zeeman corrected atomic absorption spectrometry. The flame was used for zinc determination. A graphite furnace and Palladium-modifier were used for the determination of selenium.

F2-Isoprostane [8-iso-Prostaglandin (PG) $F_{2\alpha}$]: Urine was collected in plastic tubes and stored at -80℃ until analysis. Urine 8-iso PGF 2α concentrations were determined using LC-MS/MS. Prior to analysis, the urine samples were thawed, mixed and centrifuged. Subsequently 0.1 mL of the labelled internal standard (10 ng/mL; 8-iso PGF 2α-d4, Cayman cat. 316350) was added to 1 mL of urine. The 'sample clean up procedure' was performed according to the method described by Bohnstedt *et al*[28]. Thereafter the samples were redissolved in 0.1 mL of 10% acetonitrile and 40 μL was injected into a Waters X-terra MS C18 column (3.5 μm, 2.1 mm × 100 mm; cat. 186000404) connected to a Quattro Micro (Waters, Milford, MA, USA) triple quadruple mass spectrometer running in negative electrospray ionization mode. Separation took place using a gradient from 7% to 33% acetonitrile, containing 0.3% ammonia. With this gradient the internal standard (357.2 > 197.3 amu) and 8-iso PGF 2α (353.2 > 193.3 amu) eluted at approximately nine minutes. The peak areas were then integrated and the ratios were calculated. The unknown samples were compared with a standard/internal standard calibrator (50 and 10 ng/mL, respectively) in order to calculate the 8-iso PGF 2α concentration in the urine samples. The within and between assay coefficients of variation were < 8% and < 9%, respectively. Finally, 8-iso PGF 2α was calculated in ng per mg creatinine in urine.

MDA: Serum was separated from the blood by centrifugation (2000 g) at 20℃ for ten minutes and stored at

-80℃ until analysis. Serum MDA concentrations were determined by high-performance liquid chromatography with fluorescence detection as described by van de Kerkhof *et al*[29].

GSH-Px: GSH-Px activity was determined in red cell hemolysate, left behind after separation of EDTA plasma and stored at -80℃. GSH-Px was measured as described by Karsdorp *et al*[30], using an Elan analyser (Merck, Germany).

WBC: WBC was measured using a Sysmex SE9000 analyzer (Sysmex Corporation, Kobe, Japan).

IL-6: IL-6 was measured with a commercially available automated solid-phase, two-site, two-step chemiluminescent immunometric assay according to the specifications of the manufacturer (Immulite®; DPC, Los Angeles, CA, USA). This assay employs a murine mAb against the IL-6 (capture antibody) and a polyclonal anti-IL-6-detecting antibody. The values were expressed in pg/mL based on the reference standard supplied by the manufacturer, with limits of detection between 2 and 1000 pg/mL.

LBP: LBP was measured in EDTA plasma with a commercially available automated solid-phase, two-site, two-step chemiluminescent immunometric assay according to the specifications of the manufacturer (Immulite®; DPC, Los Angeles, CA, USA). This assay employs a murine mAb against the LBP (capture antibody) and a polyclonal anti-LBP-detecting antibody. The values were expressed in μg/mL based on the reference standard supplied by the manufacturer, with the limits of detection between 0.2 and 200 μg/mL.

Statistical analysis

The interval/ratio variables were expressed as mean and standard deviation. The Mann-Whitney *U* test was performed to analyse patient characteristics, the tumour characteristics and the difference between the control and treatment group over time. Fischer's Exact test was performed to analyse the nominal variables of the patient and tumour characteristics. The Wilcoxon Signed Ranks Test was performed per group of patients to obtain the effect of the surgical intervention between the day before surgery (-1) and the first postoperative day (1). Differences between the control and the treatment group in the development of postoperative antioxidant and oxidant parameters were analysed using the general estimating equations (GEE) population averaged model. GEE is a linear regression technique, which is suitable for analysing results from a longitudinal study in which outcome variables are repeatedly measured in each individual[31]. Time is treated as a categorical variable, represented by dummies. In a single analysis the differences between the treatment and control group over time were analysed, corrected for baseline. The GEE analysis was performed following corrections for gender, age, smoking, chemotherapy before surgery, surgery (open or laparoscopic), duration of surgery, blood loss, tumour size or intake of calories. The Wilcoxon Signed Ranks test, the Mann-Whitney *U* test and the Fischer's Exact test were performed with SPSS 14.0 for Windows® (SPSS Inc. Chicago, IL, USA). GEE-analysis was performed with STATA® (version 7.0)[31]. For all analyses a *P*-value < 0.05 was considered significant.

RESULTS

No side-effects were observed following the administration of Module AOX. With regard to practicalities, the set-up of Module AOX was not found to be difficult by any of the nurses involved in the study. No serious events, such as occlusion of the tube were observed. As for tolerance, the daily weight of stools was not different between the control and the treatment group, the consistency of stools (liquid or soft, formed or hard) did not differ between the groups, nor did abdominal pain, but flatulence was less intense in the treatment group (data not shown).

Patient characteristics and follow-up

In total 27 patients were considered eligible for enrolment in the study, of which eleven were included in the control group and ten in the treatment group. Six patients were excluded from the study before randomization because no jejunostomy was available, which was necessary for the feeding route. In the control group, one patient died on the second day after surgery, before receiving enteral feeding. Another patient in the control group refused further blood sampling. According to the principle of 'intention to treat analysis', the results of all 21 patients were analyzed.

Patients received upper gastrointestinal tract surgery. No differences in the occurrence of postoperative complications, infectious and non-infectious, were found between the control and treatment group in the first week after surgery. Patients in the control group stayed an average of 2.5 ± 0.7 d and patients in the treatment group stayed an average of 3.7 ± 0.8 d on an intensive or medium care unit ($P = 0.236$). Baseline patient characteristics and tumour characteristics are given in Tables 2 and 3, respectively. The control and treatment groups were comparable with respect to anthropometrics, surgery and tumour characteristics.

Intake

Intake of Sondalis ISO® with or without Module AOX was started as soon as possible after surgery. Caloric intake was based on caloric requirements. However, the patients reached on average 60% of their daily caloric requirements, as measured on the day before surgery. In both groups, patients received a similar amount of calories during the treatment period (Figure 1). A plateau in intake was reached in both groups between three and five days after surgery. Table 4 shows the absolute intake of nutrients in the control and the treatment group. The

Table 2 Patient characteristics

	Control group $n = 11$	Treatment group $n = 10$	*P*-value
Male/Female	9/2	6/4	0.361[1]
Age (yr)	62 (8)	57 (10)	0.230[2]
Smoking/not smoking	3/8	6/4	0.198[1]
Bodyweight (kg)	74 (19)	67 (12)	0.245[2]
Height (cm)	174 (5)	174 (12)	0.697[2]
Body Mass Index (kg/m^2)	24 (5)	22 (3)	0.181[2]
Albumin (g/L)	38 (5)	39 (5)	0.426[2]
Chemotherapy prior to surgery (Yes/No)	4/7	5/5	0.670[1]
Surgery type (laparoscopic/open)	4/7	2/8	0.635[1]
Duration of surgery (min)	337 (100)	353 (155)	0.888[2]
Blood loss during surgery (mL)	2168 (1726)	1720 (1232)	0.647[2]
Admission ICU/MCU (d)	2.5 (0.7)	3.7 (0.8)	0.236[2]

[1]Fischer's Exact Test; [2]Mann-Whitney *U* Test. Data expressed as mean (standard deviation).

Table 3 Tumour characteristics (mean)

	Control group	Treatment group	*P*-value
Tumour size (cm)	5.93 ($n = 11$)	5.11 $n = 9$[2]	0.970[4]
Positive lymph nodes (number if present)	1.36 ($n = 7$)	3.00 $n = 5$[1]	0.412[4]
Metastasis (observed during surgery/post-surgery histology)	27.3% ($n = 11$)	30% ($n = 10$)	1.000[3]

[1]One patient had 10 positive lymph nodes; [2]No information on one patient; [3]Fischer's Exact Test; [4]Mann-Whitney *U* Test.

treatment group consumed significantly more glutamine, cysteine, zinc, selenium, Vitamin C, Vitamin E and β-carotene than the control group ($P < 0.001$).

First postoperative day

Significant decreases in the levels of antioxidants were noticed in both groups on the first day after surgery (Table 5). Total cysteine was also reduced significantly by surgery in both groups on the first day after surgery (Table 5). GSH-Px, F2-isoprostane (Figure 2) and MDA were not affected on the first day after surgery in both groups. As for the inflammatory markers, IL-6 and LBP increased in both the control and treatment group. WBC count increased in the treatment group only (Table 5).

Levels of oxidative stress parameters when starting with Module AOX or standard nutrition

Changes in plasma or serum levels of the oxidative stress parameters and the antioxidant/oxidant parameters are shown in Table 6. Glutamine increased significantly in both groups ($P = 0.001$). Total cysteine increased after surgery in both groups ($P < 0.001$). Vitamin E increased in both groups ($P < 0.001$) and Vitamin C increased in the treatment group only ($P = 0.014$). Zinc increased significantly in both groups ($P < 0.001$), as well as selenium ($P = 0.003$), with a greater rise in the treatment group ($P = 0.006$). Only selenium and glutamine preoperative levels were attained one week after surgery. For all other parameters, the levels were below preoperative values after one week. During the first three days after surgery F2-isoprostane significantly increased in the treatment group compared with the control group ($P = 0.007$) (Figure 2). MDA did not change after surgery in both groups. GSH-Px eventually decreased in the control group ($P = 0.013$), as well as in the treatment group. IL-6 tended to decrease after surgery in both groups ($P = 0.062$). No change was observed in WBC after surgery, in either group. Lipopolysaccharide-binding protein showed a peak value at day three in the control group that was significantly higher than that in the treatment group ($P = 0.018$). In the treatment group LBP levels remained at the level of the first postoperative day (Figure 3).

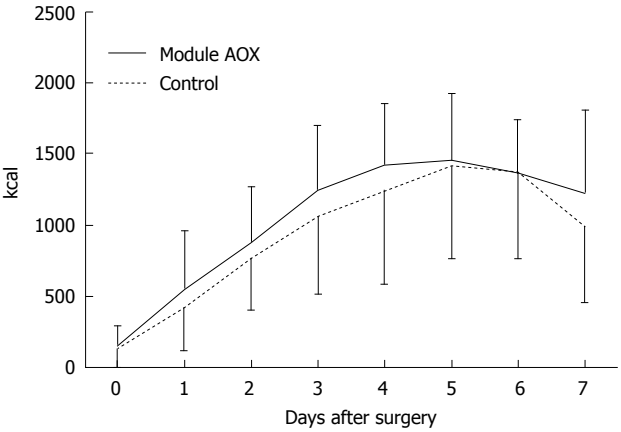

Figure 1 Intake of Kcal after surgery (mean ± SD). Caloric intake on the day before and on 7 d after surgery in the control and treatment groups. No significant difference in intake was observed between the groups.

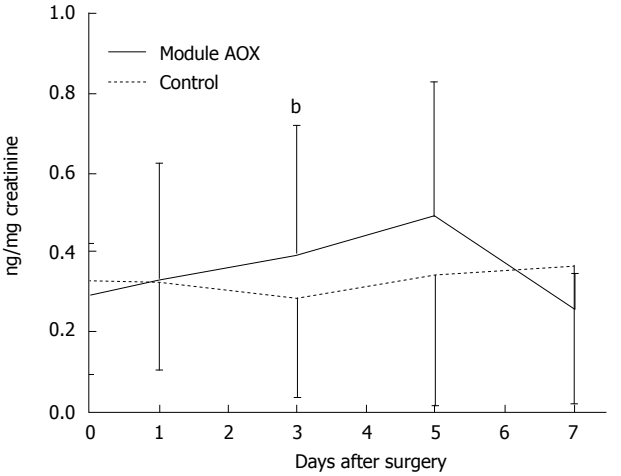

Figure 2 F2-Isoprostane after surgery (mean ± SD). Development of F2-isoprostane in urine (ng/mg creatinine) after surgery in the control and treatment groups. The difference between the two groups over time was observed to be significant between the first and the third day after surgery, $^bP < 0.01$.

DISCUSSION

The present study reports on the effects of an antioxidant

Table 4 Intake of anti-oxidant nutrients per day (mean)

		Days after surgery						
		1	2	3	4	5	6	7
Glutamine (g)	Control	1.2	2.5	3.4	3.9	4.3	4.1	3.2
	Treatment	4.5	7.9	11	11	12	11	7.4
Cysteine (g)	Control	0.1	0.2	0.3	0.3	0.4	0.4	0.3
	Treatment	1.4	2.5	3.2	3.4	3.5	3.5	2.2
Vitamin C (mg)	Control	18	40	54	62	68	65	51
	Treatment	94	167	219	236	239	233	153
Vitamin E (mg)	Control	3.4	7.2	10	12	13	12	9.4
	Treatment	20	35	45	49	50	48	32
β-carotene (mg)	Control	-	-	-	-	-	-	-
	Treatment	3.1	5.5	7.1	7.4	7.6	7.5	4.9
Zinc (mg)	Control	3.4	7.2	10	12	13	12	9.4
	Treatment	7.1	13	17	19	19	18	12
Selenium (μg)	Control	15	32	44	50	55	53	41
	Treatment	43	77	103	113	114	110	73
Module AOX units given	Control	-	-	-	-	-	-	-
	Treatment	0.57	1.01	1.29	1.36	1.39	1.38	0.89

Table 5 Surgery-induced response [difference between preoperative day (-1) and day after surgery (1) & difference between treatment and control groups over time, before start of intervention]

	Group	Day -1	Day 1	*P*-value[1]	Δ control and treatment group over time[2], *P*-value
Glutamine (μmol/L)	Control	557 (99)	434 (149)	0.021	0.888
	Treatment	596 (81)	457 (106)	0.007	
Vitamin C (μmol/L)	Control	53.5 (24.9)	22.3 (10.3)	0.003	0.379
	Treatment	68.6 (27.9)	29.4 (15.5)	0.007	
Vitamin E (μmol/L)	Control	27.4 (6.7)	11.0 (5.9)	0.003	0.458
	Treatment	29.1 (6.5)	10.9 (4.6)	0.005	
β-carotene (μmol/L)	Control	0.67 (0.78)	0.20 (0.23)	0.003	0.916
	Treatment	0.81 (1.09)	0.32 (0.45)	0.005	
Zinc (μmol/L)	Control	11.7 (2.1)	4.5 (2.1)	0.003	0.761
	Treatment	11.4 (2.6)	4.7 (2.0)	0.008	
Selenium (μmol/L)	Control	1.27 (0.28)	0.77 (0.23)	0.003	0.305
	Treatment	1.19 (0.25)	0.78 (0.21)	0.008	
MDA (μmol/L)	Control	10.29 (2.6)	12.61 (5.3)	0.091	0.035
	Treatment	10.32 (1.9)	9.35 (2.0)	0.285	
GSH-Px (U/g Hb)	Control	12.67 (3.55)	12.00 (2.54)	0.423	0.359
	Treatment	11.75 (2.08)	12.05 (1.63)	0.415	
Cysteine total (μmol/L)	Control	335 (46)	193 (40)	0.012	0.817
	Treatment	324 (39)	196 (56)	0.018	
WBC (E9/L)	Control	9 (2.7)	11.5 (5.2)	0.142	0.751
	Treatment	6.5 (1.6)	9.9 (3.4)	0.022	
IL-6 (pg/mL)	Control	10.7 (17.5)	261.7 (424.8)	0.003	1.000
	Treatment	3.0 (1.8)	337.2 (545.8)	0.005	

Data expressed as mean (standard deviation); MDA = malondialdehyde; GSH-Px = glutathione peroxidase; creat = creatinine; Hb = hemoglobin; WBC = white blood cell count; IL-6 = interleukin 6; [1]Wilcoxon Signed Rank Test; [2]Mann-Whitney *U* Test.

supplement for enteral nutrition on the indicators of oxidative stress and levels of antioxidants after major abdominal surgery. Major abdominal surgery induces oxidative stress that is associated with cellular dysfunction which may impair recovery. The rapid decrease in antioxidant levels on the first postoperative day indicates consumption to counter surgery-induced oxidative stress and is in accordance with earlier reports[32,33]. The levels of antioxidants could not be restored by the administration of Module AOX in the first five postoperative days, except for levels of selenium and glutamine (Table 5). These findings are in line with the results reported by Schroeder *et al*[33] who investigated the antioxidant enteral supplement Intestamin® (Fresenius Kabi) in similar major gastrointestinal tract surgical patients. They found that even at the higher dosage of glutamine, selenium, zinc, Vitamin C, Vitamin E and beta-carotene in Intestamin® compared to Module AOX, the antioxidant levels could

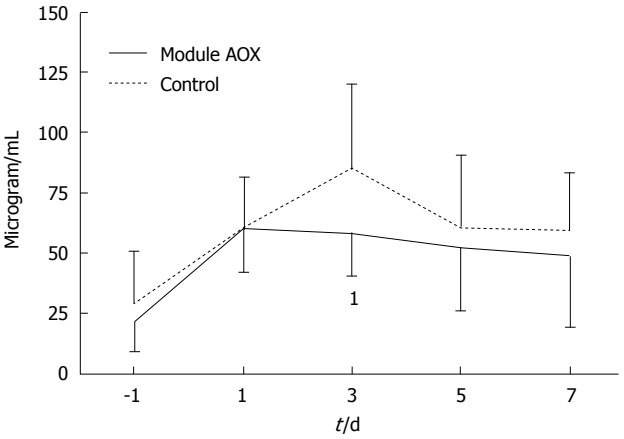

Figure 3 Development of LBP plasma concentration. Data represent means ± SD. [1]Indicates statistically significant difference between the control and treatment groups with respect to day 1.

not be raised to preoperative levels after five days of enteral nutrition. In contrast, in surgical critically ill patients, Intestamin® raised the plasma levels of glutamine, Vitamin C, Vitamin E and beta-carotene to normal levels at the third postoperative day[32]. A possible explanation for this discrepancy may be related to differences in the flow of antioxidants between cellular compartments in different patient populations and the capacity to absorb and metabolize supplemented antioxidants[34]. It should be noted that blood measurements only provide an approximation of the actual endogenous antioxidant defence status. Assessing antioxidant levels in other compartments or tissues is more difficult, but may better reflect the antioxidant defence status.

The blood concentrations of antioxidants present in Module AOX were significantly decreased on the first day after surgery. Only selenium and glutamine blood levels could be restored to preoperative levels, suggesting that the dosage of antioxidants was too low to influence blood levels. Theoretically, supplementing a combination of antioxidants that are depleted after surgery may have greater effects than supplying each antioxidant itself[10,35]. However, studies using some single antioxidant nutrients have shown important clinical results. This is especially true for glutamine which is considered a pharmaconutrient indispensable in critical illness. Glutamine appears beneficial in several patient groups, especially in those with burns, major trauma and after major surgery[36-39]. As an antioxidant, glutamine attenuates the inflammatory and oxidative stress response by enhancing plasma and tissue levels of glutathione[32]. The amount of glutamine supplementation recommended in a meta-analysis was 0.2-0.5 g per kg body weight which exceeds the amount of glutamine in Module AOX[39]. This may explain why there were no differences in glutamine levels between the control and Module AOX group after five days of nutrition.

As a single supplement, selenium is associated with decreased morbidity[16] and mortality[10] in critically ill patients. As a component of GSH-Px selenoenzymes,

Table 6 Postoperative response; 3, 5, 7 d after surgery

	Group	Day 3, *P*	Day 5, *P*	Day 7, *P*
Glutamine (μmol/L)	Control	494 (82)	566 (137)	624 (279)
		0.821	0.349	0.151
	Treatment	503 (91)	512 (108)	544 (83)
Vitamin C (μmol/L)	Control	18.3 (7.3)	23.1 (11.2)	32.2 (32.7)
		0.123	0.014	0.602
	Treatment	31.9 (13.5)	41.9 (13.9)	46.8 (16.6)
Vitamin E (μmol/L)	Control	17.6 (8.7)	21.9 (9.6)	20.6 (9.1)
		0.130	0.293	0.185
	Treatment	19.5 (4.7)	23.8 (8.7)	25.8 (11.6)
β-carotene (μmol/L)	Control	0.28 (0.31)	0.30 (0.28)	0.19 (0.23)
		0.458	0.301	0.087
	Treatment	0.34 (0.46)	0.39 (0.43)	0.45 (0.45)
Zinc (μmol/L)	Control	6.6 (2.9)	8.7 (3.4)	9.7 (3.6)
		0.704	0.426	0.280
	Treatment	6.9 (2.5)	8.5 (3.1)	10.3 (3.0)
Selenium (μmol/L)	Control	0.74 (0.26)	0.98 (0.25)	0.99 (0.25)
		0.123	0.002	0.006
	Treatment	0.89 (0.32)	1.15 (0.36)	1.29 (0.39)
MDA (μmol/L)	Control	11.55 (3.94)	11.71 (2.19)	10.69 (2.58)
		0.104	0.392	0.095
	Treatment	11.04 (4.21)	10.13 (1.98)	11.01 (2.73)
GSH-Px (U/g Hb)	Control	11.29 (1.39)	11.44 (1.76)	10.85 (1.51)
		0.648	0.945	0.165
	Treatment	11.84 (1.63)	11.83 (1.72)	11.78 (1.63)
Cysteine total (μmol/L)	Control	253 (49)	295 (50)	307 (84)
		0.836	0.278	0.419
	Treatment	251 (52)	308 (73)	320 (85)
WBC (E9/L)	Control	10.8 (2.1)	9.7 (2.9)	10.9 (4.1)
		0.595	0.447	0.893
	Treatment	8.2 (2.4)	6.3 (1.3)	10.0 (3.8)
IL-6 (pg/mL)	Control	91.6 (165)	25.1 (25.8)	33.7 (39)
		0.547	0.708	0.650
	Treatment	43.5 (37.6)	28.1 (27.6)	20.1 (16.3)

Data expressed as mean (standard deviation); MDA = malondialdehyde; GSH-Px = glutathione peroxidase; creat = creatinine; Hb = hemoglobin; GEE-analysis was used for statistical analysis; *P*, difference between the treatment and control group over time, corrected for baseline.

selenium inhibits nuclear factor kappa b (NFκB) which has a key role in the regulation of the expression of numerous cellular genes, particularly those involved in immune, inflammatory and stress responses. Therefore, by its effect on NFκB, selenium not only reduces inflammation, but also reduces oxidative stress and improves the defence mechanisms[40,41]. It is known that plasma GSH-Px is a sensitive marker of the response to antioxidant supplementation, especially selenium. Plasma GSH-Px declines in parallel with plasma selenium, while selenium supplementation restores the activity of the enzyme[16]. This is in contrast with the present study findings. Although the selenium plasma levels were raised after surgery in the treatment group, no difference in the plasma GSH-Px concentration was found. This suggests that the dosage of selenium given was insufficient to restore the activity of plasma GSH-Px.

Considering the above, one could argue that the dosages of glutamine and selenium are too low in Module AOX. However, increasing the dosages of antioxidants may be hazardous because at high intake some antioxidants may be toxic. In addition, as a consequence of their physical properties, some antioxidants also have pro-

oxidant effects. The capacity to scavenge free radicals is associated with the transformation of the scavenger into a free radical itself[16,34]. The possibility of an antioxidant acting as a pro-oxidant as well, could explain the unexpected increase in F2-isoprostane in the treatment group, although the dosages of the antioxidants used were low and established as safe. As for the other indicator of oxidative stress measured, MDA, no changes were observed in either of the groups. Similar to our findings, Preiser *et al*[42] also could not demonstrate any effect of an antioxidant-containing diet on levels of MDA in a randomised, double-blind, placebo-controlled study with critically ill patients during a seven day study period.

In the present study, malnutrition was an exclusion criterion, because it is an independent risk factor for the occurrence of postoperative infectious complications[43,44]. Kondrup *et al*[45] using the Nutritional Risk Screening (NRS 2002) examined how severity of illness and nutritional risk affected results in nutritional intervention studies. They found that better clinical effects were achieved in patients with a greater severity of illness and malnutrition[45]. It is possible that in the present study the patients who would have benefited most from antioxidant-enriched enteral nutrition, were excluded.

Lipopolysaccharide binding protein is an acute phase protein that is mainly synthesized by hepatocytes and its concentration in the circulation increases during inflammation[46]. At times of gut injury, such as during major surgery, LBP strongly modulates the response to endotoxins, which are present at the outer membrane of gram-negative bacteria (GNB). LBP-coated GNB are taken up mainly by monocytes and macrophages[46,47]. Endotoxins induce a receptor-mediated signalling cascade that leads to NF-κB activation and the transcription and subsequent release of cytokines and other proinflammatory mediators by monocytes and macrophages. Reactive oxygen species may be involved in the endotoxin-induced inflammatory response in two ways. Firstly, ROS may impair gut integrity by damaging the gut wall[48] inducing endotoxin translocation, and secondly ROS mediate endotoxin-induced NF-κB activation. It was demonstrated that neutralizing endotoxin, by recombinant bactericidal permeability increasing protein, lowered LBP levels in patients undergoing major liver resection[49]. In the Module AOX group, LBP levels decreased significantly compared to the control group which suggests that antioxidant supplementation modulates this acute phase response. This effect may be related to protection of gut integrity against ROS damage.

In major gastrointestinal tract surgical patients the oxidative stress parameters were not increased on the first postoperative day. Interestingly, in the antioxidant-supplemented group an increase in F2-isoprostane was observed during the first three postoperative days. This observation questions the use of antioxidants and further studies on the underlying mechanism are needed. A postoperative decrease in antioxidant levels occurred which could not be restored to the preoperative levels by Module AOX except for selenium levels. Larger multicenter studies are needed to further elucidate the effects of antioxidant-supplemented enteral nutrition in major gastrointestinal tract surgical patients. In such a trial, malnourished patients should also be included.

COMMENTS

Background

Major surgery and critical illness induce an immuno-inflammatory response, which is accompanied by the production of reactive oxygen species (ROS) at the site of injury. Reactive oxygen species can cause direct cellular injury by damaging lipids, proteins and DNA. In situations of major surgery and critical illness, a redistribution of antioxidants occurs to tissues or organs in need. This results in a depletion of antioxidant stores that may be deleterious when oxidative stress is prolonged. In these situations the supplementation of certain antioxidant amino acids (glutamine, cysteine) and antioxidant micronutrients (zinc, Vitamin C, Vitamin E, β-carotene, selenium) may improve outcome. The present study investigates the effect of antioxidant supplemented enteral feeding on circulating factors of the antioxidant defence system and markers of oxidative stress in patients after major upper gastrointestinal tract surgery.

Research frontiers

Theoretically, supplementing a combination of the antioxidants that are depleted after surgery may have greater effects than supplying each antioxidant itself. However, studies using some single antioxidant nutrients, like glutamine and selenium, have shown important clinical results, The effects of supplementation with combinations of antioxidants are still scarce, but the effects which are known are promising. Little is known about the effects of major abdominal surgery and antioxidant supplementation on the blood levels of parameters of antioxidant capacity and oxidative stress, which are important to know.

Innovations and breakthroughs

This study showed that oxidative stress parameters were not increased on the first postoperative day. A postoperative decrease in antioxidant levels occurred which could not be restored to the preoperative levels by Module AOX except for selenium levels. Interestingly, in the antioxidant-supplemented group, an increase in F2-isoprostane was observed during the first three postoperative days. This observation questions the use of antioxidants and further studies on the underlying mechanism are needed.

Applications

Larger multicenter studies are needed to further elucidate the effects of antioxidant-supplemented enteral nutrition in major gastrointestinal tract surgical patients. In such a trial, malnourished patients should also be included, since they might benefit most from such supplementation.

Peer review

This study investigates the effects of antioxidant enriched enteral nutrition on oxidative stress after major upper gastro-intestinal tract surgery. This study is well investigated and has some interesting findings.

REFERENCES

1 **Anup R**, Aparna V, Pulimood A, Balasubramanian KA. Surgical stress and the small intestine: role of oxygen free radicals. *Surgery* 1999; **125**: 560-569

2 **Cadenas S**, Cadenas AM. Fighting the stranger-antioxidant protection against endotoxin toxicity. *Toxicology* 2002; **180**: 45-63

3 **Nathens AB**, Neff MJ, Jurkovich GJ, Klotz P, Farver K, Ruzinski JT, Radella F, Garcia I, Maier RV. Randomized, prospective trial of antioxidant supplementation in critically ill surgical patients. *Ann Surg* 2002; **236**: 814-822

4 **Prabhu R**, Thomas S, Balasubramanian KA. Oral glutamine attenuates surgical manipulation-induced alterations in the intestinal brush border membrane. *J Surg Res* 2003; **115**: 148-156

5 **Thomas S**, Prabhu R, Balasubramanian KA. Surgical manipulation of the intestine and distant organ damage-protection by oral glutamine supplementation. *Surgery* 2005; **137**: 48-55

6 **Dahlgren C**, Karlsson A. Respiratory burst in human

neutrophils. *J Immunol Methods* 1999; **232**: 3-14
7 **Bautista AP**, Schuler A, Spolarics Z, Spitzer JJ. Tumor necrosis factor-alpha stimulates superoxide anion generation by perfused rat liver and Kupffer cells. *Am J Physiol* 1991; **261**: G891-G895
8 **Haddad IY**, Pataki G, Hu P, Galliani C, Beckman JS, Matalon S. Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *J Clin Invest* 1994; **94**: 2407-2413
9 **Kooy NW**, Lewis SJ, Royall JA, Ye YZ, Kelly DR, Beckman JS. Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite. *Crit Care Med* 1997; **25**: 812-819
10 **Heyland DK**, Dhaliwal R, Suchner U, Berger MM. Antioxidant nutrients: a systematic review of trace elements and vitamins in the critically ill patient. *Intensive Care Med* 2005; **31**: 327-337
11 **Bulger EM**, Maier RV. Antioxidants in critical illness. *Arch Surg* 2001; **136**: 1201-1207
12 **Radi R**, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 1991; **288**: 481-487
13 **Berger MM**, Shenkin A. Update on clinical micronutrient supplementation studies in the critically ill. *Curr Opin Clin Nutr Metab Care* 2006; **9**: 711-716
14 **Roth E**, Manhart N, Wessner B. Assessing the antioxidative status in critically ill patients. *Curr Opin Clin Nutr Metab Care* 2004; **7**: 161-168
15 **Therond P**, Bonnefont-Rousselot D, Davit-Spraul A, Conti M, Legrand A. Biomarkers of oxidative stress: an analytical approach. *Curr Opin Clin Nutr Metab Care* 2000; **3**: 373-384
16 **Berger MM**, Chiolero RL. Antioxidant supplementation in sepsis and systemic inflammatory response syndrome. *Crit Care Med* 2007; **35**: S584-S590
17 **Clavel JP**, Emerit J, Thuillier A. [Lipid peroxidation and free radicals. Role in cellular biology and pathology] *Pathol Biol* (Paris) 1985; **33**: 61-69
18 **Inal ME**, Eguz AM. The effects of isosorbide dinitrate on methemoglobin reductase enzyme activity and antioxidant states. *Cell Biochem Funct* 2004; **22**: 129-133
19 **Chan WH**, Yu JS, Yang SD. PAK2 is cleaved and activated during hyperosmotic shock-induced apoptosis via a caspase-dependent mechanism: evidence for the involvement of oxidative stress. *J Cell Physiol* 1999; **178**: 397-408
20 **Tapiero H**, Townsend DM, Tew KD. The role of carotenoids in the prevention of human pathologies. *Biomed Pharmacother* 2004; **58**: 100-110
21 **Berger MM**. Antioxidant micronutrients in major trauma and burns: evidence and practice. *Nutr Clin Pract* 2006; **21**: 438-449
22 **Meagher EA**, FitzGerald GA. Indices of lipid peroxidation in vivo: strengths and limitations. *Free Radic Biol Med* 2000; **28**: 1745-1750
23 **Teerlink T**, van Leeuwen PA, Houdijk A. Plasma amino acids determined by liquid chromatography within 17 minutes. *Clin Chem* 1994; **40**: 245-249
24 **Malloy MH**, Rassin DK, Gaull GE. A method for measurement of free and bound plasma cyst(e)ine. *Anal Biochem* 1981; **113**: 407-415
25 **Miller KW**, Yang CS. An isocratic high-performance liquid chromatography method for the simultaneous analysis of plasma retinol, alpha-tocopherol, and various carotenoids. *Anal Biochem* 1985; **145**: 21-26
26 **Margolis SA**, Paule RC, Ziegler RG. Ascorbic and dehydroascorbic acids measured in plasma preserved with dithiothreitol or metaphosphoric acid. *Clin Chem* 1990; **36**: 1750-1755
27 **Speek AJ**, Schrijver J, Schreurs WH. Fluorometric determination of total vitamin C in whole blood by high-performance liquid chromatography with pre-column derivatization. *J Chromatogr* 1984; **305**: 53-60
28 **Bohnstedt KC**, Karlberg B, Wahlund LO, Jonhagen ME, Basun H, Schmidt S. Determination of isoprostanes in urine samples from Alzheimer patients using porous graphitic carbon liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003; **796**: 11-19
29 **van de Kerkhof J**, Schalkwijk CG, Konings CJ, Cheriex EC, van der Sande FM, Scheffer PG, ter Wee PM, Leunissen KM, Kooman JP. Nepsilon-(carboxymethyl)lysine, Nepsilon-(carboxyethyl)lysine and vascular cell adhesion molecule-1 (VCAM-1) in relation to peritoneal glucose prescription and residual renal function; a study in peritoneal dialysis patients. *Nephrol Dial Transplant* 2004; **19**: 910-916
30 **Karsdorp VH**, Dekker GA, Bast A, van Kamp GJ, Bouman AA, van Vugt JM, van Geijn HP. Maternal and fetal plasma concentrations of endothelin, lipidhydroperoxides, glutathione peroxidase and fibronectin in relation to abnormal umbilical artery velocimetry. *Eur J Obstet Gynecol Reprod Biol* 1998; **80**: 39-44
31 **Twisk JCR**. Applied longitudinal data analysis for epidemiology. A practical guide. Cambridge, UK: Cambridge University Press, 2003
32 **Beale RJ**, Sherry T, Lei K, Campbell-Stephen L, McCook J, Smith J, Venetz W, Alteheld B, Stehle P, Schneider H. Early enteral supplementation with key pharmaconutrients improves Sequential Organ Failure Assessment score in critically ill patients with sepsis: outcome of a randomized, controlled, double-blind trial. *Crit Care Med* 2008; **36**: 131-144
33 **Schroeder J**, Alteheld B, Stehle P, Cayeux MC, Chiolero RL, Berger MM. Safety and intestinal tolerance of high-dose enteral antioxidants and glutamine peptides after upper gastrointestinal surgery. *Eur J Clin Nutr* 2005; **59**: 307-310
34 **Berger MM**. Can oxidative damage be treated nutritionally? *Clin Nutr* 2005; **24**: 172-183
35 **Crimi E**, Sica V, Williams-Ignarro S, Zhang H, Slutsky AS, Ignarro LJ, Napoli C. The role of oxidative stress in adult critical care. *Free Radic Biol Med* 2006; **40**: 398-406
36 **Kreymann KG**, Berger MM, Deutz NE, Hiesmayr M, Jolliet P, Kazandjiev G, Nitenberg G, van den Berghe G, Wernerman J, Ebner C, Hartl W, Heymann C, Spies C. ESPEN Guidelines on Enteral Nutrition: Intensive care. *Clin Nutr* 2006; **25**: 210-223
37 **Calder PC**. Immunonutrition in surgical and critically ill patients. *Br J Nutr* 2007; **98** Suppl 1: S133-S139
38 **Houdijk AP**, Rijnsburger ER, Jansen J, Wesdorp RI, Weiss JK, McCamish MA, Teerlink T, Meuwissen SG, Haarman HJ, Thijs LG, van Leeuwen PA. Randomised trial of glutamine-enriched enteral nutrition on infectious morbidity in patients with multiple trauma. *Lancet* 1998; **352**: 772-776
39 **Novak F**, Heyland DK, Avenell A, Drover JW, Su X. Glutamine supplementation in serious illness: a systematic review of the evidence. *Crit Care Med* 2002; **30**: 2022-2029
40 **Mishra V**, Baines M, Perry SE, McLaughlin PJ, Carson J, Wenstone R, Shenkin A. Effect of selenium supplementation on biochemical markers and outcome in critically ill patients. *Clin Nutr* 2007; **26**: 41-50
41 **Kretz-Remy C**, Arrigo AP. Selenium: a key element that controls NF-kappa B activation and I kappa B alpha half life. *Biofactors* 2001; **14**: 117-125
42 **Preiser JC**, Van Gossum A, Berre J, Vincent JL, Carpentier Y. Enteral feeding with a solution enriched with antioxidant vitamins A, C, and E enhances the resistance to oxidative stress. *Crit Care Med* 2000; **28**: 3828-3832
43 **Kudsk KA**. Immunonutrition in surgery and critical care. *Annu Rev Nutr* 2006; **26**: 463-479
44 **Dominioni L**, Rovera F, Pericelli A, Imperatori A. The rationale of early enteral nutrition. *Acta Biomed* 2003; **74** Suppl 2: 41-44
45 **Kondrup J**, Rasmussen HH, Hamberg O, Stanga Z.

Nutritional risk screening (NRS 2002): a new method based on an analysis of controlled clinical trials. *Clin Nutr* 2003; **22**: 321-336

46 **Vreugdenhil AC**, Snoek AM, Greve JW, Buurman WA. Lipopolysaccharide-binding protein is vectorially secreted and transported by cultured intestinal epithelial cells and is present in the intestinal mucus of mice. *J Immunol* 2000; **165**: 4561-4566

47 **Weiss J**. Bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP): structure, function and regulation in host defence against Gram-negative bacteria. *Biochem Soc Trans* 2003; **31**: 785-790

48 **Wischmeyer PE**. Glutamine: role in gut protection in critical illness. *Curr Opin Clin Nutr Metab Care* 2006; **9**: 607-612

49 **Wiezer MJ**, Meijer C, Sietses C, Prins HA, Cuesta MA, Beelen RH, Meijer S, van Leeuwen PA. Bactericidal/permeability-increasing protein preserves leukocyte functions after major liver resection. *Ann Surg* 2000; **232**: 208-215

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RAPID COMMUNICATION

Risk factors affecting pancreatic fistulas after pancreaticoduodenectomy

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Abstract

AIM: To analyze the risk factors of pancreatic leakage after pancreaticoduodenectomy.

METHODS: We retrospectively reviewed 172 consecutive patients who had undergone pancreaticoduodenectomy at Inha University Hospital between April 1996 and March 2006. We analyzed the pancreatic fistula rate according to the clinical characteristics, the pathologic and laboratory findings, and the anastomotic methods.

RESULTS: The incidence of developing pancreatic fistulas in patients older than 60 years of age was 21.7% (25/115), while the incidence was 8.8% (5/57) for younger patients; the difference was significant (P = 0.03). Patients with a dilated pancreatic duct had a lower rate of post-operative pancreatic fistulas than patients with a non-dilated duct (P = 0.001). Other factors, including clinical features, anastomotic methods, and pathologic diagnosis, did not show any statistical difference.

CONCLUSION: Our study demonstrated that pancreatic fistulas are related to age and a dilated pancreatic duct. The surgeon must take these risk factors into consideration when performing a pancreaticoduodenectomy.

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Key words: Pancreaticoduodenectomy; Pancreatic fistula; Pancreatic leakage

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INTRODUCTION

Pancreaticoduodenectomy (PD) is a commonly performed surgical procedure for managing duodenal trauma and various benign and malignant diseases of the periampullary region.

This procedure was first described by Whipple *et al*[1] in 1935. At that time, PD was technically difficult to perform and the mortality rate was reported to be > 30%[1]. Despite marked progress in the procedure and in the treatment of perioperative patients, the mortality rate is still reported to be 2%-10% in most hospitals[2,3]. The incidence of pancreatic fistulas remains a major cause of postoperative complications; it is reported that the incidence of pancreatic fistulas after PD is 6%-25%[4-12]. It is known that such pancreatic fistulas induce abscess formation, vascular injuries, rupture of pseudoaneurysms, and postoperative delayed hemorrhage, all resulting from inflammation around leakage sites due to stasis of fluid, including active pancreatic enzymes[13-16].

The aim of this study was to analyze the independent risk factors for pancreatic fistulas after PD.

MATERIALS AND METHODS

Between April 1996 and March 2006, 172 consecutive patients who had undergone PD at Inha University Hospital were retrospectively reviewed. The operations were performed by five surgeons, and pancreatic fistulas were investigated retrospectively by review of the patients'

medical records. A pancreatic fistula was defined as follows: from the 7th postoperative day on, the drainage output was > 50 cc a day and the drainage fluid amylase level was 3 times higher than the serum level[6].

We compared the pancreatic fistula rates based on gender, age, anastomotic method, preoperative serum total bilirubin level, serum albumin level, white blood cell (WBC) count, histologic diagnosis, texture of the remnant pancreas, and size of the pancreatic duct. The methods used for anastomosis included end-to-end anastomosis (dunking) between cross-sections of the jejunum and the pancreatic stump, and a pancreatic duct-to-jejunal mucosal anastomosis. The results were compared based on whether or not a feeding tube was placed into the duct, serving as a stent for the reconstruction and exteriorization of the duct from the anastomotic site through the lateral abdominal wall.

In all cases, somatostatin was used prophylactically for 7 d postoperatively. The statistical analyses of the correlations among multiple clinical factors were performed using independent t-tests and χ^2 tests, and a significant difference was considered when $P < 0.05$. The assessment of the statistical significance was carried out using multivariate analyses.

RESULTS

Leakage of the pancreaticojejunostomy occurred in 30 of 172 patients (17.4%), and the frequency of such leakage was analyzed and compared according to gender, age, anastomotic methods, operative findings, preoperative serum total bilirubin and albumin levels, preoperative WBC, and histopathologic diagnosis (Table 1).

Reoperations were carried out in 5 of 30 patients (16.7%) with leakage of the pancreaticojejunostomy; 4 patients had total pancreatectomies and 1 patient had a segmental resection of the small bowel. Among 172 patients, there were 4 deaths and the mortality rate was 2.3%; 1 patient underwent reoperation and 3 patients died during conservative management. There were 4 deaths in groups with leakage of the pancreaticojejunostomy.

Five surgeons operated on 95, 26, 19, 19, and 13 patients, respectively, in our hospital. The occurrences of pancreatic fistulas according to the surgeons were 17.9% (17/95), 23.1% (6/26), 15.8% (3/19), 10.5% (2/19), and 15.4% (2/13), respectively. There was no significant correlation between the surgeons and pancreatic fistulas ($P = 0.867$).

The mean age of the patients was 62.2 years, with a range of 33-87 years. Pancreatic fistulas occurred in 25 patients over 60 years of age (21.7%), and in 5 of 57 patients under 60 years of age (8.8%). The difference in the pancreatic fistula rates between the two groups was significant ($P = 0.016$).

With regard to gender, anastomotic method, pancreatic stenting, texture of the remnant pancreas, preoperative serum albumin level, total bilirubin level, and WBC count, there were no significant differences in pancreatic fistula rates.

The pancreatic duct size was included in the pathology reports and the patients were divided into 2 groups based on the main duct size, as follows: (1) patients with a dilated pancreatic main duct, defined as having a visible main duct and (2) a non-dilated pancreatic main duct, defined as having a non-visible main duct. Only 136 of 172 patients were classified according to pancreatic duct size. The number of patients with dilated and non-dilated pancreatic main ducts was 60 and 76, respectively. Pancreatic fistulas developed in 4 of 60 patients in the group with a dilated pancreatic main duct (6.6%), and in 21 of 76 patients in the group with a non-dilated pancreatic main duct (27.6%); there was a significant difference between the two groups ($P = 0.001$; Table 1).

Table 1 Pancreatic fistula rate based on clinical factors

Factors		Number of patients (%)	Leakage (%)	*P* value
Age (yr)	≥ 60	115 (66.9)	25 (21.7)	0.016
	< 60	57 (32.1)	5 (8.8)	
Gender	Male	116 (67.4)	19 (16.4)	0.606
	Female	56 (32.6)	11 (19.6)	
Type of anastomosis	Duct-to-mucosa	133 (77.3)	22 (16.5)	0.582
	End-to-end	39 (22.7)	8 (19.6)	
Pancreatic stent	Yes	58 (33.7)	11 (19.0)	0.712
	No	114 (66.3)	19 (16.7)	
Texture of remnant pancreas	Hard	61 (58.5)	7 (16.3)	0.392
	Soft	43 (41.5)	7 (23.0)	
Pancreatic duct size	Dilated	60 (44.1)	4 (6.6)	0.001
	Non-dilated	76 (55.9)	21 (27.6)	
Pre-op bilirubin	≥ 1.3 mg/dL	92 (54.5)	15 (16.3)	0.674
	< 1.3 mg/dL	80 (46.5)	15 (18.8)	
Pre-op albumin	≥ 3.1 g/dL	138 (80.2)	23 (16.7)	0.607
	< 3.1 g/dL	34 (19.8)	7 (20.6)	
Pre-op WBC	≥ 10000/mm^3	29 (16.9)	7 (24.1)	0.349
	< 10000/mm^3	143 (83.1)	23 (16.1)	

When the incidence of pancreatic fistulas was compared based on histopathologic diagnosis, pancreatic fistulas occurred in 6 of 37 patients (16.2%) diagnosed with carcinomas in the pancreatic head, in 8 of 46 patients (17.4%) diagnosed with carcinomas in the common bile duct (CBD), in 8 of 34 patients (23.5%) diagnosed with carcinomas of the ampulla of Vater, in 1 of 10 patients (10.0%) diagnosed with duodenal cancer, in 3 of 9 patients (33.3%) diagnosed with intraductal papillary mucinous tumors, in 0 of 6 patients (0%) diagnosed with chronic pancreatitis, in 2 of 22 patients (9.1%) diagnosed with gastric cancer and pancreatic invasion, in 1 of 4 patients (25.0%) with trauma to the pancreas, and in 1 of 4 patients (25.0%) diagnosed with gallbladder (GB) cancer (Table 2). Although patients diagnosed with chronic pancreatitis and gastric cancer with pancreatic invasion tended to develop pancreatic fistulas less frequently than other patients, there was no significant difference in the correlation between histopathologic diagnosis and pancreatic fistulas.

DISCUSSION

PD is technically difficult, and as a result, relatively high mortality (15%-30%) and complication rates (50%-75%) were reported before the 1980s. With advances in surgi-

Table 2 Pancreatic fistula based on histopathologic diagnosis

Name of disease	Number of patients (%)	Leakage (%)
Pancreatic head cancer	37 (21.5)	6 (16.2)
CBD cancer	46 (26.7)	8 (17.4)
Ampulla of Vater cancer	34 (19.8)	8 (23.5)
Duodenal cancer	10 (5.8)	1 (10.0)
Chronic pancreatitis	6 (3.5)	0 (0)
Intraductal papillary mucinous tumor	9 (5.2)	3 (33.2)
Gastric cancer	22 (12.8)	2 (9.1)
GB cancer	4 (2.3)	1 (25)
Trauma	4 (2.3)	1 (25)

cal techniques and perioperative care, the mortality rate associated with PD has since improved[17].

Most complications after PD commonly arise from failure in healing of the pancreaticojejunostomy, and have been described as pancreatic fistulas or anastomotic leakages by various authors.

Berberat *et al*[18] defined a pancreatic fistula as an anastomotic leak of the pancreaticojejunostomy demonstrated radiographically or intraoperatively, and as a prolonged or elevated output of amylase-rich fluid through an intraoperatively-placed drain (> 3 times the normal serum amylase level). Lowy *et al*[19] divided pancreatic fistulas into clinical leakage and biochemical leakage, in which the former referred to the amylase level of the fluid obtained through an intraoperatively-placed drain to be > 3 times the normal serum amylase level, with a high fever, leukocytosis, sepsis, and the need for drainage, while the latter referred to asymptomatic patients. We favor the definition described by Yeo *et al*[6] i.e. from the 7th postoperative day on, the drain output is > 50 cc a day and the drain fluid amylase level is 3 times higher than the serum amylase level. Using the Yeo *et al*[6] definition, we analyzed the risk factors for pancreatic leakage.

To place a tube as a stent and to determine how a pancreaticojejunostomy relates to pancreatic fistula formation requires more research[20]. Yeo *et al*[21] reported that pancreatic fistulas were correlated with anastomotic technique, operative time, a surgeon's skills and experience in performing a PD, tumor location, and co-morbid illnesses[21]. Bartoli *et al*[17] reported a difference in the degree of fibrosis of the remnant pancreas, and that anastomotic leakage occurred in 5% of patients with chronic pancreatitis and in 33% of patients with carcinoma of the CBD. Patients in whom the pancreatic texture has a hard consistency have been reported to be at lower risk for pancreatic leakage than those patients who have a pancreatic parenchyma with a soft or intermediate consistency[22,23]. The texture of the pancreatic parenchyma has been reported to be correlated with the pancreatic duct diameter[24], in considering the ease in performing a pancreatic duct-to-jejunum mucosa anastomosis, such a simple comparison requires more consideration. The pancreatic duct diameter has been correlated with pancreatic leakage[5], and our study showed that 136 (79.1%) patients had evidence of pancreatic duct dilatation by histopathologic reports, confirming the correlation between pancreatic duct size and pancreatic fistula development ($P = 0.001$). None of the 6 patients with chronic pancreatitis developed pancreatic leakage, and chronic pancreatitis induced pancreatic fistula less often than other pancreatic diseases; however, there was no statistical significance. We considered this result to reflect a small number of the population and because of the possible prediction of pancreatic duct dilatation in chronic pancreatitis, pancreatic duct diameter could be correlated with pancreatic leakage. However, in considering factors related to the texture of the remnant pancreas, the incidence of pancreatic fistulas in patients with a hard texture of the remnant pancreatic parenchyma was 16.3% (7/43) and was lower than that in patients with a soft texture [23% (14/61)], but this finding lacked statistical significance ($P = 0.392$). These results are considered to have no statistical significance because pancreatic texture was demonstrated in only 104 of 172 patients (60.5%).

In this study, the incidence of pancreatic fistulas in patients with gastric cancer with pancreatic invasion was 9.1% and was lower than that in patients with other diseases, again showing no statistical significance. Among 22 patients with gastric cancer with pancreatic invasion, 13 patients were < 60 years of age, suggesting that gastric cancer with pancreatic invasion affects a younger age group when compared with 57 patients < 60 years of age in the total population of 172 patients. These results were considered to be influenced by the bias arising from the difference between the older age group, a significant factor in our study, and the younger age group of patients with gastric cancer with pancreatic invasion.

Pancreatic leakage has been related to the presence or absence of co-morbid illnesses, and age has been correlated with the occurrence of pancreatic fistulas[21-23]. In our study, there was a significant correlation between age and pancreatic fistulas ($P = 0.03$).

With regard to the pancreaticojejunostomy technique, binding pancreaticojejunostomy significantly decreased postoperative complications and the pancreaticojejunostomy leakage rate[25-27]. In our study, the methods used for anastomosis were divided into end-to-end dunking anastomosis between cross-sections of the jejunum and the pancreatic stump, and pancreatic duct-to jejunal mucosal anastomosis. The results were compared based on whether or not a feeding tube, which serves as a stent for the reconstruction and exteriorization, was placed from the anastomotic site through the lateral abdominal wall.

Many previous reports have proposed that hard texture of the pancreatic parenchyma and a dilated pancreatic duct have a lower risk of pancreatic fistula formation owing to an ability to prevent pancreatic duct dilatation and shrinkage of the pancreaticojejunostomy after PD[16]. Conversely, in the case of a small pancreatic duct and a soft pancreas, an end-to-end invagination anastomosis or binding pancreaticojejunostomy significantly decreases postoperative complications[20,27]. In our study there was no significant difference in the incidence of pancreatic fistulas as a function of anastomotic technique.

Although there is a report that the preoperative serum total bilirubin level, duration of jaundice, surgery performed under emergent conditions, and preoperative serum albumin level can affect the occurrence of pancreatic fistulas[28], there was no significant difference among the preoperative serum total bilirubin level, albumin level, and leukocytosis in our study. The patients with pancreatic parenchyma of soft consistency produce a larger amount of pancreatic juice and have a higher risk of pancreatic leakage than those with a hard consistency[14]. Therefore, a variety of surgical methods have been attempted. Specifically, efforts to exteriorize the pancreatic fluid with a tube, to place a tube into the pancreatic duct, and to use synthetic somatostatin prophylactically have been attempted, but the reports have failed to show a statistically significant difference among the techniques[26,27].

In this study, it was demonstrated that there were no significant differences in the incidence of pancreatic fistulas based on surgical technique. Therefore, we are of the opinion that the surgical technique should be individualized based on the patient's condition and the surgeon's preferences.

We demonstrated several risk factors related to pancreatic leakage after PD; age and pancreatic duct size were significantly correlated with an increased incidence of pancreatic fistula. In conclusion, it is important that surgeons are aware of these risk factors for pancreatic fistula formation when performing a PD.

COMMENTS

Background

Pancreaticoduodenectomy is technically difficult, and as a result, relatively high mortality (15%-30%) and complication rates (50%-75%) were reported before the 1980s. With advances in surgical techniques and perioperative care, the mortality rate associated with PD has since improved.

Research frontiers

Most complications after PD commonly arise from failure in healing of the pancreaticojejunostomy, and have been described as pancreatic fistulas or anastomotic leakages by various authors.

Innovations and breakthroughs

The incidence of developing pancreatic fistulas in patients older than 60 years of age was 21.7% (25/115), while the incidence was 8.8% (5/57) for younger patients; the difference was significant (P = 0.03). The patients with a dilated pancreatic duct had a lower rate of post-operative pancreatic fistulas than the patients with a non-dilated duct (P = 0.001).

Applications

This study demonstrated that pancreatic fistulas are related to age and a dilated pancreatic duct. The surgeon must take these risk factors into consideration when performing a pancreaticoduodenectomy.

Peer review

A well organized paper about the risk factors affecting pancreatic fistulas after pancreaticoduodenectomy.

REFERENCES

1 **Whipple AO**, Parsons WB, Mullins CR. Treatment of carcinoma of the ampulla of vater. *Ann Surg* 1935; **102**: 763-779

2 **Marcus SG**, Cohen H, Ranson JH. Optimal management of the pancreatic remnant after pancreaticoduodenectomy. *Ann Surg* 1995; **221**: 635-645; discussion 645-648

3 **Bottger TC**, Junginger T. Factors influencing morbidity and mortality after pancreaticoduodenectomy: critical analysis of 221 resections. *World J Surg* 1999; **23**: 164-171; discussion 171-172

4 **Neoptolemos JP**, Russell RC, Bramhall S, Theis B. Low mortality following resection for pancreatic and periampullary tumours in 1026 patients: UK survey of specialist pancreatic units. UK Pancreatic Cancer Group. *Br J Surg* 1997; **84**: 1370-1376

5 **Miedema BW**, Sarr MG, van Heerden JA, Nagorney DM, McIlrath DC, Ilstrup D. Complications following pancreaticoduodenectomy. Current management. *Arch Surg* 1992; **127**: 945-949; discussion 949-950

6 **Yeo CJ**, Cameron JL, Sohn TA, Lillemoe KD, Pitt HA, Talamini MA, Hruban RH, Ord SE, Sauter PK, Coleman J, Zahurak ML, Grochow LB, Abrams RA. Six hundred fifty consecutive pancreaticoduodenectomies in the 1990s: pathology, complications, and outcomes. *Ann Surg* 1997; **226**: 248-257; discussion 257-260

7 **Rosenberg L**, MacNeil P, Turcotte L. Economic evaluation of the use of octreotide for prevention of complications following pancreatic resection. *J Gastrointest Surg* 1999; **3**: 225-232

8 **Buchler MW**, Friess H, Bittner R, Roscher R, Krautzberger W, Muller MW, Malfertheiner P, Beger HG. Duodenum-preserving pancreatic head resection: Long-term results. *J Gastrointest Surg* 1997; **1**: 13-19

9 **Lieberman MD**, Kilburn H, Lindsey M, Brennan MF. Relation of perioperative deaths to hospital volume among patients undergoing pancreatic resection for malignancy. *Ann Surg* 1995; **222**: 638-645

10 **Trede M**, Schwall G. The complications of pancreatectomy. *Ann Surg* 1988; **207**: 39-47

11 **Cullen JJ**, Sarr MG, Ilstrup DM. Pancreatic anastomotic leak after pancreaticoduodenectomy: incidence, significance, and management. *Am J Surg* 1994; **168**: 295-298

12 **Strasberg SM**, Drebin JA, Soper NJ. Evolution and current status of the Whipple procedure: an update for gastroenterologists. *Gastroenterology* 1997; **113**: 983-994

13 **Brodsky JT**, Turnbull AD. Arterial hemorrhage after pancreatoduodenectomy. The 'sentinel bleed'. *Arch Surg* 1991; **126**: 1037-1040

14 **Hamanaka Y**, Nishihara K, Hamasaki T, Kawabata A, Yamamoto S, Tsurumi M, Ueno T, Suzuki T. Pancreatic juice output after pancreatoduodenectomy in relation to pancreatic consistency, duct size, and leakage. *Surgery* 1996; **119**: 281-287

15 **van Berge Henegouwen MI**, De Wit LT, Van Gulik TM, Obertop H, Gouma DJ. Incidence, risk factors, and treatment of pancreatic leakage after pancreaticoduodenectomy: drainage versus resection of the pancreatic remnant. *J Am Coll Surg* 1997; **185**: 18-24

16 **Tani M**, Onishi H, Kinoshita H, Kawai M, Ueno M, Hama T, Uchiyama K, Yamaue H. The evaluation of duct-to-mucosal pancreaticojejunostomy in pancreaticoduodenectomy. *World J Surg* 2005; **29**: 76-79

17 **Bartoli FG**, Arnone GB, Ravera G, Bachi V. Pancreatic fistula and relative mortality in malignant disease after pancreaticoduodenectomy. Review and statistical meta-analysis regarding 15 years of literature. *Anticancer Res* 1991; **11**: 1831-1848

18 **Berberat PO**, Friess H, Uhl W, Buchler MW. The role of octreotide in the prevention of complications following pancreatic resection. *Digestion* 1999; **60** Suppl 2: 15-22

19 **Lowy AM**, Lee JE, Pisters PW, Davidson BS, Fenoglio CJ, Stanford P, Jinnah R, Evans DB. Prospective, randomized trial of octreotide to prevent pancreatic fistula after pancreaticoduodenectomy for malignant disease. *Ann Surg* 1997; **226**: 632-641

20 **Poon RT**, Lo SH, Fong D, Fan ST, Wong J. Prevention of pancreatic anastomotic leakage after pancreaticoduodenectomy. *Am J Surg* 2002; **183**: 42-52

21 **Yeo CJ**, Cameron JL, Maher MM, Sauter PK, Zahurak

ML, Talamini MA, Lillemoe KD, Pitt HA. A prospective randomized trial of pancreaticogastrostomy versus pancreaticojejunostomy after pancreaticoduodenectomy. *Ann Surg* 1995; **222**: 580-588; discussion 588-592

22 **Reid-Lombardo KM**, Farnell MB, Crippa S, Barnett M, Maupin G, Bassi C, Traverso LW. Pancreatic anastomotic leakage after pancreaticoduodenectomy in 1,507 patients: a report from the Pancreatic Anastomotic Leak Study Group. *J Gastrointest Surg* 2007; **11**: 1451-1458; discussion 1459

23 **Yang YM**, Tian XD, Zhuang Y, Wang WM, Wan YL, Huang YT. Risk factors of pancreatic leakage after pancreaticoduodenectomy. *World J Gastroenterol* 2005; **11**: 2456-2461

24 **Suzuki Y**, Fujino Y, Tanioka Y, Hiraoka K, Takada M, Ajiki T, Takeyama Y, Ku Y, Kuroda Y. Selection of pancreaticojejunostomy techniques according to pancreatic texture and duct size. *Arch Surg* 2002; **137**: 1044-1047; discussion 1048

25 **Peng S**, Mou Y, Cai X, Peng C. Binding pancreaticojejunostomy is a new technique to minimize leakage. *Am J Surg* 2002; **183**: 283-285

26 **Yeo CJ**, Cameron JL, Lillemoe KD, Sauter PK, Coleman J, Sohn TA, Campbell KA, Choti MA. Does prophylactic octreotide decrease the rates of pancreatic fistula and other complications after pancreaticoduodenectomy? Results of a prospective randomized placebo-controlled trial. *Ann Surg* 2000; **232**: 419-429

27 **Peng SY**, Wang JW, Lau WY, Cai XJ, Mou YP, Liu YB, Li JT. Conventional versus binding pancreaticojejunostomy after pancreaticoduodenectomy: a prospective randomized trial. *Ann Surg* 2007; **245**: 692-698

28 **Billingsley KG**, Hur K, Henderson WG, Daley J, Khuri SF, Bell RH Jr. Outcome after pancreaticoduodenectomy for periampullary cancer: an analysis from the Veterans Affairs National Surgical Quality Improvement Program. *J Gastrointest Surg* 2003; **7**: 484-491

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RAPID COMMUNICATION

Clinical outcome of Fitz-Hugh-Curtis syndrome mimicking acute biliary disease

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Abstract

AIM: To analyze the clinical characteristics of patients diagnosed with Fitz-Hugh-Curtis syndrome.

METHODS: The clinical courses of patients that visited St. Mary's Hospital with abdominal pain from January 2005 to December 2006 and were diagnosed with Fitz-Hugh-Curtis syndrome were examined.

RESULTS: Fitz-Hugh-Curtis syndrome was identified in 22 female patients of childbearing age; their mean age was 31.0 ± 8.1 years. Fourteen of these cases presented with pain in the upper right abdomen alone or together with pain in the lower abdomen, and six patients presented with pain only in the lower abdomen. The first impression at the time of visit was acute cholecystitis or cholangitis in 10 patients and acute appendicitis or pelvic inflammatory disease in eight patients. Twenty-one patients were diagnosed by abdominal computer tomography (CT), and the results of abdominal sonography were normal for 10 of these patients. *Chlamydia trichomatis* was isolated from 18 patients. Two patients underwent laparoscopic adhesiotomy and 20 patients were completely cured by antibiotic treatment.

CONCLUSION: For women of childbearing age with acute pain in the upper right abdomen alone or together with pain in the lower abdomen, Fitz-Hugh-Curtis syndrome should be considered during differential diagnosis. Moreover, in cases suspected to be Fitz-Hugh-Curtis syndrome, abdominal CT, rather than abdominal sonography, assists in the diagnosis.

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Key words: *Chlamydia trichomatis*; Abdominal pain; Fitz-Hugh-Curtis syndrome

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INTRODUCTION

Acute abdominal pain is one of the most common symptoms experienced by patients visiting hospitals, particularly the emergency room. Pain in the right upper abdomen is a symptom of biliary diseases, such as gall bladder (GB) stones or cholecystitis, and it may also be present in duodenal ulcers, liver abscess, subphrenic abscess, herpes zoster infection, *etc*. In addition, pain in the right upper abdomen, alone or together with pain in the lower abdomen, is associated with Fitz-Hugh-Curtis syndrome, although this condition is infrequent among hospital patients.

Fitz-Hugh-Curtis syndrome is characterized by inflammation in perihepatic capsules with concomitant pelvic inflammation without involvement of hepatic parenchyma[1-3]. Most Fitz-Hugh-Curtis syndrome patients are women of childbearing age who visit hospitals because of acute pain and tenderness in the right upper abdomen. The pain in the right upper abdomen is caused by adhesion of the anterior hepatic surface and the abdominal wall[4]. The pain tends to

become more severe upon body movement, breathing, *etc*, and, thus, it is difficult to distinguish it from acute cholecystitis, pleurisy, right pyelonephritis, *etc* in many cases[5]. Furthermore, in some cases, it presents with pain in the lower abdomen only, without the characteristic pain in the right upper abdomen, and it is thus misdiagnosed as acute appendicitis or some other form of peritonitis[6].

Recently, with the development of imaging tests and antibiotics, Fitz-Hugh-Curtis syndrome has been classified as a benign disease that can be diagnosed and treated readily by non-invasive methods[7], for example, by abdominal computer tomography (CT) scan and oral antibiotics, respectively. Nevertheless, without sufficient understanding of this disease, it could be misdiagnosed as another acute disease with similar clinical symptoms, and thus patients may undergo unnecessary surgery or tests.

Until now, Fitz-Hugh-Curtis syndrome has been considered a gynecological disease; however, the major complaint is pain in the right upper abdomen. Hence, clinicians that focus on the digestive tract frequently encounter such patients during the primary diagnosis. This study analyzed the clinical characteristics of patients diagnosed with Fitz-Hugh-Curtis syndrome at our hospital.

MATERIALS AND METHODS

Patients

This study was performed with 22 patients that visited St. Mary's Hospital, Catholic University, from January 2005 to December 2006, due to abdominal pain and were diagnosed with Fitz-Hugh-Curtis syndrome. In the study, the diagnostic standard of Fitz-Hugh-Curtis syndrome was the following: (1) abdominal CT scan showed pelvic inflammation with contrast enhancement of hepatic capsules; (2) patients had an adhesion between the liver and the diaphragm or the liver and the anterior abdominal wall detected by laparoscopic surgery. CT images were acquired using multi-detector scanners, Lightspeed VCT (General Electric, Milwaukee, WI, USA). A total of 120 mL of iopromide, Ultravist 300 (Bayer Schering Pharma AG, Berlin, Germany) was administrated at a rate of 3 mL/s with an automatic power injector. Images were obtained before and at 60-70 s after iv contrast material injection. In all phases, scanning was initiated at the dome of the right hemidiaphragm and scans of the entire abdomen to the symphysis pubis were obtained. Slice thickness was 5 mm. To gain a definitive diagnosis of pathogens, we performed PCR tests, used sexually transmitted disease detection kits to detect *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Mycoplasma genitalium*, and performed serological tests, including enzyme immunoassay for Chlamydia IgM and ELISA for Chlamydia IgG.

Study design

We retrospectively reviewed the records of 22 patients who were diagnosed with Fitz-Hugh-Curtis syndrome. Patients' medical records, disease histories, physical and systemic examinations, and first impression by the clinicians that performed the initial diagnosis were examined. Laboratory tests, blood chemistry, serological tests, direct smears of specimens, and bacterial culture tests were performed. For radiological tests, the results of ultrasound tests and CT were examined. Diagnostic and therapeutic laparoscopic surgery, antibiotic treatments after diagnosis, and prognosis were examined for each patient.

RESULTS

Characteristic of patients

Our study sample included 22 Fitz-Hugh-Curtis syndrome patients diagnosed at our hospital. The patients were all women of childbearing age with a mean age of 31.0 ± 8.1 years (range 19-49 years). Their most recent sexual activity was between 3 d and 1 mo prior to the visit and the frequency of sexual activity was also provided. Except for two patients for whom symptoms were relatively meager and who were thus admitted through outpatient clinics, 20 patients visited the emergency room because of acute abdominal pain. The interval from the development of symptoms to a hospital visit was diverse, ranging from the same day to 2 wk, with an average of 5.8 ± 4.5 d (Table 1).

Chief complaint

The chief complaint requiring a visit to the hospital was pain in the right upper abdomen in seven patients (32%), pain in the right upper abdomen and pain in the lower abdomen in seven cases (32%), pain in the lower abdomen in six cases (27%), fever in one case (4.5%), and epigastric pain in one case (4.5%). With the exception of one case, fever was absent in the patients (Table 1).

First impression

After the first impression of the clinicians that examined the patients initially, 10 patients were diagnosed with acute cholecystitis or a GB stone, eight patients were diagnosed with acute appendicitis or pelvic inflammatory disease, and four patients were diagnosed with acute pyelonephritis, acute hepatitis, acute gastritis, or peptic ulcer disease (Table 1).

Test results

Various test methods were used for diagnosis. In 21 patients (95.5%), an abdominal CT scan was performed and Fitz-Hugh-Curtis syndrome was diagnosed according to its characteristics, the finding of contrast enhancement in the hepatic capsules. One of the patients was diagnosed as having Fitz-Hugh-Curtis syndrome by an abdominal CT scan and was treated; however, due to deterioration in her symptoms, laparoscopic surgery was performed and adhesion between the anterior side of the liver and the abdominal wall was confirmed. The remaining patient developed acute peritonitis during

Table 1 Clinical manifestation of 22 patients with Fitz-Hugh-Curtis syndrome

	mean ± SD (range)
Age (yr)	31.0 ± 8.1 (19-49)
Last intercostal history (d)	11.0 ± 10.9 (3-30)
Hospital visit duration (d)	5.8 ± 4.5 (0-14)
	Number (%)
Chief complaints	
RUQ pain	7 (32)
RUQ and lower abdominal pain	7 (32)
Lower abdominal pain	6 (27)
Epigastric pain	1 (4.5)
Fever and lymphadenopathy	1 (4.5)
First impression	
Acute cholecystitis	10 (46)
Acute appendicitis	5 (23)
Pelvic inflammatory disease	3 (13)
Peritonitis	1 (4.5)
Hepatitis	1 (4.5)
Gastric ulcer	2 (9)

pregnancy and was diagnosed by laparoscopic surgery without abdominal CT scan. Among the 21 patients diagnosed by abdominal CT scan, abdominal sonography was performed in 10 of them prior to abdominal CT scan and the results were normal in all cases.

According to blood chemistry tests, the number of leukocytes averaged 11.532/mm^3, which was slightly higher than normal. Liver function test results were within the normal range in most patients, with the exception of 1 patient with a result 4 times greater than the upper normal limit. ESR averaged 51.3 mm/h which was elevated five times more than normal (normal value, 0-10 mm/h). C-reactive protein (CRP) averaged 52.3 mg/L, which was over 10 times higher than normal (normal value, less than 5 mg/L). The serological antibody tests to *C. trichomatis* and PCR tests to six common sexually transmitted disease pathogens indicated the presence of *C. trichomatis* in 18 patients, *T. vaginalis* in one patient, *U. urealyticum* in one patient, and *M. hominis* in one patient. One patient was diagnosed during emergency surgery and, thus, the serology tests were not performed. Direct specimen smears were all negative. A cell culture test was performed for seven patients (three cases for *Streptococcus agalactiae*, one case for *U. urealyticum*, one case for *Staphylococcus aureus*, one case for *Candida albicans*, and one case for *Escherichia coli*) and the findings concurred with the results of the serological chlamydia antibody tests of and PCR tests performed to determine the presence of pathogens (Table 2).

Treatment and progress

Of 22 patients, 20 patients (91%) improved following general or combinatorial antibiotic therapy and conservative care. One patient did not respond to antibiotic therapy; her pain became more severe but her symptoms improved after adhesiotomy by laparoscopic surgery. One patient developed acute peritonitis during pregnancy and, thus, laparoscopic surgery was performed for the purpose of diagnosis and treatment; she improved after adhesiotomy. Doxycycline was administered as a single antibiotic to five patients, and in combination to 14 patients. For those patients that received combination therapy, triple drug therapy consisted of metronidazole and aminoglycoside with ampicillin/sulbactam or cephalosporin and dual drug therapy consisted of an aminoglycoside with ampicillin/sulbactam or metronidazole with cephalosporin. Three patients received 2 drug therapy for 1 wk and their clinical symptoms were improved (Table 3).

Table 2 Laboratory and radiographic study of 21[1] patients with Fitz-Hugh-Curtis syndrome, which was diagnosed by abdominal CT

	mean ± SD (range)
Laboratory study (n = 21)	
WBC (1000/mm^3)	11.5 ± 3.4 (3.5-16.2)
AST (IU/L)	22.1 ± 30.1 (10-156)
ALT (IU/L)	17.0 ± 24.8 (5-126)
ESR (mm/h)	51.2 ± 28.9 (18-120)
CRP (mg/L)	52.1 ± 41.9 (2-154)
	Positive result of number (%)
Serologic test or PCR (n = 21)	
C. trichomatis	18 (85.7)
T. vaginalis	1 (4.8)
U. urealyticum	1 (4.8)
M. hominis	1 (4.8)
Microbiologic culture (n = 21)	
S. agalactiae	3 (14.3)
U. urealyticum	1 (4.8)
S. aureus	1 (4.8)
C. albicans	1 (4.8)
E. coli	1 (4.8)
	Positive result of number (%)
Radiographic study[2]	
Abdomen CT (n = 21)	21 (100)
Abdomen ultrasonography (n = 10)	0 (0)

[1]Among the 22 patients, one was diagnosed by laparoscopic surgery without a study; [2]Abdominal CT was performed in 21 patients, and only 10 patients among them were investigated by abdominal ultrasonography.

DISCUSSION

Fitz-Hugh-Curtis syndrome is characterized by perihepatic inflammation appearing with pelvic inflammation primarily in women of childbearing age. It occurs in 12.0%-13.8% of pelvic inflammation cases[8,9]. In 1930, Curtis[1] reported the violin-string appearance between the anterior hepatic surface and the abdominal wall in gonorrhea patients. Furthermore, in 1934, Fitz-Hugh[2] reported gonococcal peritonitis accompanied by pain in the right upper abdomen. Previously, *N. gonorrhoeae* was considered to be the major pathogen of this syndrome; however, in 1985, Lopes-Zeno *et al*[4] showed that *C. trachomatis*, not *N. gonorrhoeae*, was the major pathogen. In the past, the definitive diagnosis was made using methods that confirmed adhesion in the vicinity of the liver by laparoscopic surgery or open abdominal surgery. Recently, the disease was diagnosed and experienced infrequently as it was diagnosed by non-invasive methods such as serological tests for specific antibodies to pathogens that induce pelvic inflammation,

Table 3 Clinical characteristics of 22 patients with Fitz-Hugh-Curtis syndrome

No.	Age	Symptom (pain)	Duration	Impression	Diagnosis	Treatment	Prog
1	39	Low abdomen	5	PID	CT	Doxycycline	
2	23	RUQ	7	Cholecystitis	CT	Doxycycline	
3	35	RLQ	5	Appendicitis	CT + Sono	Cepha + Amino + Metro	
4	19	Low abdomen	5	Appendicitis	CT + Sono	Doxycycline	
5	22	RUQ	7	Cholecystitis	CT + Sono	Cepha + Amino + Metro	
6	29	RUQ + low abdomen	6	Appendicitis	CT + Sono	Cepha + Metro	
7	26	RUQ	3	Cholecystitis	CT	Cepha + Amino + Metro	
8	21	Epigastrium	3	Gastric ulcer	CT + Sono	Cepha + Amino + Metro	
9	37	Fever	10	Hepatitis	CT	Doxycycline	
10	41	RUQ	14	Cholecystitis	CT	Amp + Amino + Metro	
11	49	Low abdomen	10	PID	CT	Amp + Amino + Metro	
12	33	RUQ + low abdomen	0	Cholecystitis	CT	Doxycycline	
13	45	RUQ	1	Cholecystitis	CT + Sono	Cepha + Metro	
14	32	RUQ	5	GB stone	CT + Sono	Amp + Amino+ Metro	
15	34	RUQ + low abdomen	14	PID	CT + Sono	Cepha + Amino + Metro	Lapa
16	27	RUQ + low abdomen	2	GB stone	CT + Sono	Cepha + Amino + Metro	
17	31	Low abdomen	14	Pyelonephritis		Amp + Amino	Lapa
18	25	RUQ + low abdomen	1	Cholecystitis	CT	Amp + Amino + Metro	
19	38	RUQ + low abdomen	1	Appendicitis	CT + Sono	Amp + Amino + Metro	
20	33	RLQ	4	Appendicitis	CT	Amp + Amino + Metro	
21	22	RUQ + low abdomen	1	Cholecystitis	CT	Amp + Amino + Metro	
22	27	RUQ pain	10	Gastritis	CT	Amp + Amino + Metro	

RUQ: Right upper quadrant abdominal; RLQ: Right lower quadrant abdominal; PID: Pelvic inflammatory disease; GB: Gallbladder; CT: Computed tomography; Sono: Ultrasonography; Cepha: Cephalosporin; Amino: Aminoglycoside; Metro: Metronidazole; Amp: Ampicillin/sulbactam; Prog: Prognosis; Lapa: Laparoscopic adhesiolysis.

PCR, and abdominal CT scan.

It has been suggested that the mechanisms of development of Fitz-Hugh-Curtis syndrome include inflammation in hepatic capsules caused by inflammation in the reproductive system through the peritoneal cavity; migration of pathogens from the peritoneal membrane to the liver via blood; migration of pathogens from the peritoneal membrane to the liver through lymph ducts; and a hyperimmune response to *C. trichomatis*; nonetheless, the precise mechanism has not been elucidated yet[10].

The pain in the right upper abdomen that appears as the main symptom during the acute phase develops as a sudden sharp pain that becomes more severe in response to deep breathing, body movements, coughing, *etc*, and it develops as a result of congestion of hepatic capsules, spotted hemorrhage, and fibrous exudates. Occasionally, the pain may radiate to the right shoulder. Lower abdominal pain may appear simultaneously with the right upper abdomen pain or intermittently with the abdominal pain. If pain progresses to a chronic state without pain in the lower abdomen, the pain in the right upper abdomen generally appears continuously or may become dull.

In our study, 14 of 22 patients (64%) showed right upper quadrant (RUQ) pain; the typical symptom of Fitz-Hugh-Curtis syndrome. Seven of 14 patients displayed only RUQ pain, and in the other seven patients RUQ pain was accompanied by pain in the lower abdomen. The other eight patients (36%) had no typical RUQ pain symptom but experienced pain in the epigastrium or only in the lower abdomen, or they had fever and other systemic symptoms. Even when there is no pain in the right upper abdomen, which is the characteristic of Fitz-Hugh-Curtis syndrome when it occurs as perihepatic inflammation, the possibility of Fitz-Hugh-Curtis syndrome can not be ruled out completely[11,12]. Though it is not typical, diagnosis of Fitz-Hugh-Curtis syndrome with the major symptom of pain in the lower abdomen with pelvic inflammation or systemic symptoms should be considered. It has been reported that cases in which pain in the upper abdomen develops without pain in the lower abdomen are rare[13,14]; however, in this study, seven patients (32%) developed pain only in the right upper abdomen. This takes place in patients who have recovered from an acute episode of pelvic inflammatory disease without appropriate treatment[10]. These cases have to be cautiously differentiated from diseases for which the major symptom is pain in the right upper abdomen, such as acute cholecystitis. Although our study did not explore this, there are cases that display pain in the left upper abdomen by perisplenitis as the main symptom of Fitz-Hugh-Curtis syndrome. Hence, we think that cases of non-typical symptoms should be fully considered[15].

For the diagnosis of Fitz-Hugh-Curtis syndrome and the earlier findings of laboratory tests, the only method for definite diagnosis used to be assessment of adhesions in the vicinity of the liver by invasive laparoscopic surgery; however, diagnosis has recently been made possible by a non-invasive abdominal CT scan resulting in contrast enhancement in hepatic capsules caused by perihepatic inflammation during the acute phase of Fitz-Hugh-Curtis syndrome[7,16,17]. In this study, 21 cases (95.5%) were diagnosed by abdominal CT scan. Laparoscopic surgery was performed in only two patients for the purpose of diagnosis and

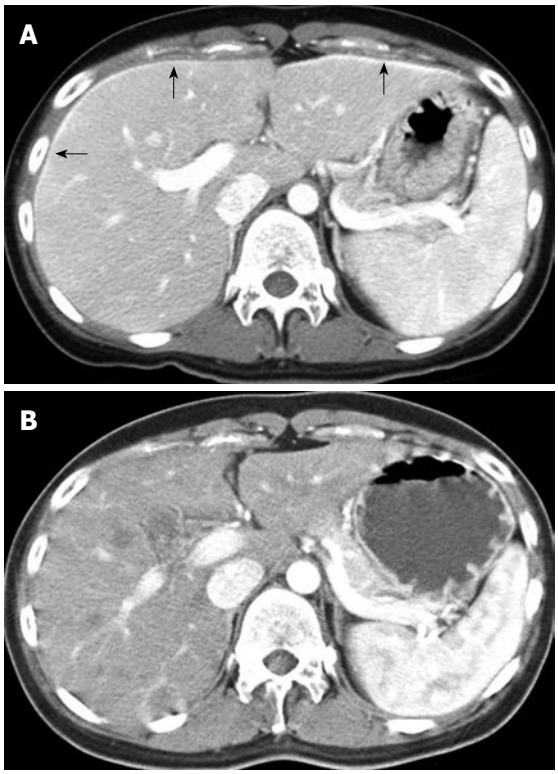

Figure 1 Contrast-enhanced CT. A: Linear enhancement of the surface at both lobes of the liver (arrow); B: CT images 1 mo after treatment indicate normal liver.

treatment. In addition, differential diagnosis of Fitz-Hugh-Curtis syndrome from hepato-biliary diseases was performed for patients presenting with pain in the right upper abdomen by abdominal sonography. In our study, abdominal sonography was performed on 10 patients in the emergency room. All of these patients showed normal findings. Because abdominal sonography observations are mainly concentrated on the GB or liver, which are usually considered major causes of pain in the right upper abdomen, there is a failure to notice pelvic inflammatory disease which can be observed in the pelvic cavity. There may also be discrepancies in the expertise of different sonographers. Although abdominal sonography is of great help as a primary diagnostic tool to prove the causality of pain in the right upper abdomen, there are clear limitations in using only abdominal sonography to diagnose Fitz-Hugh-Curtis syndrome. Therefore, in sexually active women of childbearing age presenting with pain in the right upper abdomen or pain in the right upper abdomen together with pain in the lower abdomen, that have normal liver function test results and for whom Fitz-Hugh-Curtis syndrome is strongly suspected, an abdominal CT scan may diagnose the syndrome more rapidly and accurately. For the identification of causative pathogens, uterine cervical specimens have been used most frequently, although rectal, urinary tract and salivary specimens can be used[10]. To confirm the presence of the major pathogen *C. trachomatis*, a culture test is widely applied and, more recently, ligase chain reaction (LCR), PCR, and a specific antibody test have been used to identify the pathogen. In our study, PCR and specific antibody tests were performed to identify the pathogen; tests were positive for *C. trichomatis* in 18 patients (82%), and testing for *N. gonorrhoeae*, which has been known to be the most prevalent pathogen in the past, was negative. Other pathogens were detected in the remaining three patients and it is thought that pathogens other than *C. trichomatis* that induce pelvic inflammation could be causative of Fitz-Hugh-Curtis syndrome. Lactobacilli maintain normal vagina flora, but if normal vagina flora is altered, an inflammation is usually induced by *G. vaginalis*, *C. albicans*, *T. vaginalis*, *M. hominis*, and the cervix becomes inflamed due to *C. trichomatis*, *N. gonorrhoeae*, *T. vaginalis*, and *C. albicans*. *C. trichomatis* and *N. gonorrhoeae* cause pelvic inflammation. Generally, the culture test is the basic test for the identification of causative pathogens; however, in our study, the major causative pathogen *C. trichomatis* was not identifiable by culture tests. *C. trichomatis* cannot be identified in general bacterial culture tests as culture tests for *C. trichomatis* require special media that is not used in general bacterial culture. Therefore, for cases suspected to be Fitz-Hugh-Curtis syndrome, culture tests should be performed using the special media for *C. trichomatis* to enable accurate diagnosis. Hepatic enzyme values are normal or slightly elevated in Fitz-Hugh-Curtis syndrome, which is of help in differentiating it from hepatitis[18]. In Fitz-Hugh-Curtis syndrome, the liver function test is normal or slightly elevated and the ESR, although still controversial, has been reported to be increased in some cases[19]. Recently, CRP has been reported to be a marker that reflects the clinical course of this disease[20,21]. In most of our cases, the results of liver function tests were normal except for one patient with a result four times greater than the upper normal limit, and leukocyte values were slightly increased. ESR were elevated five times and CRP was elevated over 10 times more than the normal in our study. However, it is difficult to diagnose Fitz-Hugh-Curtis syndrome definitely by serum biochemistry and serological tests; therefore, they are only partially able to aid in the diagnosis of Fitz-Hugh-Curtis syndrome.

Antibiotics against the identified causative bacteria were administered as treatment and, for cases that were unresponsive to antibiotic therapy, surgery was performed to remove the adhesion in the vicinity of the liver. Standardized treatments were not available so antibiotics targeting *C. trichomatis*, *N. gonorrhoeae*, gram negative bacilli, and anaerobic bacteria were administered. Oral antibiotics were administered for 2 wk and non-oral antibiotics were administered for 48 h after the improvement of clinical symptoms[22]. It has been reported that treatment reactions in most patients are good. This was the case in our study, with the exception of one case in which laparoscopic surgery was performed following the deterioration of symptoms. Most patients improved following antibiotic treatment. Moreover, the lesions were absent on abdominal CT scan performed after the treatment (Figure 1).

The diagnosis rate of Fitz-Hugh-Curtis syndrome has increased due to the recent development of

imaging tests. Most patients recover completely after treatment with appropriate antibiotics. Nevertheless, this syndrome is often misdiagnosed as other diseases with similar clinical symptoms which results in unnecessary treatment and hospital stays. Therefore, if sexually active women visit a hospital because of pain in the right upper abdomen or pain in the right upper abdomen and pain in the lower abdomen, Fitz-Hugh-Curtis syndrome should be considered. For patients suspected to have Fitz-Hugh-Curtis syndrome, an abdominal CT scan, rather than abdominal sonography, may be helpful since it provides a more rapid and accurate diagnosis.

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COMMENTS

Background

Fitz-Hugh-Curtis syndrome has been considered a gynecological disease; however, the major complaint is pain in the right upper abdomen. Hence, clinicians that focus on the digestive tract frequently encounter such patients during primary diagnosis.

Research frontiers

Fitz-Hugh-Curtis syndrome has been classified as a benign disease. Nevertheless, without sufficient understanding of this disease, it may be misdiagnosed as other acute diseases with similar clinical symptoms. This study analyzed the clinical characteristics of patients diagnosed with Fitz-Hugh-Curtis syndrome.

Innovations and breakthroughs

Abdominal sonography is of great help as a primary diagnostic tool to prove the causality of pain in the right upper abdomen. However, there is a clear limitation to diagnosis of Fitz-Hugh-Curtis syndrome by using only abdominal sonography. Because the observations are mainly concentrated on the hepato-biliary disease, there is a failure to notice pelvic inflammatory disease. For patients suspected to have Fitz-Hugh-Curtis syndrome, an abdominal CT scan, rather than abdominal sonography, may be helpful.

Applications

The results show that in sexually active women of childbearing age presenting with pain in the right upper abdomen or pain in the right upper abdomen together with pain in the lower abdomen and normal liver function test results, Fitz-Hugh-Curtis syndrome should be considered during differential diagnosis.

Terminology

Fitz-Hugh-Curtis syndrome is characterized by inflammation in perihepatic capsules with concomitant pelvic inflammation. Most Fitz-Hugh-Curtis syndrome patients visit hospitals due to acute pain and tenderness in the right upper abdomen. The diagnosis is made by a non-invasive abdominal CT scan resulting in contrast enhancement in hepatic capsules caused by perihepatic inflammation. The treatment is antibiotic therapy against the identified causative bacteria.

Peer review

This is an interesting study which was well organized. It demonstrated that although Fitz-Hugh-Curtis syndrome is a benign disease that can be diagnosed by non-invasive methods and treated by antibiotics, it is often misdiagnosed as other acute diseases which results in unnecessary treatment and hospital stays.

REFERENCES

1 **Curtis AH**. A cause of adhesions in the right upper quadrant. *JAMA* 1930; **94**: 1221-1222
2 **Fitz-Hugh T Jr**. Acute gonococcic peritonitis of the right upper quadrant in women. *JAMA* 1934; **102**: 2094-2096
3 **Hyun JJ**, Kim JY, Bak YT, Lee CH, Choi SY. Education and imaging. Gastrointestinal: Fitz-Hugh-Curtis syndrome. *J Gastroenterol Hepatol* 2006; **21**: 1493
4 **Lopez-Zeno JA**, Keith LG, Berger GS. The Fitz-Hugh-Curtis syndrome revisited. Changing perspectives after half a century. *J Reprod Med* 1985; **30**: 567-582
5 **Wood JJ**, Bolton JP, Cannon SR, Allan A, O'Connor BH, Darougar S. Biliary-type pain as a manifestation of genital tract infection: the Curtis-Fitz-Hugh syndrome. *Br J Surg* 1982; **69**: 251-253
6 **Shanahan D**, Gau D. Chlamydial Fitz-Hugh/Curtis syndrome. *Lancet* 1986; **1**: 1216
7 **Nishie A**, Yoshimitsu K, Irie H, Yoshitake T, Aibe H, Tajima T, Shinozaki K, Nakayama T, Kakihara D, Matsuura T, Takahashi M, Kamochi N, Onitsuka H, Honda H. Fitz-Hugh-Curtis syndrome. Radiologic manifestation. *J Comput Assist Tomogr* 2003; **27**: 786-791
8 **Semchyshyn S**. Fitz-Hugh and Curtis syndrome. *J Reprod Med* 1979; **22**: 45-48
9 **Onsrud M**. Perihepatitis in pelvic inflammatory disease--association with intrauterine contraception. *Acta Obstet Gynecol Scand* 1980; **59**: 69-71
10 **Peter NG**, Clark LR, Jaeger JR. Fitz-Hugh-Curtis syndrome: a diagnosis to consider in women with right upper quadrant pain. *Cleve Clin J Med* 2004; **71**: 233-239
11 **Ricci P**, Lema R, Sola V, Fernandez C, Fabres C, Fernandez E, Pardo J. Fitz-Hugh-Curtis syndrome: Three cases of incidental diagnosis during laparoscopy. *J Obstet Gynaecol* 2008; **28**: 352-354
12 **Counselman FL**. An unusual presentation of Fitz-Hugh-Curtis syndrome. *J Emerg Med* 1994; **12**: 167-170
13 **Muller-Schoop JW**, Wang SP, Munzinger J, Schlapfer HU, Knoblauch M, Tammann RW. Chlamydia trachomatis as possible cause of peritonitis and perihepatitis in young women. *Br Med J* 1978; **1**: 1022-1024
14 **Katzman DK**, Friedman IM, McDonald CA, Litt IF. Chlamydia trachomatis Fitz-Hugh-Curtis syndrome without salpingitis in female adolescents. *Am J Dis Child* 1988; **142**: 996-998
15 **Gatt D**, Jantet G. Perisplenitis and perinephritis in the Curtis-Fitz-Hugh syndrome. *Br J Surg* 1987; **74**: 110-112
16 **Cho HJ**, Kim HK, Suh JH, Lee GJ, Shim JC, Kim YH. Fitz-Hugh-Curtis syndrome: CT findings of three cases. *Emerg Radiol* 2008; **15**: 43-46
17 **Nozu T**, Komiyama H. Fitz-Hugh-Curtis syndrome. *Intern Med* 2006; **45**: 221-222
18 **Litt IF**, Cohen MI. Perihepatitis associated with salpingitis in adolescents. *JAMA* 1978; **240**: 1253-1254
19 **Keane JA**, McKimm RJ, David CM. Perihepatitis associated with pelvic infection: the Fitz-Hugh-Curtis syndrome. *N Z Med J* 1982; **95**: 725-728
20 **Lim SC**, Park YW, Choi HJ, Kim YH. Clinical experiences of Fitz-Hugh-Curtis syndrome. *Korean J Obstet Gynecol* 2006; **49**: 1738-1744
21 **Chung HJ**, Choi HY, Cho YJ, Han KH, Kim YD, Jung SM, Kim JU, Cheon GJ. [Ten cases of Fitz-Hugh-Curtis syndrome] *Korean J Gastroenterol* 2007; **50**: 328-333
22 **McCormack WM**. Pelvic inflammatory disease. *N Engl J Med* 1994; **330**: 115-119

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RAPID COMMUNICATION

Effect of mucin production on survival in colorectal cancer: A case-control study

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Abstract

AIM: To investigate the impact of mucin production on prognosis in colorectal cancer, in terms of overall survival (OS) and time to disease progression (TTP) in patients with mucinous compared to those with non-mucinous colorectal cancer (NMCRC), matched for age, gender, and tumor stage.

METHODS: Thirty five patients with mucinous colorectal cancer (MCRC) were matched for age, gender, and tumor stage with 35 controls having NMCRC. OS and TTP were compared among 4 groups divided according to mucin content: group A (50%-75% mucin), group B (75%-100% mucin), group C or controls (< 50% mucin). Group D consisted of all patients with tumors having < 75% mucin (controls and groups A together).

RESULTS: Median survival in MCRC and NMCRC groups was 46.2 and 112.9 mo, respectively (P = 0.26). OS in groups A and B was 70.1 and 32.8 mo (P = 0.46), and in groups B and D was 32.8 and 70.1 mo, respectively (P = 0.143). TTP in MCRC and NMCRC was 50.17 and 44.77 mo, respectively (P = 0.795). TTP in groups A, B, and D was 70.1, 24.8, and 65.5 mo, respectively. Twenty-eight percent of patients with MCRC had poorly differentiated adenocarcinoma versus 8.6% in NMCRC patients (P = 0.028).

CONCLUSION: MCRC is associated with a non-significant decrease in median survival and TTP, particularly when mucin content is > 75% of tumor volume. However, it tends to be more poorly differentiated. A larger study matching for stage and grade is needed.

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Key words: Colorectal cancer; Adenocarcinoma; Mucin; Mucinous; Prognosis

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INTRODUCTION

Mucinous adenocarcinoma is a histological variant that accounts for 5% to 15% of cases of primary colorectal cancer[1]. It is defined as a tumor displaying extracellular mucin in more than 50% of the tumor volume. There is evidence that mucinous colorectal cancer (MCRC) may be a distinct biological and genetic entity compared with non-mucinous adenocarcinoma (NMCRC)[2,3]. Thus patients with MCRC may be younger[4,5], their tumors are more likely to be right-sided[6-8], and they present at a more advanced stage[3,4,6,8]. In addition, these tumors may have distinct mutations and cytogenetic abnormalities[2,9] and may be less likely to respond to chemotherapy[10-12]. There is, however, a lack of consensus in the published literature on whether the presence of mucin in colorectal tumors portends a poorer prognosis and worse survival or not.

Many studies suggest that MCRC is associated with poorer outcome and lower survival rates[10,13-15], but others deny this association[3,8,16,17]. The reasons for the conflicting reports are not clear. In many studies, there was no control for, age, sex, tumor stage or location[4,6,7,10]. In addition, the prognostic significance of having an extracellular mucinous component that is much larger than 50% is not known.

The aim of our study was to compare time to disease progression and overall survival (OS) of patients

with mucinous to those with NMCRC after matching all patients for age, gender, and tumor stage. We also evaluated the effect of increasing mucin percentage in tumor body on survival.

MATERIALS AND METHODS

A retrospective review of patients with colorectal cancer treated at the American University of Beirut-Medical Center between 1986 and 2003 identified 35 patients with MCRC and 750 patients with NMCRC.

For each patient with MCRC, a control with NMCRC was matched for age, gender and tumor stage, resulting in 70 cases and controls.

The patients' gender, age, comorbid medical illnesses, tumor location, stage, grade, organ metastasis, surgical resection, chemotherapy regimen, radiological intervention, and survival data were obtained from hospital medical records and recorded for each patient.

Patients were classified as having MCRC if mucin constituted more than 50% of the tumor volume by histological examination.

An average of 4 slides per patient was reviewed to determine mucin content.

To determine the correlation between the percentage of mucin component and tumor stage and survival, histological slides from all patients (cases and controls) initially reported as mucinous tumors were reviewed by two pathologists from AUB-MC to determine the percentage of mucin in the studied sections.

To study the impact of increasing mucin content on survival, two cut-off points (50% and 75% mucin) were chosen. Accordingly, MCRC patients were further divided into 2 subgroups: group A (50%-75% mucin) and group B (75%-100% mucin). Furthermore, NMCRC patients (controls) were designated as group C (< 50% mucin). Moreover, all patients with less than 75% mucin (including groups A and C) were combined into a new group (group D).

Primary tumors were divided into four locations: right-sided (if arising in the cecum, ascending colon or hepatic flexure), transverse colon, left-sided (arising in the splenic flexure, descending colon, or sigmoid colon), and tumors arising in the rectum or rectosigmoid junction of the colon.

Patients with signet-ring colorectal cancer were excluded from the study.

Statistical analysis

The study design was a retrospective case-control study and the case-to-control ratio was 1:1. The Kaplan-Meier method was used to estimate survival. Univariate analysis was performed using the chi-squared testing in SPSS 14.0 software. $P < 0.05$ was considered statistically significant. Univariate analysis was performed to determine if increasing mucin production had an effect on survival that is independent of age, gender, and tumor stage. The median follow-up time was 42 ± 31 mo for non-mucinous (median: 27) and 38 ± 23 (median: 32) for mucinous tumors.

Table 1 Demographic data of patients with MCRC and controls

	MCRC (%), n = 35	NMCRC (%), n = 35	P
Mean age	56.8 (22-83)	56.7 (28-81)	
Gender (M:F)	20:15	20:15	
Comorbid conditions			
Heart disease	4 (11.4)	2 (5.7)	0.673
Diabetes mellitus	2 (5.7)	2 (5.7)	1.000
Hypertension	3 (8.6)	9 (25.7)	0.110
Other cancer	2 (5.7)	1 (2.9)	
Family history			
Colon cancer	4 (11.4)	2 (5.7)	0.673
Other cancer	8 (22.9)	11 (28.6)	0.785

Table 2 Distribution of the tumor by location in the colon

	MCRC (%), n = 35	NMCRC (%), n = 35
Location		
Right colon	8 (22.8)	7 (20)
Transverse colon	2 (5.7)	1 (2.9)
Left colon	14 (40)	16 (45.7)
Rectum/Rectosigmoid	10 (28.6)	10 (28.6)
Grade		
Poor	10 (28.6)	3 (8.6)
Moderate	16 (45.7)	26 (74.3)
Well	6 (17.1)	3 (8.6)
Missing	3 (8.6)	3 (8.6)
Stage		
Ⅰ	2 (5.7)	2 (5.7)
Ⅱ	12 (34.3)	12 (34.3)
Ⅲ	14 (40)	13 (37.1)
Ⅳ	7 (20)	8 (22.8)

RESULTS

Demographic data

Patients with MCRC in our study accounted for 4.7% of all colorectal cancers. The study included 70 patients, 40 males and 30 females. The mean age at diagnosis for all patients was 56.8 (22-83) years, 56.8 (28-81) years for the MCRC group, and 56.7 (28-81) years for the NMCRC group. Males constituted 57% of the patients in both groups.

Patients in the two groups showed similar concomitant medical problems, mostly heart disease, diabetes mellitus type Ⅱ, and hypertension (Table 1).

Clinical characteristics of the tumor

Both MCRC and NMCRC subgroups showed similar distributions of tumor location; the majority of tumors in both groups were located in the left colon and rectum (Table 2). The location of the tumor was not available in one patient.

Most tumors in both the MCRC and NMCRC groups were moderately or poorly differentiated (Table 2). Interestingly, 28.6% of tumors in the MCRC group were poorly differentiated versus 8.6% of those in the NMCRC group. ($P = 0.0028$). Moreover, 40% of group B tumors were of poor grade as compared to 11% of group D tumors ($P = 0.001$). Lymph nodes were involved in 37% of

Table 3 Colorectal recurrence and patterns of metastasis in patients with MCRC and controls

	MCRC (%), *n* = 35	NMCRC (%), *n* = 35
Colorectal recurrence	6 (17)	7 (20)
Metastasis on presentation	5 (14.3)	4 (11.4)
Metastasis on follow up	7 (20)	14 (40)
Sites involved by metastasis		
Liver	8 (22.8)	12 (34.3)
Lung	4 (11.4)	4 (11.4)
Bone	2 (5.7)	1 (2.9)
Brain	1 (2.9)	2 (5.7)
Other	7 (20)	6 (17.1)
Lymph node involvement	13 (37.1)	13 (37.1)

Table 4 Treatment offered to patients with MCRC and controls

	MCRC (%), *n* = 35	NMCRC (%), *n* = 35
Treatment		
Surgery	35 (100)	35 (100)
Chemotherapy	20 (57.1)	19 (54.3)
Neoadjuvant	5 (14.3)	3 (8.6)
Adjuvant	15 (42.9)	16 (45.7)
Type	5-Fu + leucovorin ± oxaliplatin	5-Fu + leucovorin ± oxaliplatin
Radiotherapy	11 (31.4)	8 (22.9)

patients in both subgroups (Table 3).

The liver was the most common organ affected by distant metastasis (22% *vs* 34%) followed by the lungs (11%) in cases and controls, respectively.

The two groups were similar in terms of local disease recurrence: 17% of MCRC patients versus 20% of NMCRC patients ($P > 0.05$).

Therapy

Patients in both groups were treated similarly. All patients were treated with surgical resection. Twenty patients from the mucinous group (57%) and 19 patients (54%) from the control group received neoadjuvant or adjuvant chemotherapy. Chemotherapy regimens were all 5-FU based, as a monotherapy or in combination. Radiotherapy was mainly given to patients with rectal cancer (Table 4).

Characteristics of mucinous subgroups

The exact mucin percentage was obtained on all 35 patients with MCRC.

Eleven patients had 50%-75% of tumor volume displaying mucin and were classified as group A. Mean age at presentation was 55 (36-73) years.

Twenty-Four patients had more than 75% of tumor volume displaying mucin and were classified as group B. Mean age at diagnosis was 59.5 (24-81) years.

Subgroup analysis shows that more than half of the patients in groups A, B, and controls (group C) presented at advanced disease stages (stages Ⅲ and Ⅳ): 54.5%, 66.7%, and 60%, respectively.

Survival analysis

Compared to patients with NMCRC, there was a non-

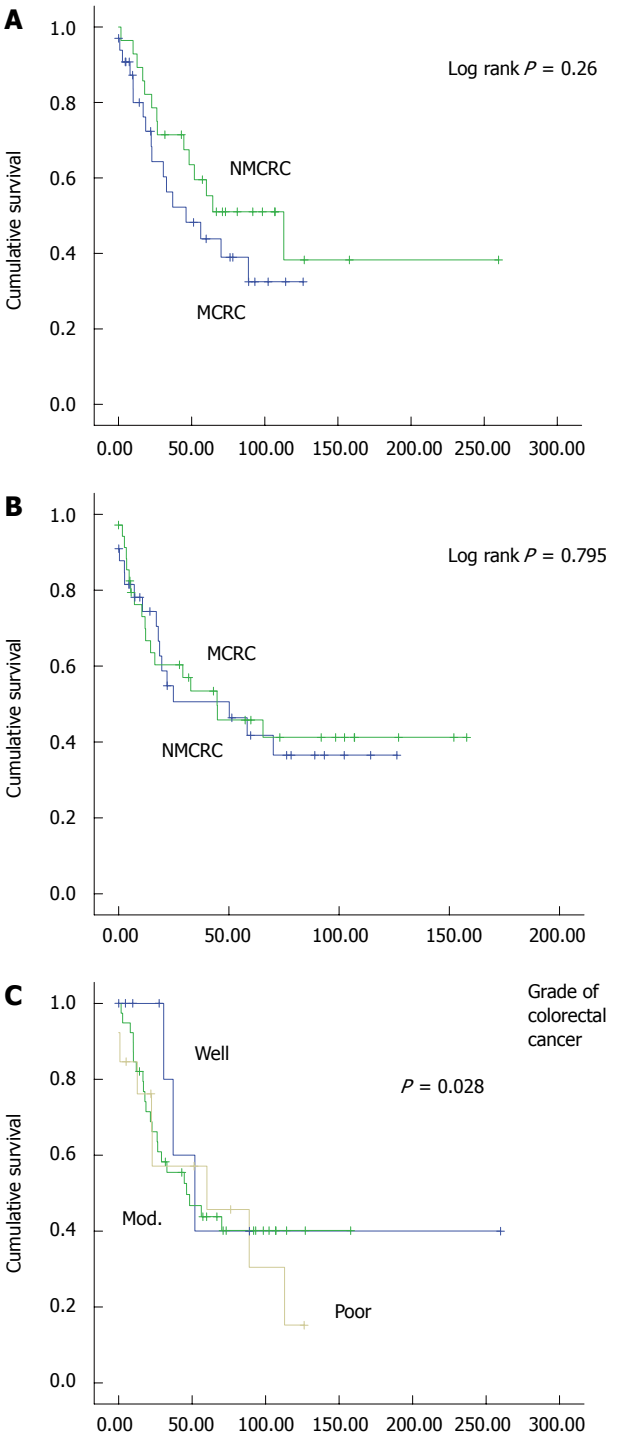

Figure 1 String diagram analyzed. A: Median survival of MCRC group: 46.2 mo compared to NMCRC group: 112.9 mo; B: Time to disease progression in MCRC and NMCRC patients; C: Survival of both MCRC and NMCRC as a function of tumor grade (well, moderate, and poor differentiation).

significant decrease in survival in patients with MCRC. Median survival of patients in the MCRC subgroup was 46.2 mo compared to 112.9 mo in the NMCRC subgroup ($P = 0.26$) (Figure 1A). Mucinous subgroups A and B had median OS of 70.1 and 32.8 mo, respectively ($P = 0.46$). Median OS was 32.8 and 70.1 mo in groups B and D, respectively ($P = 0.143$). TTP in MCRC and NMCRC was 50.17 *vs* 44.77 mo, respectively ($P = 0.795$) (Figure 1B). TTP in groups A, B, and D was 70.1, 24.8,

Table 5 Studies matching mucinous cases to non-mucinous controls

	Case: control	Matched for	Survival in MCRC *vs* NMCRC	*P*
Symonds *et al*[14]	120/120	Age, sex, stage	Decreased	Significant
Connelly *et al*[16]	60/60	Stage	Same	NS
Green *et al*[13]	52/343	Stage	Same	NS
Consorti *et al*[3]	29/54	Age, sex, location, stage	Decreased	NS
Kang *et al*[8]	16991/146115	Age, grade, stage, location	Same	NS
Farhat	35/35	Age, sex, stage	Decreased	NS

NS: Not significant.

and 65.5 mo ($P > 0.05$), respectively.

When survival of all patients was analyzed in relation with tumor grade, no difference in survival was noted among the various degrees of differentiation (Figure 1C).

DISCUSSION

Our results show that MCRC is associated with a non-significant decrease in median OS and TTP, compared to NMCRC. Similar results were obtained when OS and TTP were compared in patients with more than 75% mucin to those with less than 75% mucin.

In our report, we matched patients for age, gender, and stage of disease at presentation. Moreover, we found that patients in the two subgroups were similar in terms of tumor location. Furthermore, there was no difference in both arms with respect to presence of co-morbid illnesses, chemotherapy regimens received or radiotherapy administration. However, we noted that more MCRC patients presented with a higher tumor grade than NMCRC patients ($P = 0.028$).

The prognostic significance of mucin content in colorectal cancer remains a controversial issue. While some authors reported that patients with MCRC show no difference in survival outcome compared to patients with NMCRC[3,8,16-18], others found that MCRC is associated with worse survival[4-7,10,14].

The difference in survival was attributed to the advanced stage at which these tumors present[3,4,6,8,13], more invasion of adjacent viscera and more extensive lymph node involvement[7,15,16], increased incidence of distant metastasis[4,17], and decreased response to chemotherapy[10].

Most of the published series compared mucinous to nonmucinous subgroups of colorectal cancer without matching for age, stage, grade and location. Comparing non-matched groups may confound survival data since the difference in survival could be related to differences in tumor location, grade or stage, and not due to the presence of mucin in the tumor.

Five studies compared matched groups of patients[3,8,13,14,16] (Table 5). One study reported a statistically significant decrease in survival in MCRC[14] while the other four showed no difference in survival outcome[3,8,13,16].

The importance of matching for stage is reflected in the large population-based study done by Kang *et al*[8] where MCRC did show worse OS but similar stage-to-stage survival as the other non-mucinous subtypes of colorectal cancer. The difference in OS between MCRC and NMCRC was attributed to the difference in stage at presentation, with more mucinous tumors presenting at advanced disease stages. That study did not, however, examine the impact of high mucin content (> 75%) on survival, a factor that may be associated with an adverse prognosis.

In patients with mucin producing colon cancer, few studies have examined the clinical impact of increasing mucin content in colorectal cancer on progression free and OS. Tumors with high mucin content (80%) were associated with worse clinicopathological behavior, more aggressive phenotype, and poorer prognosis[5,19,20]. By contrast, tumors with moderate mucin content (60%-80%) were found to be indistinguishable from non-mucinous tumors. However, the different subgroups were not matched for age, stage, grade, or location.

The prognostic value of tumor grade in MCRCs has not been adequately investigated in matched studies. In a non-matched study by Enríquez *et al*[21], MCRC showed higher tumor grade that did not affect survival, which is in agreement with our data. Moreover, Connelly *et al*[16] excluded the poorly differentiated histology from their MCRC study because tumors with high grade tend to exhibit a distinct biological behavior and a worse prognosis[22,23]. However, Kang *et al*[8] controlled for tumor grade and concluded that MCRC and NMCRC patients show similar risks of dying.

Although our patients with MCRC had more poorly differentiated tumors ($P = 0.0028$), there was no apparent significant effect of grade on prognosis. Due to the small number of patients in our study, matching for tumor grade was not possible.

There are several limitations in our study. First, this was a case-control study with a relatively small number of subjects. The inability to adequately perform more subgroup analyses or to control for additional confounders because of small sample size is to be noted.

Second, this is a retrospective assessment with all the attendant limitations of this approach including the missing data, which leads to fewer patients included in multivariable models, generally increasing the risk for both type one and type two errors. Third, the cases seen represent a single tertiary center experience which might reduce the prevalence rate of MCRC reported here as compared to the higher prevalence rates in some studies that used data from national registries. Fourth, some of the trends observed in the study might have reached statistical significance if the study sample had been larger. However, despite the limitations of our study, very few studies have examined the effect of increasing mucin content on survival in colorectal patients which adds more value to the results of our study and others'.

In conclusion, high mucin content in colorectal cancer is associated with a nonsignificant decrease in OS and TTP. However, the presence of mucin in more than 75% of tumor volume may indicate a more aggressive pheno-

type that presents with a higher tumor grade and a possible subsequent decrease in OS and TTP. Larger studies in patients with MCRC with matching for stage and grade are warranted to examine the impact of mucin content on survival, especially those with more than 75% mucin content.

COMMENTS

Background

Mucinous adenocarcinoma is a histological variant that accounts for 5% to 15% of cases of primary colorectal cancer. There is evidence that mucinous colorectal cancer (MCRC) may be a distinct biological and genetic entity compared with non-mucinous adenocarcinoma.

Research frontiers

A retrospective review of patients with colorectal cancer treated at the American University of Beirut-Medical Center between 1986 and 2003 identified 35 patients with MCRC and 750 patients with NMCRC. To study the impact of increasing mucin content on survival, two cut-off points (50% and 75% mucin) were chosen. Univariate analysis was performed to determine if increasing mucin production had an effect on survival that is independent of age, gender, and tumor stage.

Innovations and breakthroughs

High mucin content in colorectal cancer is associated with a nonsignificant decrease in overall survival (OS) and time to disease progression (TTP). However, the presence of mucin in more than 75% of tumor volume may indicate a more aggressive phenotype that presents with a higher tumor grade and a possible subsequent decrease in OS and TTP.

Applications

MCRC is associated with a non-significant decrease in median survival and TTP, particularly when mucin content is > 75% of tumor volume. However, it tends to be more poorly differentiated. Larger studies in patients with MCRC with matching for stage and grade are warranted to examine the impact of mucin content on survival, especially those with more than 75% mucin content.

Peer review

It is an interesting manuscript trying to answer the question if the mucin production is related with survival in colorectal cancer.

REFERENCES

1 **American Joint Committee on Cancer: Staging Manual, 6th ed**. New York: Springer 2002. Available from: URL: http://www.cancerstaging.org/products/ajccproducts.html

2 **Zhang H**, Evertsson S, Sun X. Clinicopathological and genetic characteristics of mucinous carcinomas in the colorectum. *Int J Oncol* 1999; **14**: 1057-1061

3 **Consorti F**, Lorenzotti A, Midiri G, Di Paola M. Prognostic significance of mucinous carcinoma of colon and rectum: a prospective case-control study. *J Surg Oncol* 2000; **73**: 70-74

4 **Wu CS**, Tung SY, Chen PC, Kuo YC. Clinicopathological study of colorectal mucinous carcinoma in Taiwan: a multivariate analysis. *J Gastroenterol Hepatol* 1996; **11**: 77-81

5 **Suma KS**, Nirmala V. Mucinous component in colorectal carcinoma--prognostic significance: a study in a south Indian population. *J Surg Oncol* 1992; **51**: 60-64

6 **Papadopoulos VN**, Michalopoulos A, Netta S, Basdanis G, Paramythiotis D, Zatagias A, Berovalis P, Harlaftis N. Prognostic significance of mucinous component in colorectal carcinoma. *Tech Coloproctol* 2004; **8** Suppl 1: s123-s125

7 **Nozoe T**, Anai H, Nasu S, Sugimachi K. Clinicopathological characteristics of mucinous carcinoma of the colon and rectum. *J Surg Oncol* 2000; **75**: 103-107

8 **Kang H**, O'Connell JB, Maggard MA, Sack J, Ko CY. A 10-year outcomes evaluation of mucinous and signet-ring cell carcinoma of the colon and rectum. *Dis Colon Rectum* 2005; **48**: 1161-1168

9 **Messerini L**, Vitelli F, De Vitis LR, Mori S, Calzolari A, Palmirotta R, Calabro A, Papi L. Microsatellite instability in sporadic mucinous colorectal carcinomas: relationship to clinico-pathological variables. *J Pathol* 1997; **182**: 380-384

10 **Negri FV**, Wotherspoon A, Cunningham D, Norman AR, Chong G, Ross PJ. Mucinous histology predicts for reduced fluorouracil responsiveness and survival in advanced colorectal cancer. *Ann Oncol* 2005; **16**: 1305-1310

11 **Glasgow SC**, Yu J, Carvalho LP, Shannon WD, Fleshman JW, McLeod HL. Unfavourable expression of pharmacologic markers in mucinous colorectal cancer. *Br J Cancer* 2005; **92**: 259-264

12 **Takemura M**, Osugi H, Lee S, Kaneko M, Tanaka Y, Fujiwara Y, Nishizawa S, Iwasaki H. [Choice of chemotherapeutic drugs for colorectal cancers by DPD and OPRT activities in cancer tissues] *Gan To Kagaku Ryoho* 2004; **31**: 1053-1056

13 **Green JB**, Timmcke AE, Mitchell WT, Hicks TC, Gathright JB Jr, Ray JE. Mucinous carcinoma--just another colon cancer? *Dis Colon Rectum* 1993; **36**: 49-54

14 **Symonds DA**, Vickery AL. Mucinous carcinoma of the colon and rectum. *Cancer* 1976; **37**: 1891-1900

15 **Yamamoto S**, Mochizuki H, Hase K, Yamamoto T, Ohkusa Y, Yokoyama S, Ushitani Y, Tamakuma S. Assessment of clinicopathologic features of colorectal mucinous adenocarcinoma. *Am J Surg* 1993; **166**: 257-261

16 **Connelly JH**, Robey-Cafferty SS, Cleary KR. Mucinous carcinomas of the colon and rectum. An analysis of 62 stage B and C lesions. *Arch Pathol Lab Med* 1991; **115**: 1022-1025

17 **Adell R**, Marcote E, Segarra MA, Pellicer V, Gamon R, Bayon AM, Canales M, Torner A. [Is mucinous colorectal adenocarcinoma a distinct entity?] *Gastroenterol Hepatol* 2002; **25**: 534-540

18 **Du W**, Mah JT, Lee J, Sankila R, Sankaranarayanan R, Chia KS. Incidence and survival of mucinous adenocarcinoma of the colorectum: a population-based study from an Asian country. *Dis Colon Rectum* 2004; **47**: 78-85

19 **Sasaki O**, Atkin WS, Jass JR. Mucinous carcinoma of the rectum. *Histopathology* 1987; **11**: 259-272

20 **Umpleby HC**, Ranson DL, Williamson RC. Peculiarities of mucinous colorectal carcinoma. *Br J Surg* 1985; **72**: 715-718

21 **Enriquez JM**, Diez M, Tobaruela E, Lozano O, Dominguez P, Gonzalez A, Muguerza JM, Ratia T. Clinical, histopathological, cytogenetic and prognostic differences between mucinous and nonmucinous colorectal adenocarcinomas. *Rev Esp Enferm Dig* 1998; **90**: 563-572

22 **Compton CC**, Fielding LP, Burgart LJ, Conley B, Cooper HS, Hamilton SR, Hammond ME, Henson DE, Hutter RV, Nagle RB, Nielsen ML, Sargent DJ, Taylor CR, Welton M, Willett C. Prognostic factors in colorectal cancer. College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med* 2000; **124**: 979-994

23 **O'Connell JB**, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. *J Natl Cancer Inst* 2004; **96**: 1420-1425

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RAPID COMMUNICATION

Interaction of methylenetetrahydrofolate reductase C677T, cytochrome P4502E1 polymorphism and environment factors in esophageal cancer in Kazakh population

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Abstract

AIM: To evaluate the association and interaction of genetic polymorphisms in methylenetetrahydrofolate reductase (MTHER) and cytochrome P4502E1 (CYP4502E1), environment risk factors with esophageal cancer (EC) in Kazakh, a high EC incidence area of Xinjiang Uygur Autonomous Region, China.

METHODS: A 1:2 matched case-control study was conducted with 120 cases of EC and 240 population- or hospital-based controls. The controls were matched for sex, nationality, area of residence and age within a 5-year difference. MTHER and CYP4502E1 genotypes were identified by PCR-based restriction fragment length polymorphism (RFLP). A conditional logistic regression model was established to identify risk factors. The strata method was adopted in interaction analysis.

RESULTS: Low consumption of green vegetables and fresh fruits, alcohol drinking, and unsafe water (shallow well, or river) were found to be the risk factors for EC. Individuals with the MTHFR677 (C/T + T/T) genotype had a 2.62-fold (95% CI: 1.61-4.28) risk of developing EC compared with those who carried the C/C genotype. Individuals with the CYP4502E1C1/C1 genotype had a 3.00-fold (95% CI: 1.82-4.96) risk compared with those who carried the CYP4502E1 (C1/C2 + C2/C2) genotype. Gene-environment interaction analysis showed that MTHFR677 gene polymorphism was correlated with consumption of green vegetables and fresh fruit, while CYP4502E1 C1/C1 was correlated with alcohol drinking and unsafe drinking water. MTHFR and CYP4502E1 analysis of gene-gene interaction showed that individuals with the MTHFR677 (C/T + T/T) and CYP4502E1C1/C1 genotypes had a 7.41-fold (95% CI: 3.60-15.25) risk of developing EC compared with those who carried the MTHFR677C/C and CYP4502E1 RsaI C1/C2 + C2/C2 genes, and the interaction rate was higher than that of the two factors alone.

CONCLUSION: Low consumption of green vegetables and fresh fruits, alcohol drinking, and unsafe water (shallow well, or river) and polymorphisms in MTHFR and CYP4502E1 genes are important risk factors for EC. There is a synergistic interaction among polymorphisms in MTHFR and CYP4502E1 genes and environment factors. MTHFR and CYP4502E1 genes can be used as biomarkers for prevention of EC in Kazakh, Xinjiang Uygur Autonomous Region, China.

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Key words: Kazakh; Esophageal Cancer; Methylenetetrahydrofolate reductase C677T; Cytochrome P4502E1; Genetic polymorphism; Environment risk factors; Interaction; Case control study

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INTRODUCTION

Kazakh is an ethnic group with a high incidence and mortality rate of esophageal cancer (EC) in China. A national survey in 1990-1992 showed that the age-adjusted

mortality rate was 68.88 per 100 000 population for EC in Kazakh, and the rate was 14.95 per 100 000 population in Chinese nationality. The mortality rate for the same Kazakh population was 67.60 per 100 000 population in 2004-2005. The mortality rate of esophageal cancer in Kazakh has never been decreased over the last 15 years. Different risk factors for esophageal cancer have been reported in the world[1-5]. It was reported that deficiency in folate is caused by low consumption of green vegetables and fresh fruits, unsanitary drinking water, smoking, alcohol drinking, fast and irregular eating, eating of peppery food, frequent engorgement, eating out of date cake, history of esophagus or stomach illness and family history of EC are the risk factors for EC in Kazakh[6]. EC is caused by multi-factors, including environmental risk factors and genetic factors. In recent years, environmental and genetic susceptibilities and their interactions were used in evaluating the risks of EC[7-11]. Primary candidates for gene-environment interaction studies are those encoding enzymes related to the metabolism of established risk factors for cancer.

Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate metabolism, which catalyzes 5, 10-methylene-tetrahydrofolate to 5-methyltetrahydrofolate[12]. A substitution of C to T at nucleotide 677 in MTHFR results in an alanine to valine substitution, which alters enzyme activity[13]. The *MTHFR C677T* polymorphism influences DNA methylation through an interaction with folate[14]. Alteration in DNA methylation, disruption of DNA integrity and DNA repair are believed to enhance carcinogenesis by altering the expression of critical tumor suppressor genes and proto-oncogenes[15]. Cytochrome P4502E1 (CYP4502E1), a member of the cytochrome P450 superfamily, is involved in the metabolic activation of many low molecular weight compounds, such as N-nitrosamines, aniline, vinyl chloride, and urethane[16-17]. N-nitrosamines present in tobacco and diet are well-recognized carcinogens involving cancer development at various sites, including the esophagus and stomach[18-19].

The high mortality rate in Kazakh population indicates that environment factors play an important role in the development of EC. However, only a few individuals in the high-risk Kazakh population develop EC, although all residents share very similar environment-related risk factors and life style, suggesting that host susceptibility factors, such as the MTHFR677 and CYP4502E1 gene polymorphisms, may play an important role in the increased risk for EC. This study analyzed the gene-environment and gene-gene interaction among the MTHER and CYP4502E1 gene polymorphisms, and environmental risk factors for EC in order to determine their relevance to EC prevention.

MATERIALS AND METHODS

Specimens

The 120 cases of esophageal cancer (confirmed by pathological diagnosis) came from inpatients and outpatients of six hospitals in the north of Xinjiang Uygur Autonomous Region between March 2005 and May 2007. Two hundred and forty population- or hospital-based controls were randomly selected and matched for sex, nationality, residence and age within a 5-year difference. The controls were confirmed to have no history of cancer and digestive system diseases. Specially trained interviewers administered a standardized questionnaire that included demographic characteristics (sex, age, area of birth and residence), lifestyle, smoking status, drinking alcohol status, history of stomach or esophagus diseases and family history of EC. Blood samples were collected and DNA was extracted for genotyping of *MTHFR* and CYP4502E1 genes.

Genotyping of MTHFR and CYP2E1

Sites MTHFR C677T and CYP4502E1 genotypes were analyzed by PCR-based restriction fragment length polymorphism (RFLP). The primers for MTHFR/CYP4502E1 were synthesized by Shengong Biotechnology Company (Shanghai, China). The sequences of PCR primers for the C677T site are 5'-TTTGAGGCTGACCTG AAGCACTTGAAGGAG-3' and 5'-GAGTGGTAGCCCTGGATGGGAAAGATCCCG-3'[20,21]. PCR was carried out in 25.0 μL reaction mixture containing 2.5 μL 10 × PCR buffer, 1.5 μL $MgCl_2$ (25 mmol/L), 0.5 μL dNTP (10 mmol/L), 0.5 μL forprimer (20 μmol/L), 0.5 μL revprimer (20 μmol/L), 0.2 μL Taq DNA polymerase (5 U/μL), 1.5 μL template DNA, and 17.8 μL nuclease free water. The reaction was initially carried out at 94℃ for 5 min followed by 35 cycles at 94℃ for 1 min, at 61℃ for 1 min, at 72℃ for 1 min, and a final extension at 72℃ for 8 min. The PCR products were digested with Hinf I at 37℃ for 13 h. The digested products were separated by electrophoresis on a 2.0% agarose gel. The 677C/C wild-type homozygotes were identified by the presence of only a 173-bp fragment; 677C/T heterozygotes were identified by the presence of 173-, 125-, and 48-bp fragments, and 677T/T homozygotes were identified by the presence of 125- and 48-bp fragments.

The CYP4502E1 was identified by amplifying genomic DNA with the forward primer 5'-CCAGTC GAGTCTACATTGTCA-3' and the reverse primer 5'-TTCATTCTGTCTTCTAACTGG-3'. PCR was performed in 25.0 μL mixture containing 2.5 μL 10 × PCR buffer (including $MgCl_2$), 2.0 μL dNTP (2.5 mmol/L), 0.5 μL forprimer (20 μmol/L), 0.5 μL revprimer (20 μmol/L), 0.2 μL Taq DNA polymerase (5 U/μL), 2.0 μL template DNA, and 17.3 μL nuclease free water. The reaction was initially carried out at 95℃ for 8 min followed by 35 cycles at 94℃ for 1 min, at 56℃ for 1 min, at 72℃ for 1 min, and a final extension at 72℃ for 8 min. The PCR products were digested with restriction enzyme RsaI at 37℃ for 4 h. The digested products were separated by electrophoresis on a 2.0% agarose gel. Three genotypes of CYP4502E1 resulting from digestion with the restriction enzyme RsaI were found: common homozygote C1/C1, heterozygote C1/C2, and rare homozygote C2/C2.

Quality control

DNA extraction and PCR were conducted at different time points. The genotypes of DNA samples were iden-

Table 1 Environmental risk factors for EC in Kazakh population

Environmental risk factors	Case *n* (%)	Control, *n* (%)	*P*[1]	OR (95% CI)[1]
Consumption of green vegetables				
Frequently	42 (35.00)	126 (52.50)	-	1.00
Occasionally	45 (37.50)	90 (37.50)	0.106	2.54 (0.91-2.59)
Less	33 (27.50)	24 (10.00)	0.000	4.66 (2.33-9.30)
Consumption of fresh fruits				
Frequently	13 (10.83)	71 (29.58)	-	1.00
Occasionally	71 (59.17)	145 (60.42)	0.003	2.79 (1.43-5.43)
Less	36 (30.00)	24 (10.00)	0.000	9.03 (3.90-20.89)
Smoking status				
Never	50 (41.67)	100 (41.67)	-	1.00
Former	29 (24.17)	42 (17.50)	0.309	1.39 (0.74-2.62)
Current	41 (34.16)	98 (40.83)	0.509	0.83 (0.47-1.45)
Alcohol drinking frequency				
Never	50 (41.67)	152 (63.33)	-	1.00
1-2 times/wk	34 (28.33)	61 (25.42)	0.002	3.28 (1.54-7.01)
3-4 times/wk	29 (24.17)	21 (8.75)	0.000	7.31 (3.14-17.03)
⩾ 5 times/wk	7 (5.83)	6 (2.50)	0.005	6.00 (1.71-21.02)
Drinking water source				
Safe water	43 (35.83)	163 (67.92)	-	1.00
Shallow well water	20 (16.67)	25 (10.41)	0.002	2.88 (1.47-5.66)
River water	57 (47.50)	52 (21.67)	0.000	4.40 (2.55-7.60)

[1]ORs, 95% CIs and *P* values were calculated in a conditional logistic regression model.

tified without knowledge of the case-control status, and a 10% random sample set of case and controls was genotyped by different investigators and the reproducibility was 100%. Each PCR was performed with the controls as blank (without DNA template), positive and negative controls, respectively. When any of these controls failed, PCR was re-conducted. Twenty percent of the questionnaires were re-administered by different investigators and the consistency was 100%.

Statistical analysis

Statistical analyses were performed using the SPSS software. Cases and controls were compared for any differences in gender and age using χ^2 test and Mann-Whitney test, respectively. The probability of Hardy Weinberg equilibrium was assessed by χ^2 test. Conditional logistic regression was employed to calculate the odds ratio (OR) of MTHFR/CYP4502E1 polymorphisms. Gene-environment and gene-gene interactions were calculated by stratified analysis.

RESULTS

The number of male and female patients with EC was 81 and 39, respectively, who were matched for 162 male and 78 female controls. The mean age (± SD) of cases and controls was 59.0 ± 10.0 years and 58.4 ± 10.1 years, respectively. There was no significant difference in age between cases and controls ($t = 0.586$, $\nu = 358$, $P = 0.558$).

The distributions of environmental risk factors in cases and controls are summarized in Table 1. Low consumption of green vegetables and fresh fruits, alcohol drinking, and unsafe water (shallow well, or river) were found to be the risk factors for EC. The percentage of tobacco smoking in cases and controls was not significantly different.

Table 2 Genotype risk assessment of EC in cases and controls

Genotype	Case *n* (%)	Control *n* (%)	*P*[1]	OR (95% CI)[1]
MTHFR677				
C/C	60 (50.00)	170 (70.83)	-	1.00
C/T	53 (44.17)	59 (24.59)	0.000	2.69 (1.63-4.44)
T/T	7 (5.83)	11 (4.58)	0.144	2.15 (0.77-5.98)
C/C	60 (50.00)	170 (70.83)	-	1.00
C/T + T/T	60 (50.00)	70 (29.17)	0.000	2.62 (1.61-4.28)
CYP2E1 RsaI				
C1/C1	94 (78.33)	128 (53.33)	-	1.00
C1/C2	23 (19.17)	90 (37.50)	0.000	0.37 (0.22-0.62)
C2/C2	3 (2.50)	22 (9.17)	0.009	0.19 (0.05-0.66)
C1/C2 + C2/C2	26 (21.67)	112 (46.67)	-	1.00
C1/C1	94 (78.33)	128 (53.33)	0.000	3.00 (1.82-4.96)

[1]ORs, 95% CIs and *P* values were calculated in a conditional logistic regression model.

The genotype distributions of the MTHFR677 and CYP4502E1 RsaI in cases and controls are summarized in Table 2. The observed frequencies of three MTHFR677 genotypes in controls (C/C = 70.83%, C/T = 24.59%, and T/T = 4.58%) were not different from those detected in Hardy-Weinberg equilibrium ($\chi^2 = 3.34$, $\nu = 2$, $P = 0.188$). However, they were significantly different from those observed in cases (C/C = 50.00%, C/T = 44.17%, and T/T = 5.83%, $\chi^2 = 15.55$, $\nu = 2$, $P = 0.000$). Subjects who carried the MTHFR677C/T genotype had a 2.69-fold (95% CI: 1.63-4.44) risk of developing EC compared with those who carried the MTHFR677C/C genotype. The MTHFR677T/T genotype, which was rare both in cases and in controls, was associated only with a slightly increased risk of EC, without statistical significance (OR = 2.15, 95% CI = 0.77-5.98). Individuals with the MTHFR677 T allele were more prone to develop EC (OR = 2.62, 95% CI = 1.61-4.28).

The distribution of the CYP4502E1 RsaI genotypes in controls (C1/C1 = 53.33%, C1/C2 = 37.50%, and C2/C2 = 9.17%) was also in accordance with Hardy-Weinberg equilibrium ($\chi^2 = 0.921$, $\nu = 2$, $P = 0.631$) and significantly different from that of the CYP4502E1 RsaI genotypes in cases (C1/C1 = 78.33%, C1/C2 = 19.17%, and C2/C2 = 2.50%, $\chi^2 = 21.79$, $\nu = 2$, $P = 0.000$). The Odds ratio (OR) of developing esophageal cancer for the CYP4502E1 RsaI C1/C2 and C2/C2 genotypes was 0.37 (95% CI = 0.22-0.62) and 0.19 (95% CI = 0.05-0.66), respectively, compared to the CYP4502E1 RsaI C1/C1 genotype. Individuals with the CYP4502E1 RsaI C1/C1 genotype had a 3.00-fold (95% CI = 1.82-4.96) risk of developing EC compared with those who carried the CYP4502E1 RsaI C1/C2 + C2/C2 genotype.

The results of interaction between MTHFR677 gene polymorphism and consumption of green vegetables

Table 3 Interaction between MTHFR 677 and consumption of green vegetables, fresh fruits in EC

Consumption of vegetables and fruits	MTHFR 677 genotype	Case (*n*)	Control (*n*)	χ^2	*P*	OR (95% CI)
Green vegetables[1]						
Frequently	C/C	19	89	-	-	1.00
Occasionally or less	C/C	41	81	7.62	0.006	2.37 (1.27-4.42)
Frequently	C/T + T/T	23	37	8.85	0.003	2.91 (1.42-5.97)
Occasionally or less	C/T + T/T	37	33	24.50	0.000	5.25 (2.66-10.39)
Freshfruits[2]						
Frequently	C/C	6	54	-	-	1.00
Occasionally or less	C/C	54	116	10.90	0.001	4.19 (1.70-10.34)
Frequently	C/T + T/T	7	17	4.81	0.028	3.71 (1.10-12.54)
Occasionally or less	C/T + T/T	53	53	26.76	0.000	9.00 (3.57-22.71)

SIA: Synergic index of addition; RERI: Relative excess risk of interaction; API: Attributable proportion of interaction. $SIA^1 = 5.25/(2.37 + 2.91 - 1.00) = 1.23$, $RERI^1 = 5.25 - (2.37 + 2.91) + 1 = 0.97$, $API^1 = [5.25 - (2.37 + 2.91) + 1]/5.25 = 18.48\%$; $SIA^2 = 9.00/(4.19 + 3.71 - 1.00) = 1.30$, $RERI^2 = 9.00 - (4.19 + 3.71) + 1 = 2.10$, $API^2 = [9.00 - (4.19 + 3.71) + 1]/9.00 = 23.33\%$.

Table 4 Interaction between CYP4502E1 RsaI 677 and alcohol drinking, safety drinking water in EC

		CYP4502E1 rsal genotype	Case (*n*)	Control (*n*)	χ^2	*P*	OR (95% CI)
Alcohol drinking[1]	Never	C1/C2 + C2/C2	12	70	-	-	1.00
	Yes	C1/C2 + C2/C2	14	42	2.34	0.126	1.94 (0.82-4.60)
	Never	C1/C1	38	82	7.59	0.006	2.70 (1.31-5.57)
	Yes	C1/C1	56	46	31.64	0.000	7.10 (3.44-14.68)
Safe drinking water[2]	Yes	C1/C2 + C2/C2	9	78	-	-	1.00
	No	C1/C2 + C2/C2	17	34	11.12	0.001	4.33 (1.76-10.69)
	Yes	C1/C1	34	85	10.11	0.001	3.47 (1.56-7.69)
	No	C1/C1	60	43	46.80	0.000	12.09 (5.47-26.74)

SIM: Synergic index of multiplication; $SIM1 = 7.10/(1.94 \times 2.70) = 1.36$, $RERI^1 = 7.10 - (1.94 + 2.70) + 1 = 3.46$, $API^1 = [7.10 - (1.94 + 2.70) + 1]/7.10 = 48.73\%$; $SIA^2 = 12.09/(4.33 + 3.47 - 1.00) = 1.78$, $RERI^2 = 12.09 - (4.33 + 3.47) + 1 = 5.29$, $API^2 = [12.09 - (4.33 + 3.47) + 1]/12.09 = 43.76\%$.

Table 5 Interaction between MTHFR 677 and CYP4502E1 Rsal gene polymorphisms in EC

MTHFR 677 genotype	CYP2E1 rsal genotype	Case (*n*)	Control (*n*)	χ^2	*P*	OR (95% CI)
C/C	C1/C2 + C2/C2	13	82	-	-	1.00
C/T + T/T	C1/C2 + C2/C2	13	30	5.30	0.021	2.73 (1.14-6.56)
C/C	C1/C1	47	88	12.91	0.000	3.37 (1.70-6.68)
C/T + T/T	C1/C1	47	40	33.44	0.000	7.41 (3.60-15.25)

$SIA = 7.41/(2.73 + 3.37 - 1.00) = 1.45$; $RERI = 7.41 - (2.73 + 3.37) + 1 = 2.31$; $API = [7.41 - (2.73 + 3.37) + 1]/7.41 = 31.17\%$.

and fresh fruit are listed in Table 3. Less consumption of green vegetables increased the OR in MTHFR677 T allele carriers (OR = 5.25, 95% CI = 2.66-10.39). The corresponding SIA, RERI and API were 1.23, 0.97, and 18.48%, respectively. Among carriers of the MTHFR677 T allele with occasional or less consumption of fresh fruits was significantly associated with an elevated risk of developing EC (OR = 9.00, 95% CI = 3.57-22.71) and the interaction rate was higher than that of the two factors alone. The corresponding SIA, RERI, and API were 1.30, 2.10, and 23.33%, respectively.

The results of interaction between the CYP4502E1 RsaI gene polymorphism and alcohol drinking as well as safe drinking water are shown in Table 4. Among carriers of the CYP4502E1C1/C1 genotype, alcohol drinking was significantly associated with an elevated risk of developing EC (OR = 7.10, 95% CI = 3.44-14.68), and the interaction rate was higher than the sum of the two factors alone. The corresponding SIM, RERI, and API were 1.36, 3.46, and 48.73%, respectively. Unsafe water increased the risk of developing EC among carriers of the CYP4502E1C1/C1 genotype (OR = 12.09, 95% CI = 5.47-26.74), and the interaction rate was higher than that of the other two factors. The corresponding SIA, RERI and API were 1.78, 5.29, and 43.76%, respectively.

A significant interaction between the MTHFR 677 genotype and CYP4502E1 RsaI genotype was found in EC risk (Table 5). Individuals who had both MTHFR677 (C/T + T/T) and CYP4502E1 RsaI C1/C1 genotypes had a 7.41-fold risk of developing EC (95% CI = 3.60-15.25) compared with those who carried MTHFR677C/C and CYP4502E1 RsaI C1/C2 + C2/C2 genotypes. The corresponding SIA, RERI and API were 1.45, 2.31 and 31.17%, respectively.

DISCUSSION

The results of the current study indicate that MTHFR677 and CYP4502E1 RsaI gene polymorphisms are the susceptibility factors for EC[22,23]. Cohort studies that simultaneously consider multiple genetic and environmental

factors possibly involved in esophageal carcinogenesis are needed to ascertain not only the relative contribution of these factors to tumor development but also the contributions of their putative interactions[24]. We observed a significant risk of having the MTHFR677 C/T + T/T and CYP4502E1C1/C1 genotypes in EC. There is a synergistic interaction among polymorphisms in MTHFR and CYP4502E1 genes and environment factors.

The studies conducted in high-risk areas showed the MTHFR677T allele increases the risk of developing EC[25,26]. However, no risk change has been observed among Caucasians in Germany and Japan[27,28]. Regional differences in folate consumption among populations may explain this inconsistency in the impact of T alleles[25], suggesting that gene-nutrient environment interactions between folate consumption and impact of the MTHFR 677T allele vary with folate intake. When folate intake is sufficient, individuals with the MTHFR CT or TT genotype may have a decreased risk of developing cancer, since decreased MTHFR activity associated with the 677TT polymorphism can lead to elevation in 5,10-methylene-tetrahydrofolate, facilitating DNA synthesis, while adequate provision of methyl donors can be ensured. In contrast, in the presence of low folate, DNA methylation and DNA synthesis/repair may be impaired, initiating carcinogenesis. Deficiency in folate is caused by low consumption of vegetables and fruit in the Kazakh population and MTHFR677 C/T + T/T genotype has a synergic interaction with less consumption of vegetables and fruit in EC. Our results are consistent with the reported findings[25,26].

Over-representation of variant CYP4502E1 Rsa1 alleles has been reported in gastric cancer[29] and a lower frequency of the RsaI variant allele has also been observed in patients with EC than in controls[30]. Individuals with the variant Rsa1 allele (c1/c2 or c2/c2) have a lower basal CYP450 2E1 activity. It was reported that the Cyp4592E1 C2/C2 genotype is associated with the decreased enzyme activity[31], adding biological plausibility to the protective effect of CYP4502E1 C2/C2 genotype observed in this study. Our observation is in agreement with the finding of recent studies showing that the C1/C1 genotype of CYP4502E1 is associated with the increased risk of developing EC[32-35]. However, contrary results have also been reported elsewhere[36-38]. Studies have shown inconsistent findings regarding the association between the CYP4502E1 polymorphism and EC. The reasons for these inconsistent findings are unknown. However, it may be due to the differences in ethnicity and life-style which can lead to variations in enzyme activity. The present findings indirectly support the hypothesis that environmental exposure to carcinogens plays a role in the etiology of EC. The CYP4502E1 polymorphism is involved in metabolism of various nitrosamines. Cigarette smoking, and alcohol drinking, unsafe drinking water containing chemicals including nitroso compound, were found to be the risk factors for EC in this study. A significant gene-environment interaction between the CYP4502E1 polymorphism and alcohol drinking and unsafe water was also observed in this study.

Selection bias and/or systematic error may occur in a case-control study because of inappropriate selection of subjects and other confounding factors. However, our study including a relatively large number of cases diagnosed in hospitals was matched for potential confounding variables. Solid and reproducible genotyping techniques can minimize systematic errors in measurement. For these reasons, the findings of our study could not solely attribute to bias.

In summary, MTHFR677 and CYP4502E1 RsaI gene polymorphisms are significantly correlated with EC, and MTHFR677 C/T or T/T genotype and CYP4502E1 C1/C1 wild type increase the susceptibility to EC in the Kazakh population of Xinjiang Uygur Autonomous Region, China. Interaction between MTHFR677 C/T + T/T genotype and less consumption of green vegetables and fresh fruits, as well as between CYP4502E1 C1/C1 and alcohol and unsafe drinking water is associated with the risk of developing EC. Gene-gene interaction between MTHFR 677 and CYP4502E1 RsaI can serve as a useful biomarker for prevention of EC in the Kazakh population.

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COMMENTS

Background

Kazakh is an ethnic group with a high incidence and mortality rate of esophageal cancer (EC) in China. Epidemiological studies have demonstrated deficiency in folate caused by low consumption of green vegetables and fresh fruits, alcohol drinking, unsanitary drinking water (containing chemicals including nitroso compound) are the main risk factors for EC in Kazakh population. Genetic polymorphisms in the MTHFR677 and CYP4502E1 genes affect the metabolism of folate, alcohol, and N-nitrosamines. There have been some studies on the roles of folate and MTHFR677 genes, alcohol and the CYP2E1 genes in EC in Chinese Han population. However, their results were conflicting and little study in Kazakh population. Therefore, the aim of the present study was to evaluate the association and interaction of MTHER and CYP4502E1 and environment risk factors with EC in Kazakh population.

Research frontiers

Accumulating evidence from prior epidemiologic studies shows an association between deficiency in folate caused by low consumption of green vegetables and fresh fruits, alcohol drinking, unsanitary drinking water and EC in Kazakh population. The genetic polymorphisms of MTHFR affect the metabolism of folate and CYP4502E1 genetic polymorphisms also affect the metabolism of alcohol and N-nitrosamines. Polymorphisms in the MTHFR and CYP4502E1 genes are associated with the risk of EC in Kazakh.

Innovations and breakthroughs

This is the first study to show significant interactions of MTHFR677 and CYP4502E1 and consumption of green vegetables and fresh fruits, alcohol drinking, and unsafe waters (shallow well, or river) with EC in Kazakh population. Synergistic interactions were found in MTHFR677 gene polymorphism with consumption of green vegetables and fresh fruit, CYP4502E1 gene polymorphism with alcohol drinking and unsafe drinking water, MTHFR677 with CYP4502E1 genotypes for EC in Kazakh population, and the interaction rate was higher than that of the two factors alone.

Applications

The detection of MTHFR677 and CYP4502E1 genotypes may become a useful biomarker for EC in Kazakh population, and also help clinicians to diagnose EC earlier.

Terminology

Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate metabolism responsible for circulating form of folate, 5-methyl-tetrahydrofolate,

which converts methionine to S-adenosylmethionine, the universal methyl donor for various intracellular methylation reactions, particularly DNA methylation. The cytochrome P450 2E1 (CYP4502E1), a member of the cytochrome P450 superfamily metabolizes a range of small organic compounds, including aniline and benzene as well as N-nitrosamines. Individuals frequently encounter different environmental conditions, and the physiological and behavioral responses to these conditions can depend on an individual's genetic makeup. This phenomenon is known as gene-environment interaction.

Peer review

This paper is interesting, and would accumulate new data on interaction of genetic polymorphisms and environment factors for esophageal cancer in Kazakh population.

REFERENCES

1 **Zhang W**, Bailey-Wilson JE, Li W, Wang X, Zhang C, Mao X, Liu Z, Zhou C, Wu M. Segregation analysis of esophageal cancer in a moderately high-incidence area of northern China. *Am J Hum Genet* 2000; **67**: 110-119

2 **Zhou XG**, Watanabe S. Factor analysis of digestive cancer mortality and food consumption in 65 Chinese countries. *J Epidemiol* 1999; **9**: 275-284

3 **Chang F**, Syrjanen S, Wang L, Syrjanen K. Infectious agents in the etiology of esophageal cancer. *Gastroenterology* 1992; **103**: 1336-1348

4 **Lu J**, Lian S, Sun X, Zhang Z, Dai D, Li B, Cheng L, Wei J, Duan W. [A case-control study on the risk factors of esophageal cancer in Linzhou] *Zhonghua Liuxing Bingxue Zazhi* 2000; **21**: 434-436

5 **Enzinger PC**, Mayer RJ. Esophageal cancer. *N Engl J Med* 2003; **349**: 2241-2252

6 **Wang XM**, Jie ES, Ma YQ, Chen B, Guo XJ, A LMT, Qin JM, Li F, Yang L. A Case-control Study on The Risk Factors of Esophageal Cancer in Xinjiang Kazakh. *Zhongguo Gonggongweisheng Zaizhi* 2007; **23**: 737-738

7 **Lee JM**, Lee YC, Yang SY, Shi WL, Lee CJ, Luh SP, Chen CJ, Hsieh CY, Wu MT. Genetic polymorphisms of p53 and GSTP1,but not NAT2,are associated with susceptibility to squamous-cell carcinoma of the esophagus. *Int J Cancer* 2000; **89**: 458-464

8 **Liu G**, Zhou Q, Wang LD, Hong JY, Deng CJ, Wang YY, Zou JX. Blood clot as a DNA source for studying genetic polymorphism of human carcinogen-metabolizing enzymes. *World J Gastroenterol* 1998; **4** (Suppl 2): 108-109

9 **Wang AH**, Sun CS, Li LS, Huang JY, Chen QS, Xu DZ. Genetic susceptibility and environmental factors of esophageal cancer in Xi'an. *World J Gastroenterol* 2004; **10**: 940-944

10 **Gao CM**, Takezaki T, Wu JZ, Chen MB, Liu YT, Ding JH, Sugimura H, Cao J, Hamajima N, Tajima K. CYP2E1 Rsa I polymorphism impacts on risk of colorectal cancer association with smoking and alcohol drinking. *World J Gastroenterol* 2007; **13**: 5725-5730

11 **Gao C**, Takezaki T, Wu J, Li Z, Wang J, Ding J, Liu Y, Hu X, Xu T, Tajima K, Sugimura H. Interaction between cytochrome P-450 2E1 polymorphisms and environmental factors with risk of esophageal and stomach cancers in Chinese. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 29-34

12 **Bailey LB**, Gregory JF 3rd. Polymorphisms of methylene tetrahydrofolate reductase and other enzymes: metabolic significance, risks and impact on folate requirement. *J Nutr* 1999; **129**: 919-922

13 **Goyette P**, Sumner JS, Milos R, Duncan AM, Rosenblatt DS, Matthews RG, Rozen R. Human methylenetetrahydrof olate reductase: isolation of cDNA, mapping and mutation identification. *Nat Genet* 1994; **7**: 195-200

14 **Frosst P**, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; **10**: 111-113

15 **Choi SW**, Mason JB. Folate and carcinogenesis: an integrated scheme. *J Nutr* 2000; **130**: 129-132

16 **Bartsch H**, Montesano R. Relevance of nitrosamines to human cancer. *Carcinogenesis* 1984; **5**: 1381-1393

17 **Guengerich FP**, Kim DH, Iwasaki M. Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* 1991; **4**: 168-179

18 **N-nitrosodiethanolamine**. *IARC Monogr Eval Carcinog Risks Hum* 2000; **77**: 403-438

19 **Pickled vegetables**. *IARC Monogr Eval Carcinog Risks Hum* 1993; **56**: 83-113

20 **Radha Rama Devi A**, Govindaiah V, Ramakrishna G, Naushad SM. Prevalence of methylene tetrahydro-folate reductase polymorphism in South Indian population. *Cur Sci* 2004; **86**: 440-443

21 **Xing D**, Tan W, Lin D. Genetic polymorphisms and susceptibility to esophageal cancer among Chinese population (review). *Oncol Rep* 2003; **10**: 1615-1623

22 **Qin JM**, Wang XM, Chen B, Yang L, Li F, He L, Liao PH. [Study on the ingestion of folate and polymorphism of MTHFR C677T with esophageal cancer in Xinjiang Kazakh] *Zhonghua Liuxing Bingxue Zazhi* 2008; **29**: 30-33

23 **Chen B**, Ma YQ, Yang L, Li F, Wang XM, Liao PH, He L, Qin JM. Relationship of CYP2E1 gene polymorphism and tobacco and alcohol consumption with susceptibility to esophageal cancer in Kazakh. *Shijie Huaren Xiaohua Zazhi* 2007; **15**: 3852-3855

24 **Hiyama T**, Yoshihara M, Tanaka S, Chayama K. Genetic polymorphisms and esophageal cancer risk. *Int J Cancer* 2007; **121**: 1643-1658

25 **Song C**, Xing D, Tan W, Wei Q, Lin D. Methylenetetrahydro folate reductase polymorphisms increase risk of esophageal squamous cell carcinoma in a Chinese population. *Cancer Res* 2001; **61**: 3272-3275

26 **Stolzenberg-Solomon RZ**, Qiao YL, Abnet CC, Ratnasinghe DL, Dawsey SM, Dong ZW, Taylor PR, Mark SD. Esophageal and gastric cardia cancer risk and folate- and vitamin B(12)-related polymorphisms in Linxian, China. *Cancer Epidemiol Biomarkers Prev* 2003; **12**: 1222-1226

27 **Zhang J**, Zotz RB, Li Y, Wang R, Kiel S, Schulz WA, Wen D, Chen Z, Zhang L, Wang S, Gabbert HE, Sarbia M. Methylenetetrahydrofolate reductase C677T polymorphism and predisposition towards esophageal squamous cell carcinoma in a German Caucasian and a northern Chinese population. *J Cancer Res Clin Oncol* 2004; **130**: 574-580

28 **Yang CX**, Matsuo K, Ito H, Shinoda M, Hatooka S, Hirose K, Wakai K, Saito T, Suzuki T, Maeda T, Tajima K. Gene-environment interactions between alcohol drinking and the MTHFR C677T polymorphism impact on esophageal cancer risk: results of a case-control study in Japan. *Carcinogenesis* 2005; **26**: 1285-1290

29 **Nishimoto IN**, Hanaoka T, Sugimura H, Nagura K, Ihara M, Li XJ, Arai T, Hamada GS, Kowalski LP, Tsugane S. Cytochrome P450 2E1 polymorphism in gastric cancer in Brazil: case-control studies of Japanese Brazilians and non-Japanese Brazilians. *Cancer Epidemiol Biomarkers Prev* 2000; **9**: 675-680

30 **Shi Y**, Zhou XW, Zhou YK, Ren X. Analysis of CYP2 E1, gSTM1 genetic polymorphisms in relation to human lung cancer and esophageal carcinoma. *J Huazhong Univ Sci Tech* 2002; **31**: 14-17

31 **Marchand LL**, Wilkinson GR, Wilkens LR. Genetic and dietary predictors of CYP2E1 activity: a phenotyping study in Hawaii Japanese using chlorzoxazone. *Cancer Epidemiol Biomarkers Prev* 1999; **8**: 495-500

32 **Lin DX**, Tang YM, Peng Q, Lu SX, Ambrosone CB, Kadlubar FF. Susceptibility to esophageal cancer and genetic polymorphisms in glutathione S-transferases T1, P1, and M1 and cytochrome P450 2E1. *Cancer Epidemiol Biomarkers Prev* 1998; **7**: 1013-1018

33 **Tan W**, Song N, Wang GQ, Liu Q, Tang HJ, Kadlubar FF,

Lin DX. Impact of genetic polymorphisms in cytochrome P450 2E1 and glutathione S-transferases M1, T1, and P1 on susceptibility to esophageal cancer among high-risk individuals in China. *Cancer Epidemiol Biomarkers Prev* 2000; **9**: 551-556

34 **Gao CM**, Li SP, Su P, Li ZY, Wan JD, Hu X, Wu JZ, Ding JH, Liu YL. The Impact of CYP2E1 Rsa I, GSTT1 and GSTM1 polymorphisms on the risk of esophageal cancer. *Zhongguo Zhongliu Zazhi* 2001; **10**: 346-349

35 **Lu XM**, Zhang YM, Lin RY, Arzi G, Wang X, Zhang YL, Zhang Y, Wang Y, Wen H. Relationship between genetic polymorphisms of metabolizing enzymes CYP2E1, GSTM1 and Kazakh's esophageal squamous cell cancer in Xinjiang, China. *World J Gastroenterol* 2005; **11**: 3651-3654

36 **Gao C**, Takezaki T, Wu J, Li Z, Wang J, Ding J, Liu Y, Hu X, Xu T, Tajima K, Sugimura H. Interaction between cytochrome P-450 2E1 polymorphisms and environmental factors with risk of esophageal and stomach cancers in Chinese. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 29-34

37 **Ribeiro Pinto LF**, Teixeira Rossini AM, Albano RM, Felzenszwalb I, de Moura Gallo CV, Nunes RA, Andreollo NA. Mechanisms of esophageal cancer development in Brazilians. *Mutat Res* 2003; **544**: 365-373

38 **Casson AG**, Zheng Z, Chiasson D, MacDonald K, Riddell DC, Guernsey JR, Guernsey DL, McLaughlin J. Associations between genetic polymorphisms of Phase I and II metabolizing enzymes, p53 and susceptibility to esophageal adenocarcinoma. *Cancer Detect Prev* 2003; **27**: 139-146

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RAPID COMMUNICATION

Effects of fluoxetine on mast cell morphology and protease-1 expression in gastric antrum in a rat model of depression

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Abstract

AIM: To investigate the effects of fluoxetine on depression-induced changes of mast cell morphology and protease-1 (rMCP-1) expression in rats.

METHODS: A Sprague-Dawley rat model of chronic stress-induced depression was established. Fifty experimental rats were randomly divided into the following groups: normal control group, fluoxetine + normal control group, depressed model group, saline + depressed model group, and fluoxetine + depressed model group. Laser scanning confocal microscopy (LSCM) immunofluorescence and RT-PCR techniques were used to investigate rMCP-1 expression in gastric antrum. Mast cell morphology was observed under transmission electron microscopy. ANOVA was used for statistical analysis among groups.

RESULTS: Morphologic observation indicated that depression induced mast cell proliferation, activation, and granule hyperplasia. Compared with the normal control group, the average immunofluorescence intensity of gastric antrum rMCP-1 significantly increased in depressed model group (37.4 ± 7.7 *vs* 24.5 ± 5.6, $P < 0.01$) or saline + depressed model group (39.9 ± 5.0 *vs* 24.5 ± 5.6, $P < 0.01$), while there was no significant difference between fluoxetine + normal control group (23.1 ± 3.4) or fluoxetine + depressed model group (26.1 ± 3.6) and normal control group. The average level of rMCP-1mRNA of gastric antrum significantly increased in depressed model group (0.759 ± 0.357 *vs* 0.476 ± 0.029, $P < 0.01$) or saline + depressed model group (0.781 ± 0.451 *vs* 0.476 ± 0.029, $P < 0.01$), while no significant difference was found between fluoxetine + normal control group (0.460 ± 0.027) or fluoxetine + depressed model group (0.488 ± 0.030) and normal control group. Fluoxetine showed partial inhibitive effects on mast cell ultrastructural alterations and de-regulated r*MCP-1* expression in gastric antrum of the depressed rat model.

CONCLUSION: Chronic stress can induce mast cell proliferation, activation, and granule hyperplasia in gastric antrum. Fluoxetine counteracts such changes in the depressed rat model.

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Key words: Depression model; Gastric antrum; Mast cell protease-1; Mast cells; Morphology; Fluoxetine hydrochloride

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INTRODUCTION

Mast cells are now recognized as "granular cells of the connective tissue", whose activation exacerbates allergic immune responses and as key players in the establishment of innate immunity as well as modulators of adaptive immune responses[1]. The role of mast cells in the gastrointestinal mucosa is not only to react to antigens, but also to actively regulate the barrier and transport properties of the intestinal epithelium. Mucosal mast cells respond to both IgE/antigen-dependent and non-IgE-dependent stimulation, releasing bioactive mediators into adjacent tissues where they induce physiological responses. Studies in models of hypersensitivity and stress

have provided evidence that changes in mucosal function are due to either direct action of mast cell mediators on epithelial receptors and/or indirect action *via* nerves/neurotransmitters[2]. Intestinal anaphylaxis is associated with disturbances in gut function that are antigen-specific and dependent on mast cell degranulation. During mucosal immunoglobulin E-mediated reactions, rat mast cell protease Ⅱ (rMCP-Ⅱ) is released and is associated with ultrastructural changes in the intestinal mucosa. The systemic appearance of this specific protease provides a serum marker of intestinal anaphylaxis. Psychological stress may trigger this sensitive alarm system *via* the brain-gut axis[3]. In clinical studies, it has become clear that psychological factors, especially anxiety and depression, play an important role in gastrointestinal diseases by precipitating exacerbation of symptoms[4,5]. Several studies have shown that the prevalence of chronic stressed disorders in patients with gastrointestinal symptoms is 60%-85%[6,7]. Stress often worsens the symptoms of gastrointestinal diseases, which might be explained by altered neuroendocrine and visceral sensory responses to stress[8].

Fluoxetine hydrochloride (fluoxetine) is a kind of selective serotonin reuptake inhibitors (SSRIs), which belong to a class of antidepressants used in the treatment of depression and anxiety disorders. SSRIs increase the extracellular expression of the neurotransmitter serotonin by inhibiting its reuptake into the presynaptic cells. Studies have suggested that SSRIs may promote the growth of new neural pathways or neurogenesis[9]. SSRIs may also protect against neurotoxicity caused by other compounds as well as from depression itself. Recent studies showed that pro-inflammatory cytokine processes took place during depression in addition to somatic diseases and it was possible that symptoms manifested in these psychiatric illnesses were being attenuated by the pharmacological effects of antidepressants on the immune system[10] SSRIs have been found to be immunomodulatory and anti-inflammatory against pro-inflammatory cytokine processes[11,12].

The aim of this study is to investigate the effects of fluoxetine hydrochloride (fluoxetine) on mast cell morphology and rMCP-1 expression in gastric antrum in a rat model of depression.

MATERIALS AND METHODS

Animals

Fifty healthy male Sprague-Dawley rats, weighing 250 ± 300 g, from the Animal Center, Hubei Academy of Preventive Medical Sciences, were used in the present study. The animals were fed standard rat chow, allowed access to tap water and acclimatized to the surroundings for 1 wk prior to the experiments.

Reagents

Cy3-conjugated goat anti-rabbit IgG, rMCP-1 rabbit anti-mouse antibody were purchased from Sigma Co., USA. Fluoxitine hydrochloride capsule was purchased from Lilly Co. Ltd. Other reagents used in the study were all of analytical grade.

Experimental protocols

All procedures were approved by the Animal Care Committee at the Medical Department of Wuhan University. A rat model of chronic stress-induced depression was established[13,14]. The rats received a variety of stressors for 21 d, including tail nip for 1 min, cold water swimming at 4℃ for 5 min, heat stress at 45℃ for 5 min, water deprivation for 24 h, food deprivation for 24 h, 12-h inverted light/dark cycle (7:00 a.m. lights off and 7:00 p.m. lights on), paw electric shock (electric current 1.0 mA10 s, every 1 min, lasting 10 s, 30 times). The animals were randomly divided into five groups (10 rats per group): normal control, fluoxetine + normal control, depressed model control, saline + depressed model, and fluoxetine + depressed model. The depressed animals were treated with saline and fluoxetine (10 mg/kg), respectively. A normal control group of rats without receiving any stress was included and housed in a separate room; food and water were freely available in their home cage.

Immunofluorescence histochemistry

The rats were anesthetized with urethane (5 mg/kg ip.) and rapidly killed by decapitation. The gastric antrum samples (1 cm × 1 cm) were perfused with 4% paraformaldehyde for immunofluorescence histology from each group. Each sample was cut into 30 sections and each section was cut 50-μm thick using a vibratome. Serial sections were placed on slides, three to a slide. The sections were numbered from 1 to 30. Ten sections were incubated. The staining procedure was as follows: (1) the sections were washed in phosphate-buffered saline (PBS), then pretreated with 0.25% Triton X-100 for 30 min at 37℃ and rinsed in PBS; (2) incubation for 12 h at 4℃ in a 1:100 dilution of the primary antibody of rMCP-1 in PBS; and (3) incubation with 1:200 diluted secondary antibody (Cy3 -conjugated goat anti-rabbit IgG) in PBS for 1 h at 37℃.The sections were washed three times for 10 min after incubation steps 1 to 3, respectively, and were finally mounted in 50 g/L glycerin.

Detection was carried out according to the kit instructions (Leica SP2 TCS AOBS made in Germany). The specimens were excited with a laser beam at a wavelength of 492 nm (Cy3). The sections were observed under a laser scanning confocal microscope (LSCM) and analyzed with a Leica Q500IW image analysis system in terms of Cy3 fluorescent intensity.

Electron microscopic analysis

For electron microscopic analysis, gastric antrum tissue sections were fixed in modified Karnovsky's medium containing 2% paraformaldehyde, 3% glutaraldehyde and 0.1% tannic acid in 0.1 mmol/L phosphate buffer (pH 7.4) and processed as before[15]. Each electron microscopic sample was divided into 5 blocks. Each block was cut into 10 sections (200 μm thick). Five sections selected

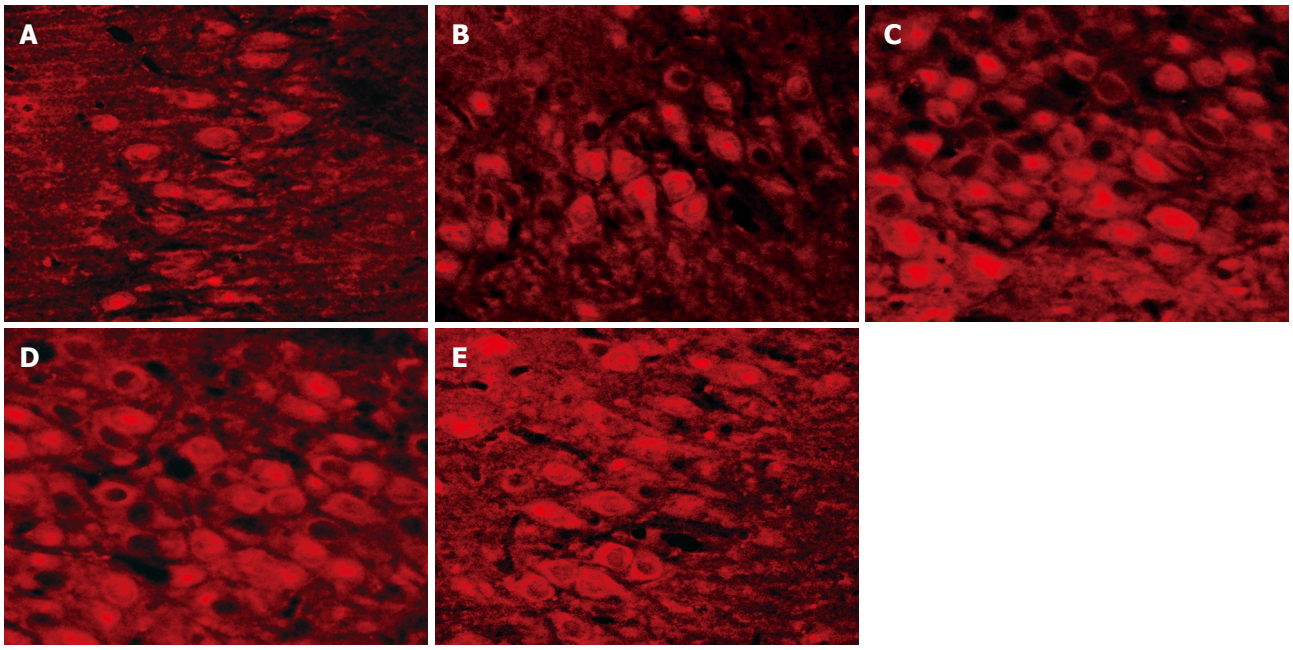

Figure 1 Gastric antrum tissue staining procedure include: (1) pretreatment with 0.25% Triton X-100; (2) incubation in the primary antibody of rMCP-1; (3) incubation with secondary antibody (Cy3 -conjugated goat anti-rabbit IgG). A: Expression of rMCP-1 in the normal control group; B: Expression of rMCP-1 in the fluoxetine + normal control group; C: Expression of rMCP-1 in the depressed model control group; D: Expression of rMCP-1 in the saline + depressed model group; E: Expression of rMCP-1 in the fluoxetine + depressed model group.

from 10 sections were observed. Ultrathin sections were placed onto copper gride, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (Hitachi H-600, Japan). Mast cells were evaluated according to Letourneau[16]. Mast cells containing many intact electron-dense granules or containing empty granules were categorized as inactive and active cells, respectively. All mast cells were counted at magnification × 4000 in 30 visual fields.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To quantify the expression of rMCP-1 RNA, we performed a RT-PCR assay as described previously[17]. Total mRNA was isolated with TRIZOL (Invitrogen) according to the instructions of manufacturer. The primers for RT-PCR were as follows: rMCP-1 (237 bp), forward primer 5'-GCCTGTAAAAACTATTTT-3'; reverse primer 5'-CAGGCTGGTCAGATCCTGC-3'. GAPDH (217bp), forward primer 5'-GAAACCTGCCAAGTATGATG-3'; reverse primer 5'-ACCAGGAAATGAGCTTGAGA-3'. The reaction mixture was added to the RNA solution and incubated at 42℃ for 1 h, heated at 94℃ for 5 min, and chilled at 48℃. For PCR, the cDNA reaction mixture was diluted with 40 ml of PCR buffer and mixed with 50 pmol of the primers. The reaction was carried out in a DNA thermal cycler under the following conditions: 94℃ for 30 s, 58℃ for 30 s and 72℃ for 45 s. Following the reaction, the amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized using ultravioletuorescence after staining with ethidium bromide. The relative content of rMCP-1 mRNA was calculated densitometrically based on the densitometric ratio between rMCP-1 and GAPDH.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was performed using one-way ANOVA and the non-parametric Mann-Whitney *U* test between groups. *P* values less than 0.05 were considered statistically significant.

RESULTS

Immunofluorescence histochemical assay

LSCM was used to prepare immunofluorescence picture, and LSCM imaging system was used to analyze the rMCP-1 immunofluorescence intensity among groups. Compared with the normal control group, the average immunofluorescence intensity of gastric antrum rMCP-1 significantly increased in depressed model group or saline + depressed model group (Figure 1, Table 1, $P < 0.01$), while there was no significant difference between fluoxetine + normal control group or fluoxetine + depressed model group and normal control group. Compared with depressed model group, the average immunofluorescence intensity of gastric antrum rMCP-1 significantly decreased in fluoxetine + depressed model group (Figure 1, Table 1, $P < 0.01$), while there was no significant difference between saline + depressed model group and depressed model group. This confirmed that chronic stress induced mast cells to secrete rMCP-1 and fluoxetine inhibited this effect.

Ultrastructural morphology analysis

Compared with the normal control rats, the total number of mast cells/30 visual fields and the percentage of activated mast cells increased significantly, while the percentage of normal mast cells decreased significantly

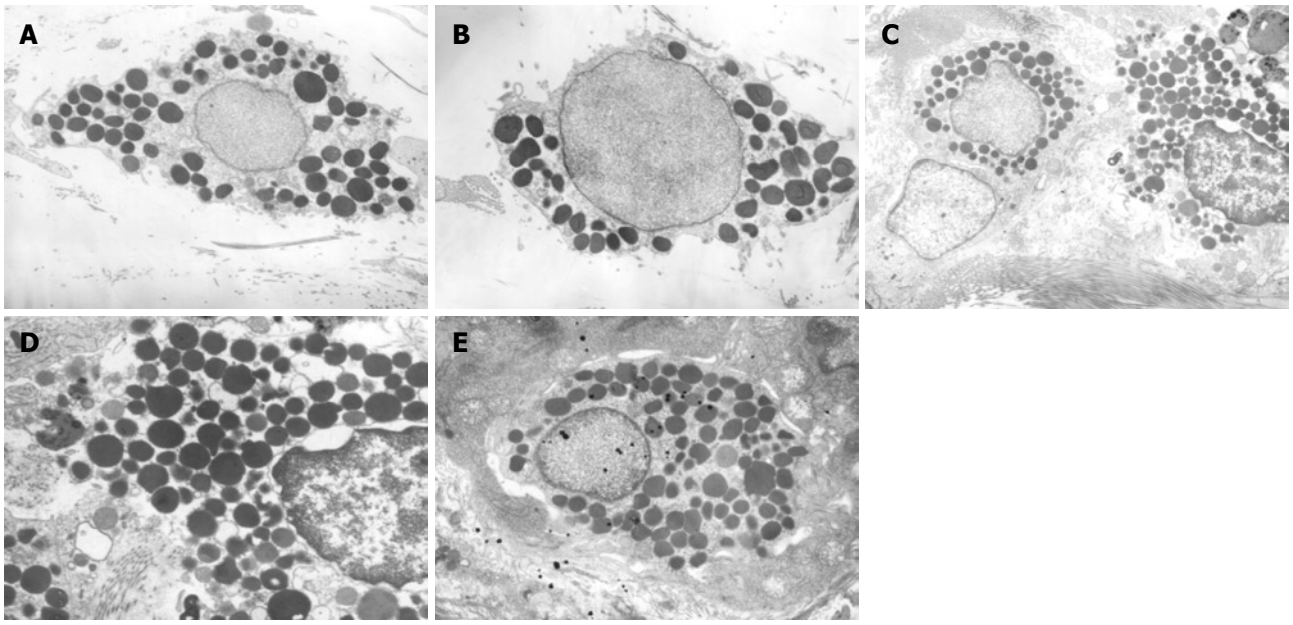

Figure 2 Electron photomicrographs of mast cells from the rat gastric antrum among groups. A: A control group (× 4000); B: A fluoxetine + normal control group (× 4000); C: A depressed model group (× 4000); D: A saline + depressed model group (× 6000); E: A fluoxetine + control group (× 4000).

Table 1 Average immunofluorescence intensity analysis of rMCP-1 alterations in gastric antrum of depressed rat model (n = 10, mean ± SE)

Group	Fluorescence intensity of MCP-1
Normal control	24.8 ± 5.6
Fluoxetine + control	23.1 ± 3.4[b]
Depressed	37.4 ± 7.7[d]
Saline + depressed	39.9 ± 5.0[d]
Fluoxetine + depressed	26.1 ± 3.6[b]

rMCP-1: rat mast cell protease- 1. [b]$P < 0.01$ *vs* depressed model control group; [d]$P < 0.01$ *vs* normal control group.

Table 2 Ultrastructural morphologic changes of gastric tissue mast cells (n = 10, mean ± SE)

Group	Mast cells/30 visual fields	Normal mast cells (%)	Activated mast cells (%)
Normal control	24.0 ± 3.5	64.6 ± 9. 9	35.4 ± 3.7
Fluoxetine + control	22.2 ± 3.4[c]	69.8 ± 6.1[c]	30.2 ± 3.7[c]
Depressed	40.6 ± 3.9[b]	33.3 ± 4.7[b]	66.7 ± 4.9[b]
Saline + depressed	42.2 ± 3.7[b]	30.4 ± 3.7[b]	69.6 ± 4.7[b]
Fluoxetine + depressed	28.1 ± 3.3[a,d]	44.4 ± 5.6[a,d]	55.6 ± 5.6[a,d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* normal control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* depressed model control group.

in chronic stress-induced depressed rats or saline + depressed model rats (Table 2, $P < 0.01$). In depressed rats treated with fluoxetine, the changes in total number of mast cells/30 visual fields and the percentage of activated or normal mast cells were between normal control group and depressed model rats (Table 2, $P < 0.05$).

In morphology, ultrastructural observations indicated that gastric antrum mast cells were rich and strictly in perivasculitis. In normal control rats or fluoxetine + normal control rats, the mast cells were spherical in shape with round nucleus, and contained electron-dense granules. Some secretary granules were intact with homogeneous electron dense content (Figure 2A and B). In chronic stress-induced depressed rats or saline + depressed rats, the mast cells were elongated with a fusiform nucleus, granules maldistributed and contained altered electron-dense content. The mast cells were proliferative, while the granules were also hyperplastic. Mast cell secretary granules exposed to the surface of the target and mast cells contained fibrillar material, empty granules and lipid bodies (Figure 2C and D). In depressed rats treated with fluoxetine, the morphological alterations were between normal control rats and depressed rats (Figure 2E).

Effects of fluoxetine on gastric antrum rMCP-1mRNA by RT-PCR

Compared with the normal control group, the average level of rMCP-1 mRNA of gastric antrum significantly increased in chronic stress-induced depressed model group or saline + depressed model group, while there was no significant difference between fluoxetine + normal control group or fluoxetine + depressed model group and normal control group. Compared with depressed model group, the average level of rMCP-1 mRNA of gastric antrum significantly decreased in fluoxetine + depressed model group, while there was no significant difference between saline + depressed model group and depressed model group (Figure 3, $P < 0.01$).

DISCUSSION

Mast cells are immunocytes, which are widely distributed throughout the gastrointestinal tract. Several stimuli (e.g.

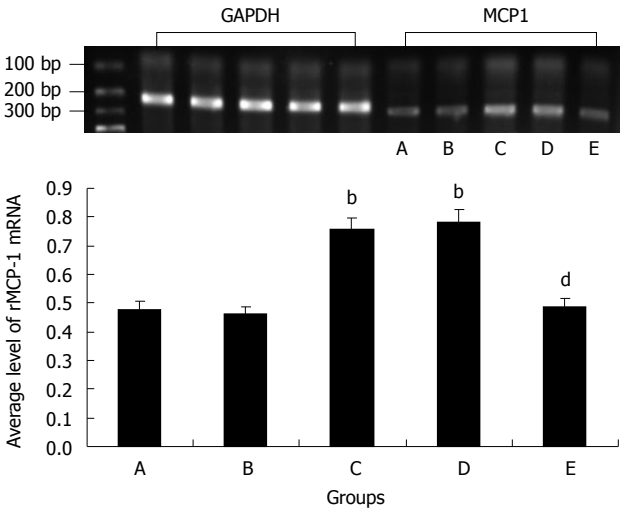

Figure 3 Photograph and the average level of rMCP-1 mRNA of rat gastric antrum by RT-PCR among groups. A: Control group; B: Fluoxetine + control group; C: Depressed model group; D: Saline + depressed model group; E: Fluoxetine + depressed model group; $^{b}P < 0.01$ *vs* normal control group; $^{d}P < 0.01$ *vs* depressed model control group. rMCP-1: rat mast cell protease-1.

allergens, neuropeptides and stress) lead to mast cell activation with consequent mediator release (e.g. histamine, protease, tryptase, chymase and prostanoids)[18,19]. A number of reports indicate that mast cells can be activated by acute stress[20]. Mast cell activation has also been reported in the intestine after repetitive exposure to odors, and acute exposure to cold, which is largely used to study gastric erosion formation induced by stress, and activates both gastric and colonic motility and transit in rats[21]. In this study, chronic stress induced rMCP-1 to release in depressed rats. Chronic unpredictable heterotypic stressors appeared to induce mast cell proliferation and activation, while mast cell granules proliferated. Mast cell secretary granules in depressed rats occurred more often, exposing to the surface of the target and mast cells contained fibrillar material, empty granules and lipid bodies. Studies on mast cells illustrated the remarkable facility of mast cell population to respond to the changes in the environment by significant alterations in multiple aspects of their phenotype, including morphology, mediator content, degranulation pattern and proliferative potential[22].

On the other hand, fluoxetine showed partial inhibitive effects on mast cell ultrastructural alterations and de-regulated r*MCP-1* mRNA expression in gastric antrum tissue in a rat model of depression. Fluoxetine is a serotonin reuptake inhibitor in the central nervous system as well as in mast cells. It depleted mast cell nuclear as well as cytoplasmic serotonin content[23]. Several researches found that antidepressants influenced histamine and serotonin secretion from rat peritoneal mast cells[24,25]. Maes[26] reported that various types of antidepressants, including SSRIs such as fluoxetine, had negative immunoregulatory effects. The negative immunoregulatory effects of antidepressants result from their effects on the cAMP-dependent protein kinase A (PKA) pathway. Chronic unpredicted mild stress can affect the PKA expression in rats and fluoxetine is antagonistic against it[27]. In addition, the effect of fluoxetine on r*MCP-1* expression was related to neuropeptide [e.g. substance P (SP), corticotropin-releasing hormone (CRH), vasoactive intestinal polypeptide (VIP)]. In severe depression, SP serum levels are increased[28]. The data indicate that SP serum levels might be related to response to antidepressant therapy[29]. In other reports, fluoxetine can improve depressed behavior, increase VIP expression and decrease CRH expression in plasma and the duodenal tissue of depressed rats. Clinically effective therapy with antidepressants normalizes the disturbed activity of the hypothalamic-pituitary-adrenal (HPA) axis, in part by decreasing SP, CRH or increasing VIP synthesis[30].

These findings will conduce to understand that chronic heterotypic stress may induce the immune responses in gastric mucosa. Treatment with fluoxetine can ameliorate pathological changes in gastric antrum of depressed rat model, suggesting that SSRIs are an effective therapeutic agent for some gastroduodenal diseases caused by psychological factors.

ACKNOWLEDGMENTS

We thank Dr. Shen-Lin Lei for his technical assistance.

COMMENTS

Background

In clinical studies, it has become clear that depression plays an important role in gastrointestinal diseases by precipitating exacerbation of symptoms. The stress may induce mast cell activation and degranulation.

Research frontiers

Some data strongly suggested that mast cells played an important role in pathophysiology of gastrointestinal diseases. Selective serotonin reuptake inhibitors (SSRIs) have been shown to be immunomodulatory and anti-inflammatory against proinflammatory cytokine processes.

Innovations and breakthroughs

The authors established a rat depression model, and observed the level of mast cell protease-1 (rMCP-1) expression in gastric antrum and the effects of fluoxetine on mast cell morphology and rMCP-1 expression in the depressed rats.

Applications

These findings will conduce to understand that chronic heterotypic stress may induce the immune responses in gastric mucosa. Treatment with fluoxetine can ameliorate pathological changes in gastric antrum of depressed rat model, suggesting that SSRIs are an effective therapeutic agent for some gastroduodenal diseases caused by psychological factors.

Terminology

Rat mast cell protease-1 (rMCP-1) is released and is associated with mast cell activation and degranulation.

Peer review

The authors of the present study showed that depression led to mast cell proliferation and activation in the gastric antrum. Fluoxetine counteracted such changes in gastric antrum in the depressed rat model.

REFERENCES

1 **Stelekati E**, Orinska Z, Bulfone-Paus S. Mast cells in allergy: innate instructors of adaptive responses. *Immunobiology* 2007; **212**: 505-519
2 **Yu LC**, Perdue MH. Role of mast cells in intestinal mucosal function: studies in models of hypersensitivity and stress. *Immunol Rev* 2001; **179**: 61-73
3 **Gui XY**. Mast cells: a possible link between psychological

stress, enteric infection, food allergy and gut hypersensitivity in the irritable bowel syndrome. *J Gastroenterol Hepatol* 1998; **13**: 980-989

4 **Mayer EA**, Craske M, Naliboff BD. Depression, anxiety, and the gastrointestinal system. *J Clin Psychiatry* 2001; **62** Suppl 8: 28-36; discussion 37

5 **Kurina LM**, Goldacre MJ, Yeates D, Gill LE. Depression and anxiety in people with inflammatory bowel disease. *J Epidemiol Community Health* 2001; **55**: 716-720

6 **Haug TT**, Mykletun A, Dahl AA. Are anxiety and depression related to gastrointestinal symptoms in the general population? *Scand J Gastroenterol* 2002; **37**: 294-298

7 **Sykes MA**, Blanchard EB, Lackner J, Keefer L, Krasner S. Psychopathology in irritable bowel syndrome: support for a psychophysiological model. *J Behav Med* 2003; **26**: 361-372

8 **Posserud I**, Agerforz P, Ekman R, Bjornsson ES, Abrahamsson H, Simren M. Altered visceral perceptual and neuroendocrine response in patients with irritable bowel syndrome during mental stress. *Gut* 2004; **53**: 1102-1108

9 **O'Brien SM**, Scully P, Scott LV, Dinan TG. Cytokine profiles in bipolar affective disorder: focus on acutely ill patients. *J Affect Disord* 2006; **90**: 263-267

10 **Obuchowicz E**, Marcinowska A, Herman ZS. [Antidepressants and cytokines--clinical and experimental studies] *Psychiatr Pol* 2005; **39**: 921-936

11 **Maes M**. The immunoregulatory effects of antidepressants. *Hum Psychopharmacol* 2001; **16**: 95-103

12 **Kubera M**, Lin AH, Kenis G, Bosmans E, van Bockstaele D, Maes M. Anti-Inflammatory effects of antidepressants through suppression of the interferon-gamma/interleukin-10 production ratio. *J Clin Psychopharmacol* 2001; **21**: 199-206

13 **Wang GH**, Dong HY, Dong WG, Wang XP, Luo HS, Yu JP. Protective effect of Radix Acanthopanacis Senticosi capsule on colon of rat depression model. *World J Gastroenterol* 2005; **11**: 1373-1377

14 **Yang PC**, Jury J, Soderholm JD, Sherman PM, McKay DM, Perdue MH. Chronic psychological stress in rats induces intestinal sensitization to luminal antigens. *Am J Pathol* 2006; **168**: 104-114; quiz 363

15 **Dimitriadou V**, Lambracht-Hall M, Reichler J, Theoharides TC. Histochemical and ultrastructural characteristics of rat brain perivascular mast cells stimulated with compound 48/80 and carbachol. *Neuroscience* 1990; **39**: 209-224

16 **Letourneau R**, Roznieckі JJ, Dimitriadou V, Theoharides TC. Ultrastructural evidence of brain mast cell activation without degranulation in monkey experimental allergic encephalomyelitis. *J Neuroimmunol* 2003; **145**: 18-26

17 **Ide H**, Itoh H, Tomita M, Murakumo Y, Kobayashi T, Maruyama H, Osada Y, Nawa Y. Cloning of the cDNA encoding a novel rat mast-cell proteinase, rMCP-3, and its expression in comparison with other rat mast-cell proteinases. *Biochem J* 1995; **311** (Pt 2): 675-680

18 **Barbara G**, Stanghellini V, De Giorgio R, Corinaldesi R. Functional gastrointestinal disorders and mast cells: implications for therapy. *Neurogastroenterol Motil* 2006; **18**: 6-17

19 **Brown JK**, Knight PA, Wright SH, Thornton EM, Miller HR. Constitutive secretion of the granule chymase mouse mast cell protease-1 and the chemokine, CCL2, by mucosal mast cell homologues. *Clin Exp Allergy* 2003; **33**: 132-146

20 **Theoharides TC**, Spanos C, Pang X, Alferes L, Ligris K, Letourneau R, Roznieckі JJ, Webster E, Chrousos GP. Stress-induced intracranial mast cell degranulation: a corticotropin-releasing hormone-mediated effect. *Endocrinology* 1995; **136**: 5745-5750

21 **Enck P**, Frieling T. Neurogastroenterology--information processing from the viscera to the brain in humans. *Dtsch Tierarztl Wochenschr* 1998; **105**: 468-471

22 **Galli SJ**. New insights into "the riddle of the mast cells": microenvironmental regulation of mast cell development and phenotypic heterogeneity. *Lab Invest* 1990; **62**: 5-33

23 **Csaba G**, Kovacs P, Pallinger E. Hormones in the nucleus. Immunologically demonstrable biogenic amines (serotonin, histamine) in the nucleus of rat peritoneal mast cells. *Life Sci* 2006; **78**: 1871-1877

24 **Ferjan I**, Erjavec F. Changes in histamine and serotonin secretion from rat peritoneal mast cells caused by antidepressants. *Inflamm Res* 1996; **45**: 141-144

25 **Purcell WM**, Hanahoe TH. The activity of amitriptyline as a differential inhibitor of amine secretion from rat peritoneal mast cells: the contribution of amine uptake. *Agents Actions* 1990; **30**: 41-43

26 **Maes M**, Kenis G, Kubera M, De Baets M, Steinbusch H, Bosmans E. The negative immunoregulatory effects of fluoxetine in relation to the cAMP-dependent PKA pathway. *Int Immunopharmacol* 2005; **5**: 609-618

27 **Wang Z**, Hu SY, Lei DL, Song WX. [Effect of chronic stress on PKA and P-CREB expression in hippocampus of rats and the antagonism of antidepressors] *Zhongnan Daxue Xuebao Yixueban* 2006; **31**: 767-771

28 **Bondy B**, Baghai TC, Minov C, Schule C, Schwarz MJ, Zwanzger P, Rupprecht R, Moller HJ. Substance P serum levels are increased in major depression: preliminary results. *Biol Psychiatry* 2003; **53**: 538-542

29 **Lieb K**, Walden J, Grunze H, Fiebich BL, Berger M, Normann C. Serum levels of substance P and response to antidepressant pharmacotherapy. *Pharmacopsychiatry* 2004; **37**: 238-239

30 **Budziszewska B**, Jaworska-Feil L, Tetich M, Basta-Kaim A, Kubera M, Leskiewicz M, Lason W. Regulation of the human corticotropin-releasing-hormone gene promoter activity by antidepressant drugs in Neuro-2A and AtT-20 cells. *Neuropsychopharmacology* 2004; **29**: 785-794

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CASE REPORT

Hydroxycut hepatotoxicity: A case series and review of liver toxicity from herbal weight loss supplements

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Abstract

Dietary supplements represent an increasingly common source of drug-induced liver injury. Hydroxycut is a popular weight loss supplement which has previously been linked to hepatotoxicity, although the individual chemical components underlying liver injury remain poorly understood. We report two cases of acute hepatitis in the setting of Hydroxycut exposure and describe possible mechanisms of liver injury. We also comprehensively review and summarize the existing literature on commonly used weight loss supplements, and their individual components which have demonstrated potential for liver toxicity. An increased effort to screen for and educate patients and physicians about supplement-associated hepatotoxicity is warranted.

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Key words: Hydroxycut; Dietary supplements; Liver; Liver failure; Toxicity; Weight loss; Medicine; Hepatitis

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INTRODUCTION

Obesity has become an increasingly important public health problem in the United States. Recent data show that more than 30% of adults are obese and 65% overweight[1]. The use of dietary supplements for weight loss has become increasingly popular, as reflected by the $55.4 billion spent in the U.S. in 2006 for weight loss and diet control[2,3]. Based on a study by the National Center for Complementary and Alternative Medicine (NCCAM), 36% of adults are using some form of complementary or alternative medicine, which rises to 62% when including megavitamins or prayer. Although dietary and herbal supplements are governed under the DSHEA act of 1994, they are not presently regulated by the U.S. Federal Drug Administration, and the safety profiles of many are unknown. An increasing number of case reports have emerged which suggest causative supplement-associated liver toxicity. Hydroxycut is an herbal weight loss supplement that has been suspected to have possible liver toxicity. Herein we present two patients who experienced severe acute hepatitis in the setting of documented Hydroxycut exposure, and without alternative etiology after comprehensive serologic liver evaluation.

CASE REPORTS

Case 1

A 40-year-old female with a prior medical history notable only for hypothyroidism and diet-controlled hyperlipidemia presented to the Emergency Department with 3 d of new-onset crampy, mid-epigastric abdominal pain and non-bloody diarrhea. She noted subjective fevers and chills, and two isolated episodes of nausea and vomiting, anorexia and profound fatigue. She did not experience jaundice, icterus, pruritus, arthralgias, acholic stools or dark urine. One week prior to presentation, she began using Hydroxycut, 6 pills daily in preparation for a bodybuilding competition. Just prior to presentation she attended an office holiday party, although no other persons in attendance became ill. She did not smoke or drink. She otherwise does not take regular medications except for levothyroxine. She denied taking any other supplements or alternative medications. She was afebrile with stable vital signs, and normal body mass index. Her exam was notable only for mild mid-epigastric tender-

ness to palpation. She had no liver enlargement and no stigmata of chronic liver disease. Her laboratory profile on admission revealed an acute hepatitis with AST 1020 U/L and ALT 1150 U/L, total bilirubin 0.67 mg/dL, alkaline phosphatase 299 U/L, INR 0.96, white cell count 5.9 × 10^3/μL, hemoglobin 11.9 g/dL, platelet count 228/μL, and creatinine 0.9 mg/dL. Diagnostic evaluation was negative for hepatitis A, B, C, cytomegalovirus and Epstein-Barr virus, autoimmune liver disorders (ANA, ASMA), alpha-1 anti-trypsin deficiency, and ehrlichiosis. On day 2 of admission, her transaminases decreased to AST 399 U/L and ALT 647 U/L. On day 3, she was clinically well and discharged from the hospital. Upon outpatient follow-up, she had returned to her usual state of health with normalization of transaminases with AST 46 U/L and ALT 48 U/L. She has not experienced any further recurrence of symptoms or liver abnormalities within 10 mo of follow-up.

Case 2

A 33-year-old female with a prior medical history of a pituitary adenoma presented to the Emergency Department with 1 mo of new-onset jaundice. She reported a flu-like illness of 2 wk duration with nausea and crampy abdominal pain and began to experience jaundice, acholic stools, dark-colored urine, pruritus, and profound fatigue. These symptoms appeared to be improving during the week prior to admission except for worsening jaundice and fatigue. She noted that during the month prior to admission, she had taken Hydroxcut supplements for 2 wk to help achieve weight loss, but discontinued this medication upon onset of symptoms. She additionally reported eating lobster during the month prior to admission, but could not recall other individuals who became ill. Her only medication was Ortho-Novum contraceptive, which she had been taking for 2.5 years. Her social history was unremarkable without regular alcohol ingestion, and the absence of risk factors for chronic viral hepatitis. She was afebrile with stable vital signs, and normal body mass index. Her exam was notable only for jaundice and scleral icterus. She had no liver enlargement and no stigmata of chronic liver disease. Her laboratory profile at admission was notable for acute hepatitis with AST 934 U/L and ALT 1 570 U/L, total bilirubin 20.9 mg/dL, direct bilirubin 14.2 mg/dL, alkaline phosphatase 112 U/L, INR 1.08, white cell count 9.2 × 10^3/μL, hematocrit 42%, platelet count 414/μL, creatinine 0.8 mg/dL. Diagnostic evaluation was negative for hepatitis A, B, C, cytomegalovirus, Epstein-Barr virus, and herpes simplex virus infections. Her autoimmune profile revealed low titer increase in antinuclear antibody (ANA) and anti-smooth muscle antibody (ASMA) suggestive of an immune-mediated drug-induced hepatitis. Her jaundice eventually resolved and her liver function normalized.

DISCUSSION

The public's increasing demand for alternative medicine, the newly found global interest in phytomedicine and herbal therapies, the rising cost of conventional prescription drugs, and a loss of faith in Western medicine, have led to a rapid rise in the use of unregulated herbal supplements and therapies. An estimated 80% of the world population uses herbal medicines, largely outside the U.S.[4]. In a study performed at an outpatient liver clinic, over 21% of patients were taking herbal supplements in the setting of chronic liver disease[5]. The FDA describes dietary supplements as a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet. The "dietary ingredients" in these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Most of these products have not been rigorously studied through placebo-controlled, blinded, randomized trials[6].

Table 1 Hydroxycut ingredients supplement facts (serving size 2 caplets)

Amount per serving	% daily value
Calcium (as hydroxycitrate) 156 mg	16[1]
Chromium (as polynicotinate) 133 mg	111[1]
Potassium (as hydroxycitrate) 218 mg	6[1]
Garcinia Cambogia (66% hydroxycitric acid)	[2]
Gymnema Sylvestre (25% gymnemic acid)	[2]
Soy Phospholipids	[2]
Rhodiola rosea extract (5% rosavins)	[2]
Green Tea as Camelia Sinensis (91 mg of EGCG)	[2]
White Tea as Camelia Sinensis (15% EGCG)	[2]
Oolong Tea as Camelia Sinensis (15% EGCG)	[2]
Caffeine anhydrous	[2]

[1]Percent daily values based on 2000 calory diet; [2]Daily values not established.

Hydroxycut is one of the most popular dietary supplements for weight loss on the market today, including two formulations Hydroxycut and Hydroxycut hardcore. Hydroxycut contains several different herbs, including: *Garcinia Cambogia* extract, chromium polynicotinate, *Gymnema sylvestre* extract and *Camellia Sinenesis* (*C. Sinenesis*) (Table 1). Both patients in this report used the dietary supplement Hydroxycut within a short time frame before presenting with acute hepatitis, suggesting Hydroxycut as the most likely etiology for acute liver injury. Neither patient had a history suggestive of other exposures or risk factors for viral hepatitis, alcoholic hepatitis, other toxin-mediated injury, or chronic liver disease. A comprehensive serologic and radiographic evaluation performed in both patients did not reveal alternative sources for liver toxicity. Although causation is difficult to confirm in cases of suspected drug-associated hepatotoxicity, the temporal relationship to acute liver injury and rapid resolution upon withdrawal of Hydroxycut make this likely. Hydroxycut has previously been associated with both a cholestatic and hepatocellular pattern of injury. The specific components likely implicated in liver toxicity include *G. Cambogia, Chromium*, and green tea root extract (*Camellia Sinensis*) based on prior data suggesting liver toxicity. Patient 1 experienced a more typical hepatocellular pattern of injury, patient 2 demonstrated an immune-mediated pattern of injury, which has not previously been described.

There has been one prior report of two cases of possible hepatotoxicity with Hydroxycut in the literature[7]; both cases were young males who had documented periods of Hydroxycut exposure and experienced similar clinical syndromes marked by fatigue, jaundice, and pruritus with marked hepatocellular or cholestatic pattern and complete resolution upon supplement withdrawal. Our case series validates the likely causative relationship between Hydroxycut exposure and liver toxicity, and further suggests that an autoimmune pattern of hepatotoxicity may be observed. Although less common, drug-associated autoimmune hepatitis has been reported in several herbal supplements including Greater Celadine, Dai-Saiko-To, and Black Cohosh. Herein we review key ingredients of Hydroxycut that have been implicated in liver toxicity.

G. Cambogia is a fruit native to southeastern Asia and western Africa used to make meals more "filling" [8]. Its main component hydroxycitric acid (HCA) is an inhibitor of the citrate cleavage enzyme (ATP citrate lyase) blocking de novo synthesis of fatty acids[9]. HCA was initially studied in rodents for the dietary treatment of obesity and the results seemed to be promising. Unfortunately randomized controlled trials in humans for this purpose showed very conflicting results. Nevertheless, HCA is a primary component of many weight loss supplements in the market, and similar to others in its class, the toxicity profile is poorly studied. In the recent literature a case of fatal liver failure was reported in a patient taking HCA and montelukast suggesting the synergistic hepatotoxic effect of these two agents[10]. There have also been reports of *G. Cambogia* toxicity by the WHO database, mostly describing an increase in hepatic enzymes[6].

Chromium is an essential trace element and cofactor to insulin most commonly occurring in hexavalent (Ⅵ) and trivalent (Ⅲ) states. The hexavalent form is found in the dye and leather industry and is responsible for occupational toxicity ranging from dermatitis to lung cancer[11]. In 1989, the National Academy of Sciences established an "estimated safe and adequate daily dietary intake" range for chromium of 50 to 200 mg[12]. In 2001 the Institute of Medicine and the National Academy of Sciences established Dietary Reference Intakes (DRI) for Chromium, ranging from 0.2 mg for infants to a maximum of 45 mg in lactating mothers[13]. Chromium is used in weight loss supplements due to purported effects of decreasing body fat and increasing basal metabolic rate[14,15]. A recent meta-analysis of available RCTs concluded that weight reduction with chromium although statistically significant was not clinically meaningful[2]. There have been case reports of chromium toxicity causing acute hepatitis, thrombocytopenia and renal failure due to both environmental[16,17] and dietary supplements[18]. Although renal failure requiring dialysis is a more common concern[19], those presenting with liver toxicity frequently elaborate aminotransferase elevations greater than 1000 mg/dL. Each Hydroxycut serving contains 133 mg of Chromium, which is taken three times daily, resulting in a cumulative daily consumption grater than twice the NAS safe maximum dose.

Camellia Sinensis is the scientific name for green tea, which is widely regarded by the public as safe, and commonly incorporated into supplements due to purported anti-cancer potential[20], weight reduction[21] and antioxidant properties[22]. Acute hepatotoxicity from *C. Sinensis* is well-described, and may range from acute hepatitis to acute liver failure. Based on 17 published cases in the literature[23-27], most cases appear to occur following large ingestions of green tea, with resolution following withdrawal, and recurrence with re-challenge[28]. Consequently, *C. Sinesis* has been banned in France and Spain, although it remains unregulated in weight loss supplements commercially available in the U.S.

Other Hydroxycut components for which liver toxicity have not been described include *Gymnema Sylvestre, Rhodiola Rosea*, and *Withania Somnifera. Gymnema Sylvestre* has been used to control hyperglycemia[29] and hyperlipidemia[30] in rats. Toxicity studies in rodents have not shown hepatotoxicity[31] and no case report of liver injury has been reported. Rhodiola Rosea extract is used to decrease fatigue[32] and improve exercise tolerance[33]. Withania Somnifera has been used for its anti-inflammatory properties[34,35]. Neither *R Rosea* or *W Somnifera* have been associated with liver toxicity, although *W Somnifera* may result in renal impairment in rats[36].

Of note, the more concentrated Hydroxycut Hard Core product contains additional herbal formulations such as White Willow extract and Yohimbine. These have not been demonstrated to result in liver injury, whereas Willow bark extract may have anti-inflammatory effects *in vitro*, this was not observed in patients with rheumatoid arthritis or osteoarthritis in a randomized controlled trial[37]. Yohimbine is an indole alkaloid from the bark of the African *Pausinystalia Yohimbe*, and serves as an alpha-2 receptor antagonist which may treat male impotence[38]. Although nausea, vomiting and abdominal pain have been described, liver toxicity has not been observed.

The FDA has issued warnings on several herbal supplements known to have hepatotoxic potential including Comfrey (2001), Kava (2002) and the dietary supplement Lipokinetix (2001). This review does not seek to provide a comprehensive review of all known hepatotoxins, but highlight a short list of ingredients within best selling weight loss supplements which have been demonstrated to have hepatotoxic potential.

Ephedra alkaloid, AKA *Ma Huang*, is the most commonly used weight loss supplement in the U.S.[4], and is well-known to have potentially deleterious cardiovascular and CNS effects[39], but has also been implicated in numerous cases of liver failure[40,41]. As such, dietary supplements containing ephedra were banned in the U.S. in April 2004[42], but remain widely available through unregulated internet sources in various forms, including the supplement *Leptoprin* (previously *Anorex*) which contains 20 mg of ephedrine, 200 mg of caffeine, 324 mg of aspirin, and unknown amounts of Green tea and cayenne. *Adipokinitex* came to the market to replace *Lipokinetix* which was removed in 2002 by Syntrax Innovations Inc. due to FDA

Table 2 Components of popular weight loss supplements

Brand name	Potentially hepatotoxic components
Hydroxycut	*Garcinia Cambogia*, Chromium, *Camelia Sinensis*
Ephedrasile Hardcore	*C. Sinensis*, Valerian, St John's wort
Zalestrim	C. Sinensis. Black Cohosh, Dong Quai
Slim-Citi	Hoodia
Ephedra	Mau Hang
Leptoril	*C. Sinenis,* Aspirin
Adipokinetix	*C. Sinensis*
Xenadrine	*C. Sinensis,* Ma Huang
Lipovox	*C. Sinensis*
Lean fire	*C. Sinensis*
Miracle Burn	Hoodia
7 DFB	Noni extract
Curvatrim	*C. Sinensis*, Dong Quai
Ambi-Slim	*C. Sinensis*, Garcinia Cambogia, Chromium, Valerian
TrimSpaX32	*C. Sinensis*, Hoodia Gordoni
Eroved	Hoodia, Garcinia Cambogia, Chromium
Zylorin	Hoodia, C. Sinensis, Chromium
Jet Fuel	*C. Sinensis,* Hoodia,
VPX redline	*C. Sinensis*
Metabolene	*C. Sinensis,* Chromium, Hoodia

Table 3 Patterns of injury in herbal hepatotoxicity

	Cholestatic	Hepatocellular	Autoimmune	Fulminant failure
Atractylis Gummifera[50]				X
Black Cohosh [51,52]			X	X
Callilepsis Laureola[53]				X
Camelia Sinensis[24-28]		X		X
Chapparral[54]	X			X
Chromium picolinate[18,19]		X		
Cascara Sagrada[55]	X			
Dai-Saiko-To[5]			X	
Garcinia Cambogia[7]	X	X		
Germander[56,57]		X		X
Greater Celadine[58,59]	X		X	
Jin Bu Huan[60]	X			
Kava[61-63]		X		
Noni[64]		X		
Paeonia[65]		X		
Paulina Cupana		X		
Penny Royal[66,67]				X
Senna[68]		X		
Shou wu pian[5]		X		
Syo-Saiko-To[69]	X	X		
Teucrium Polium[5]				X
Usnic Acid[70,71]		X		X
Valerian[72]		X		

warnings regarding reported cases of hepatotoxicity[43]. Although the Usnic acid has been removed from the formulation, this diet pill still contains Norephedrine or Norphenephrine and Green tea extracts. *Ephedrasil Hardcore*, a top selling diet pill, is a newer formulation of Ephedra following its ban in 2004, and contains many ingredients including Chromium, Yohimbine, and green tea extract.

Hoodia Gordoni, a popular supplement used for appetite suppression, and is derived from a cactus-like bush leaf native to southern Africa. Although initially isolated by Pfizer in the 1970s, research was discontinued due to liver toxicity found in early research studies. Despite these reports, this is a common ingredient in dietary supplements such as *Slim-Citi, Trim Spa X32* and *Ephedrine-P57*.

Zalestrim is another top selling supplement composed of green tea and Black Cohosh, which are described above as known sources of potential hepatotoxicity[44-48]. It also contains *Dong Quai,* which increases prothrombin time and thereby increases bleeding risk[49].

In summary, this case series and review of the literature highlight the potential hepatotoxicity of commonly used herbal supplements including Hydroxycut. Due to their loose regulation in the current drug marketplace, oversight of their use by physicians and regulators alike remains poor. Those supplements with potential liver toxicity are summarized in Tables 2 and 3, although further investigation is needed to better clarify the mechanisms and patterns of injury, and inform policy makers on those ingredients which require more vigorous regulation. The use of supplements is frequently not queried by physicians, and may not be reported in the same manner as prescription drugs by patients. As such, it is advisable that physicians ask specifically about the use of non-prescription drugs and supplements, warn patients with known liver disease about the potential consequences of their use, and to query patients specifically about possible supplement exposure in cases of acute or chronic liver injury. Increased attention to this issue by physicians and regulatory agencies may lead to more successful efforts to decrease the burden of drug-associated liver injury in the U.S.

REFERENCES

1 **National Center for Health Statistics**. Prevalence of Overweight and Obesity Among Adults: United States, 1999-2002. Available from: URL: http://www.cdc.gov/nchs/products/pubs/pubd/hestats/obese/obse99.htm

2 **Blanck HM**, Khan LK, Serdula MK. Use of nonprescription weight loss products: results from a multistate survey. *JAMA* 2001; **286**: 930-935

3 **Miles J**, Petrie C, Steel M. Slimming on the Internet. *J R Soc Med* 2000; **93**: 254-257

4 **Farnsworth NR**, Akerele O, Bingel AS, Soejarto DD, Guo Z. Medicinal plants in therapy. *Bull World Health Organ* 1985; **63**: 965-981

5 **Stickel F**, Patsenker E, Schuppan D. Herbal hepatotoxicity. *J Hepatol* 2005; **43**: 901-910

6 **Pittler MH**, Ernst E. Dietary supplements for body-weight reduction: a systematic review. *Am J Clin Nutr* 2004; **79**: 529-536

7 **Stevens T**, Qadri A, Zein NN. Two patients with acute

liver injury associated with use of the herbal weight-loss supplement hydroxycut. *Ann Intern Med* 2005; **142**: 477-478
8 **Burdock G**, Soni M, Bagchi M, Bagchi D. Garcinia cambogia toxicity is misleading. *Food Chem Toxicol* 2005; **43**: 1683-1684; author reply 1685-1686
9 **Heymsfield SB**, Allison DB, Vasselli JR, Pietrobelli A, Greenfield D, Nunez C. Garcinia cambogia (hydroxycitric acid) as a potential antiobesity agent: a randomized controlled trial. *JAMA* 1998; **280**: 1596-1600
10 **Actis GC**, Bugianesi E, Ottobrelli A, Rizzetto M. Fatal liver failure following food supplements during chronic treatment with montelukast. *Dig Liver Dis* 2007; **39**: 953-955
11 **Katz SA**, Salem H. The toxicology of chromium with respect to its chemical speciation: a review. *J Appl Toxicol* 1993; **13**: 217-224
12 **National Research Council (U.S.)**. Subcommittee on the Tenth Edition of the RDAs, National Institutes of Health (U.S.), National Research Council (U.S.). Committee on Dietary Allowances. Recommended dietary allowances. 10th rev. ed. Washington, D.C.: National Academy Press, 1989: 241-243
13 **Institute of Medicine FaNB, ebrary Inc.** DRI. dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc: a report of the Panel on Micronutrients ... and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine. 2001: xxii, 773 p. Available from: URL: http://site.ebrary.com/lib/yale/Doc?id=10032471
14 **Anderson RA**. Essentiality of chromium in humans. *Sci Total Environ* 1989; **86**: 75-81
15 **Anderson RA**. Effects of chromium on body composition and weight loss. *Nutr Rev* 1998; **56**: 266-270
16 **Fristedt B**, Lindqvist B, Schuetz A, Ovrum P. Survival in a case of acute oral chromic acid poisoning with acute renal failure treated by haemodialysis. *Acta Med Scand* 1965; **177**: 153-159
17 **van Heerden PV**, Jenkins IR, Woods WP, Rossi E, Cameron PD. Death by tanning--a case of fatal basic chromium sulphate poisoning. *Intensive Care Med* 1994; **20**: 145-147
18 **Cerulli J**, Grabe DW, Gauthier I, Malone M, McGoldrick MD. Chromium picolinate toxicity. *Ann Pharmacother* 1998; **32**: 428-431
19 **Wasser WG**, Feldman NS, D'Agati VD. Chronic renal failure after ingestion of over-the-counter chromium picolinate. *Ann Intern Med* 1997; **126**: 410
20 **Yang CS**, Yang GY, Chung JY, Lee MJ, Li C. Tea and tea polyphenols in cancer prevention. *Adv Exp Med Biol* 2001; **492**: 39-53
21 **Shixian Q**, VanCrey B, Shi J, Kakuda Y, Jiang Y. Green tea extract thermogenesis-induced weight loss by epigallocatechin gallate inhibition of catechol-O-methyltransferase. *J Med Food* 2006; **9**: 451-458
22 **Coimbra S**, Castro E, Rocha-Pereira P, Rebelo I, Rocha S, Santos-Silva A. The effect of green tea in oxidative stress. *Clin Nutr* 2006; **25**: 790-796
23 **Abu el Wafa Y**, Benavente Fernandez A, Talavera Fabuel A, Perez Ramos MA, Ramos-Clemente JI. [Acute hepatitis induced by Camellia sinensis (green tea)] *An Med Interna* 2005; **22**: 298
24 **Duenas Sadornil C**, Fabregas Puigtio S, Durandez R. [Hepatotoxicity due to Camelia sinensis] *Med Clin* (Barc) 2004; **122**: 677-678
25 **Garcia-Moran S**, Saez-Royuela F, Gento E, Lopez Morante A, Arias L. [Acute hepatitis associated with Camellia thea and Orthosiphon stamineus ingestion] *Gastroenterol Hepatol* 2004; **27**: 559-560
26 **Gloro R**, Hourmand-Ollivier I, Mosquet B, Mosquet L, Rousselot P, Salame E, Piquet MA, Dao T. Fulminant hepatitis during self-medication with hydroalcoholic extract of green tea. *Eur J Gastroenterol Hepatol* 2005; **17**: 1135-1137
27 **Molinari M**, Watt KD, Kruszyna T, Nelson R, Walsh M, Huang WY, Nashan B, Peltekian K. Acute liver failure induced by green tea extracts: case report and review of the literature. *Liver Transpl* 2006; **12**: 1892-1895
28 **Bonkovsky HL**. Hepatotoxicity associated with supplements containing Chinese green tea (Camellia sinensis). *Ann Intern Med* 2006; **144**: 68-71
29 **Yeh GY**, Eisenberg DM, Kaptchuk TJ, Phillips RS. Systematic review of herbs and dietary supplements for glycemic control in diabetes. *Diabetes Care* 2003; **26**: 1277-1294
30 **Luo H**, Kashiwagi A, Shibahara T, Yamada K. Decreased bodyweight without rebound and regulated lipoprotein metabolism by gymnemate in genetic multifactor syndrome animal. *Mol Cell Biochem* 2007; **299**: 93-98
31 **Ogawa Y**, Sekita K, Umemura T, Saito M, Ono A, Kawasaki Y, Uchida O, Matsushima Y, Inoue T, Kanno J. [Gymnema sylvestre leaf extract: a 52-week dietary toxicity study in Wistar rats] *Shokuhin Eiseigaku Zasshi* 2004; **45**: 8-18
32 **Darbinyan V**, Kteyan A, Panossian A, Gabrielian E, Wikman G, Wagner H. Rhodiola rosea in stress induced fatigue--a double blind cross-over study of a standardized extract SHR-5 with a repeated low-dose regimen on the mental performance of healthy physicians during night duty. *Phytomedicine* 2000; **7**: 365-371
33 **De Bock K**, Eijnde BO, Ramaekers M, Hespel P. Acute Rhodiola rosea intake can improve endurance exercise performance. *Int J Sport Nutr Exerc Metab* 2004; **14**: 298-307
34 **Rasool M**, Varalakshmi P. Immunomodulatory role of Withania somnifera root powder on experimental induced inflammation: An in vivo and in vitro study. *Vascul Pharmacol* 2006; **44**: 406-410
35 **Spelman K**, Burns J, Nichols D, Winters N, Ottersberg S, Tenborg M. Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. *Altern Med Rev* 2006; **11**: 128-150
36 **Arseculeratne SN**, Gunatilaka AA, Panabokke RG. Studies of medicinal plants of Sri Lanka. Part 14: Toxicity of some traditional medicinal herbs. *J Ethnopharmacol* 1985; **13**: 323-335
37 **Biegert C**, Wagner I, Ludtke R, Kotter I, Lohmuller C, Gunaydin I, Taxis K, Heide L. Efficacy and safety of willow bark extract in the treatment of osteoarthritis and rheumatoid arthritis: results of 2 randomized double-blind controlled trials. *J Rheumatol* 2004; **31**: 2121-2130
38 **Thomas C**. Westfall and David P. Westfall. Adrenergic receptor antagonist: Yohimbine. In: Goodman LS, Gilman A, Gilman AG, editors. Goodman and Gilman's the pharmacological basis of therapeutics. 11th ed. New York: The McGraw-Hill Companies, Inc, 2006: 271
39 **Haller CA**, Benowitz NL. Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids. *N Engl J Med* 2000; **343**: 1833-1838
40 **Borum ML**. Fulminant exacerbation of autoimmune hepatitis after the use of ma huang. *Am J Gastroenterol* 2001; **96**: 1654-1655
41 **Nadir A**, Agrawal S, King PD, Marshall JB. Acute hepatitis associated with the use of a Chinese herbal product, ma-huang. *Am J Gastroenterol* 1996; **91**: 1436-1438
42 **US Food and Drug Administration**. FDA Issues Regulation Prohibiting Sale of Dietary Supplements Containing Ephedrine Alkaloids and Reiterates Its Advice That Consumers Stop Using These Products. 2004 [cited 2007 8th November]; Available from: URL: http://www.fda.gov/bbs/topics/NEWS/2004/NEW01021.html
43 **Administration UFaD**. Letter to Distributor on Hazardous Dietary Supplement LipoKinetix. 2001 [cited 2007 8th November]; Available from: URL: http://www.cfsan.fda.gov/~dms/ds-ltr26.html
44 **Cohen SM**, O'Connor AM, Hart J, Merel NH, Te HS. Autoimmune hepatitis associated with the use of black cohosh: a case study. *Menopause* 2004; **11**: 575-577

45 **Levitsky J**, Alli TA, Wisecarver J, Sorrell MF. Fulminant liver failure associated with the use of black cohosh. *Dig Dis Sci* 2005; **50**: 538-539
46 **Lontos S**, Jones RM, Angus PW, Gow PJ. Acute liver failure associated with the use of herbal preparations containing black cohosh. *Med J Aust* 2003; **179**: 390-391
47 **Mahady GB**. Black cohosh (Actaea/Cimicifuga racemosa): review of the clinical data for safety and efficacy in menopausal symptoms. *Treat Endocrinol* 2005; **4**: 177-184
48 **Whiting PW**, Clouston A, Kerlin P. Black cohosh and other herbal remedies associated with acute hepatitis. *Med J Aust* 2002; **177**: 440-443
49 **Stedman C**. Herbal hepatotoxicity. *Semin Liver Dis* 2002; **22**: 195-206
50 **Larrey D**, Pageaux GP. Hepatotoxicity of herbal remedies and mushrooms. *Semin Liver Dis* 1995; **15**: 183-188
51 **Lynch CR**, Folkers ME, Hutson WR. Fulminant hepatic failure associated with the use of black cohosh: a case report. *Liver Transpl* 2006; **12**: 989-992
52 **Huntley A**. The safety of black cohosh (Actaea racemosa, Cimicifuga racemosa). *Expert Opin Drug Saf* 2004; **3**: 615-623
53 **Mokhobo KP**. Herb use and necrodegenerative hepatitis. *S Afr Med J* 1976; **50**: 1096-1099
54 **Sheikh NM**, Philen RM, Love LA. Chaparral-associated hepatotoxicity. *Arch Intern Med* 1997; **157**: 913-919
55 **Nadir A**, Reddy D, Van Thiel DH. Cascara sagrada-induced intrahepatic cholestasis causing portal hypertension: case report and review of herbal hepatotoxicity. *Am J Gastroenterol* 2000; **95**: 3634-3637
56 **Larrey D**, Vial T, Pauwels A, Castot A, Biour M, David M, Michel H. Hepatitis after germander (Teucrium chamaedrys) administration: another instance of herbal medicine hepatotoxicity. *Ann Intern Med* 1992; **117**: 129-132
57 **Mostefa-Kara N**, Pauwels A, Pines E, Biour M, Levy VG. Fatal hepatitis after herbal tea. *Lancet* 1992; **340**: 674
58 **Benninger J**, Schneider HT, Schuppan D, Kirchner T, Hahn EG. Acute hepatitis induced by greater celandine (Chelidonium majus). *Gastroenterology* 1999; **117**: 1234-1237
59 **Stickel F**, Poschl G, Seitz HK, Waldherr R, Hahn EG, Schuppan D. Acute hepatitis induced by Greater Celandine (Chelidonium majus). *Scand J Gastroenterol* 2003; **38**: 565-568
60 **Woolf GM**, Petrovic LM, Rojter SE, Wainwright S, Villamil FG, Katkov WN, Michieletti P, Wanless IR, Stermitz FR, Beck JJ, Vierling JM. Acute hepatitis associated with the Chinese herbal product jin bu huan. *Ann Intern Med* 1994; **121**: 729-735
61 **Escher M**, Desmeules J, Giostra E, Mentha G. Hepatitis associated with Kava, a herbal remedy for anxiety. *BMJ* 2001; **322**: 139
62 **Russmann S**, Lauterburg BH, Helbling A. Kava hepatotoxicity. *Ann Intern Med* 2001; **135**: 68-69
63 **Stickel F**, Baumuller HM, Seitz K, Vasilakis D, Seitz G, Seitz HK, Schuppan D. Hepatitis induced by Kava (Piper methysticum rhizoma). *J Hepatol* 2003; **39**: 62-67
64 **Millonig G**, Stadlmann S, Vogel W. Herbal hepatotoxicity: acute hepatitis caused by a Noni preparation (Morinda citrifolia). *Eur J Gastroenterol Hepatol* 2005; **17**: 445-447
65 **Galloway JH**, Marsh ID, Bittiner SB, Messenger AG, Gawkrodger DJ, Glet R, Forrest AR. Chinese herbs for eczema, the active compound? *Lancet* 1991; **337**: 566
66 **Anderson IB**, Mullen WH, Meeker JE, Khojasteh-BakhtSC, Oishi S, Nelson SD, Blanc PD. Pennyroyal toxicity: measurement of toxic metabolite levels in two cases and review of the literature. *Ann Intern Med* 1996; **124**: 726-734
67 **Bakerink JA**, Gospe SM Jr, Dimand RJ, Eldridge MW. Multiple organ failure after ingestion of pennyroyal oil from herbal tea in two infants. *Pediatrics* 1996; **98**: 944-947
68 **Seybold U**, Landauer N, Hillebrand S, Goebel FD. Senna-induced hepatitis in a poor metabolizer. *Ann Intern Med* 2004; **141**: 650-651
69 **Itoh S**, Marutani K, Nishijima T, Matsuo S, Itabashi M. Liver injuries induced by herbal medicine, syo-saiko-to (xiao-chai-hu-tang). *Dig Dis Sci* 1995; **40**: 1845-1848
70 **Durazo FA**, Lassman C, Han SH, Saab S, Lee NP, Kawano M, Saggi B, Gordon S, Farmer DG, Yersiz H, Goldstein RL, Ghobrial M, Busuttil RW. Fulminant liver failure due to usnic acid for weight loss. *Am J Gastroenterol* 2004; **99**: 950-952
71 **Sanchez W**, Maple JT, Burgart LJ, Kamath PS. Severe hepatotoxicity associated with use of a dietary supplement containing usnic acid. *Mayo Clin Proc* 2006; **81**: 541-544
72 **Mennecier D**, Saloum T, Dourthe PM, Bronstein JA, Thiolet C, Farret O. [Acute hepatitis after phytotherapy] *Presse Med* 1999; **28**: 966

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CASE REPORT

Gastrointestinal manifestations of systemic mastocytosis

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Abstract

Systemic mastocytosis (SM) is a rare disease with abnormal proliferation and infiltration of mast cells in the skin, bone marrow, and viscera including the mucosal surfaces of the digestive tract. Gastrointestinal (GI) symptoms occur in 14%-85% of patients with systemic mastocytosis. The GI symptoms may be as frequent as the better known pruritus, urticaria pigmentosa, and flushing. In fact most recent studies show that the GI symptoms are especially important clinically due to the severity and chronicity of the effects that they produce. GI symptoms may include abdominal pain, diarrhea, nausea, vomiting, and bloating. A case of predominantly GI systemic mastocytosis with unique endoscopic images and pathologic confirmation is herein presented, as well as a current review of the GI manifestations of this disease including endoscopic appearances. Issues such as treatment and prognosis will not be discussed for the purposes of this paper.

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Key words: Systemic mastocytosis; Idiopathic diarrhea; Gastrointestinal manifestations

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INTRODUCTION

Systemic mastocytosis (SM) is characterized by abnormal growth and accumulation of mast cells in various organs[1]. SM represents a heterogeneous group of disorders with varying frequencies of GI disease[2]. Different studies report a wide array of GI involvement with a range from 14%-85% and more recent studies suggest that it comes as a close second commonest symptom next only to pruritus[2,3]. The differences in reported GI symptoms are thought to reflect the heterogeneous population of SM studied, methodology of studies, and the differences in the definition of mastocytosis[2]. Indeed the involvement of the GI tract has been shown to vary with the Metcalfe type of SM[4,5]. It must be stressed that while most patients have pruritus or urtication, these symptoms generally cause less significant discomfort for patients than the GI symptoms which are more distressing chronic complaints[6,7].

CASE REPORT

A 75-year-old man with a history of colonic polyps, hypertension, childhood tonsillectomy, and a remote smoking history presented with a complaint of "excessive mucus in the throat" for the past several years. The patient had a sensation of a "sore throat" for years along with more frequent bowel movements and increased flatus. There were no other gastrointestinal complaints. The patient subsequently had maculopapular rashes which on biopsy showed increased numbers of dermal mast cells highlighted by c-kit (CD 117) immunoperoxidase staining consistent with that of dermal mastocytosis (Figure 1 A). The haematoxylin and eosin stain (HE) is also included (Figure 1 B).

When seen by the gastroenterologist, the patient had no lymphadenopathy in the head and neck region but

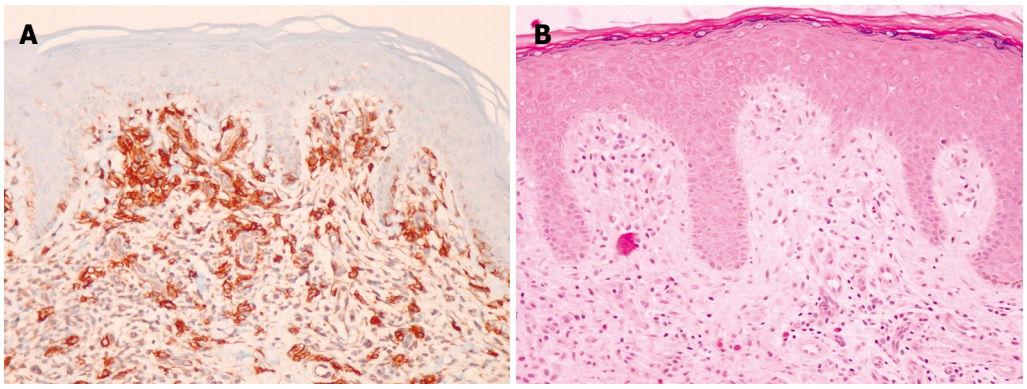

Figure 1 The patient subsequently had maculopapular rashes which on biopsy showed increased numbers of dermal mast cells highlighted by c-kit (CD 117) immunoperoxidase staining consistent with that of dermal mastocytosis. The haematoxylin and eosin stain (HE) is also included.

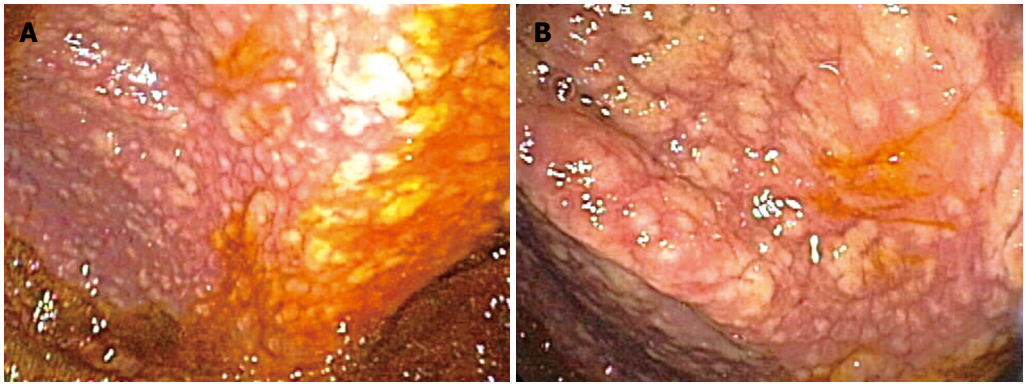

Figure 2 On colonoscopy a mucus type material adherent to the mucosa was present in the right colon along with a slightly raised appearance of the mucosa through areas of the transverse and right colon.

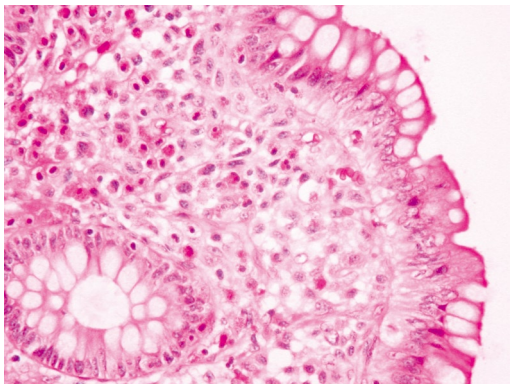

Figure 3 The biopsy in these colonic regions showed an increased number of mast cells with recruited eosinophils in the lamina propria (HE).

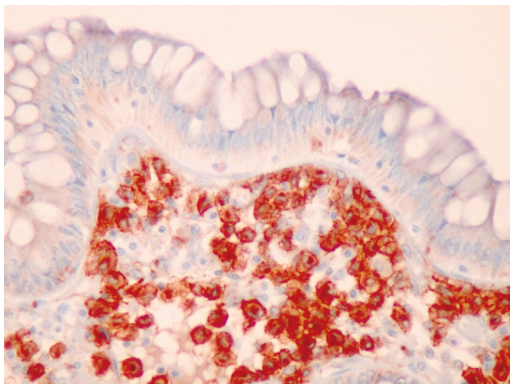

Figure 4 The mast cells are highlighted by positive c-kit staining.

did have tissues in the distal soft palate and pharynx appearing uniformly swollen and hyperemic. The respiratory, cardiovascular, and neurological exams were grossly normal. On abdominal exam there was no organomegaly, masses, tenderness, or inguinal lymphadenopathy. A ventral hernia was noted.

On colonoscopy a mucus type material adherent to the mucosa was present in the right colon along with a slightly raised appearance of the mucosa through areas of the transverse and right colon (Figure 2). The biopsy in these colonic regions showed an increased number of mast cells with recruited eosinophils in the lamina propria (Figure 3 HE). The mast cells are highlighted by positive c-kit staining (Figure 4).

Common GI complaints in SM include abdominal pain, diarrhea, nausea, and vomiting[2]. It appears that the frequency of various GI symptoms is less variable than their prevalence[2]. These symptoms are thought to be secondary to mast cell mediators on the GI tract. The most common symptom is abdominal pain (mean 51%) followed by diarrhea (mean 43%) and nausea or vomiting (mean 28%)[2]. Abdominal pain can be classified into two different types corresponding to different pathology. Epigastric dyspeptic pain is associated with ulcer disease and acid hypersecretion whereas no dyspeptic pain is characterized by lower abdominal cramps[8]. Diarrhea is thought to occur due to gastric acid hypersecretion, malabsorption caused by mucosal injury and edema, and altered bowel motility[8]. Moreover, diarrhea in SM can also be caused or contributed to by tissue infiltration by mast cells[3,9]. It is however worth noting that the mast cells themselves do not secrete gastrointestinal peptides that directly alter motility[3].

A common symptom in SM is facial flushing[10]. The flushing in SM can be distinguished from the flushing

Table 1 Endoscopic features reported in patients with systemic mastocytosis with gastrointestinal symptoms

Esophageal	Gastric and Duodenal	Small Intestine	Colon and Rectum
Esophagitis	Peptic ulcers	Thickened jejunal folds with edema	Nodular lesions
Esophageal stricture	Thickened gastric or duodenal folds	Dilated small bowel	Urticarial lesions in the rectum
Varices	Nodular mucosal lesions		Multiple polypoid lesions
	Urticarial lesions		Diffuse intestinal telangiectasias

seen in the rare but more prevalent carcinoid tumors in that carcinoid flushing is generally more widespread, patchy, violaceous, and evanescent, lasting only a few minutes, often occurs postprandially, and in the majority of cases not associated with hemodynamic changes[10]. Furthermore, the response to long acting somatostatin analogue octreotide, the specific pattern of mediator excretion, and aggravation of the flushing by epinephrine in the carcinoid case can assist in the differentiation[10,11]. In SM, the flushing tends to be more bright red, pruritic, and burning, and may be accompanied simultaneously by other symptoms of systemic mast cell degranulation including GI symptoms[10,12].

Other symptoms may also occur as a result of GI bleeding and peptic ulcer disease[2,3]. It is estimated that GI bleeding occurs in 11% of SM[2]. Contrary to previously reports it is estimated that peptic ulcer disease is an under diagnosed phenomena[2]. Indeed in a more recent prospective study within which nine patients had upper GI endoscopy, four of the nine patients had peptic ulcer disease[3]. Interestingly, hypersecretion of gastric acid is not consistently related to the level of serum histamine levels measured[3]. It may however be possible that a relationship between tissue concentration of histamine and GI manifestations may exist[3]. It is unclear what triggers the vasoactive mediators from mast cells but certain medications such as NSAIDS, opiate analgesics, procaine, and penicillin may be potential triggers[13].

Rarely, esophageal complaints in SM are made and include symptoms due to esophagitis and in some reports variceal bleeding secondary to portal hypertension that may develop in SM[14].

Signs of GI SM include steatorrhea, malabsorption, hepatomegaly, splenomegaly[2] and sometimes ascites secondary to portal hypertension. Steatorrhea can be measured by 72-h fecal fat excretion test, D-xylose tolerance test, and Shilling test. In SM steatorrhea is associated in 5%-67% of patients[7,15,16]. Hepatomegaly was found in 41%-72% of patients[12,17]. Mast cells are known to infiltrate the liver diffusely with or without portal fibrosis and rarely cirrhosis in 4% of patients[12]. Portal hypertension is also reported in association with SM[18,19] and is ascribed to pre- and post-sinusoidal block secondary to mast cell infiltration and or fibrosis[18]. Splenomegaly attributable to SM is thought to be due to mast cell infiltration that may accompany hepatomegaly[20,21]. Marked splenomegaly was found to be more common in aggressive SM[22]. Since SM is a rare entity a low clinical suspicion is required and usually the diagnosis is made on biopsy[10].

Case reports highlight some of the more unusual pathology. One such is SM and giant gastroduodenal ulcers associated with telangiectasia macularis eruptiva perstans which is thought to represent a cutaneous form of mastocytosis[8]. Rarely aggressive SM can be complicated by protein-losing enteropathy[23]. Other reports include initial misdiagnosis as SM can mimic inflammatory bowel disease[9,24] and Zollinger-Ellison syndrome[24].

Small intestinal pathology may reveal dilated loops of bowel and thickened, edematous or scalloped folds[6,25]. In the colon, urticarial lesions, diverticulitis, polypoid lesions, and diffuse intestinal telangiectasia may be seen (Figure 3)[25,26].

Diagnosis of SM is based on identification of neoplastic mast cells by morphologic, immunophenotypic, and/or genetic criteria in various organs. Specifics of the World Health Organization criteria for the diagnosis of SM based on a paper by Valent *et al*[27] will not be discussed. Currently there is no consensus GI biopsy criteria.

Needless to say, patients with obvious dermatological findings are diagnosed by clinicians more quickly as having SM. Unfortunately, patients lacking skin manifestations of SM have a delayed diagnosis even though they often have more aggressive disease, impaired liver function, ascites, malabsorption, and splenomegaly[28].

Interestingly our endoscopic images (Figure 2) from the colonic involvement look markedly different from previously published images by Scolapio *et al*[25]. In the pictures provided in this paper, the appearance of SM in the colon would be described as small mucosal nodules with focal areas of edema, urticarial, granular, and multiple purple-pigmented lesions. To date these are the first endoscopic images that contain all of these features although these findings have been reported in isolation or in pairs[25,29,30].

An earlier review by Jensen in 2000 has reported the following endoscopic findings which are displayed in a table[2]. (Table 1 adapted from Jensen[2]).

DISCUSSION

This report describes an interesting case of a rare cause of chronic diarrhea. It is important to consider this diagnosis in the work up of intractable diarrhea. The distinct endoscopic appearance of this disorder may at times be seen and it is recommended that this information be communicated to the pathologist responsible so that special stains may be performed, allowing the information to perhaps contribute to the diagnosis of SM. It is important to identify potential triggers for GI symptoms of SM as well as to initiate symptomatic treatments. Presently there is no cure for SM and it is recommended that cytoreductive therapy and ensuing management be

planned carefully with expert hematologists and hematopathologists.

REFERENCES

1 **Patnaik MM**, Rindos M, Kouides PA, Tefferi A, Pardanani A. Systemic mastocytosis: a concise clinical and laboratory review. *Arch Pathol Lab Med* 2007; **131**: 784-791
2 **Jensen RT**. Gastrointestinal abnormalities and involvement in systemic mastocytosis. *Hematol Oncol Clin North Am* 2000; **14**: 579-623
3 **Cherner JA**, Jensen RT, Dubois A, O'Dorisio TM, Gardner JD, Metcalfe DD. Gastrointestinal dysfunction in systemic mastocytosis. A prospective study. *Gastroenterology* 1988; **95**: 657-667
4 **Metcalfe DD**. Mastocytosis. *Novartis Found Symp* 2005; **271**: 232-42; discussion 242-249
5 **Metcalfe DD**. Classification and diagnosis of mastocytosis: current status. *J Invest Dermatol* 1991; **96**: 2S-4S
6 **Tebbe B**, Stavropoulos PG, Krasagakis K, Orfanos CE. Cutaneous mastocytosis in adults. evaluation of 14 patients with respect to systemic disease manifestations. *Dermatology* 1998; **197**: 101-108
7 **Horan RF**, Austen KF. Systemic mastocytosis: retrospective review of a decade's clinical experience at the Brigham and Women's Hospital. *J Invest Dermatol* 1991; **96**: 5S-13S; discussion 13S-14S, 60S-65S
8 **Arguedas MR**, Ferrante D. Systemic mastocytosis and giant gastroduodenal ulcer. *Gastrointest Endosc* 2001; **54**: 530-533
9 **Bedeir A**, Jukic DM, Wang L, Mullady DK, Regueiro M, Krasinskas AM. Systemic mastocytosis mimicking inflammatory bowel disease: A case report and discussion of gastrointestinal pathology in systemic mastocytosis. *Am J Surg Pathol* 2006; **30**: 1478-1482
10 **Butterfield JH**. Systemic mastocytosis: clinical manifestations and differential diagnosis. *Immunol Allergy Clin North Am* 2006; **26**: 487-513
11 **Adamson AR**, Grahame-Smith DG, Peart WS, Starr M. Pharmacological blockade of carcinoid flushing provoked by catecholamines and alcohol. *Lancet* 1969; **2**: 293-297
12 **Horny HP**, Kaiserling E, Campbell M, Parwaresch MR, Lennert K. Liver findings in generalized mastocytosis. A clinicopathologic study. *Cancer* 1989; **63**: 532-538
13 **Golkar L**, Bernhard JD. Mastocytosis. *Lancet* 1997; **349**: 1379-1385
14 **Silvain C**, Levillain P, Mouton P, Carretier M, Babin P, Beauchant M. [Systemic mastocytosis disclosed by rupture of esophageal varices] *Gastroenterol Clin Biol* 1989; **13**: 834-837
15 **Soter NA**, Austen KF, Wasserman SI. Oral disodium cromoglycate in the treatment of systemic mastocytosis. *N Engl J Med* 1979; **301**: 465-469
16 **Barriere H**, Dreno B, Pecquet C, Le Bodic MF, Bolze JL. [Systemic mastocytosis and intestinal malabsorption] *Sem Hop* 1983; **59**: 2925-2931
17 **Travis WD**, Li CY, Bergstralh EJ, Yam LT, Swee RG. Systemic mast cell disease. Analysis of 58 cases and literature review. *Medicine* (Baltimore) 1988; **67**: 345-368
18 **Capron JP**, Lebrec D, Degott C, Chivrac D, Coevoet B, Delobel J. Portal hypertension in systemic mastocytosis. *Gastroenterology* 1978; **74**: 595-597
19 **Fonga-Djimi HS**, Gottrand F, Bonnevalle M, Farriaux JP. A fatal case of portal hypertension complicating systemic mastocytosis in an adolescent. *Eur J Pediatr* 1995; **154**: 819-821
20 **Travis WD**, Li CY. Pathology of the lymph node and spleen in systemic mast cell disease. *Mod Pathol* 1988; **1**: 4-14
21 **Gonnella JS**, Lipsey AI. Mastocytosis manifested by hepatosplenomegaly. Report of a case. *N Engl J Med* 1964; **271**: 533-535
22 **Metcalfe DD**. The liver, spleen, and lymph nodes in mastocytosis. *J Invest Dermatol* 1991; **96**: 45S-46S
23 **Mickys U**, Barakauskiene A, De Wolf-Peeters C, Geboes K, De Hertogh G. Aggressive systemic mastocytosis complicated by protein-losing enteropathy. *Dig Liver Dis* 2007; **39**: 693-697
24 **Blonski WC**, Katzka DA, Lichtenstein GR, Metz DC. Idiopathic gastric acid hypersecretion presenting as a diarrheal disorder and mimicking both Zollinger-Ellison syndrome and Crohn's disease. *Eur J Gastroenterol Hepatol* 2005; **17**: 441-444
25 **Scolapio JS**, Wolfe J 3rd, Malavet P, Woodward TA. Endoscopic findings in systemic mastocytosis. *Gastrointest Endosc* 1996; **44**: 608-610
26 **Miner PB Jr**. The role of the mast cell in clinical gastrointestinal disease with special reference to systemic mastocytosis. *J Invest Dermatol* 1991; **96**: 40S-43S; discussion 43S-44S, 60S-65S
27 **Valent P**, Horny HP, Escribano L, Longley BJ, Li CY, Schwartz LB, Marone G, Nunez R, Akin C, Sotlar K, Sperr WR, Wolff K, Brunning RD, Parwaresch RM, Austen KF, Lennert K, Metcalfe DD, Vardiman JW, Bennett JM. Diagnostic criteria and classification of mastocytosis: a consensus proposal. *Leuk Res* 2001; **25**: 603-625
28 **Valent P**, Akin C, Sperr WR, Mayerhofer M, Fodinger M, Fritsche-Polanz R, Sotlar K, Escribano L, Arock M, Horny HP, Metcalfe DD. Mastocytosis: pathology, genetics, and current options for therapy. *Leuk Lymphoma* 2005; **46**: 35-48
29 **Dantzig PI**. Tetany, malabsorption, and mastocytosis. *Arch Intern Med* 1975; **135**: 1514-1518
30 **Mahood JM**, Harrington CI, Slater DN, Corbett CL. Forty years of diarrhoea in a patient with urticaria pigmentosa. *Acta Derm Venereol* 1982; **62**: 264-265

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CASE REPORT

Methotrexate induced sprue-like syndrome

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Abstract

A 52 year-old male patient diagnosed of ankylosing spondylitis presented with an iron deficiency anemia after a ten-month treatment of methotrexate. He did not respond to treatment with oral iron not a proton pump inhibitor and an upper endoscopy was performed. The histological study of the duodenal biopsies showed villus atrophy. After removing the methotrexate, administrating intramuscular iron and undertaking a gluten-free diet, the histological and analytical alterations progressively resolved.

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Key words: Methotrexate; Villous atrophy; Iron deficiency anemia; Paucisymptomatic; Absence of celiac antibodies

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INTRODUCTION

Methotrexate is an immunosuppressive agent commonly used in the daily practice of many specialties. Methotrexate, in a weekly dose, can be used for years where its use is primarily limited by toxicity. The main side effects include renal or liver impairment, orointestinal mucositis, bone marrow toxicity and gastrointestinal side effects, such as nausea or vomiting, many of which can often be avoided by using low-doses of the drug, or combining it with folic acid supplementation[1].

Several cases of villus atrophy, after the use of immunosuppressors[2], have been reported in the medical literature; however, only one case of intestinal villus atrophy secondary to Methotrexate, has been described[3]. We present a second case of a sprue-like syndrome secondary to methotrexate treatment, this time in a paucisymptomatic patient.

CASE REPORT

A 52 year-old male patient was referred to our Gastroenterology out-patient clinic from the Rheumatology Department, as he presented with a progressively increasing iron deficiency anemia, that did not respond to proton pump inhibitors and iron taken orally.

He had been diagnosed of ankylosing spondylitis, HLA B-27 positive, in 2000, and was initially treated with the nonsteroidal antiinflammatory drugs (NSAIDs), salazopyrine and omeprazol. In April, 2002, treatment with salazopyrine was stopped, but prednisolone and methotrexate were added in October, 2002, because the patient had severe arthralgias. The arthralgias lessened with the new treatment, but analytical alterations were progresively seen. An analysis during December, 2002, showed normal hemoglobin (Hb 13.4 g/dL, VCM 87 and HCM 30.2) and iron (70 μg/dL), but in January, 2003, the hemoglobin level had decreased to 11.7 g/dL. By May, 2003, the asymptomatic patient presented with iron deficiency anemia (Hb 9.9 g/dL, Hto. 31%, VCM 71, HCM 22.9, platelets 489000, iron 22 μg/dL,

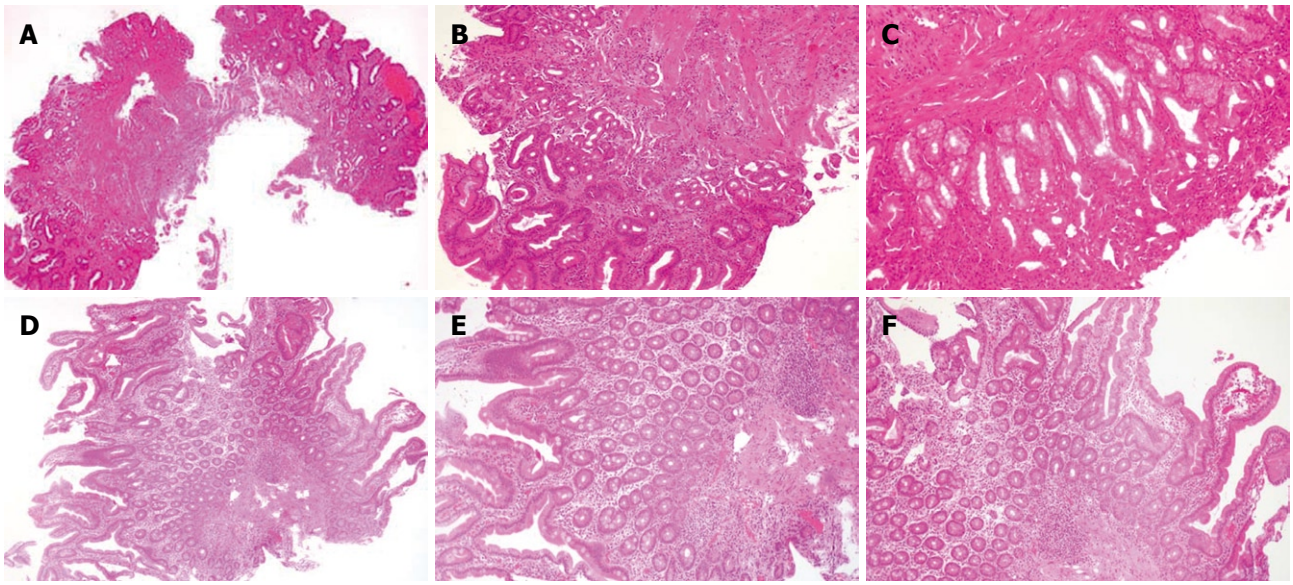

Figure 1 Biopsy of duodenum. A: Year 2003, (HE, x 40); B-C: Year 2003, (HE, x 100); D: Year 2005, (HE, x 40); E-F: Year 2005, (HE, x 100).

transferrine 366, IST 5% and ferritine 2 ng/dL), for which he received iron, taken orally, and was referred to the Gastroenterology Department.

He had no digestive symptoms, weight loss, or anorexia. The suspected diagnosis included: (1) gastric erosions secondary to NSAIDs, (2) a side-effect of Methotrexate, or (3) other causes of iron deficiency anemia. Therefore, an endoscopy and celiac sprue antibodies were requested, and treatment with oral iron and proton pump inhibitors continued.

The upper endoscopy showed a stomach with patched mucosa, alternating red and white areas, which continued in the first and second portions of the duodenum, in which many longitudinal erosions, covered with fibrin, were observed, macroscopically compatible with an Inflammatory Bowel Disease, or a Lymphoma.

However, the histological study showed intestinal biopsy with evidence of atrophy of the wall, with decreased thickness, increased collagenous fibers in the interstitium, mucosal flattening of the villi and small repare glands (Figure 1A). The mucosa shows a loss of glandular structure, with small and round reparing glands, covered by a cubical one-layer epithelium, with nuclei containing reactive atypia, with mild pleomorphism, larger in size and variable nucleoli. There was a fibrous stroma and a heterogeneous inflammatory infiltrate, with eosinophil leukocytes in moderate quantity (Figure 1B-C).

In July, since the hemogram was similar (Hb 9.2 g/dL), despite three months of oral iron, antibodies were negative, and taking into account the results of the upper endoscopy, an intestinal follow-through and a colonoscopy (which were both normal) were requested and methotrexate was discontinued.

Three months later, the iron deficiency anemia persisted and a gluten-free diet was tested. In January, 2004, after three months of a gluten-free diet and six months of not taking methotrexate, there was still a iron deficiency anemia with a hemoglobin of 9.4 g/dL, a new endoscopy was performed. The upper endoscopy showed a loss of duodenal folds and the urease test for *Helicobacter pylori* was negative. The histological study confirmed the existence of an atrophic gastritis and duodenitis. With the result of the endoscopy, the patient returned to our out-patient-clinic in March, 2004. He had an itchy eruption, made up of clusters of tiny blisters in the elbows and back, which was compatible with a Dermatitis Herpetiformis. He was referred to the Dermatology out-patient clinic, but was not visited until six weeks later where the skin lesions had spontaneously disappeared.

Treatment with intramuscular iron was initiated after the Dermatologic evaluation in April, 2004, and three months later the hemogram was almost normal; there was no anemia, (Hb 13.1 g/dL), but there was still a slight hypocromia and microcytosis. The intramuscular iron was stopped in December, 2004, when the still asymptomatic patient reached acceptable levels of iron, ferritine and transferrine (Fe 158 μg/dL, Transf. 275 mg/dL and FE 93 ng/mL) and had a normal hemogram (Hb 14.5 g/dL, Hto. 43%, VCM 84, HCM 28.5).

The patient was periodically seen in our out-patient clinic the following year. Analysis remained normal and a control upper endoscopy was carried out on December, 2005. It showed a dramatic change from the first one, which had been performed two years earlier, when the patient was undergoing treatment with methotrexate. The endoscopy was macroscopically normal.

The histological study showed an intestinal biopsy with normal wall thickness, normal size and number of villi, as well as normal gland and stromal density (Figure 1D). Many intestinal glands, covered by a one-layer cylindrical epithelium with goblet cells, were observed. Between them, the interstitium showed very mild inflammation with a decrease in the fibrous weft. Inflammatory cells were heterogeneously composed of

lymphocytes and plasmatic cells (Figure 1E-F).

The patient remains asymptomatic, from a gastrointestinal point of view, and without anemia or ferropenia. We have not rechallenged methotrexate for ethical reasons.

DISCUSSION

The interest of this case is the appearance of a sprue-like syndrome after the use of methotrexate, and its complete resolution, following the removal of the drug. Although several cases of small-bowel villus atrophy have been described with other immunosuppresive agents, like Azatioprine[2], to our knowledge, only one similar case has been described regarding methotrexate[3]. The latter presented a case of diarrhea, progressive weight loss and general malaise after two years of low-dose methotrexate. Biopsies taken during the treatment showed small-bowel villus atrophy and confirmation of mucosal healing was carried out months after removal of methotrexate.

Our patient did not display any symptoms. He only had an iron deficiency anemia. Six months after beginning low-dose methotrexate (15 mg/wk), a iron deficiency anemia developed. He showed no signs or symptoms of mucositis, or symptoms of bone marrow toxicity, or of renal or liver impairment. No diarrhea, nausea or other gastro-intestinal symptoms were present. We believe he had an iron malabsorption, secondary to duodenal atrophy, which slowly resolved after removing methotrexate, with clinical, analytical, endoscopical and microscopical confirming the healing.

This drug is frequently used in patients with rheumatological, gastroenterological and oncological illnesses. At high doses, and without folic acid supplementation, mucositis is a side-effect in which experimental studies have tried to understand[4,5] for prevention[6-7]; however, not many human cases have been described. Its presentation is usually symptomatic (weight-loss, diarrhea, nausea, general malaise, *etc*) and tends to appear with high doses.

The pathogenesis of the sprue-like syndrome is unclear. Two mechanisms might be involved, local antimetabolite toxicity and genetic predisposition[3].

Our case might allow other clinicians to think of an underlying sprue-like syndrome when a iron deficiency anemia appears when taking methotrexate, even if, like in our case, the patient is taking low doses of the drug and is completely asymptomatic.

This methotrexate-induced sprue-like syndrome is of clinical interest because of its singularity in the clinical presentation (only iron deficiency anemia), its origin (a duodenal atrophy induced by low-dose methotrexate, with no myelosuppression), and its complete resolution after withdrawal of the drug, which has been confirmed both through the periodical analysis and through the endoscopical study.

REFERENCES

1 **Hoekstra M**, van Ede AE, Haagsma CJ, van de Laar MA, Huizinga TW, Kruijsen MW, Laan RF. Factors associated with toxicity, final dose, and efficacy of methotrexate in patients with rheumatoid arthritis. *Ann Rheum Dis* 2003; **62**: 423-426

2 **Ziegler TR**, Fernandez-Estivariz C, Gu LH, Fried MW, Leader LM. Severe villus atrophy and chronic malabsorption induced by azathioprine. *Gastroenterology* 2003; **124**: 1950-1957

3 **Houtman PM**, Hofstra SS, Spoelstra P. Non-coeliac sprue possibly related to methotrexate in a rheumatoid arthritis patient. *Neth J Med* 1995; **47**: 113-116

4 **Farrell RJ**, Kelly CP. Celiac sprue. *N Engl J Med* 2002; **346**: 180-188

5 **Carneiro-Filho BA**, Lima IP, Araujo DH, Cavalcante MC, Carvalho GH, Brito GA, Lima V, Monteiro SM, Santos FN, Ribeiro RA, Lima AA. Intestinal barrier function and secretion in methotrexate-induced rat intestinal mucositis. *Dig Dis Sci* 2004; **49**: 65-72

6 **Harsha WT**, Kalandarova E, McNutt P, Irwin R, Noel J. Nutritional supplementation with transforming growth factor-beta, glutamine, and short chain fatty acids minimizes methotrexate-induced injury. *J Pediatr Gastroenterol Nutr* 2006; **42**: 53-58

7 **Yuncu M**, Eralp A, Koruk M, Sari I, Baqci C, Inaloz S. Effect of vitamin A against methotrexate-induced damage to the small intestine in rats. *Med Princ Pract* 2004; **13**: 346-352

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CASE REPORT

A subset of ulcerative colitis with positive proteinase-3 antineutrophil cytoplasmic antibody

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Abstract

A small subset of patients with active ulcerative colitis is non-responsive to major known non-biological therapies. We reported 5 patients with positive serum proteinase-3 antineutrophil cytoplasmic antibody (PR3-ANCA) and tried to (1) identify the common clinical features of these patients; (2) investigate the efficacy of a novel therapy using a Chinese medicine compound; and (3) attract more gastroenterologists to be engaged in further study of this subset of patients. The common manifestations of disease in these 5 patients included recurrent bloody diarrhea and inflammatory lesions involving the entire colorectal mucosa. Initial treatment with intravenous methylprednisolone successfully induced remission. Four of these 5 patients were steroid-dependence, and immunosuppressants, such as azathioprine and cyclophosphamide, were ineffective. In 3 patients, only the particular Chinese medicine compound could induce and maintain remission. One patient underwent colectomy. No vascular inflammatory lesions were found by histopathological examination. Although more cases are needed for confirmation, our study indicates that ulcerative colitis with positive PR3-ANCA may belong to a subtype of refractory ulcerative colitis. The particular Chinese medicine compound used in our study is by far the most effective in the management of these patients, with additional advantages of having no noticeable side-effects and less financial burden.

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Key words: Refractory ulcerative colitis; Proteinase-3 antineutrophil cytoplasmic antibody; Methylprednisolone; Steroid-dependence; Chinese medicine

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INTRODUCTION

Ulcerative colitis (UC) is a disease of chronic inflammation of colon, which is diagnosed by a combination of clinical, colonoscopic, histopathological, radiological findings and therapeutic response. Infectious and non-infectious colorectal diseases such as Crohn's disease, ischemic colitis, collagenous colitis, lymphocytic colitis and colorectal cancers should be excluded. The principle therapy is 5-aminosalicylic acid for mild to moderate disease, and corticosteroids for moderate to severe disease. If both are ineffective, cyclosporin A is considered as a salvage therapy[1]. Colectomy has to be used in those who fail on medical treatment. Either 5-aminosalicylic acid or azathioprine is recommended for maintenance of remission[2]. In clinical practice, some patients would prefer to die rather than have a colectomy as this can impair quality of life. These patients are the challenge for gastroenterologists who are trying their best to seek promising medicine.

In 2006, a refractory UC patient treated in the Division of Gastroenterology, Shanghai Renji Hospital was unexpectedly found to be serum proteinase-3 antineutrophil cytoplasmic antibody (PR3-ANCA)

positive. Therefore, all UC patients admitted from Jan 2003 to Dec 2007 were screened and the PR3-ANCA positive patients were studied and analyzed.

CASE REPORT

The clinical data of 180 UC patients admitted from Jan 2003 to Dec 2007 were analyzed, 65 patients were investigated for ANCA and only 5 patients were positive for PR3-ANCA. Their demographic and clinical characteristics are summarized in Table 1. The disease activity of UC was evaluated according to Truelove & Witts criteria. Serum myeloperoxidase (MPO)-ANCA was negative (< 1.4) and PR3-ANCA was positive (> 1.4) in all 5 patients. The common disease manifestations in these 5 patients included recurrent bloody diarrhea and inflammatory lesions involving the entire colorectal mucosa (Table 2).

Remission was initially and successfully induced in all 5 patients by treatment with iv Methylprednisolone (Table 3). Four of them relapsed when the prednisone dosage was reduced. In accordance with the principle of UC therapy, azathioprine was prescribed in 3 patients. As 75 mg/d or 100 mg/d could not be tolerated, 50 mg/d was adopted for more than 3 mo but remained without effect. Cyclophosphamide 0.6 g iv was also ineffective. Case 1 had tried cyclosporin A, but this was discontinued because of vomiting. Her life was threatened by bloody diarrhea more than twenty times per day, a high temperature of 40℃, and venous thrombosis of the left leg. She was persuaded again and again, and at last reluctantly accepted colectomy. One patient (case 2) had bloody diarrhea more than ten times per day and a high temperature when prednisone was reduced in dosage from 40 mg/d. She visited a traditional Chinese medicine physician and successfully withdrew the prednisone by taking a Chinese medicine compound prescribed by that physician. Remission was successfully maintained for more than 1 year. She discontinued the Chinese medicine compound by herself because she thought the disease was cured but bloody diarrhea recurred within 2 mo. She started to take the Chinese medicine compound again and her bloody diarrhea quickly disappeared. She returned to her work without any side effects caused by the Chinese medicine, but refused to undergo colonoscopy again.

As a result of this outcome, cases 3 and 4 were recommended to visit the same traditional Chinese medicine physician. They were able to reduce the dose of prednisone gradually after taking the Chinese medicine compound and successfully maintain the remission.

Repeat colonoscopies were performed in 4 of these 5 patients because of recurrent bloody diarrhea. Case 1 showed severe inflammatory lesions involving the entire colorectal mucosa with bleeding and many large deep irregular ulcers before therapy. After prednisone treatment, scars in the cecum and ascending colon, pseudopolyps in the transverse colon, and scattered erosions in the rectosigmoid colon were observed. When bloody diarrhea relapsed 20 times per day, colonoscopy revealed severe inflammatory lesions of entire colorectal mucosa. Case 2 initially showed inflammatory lesions involving the entire colorectal mucosa. When bloody diarrhea relapsed during the maintenance therapy with sulphasalazine 1.5 g/d, colonoscopy revealed again inflammatory lesions from the hepatic flexure to the rectum. Case 3 revealed severe inflammatory lesions involving the entire colorectal mucosa initially and limited lesions were found in the rectosigmoid colon with several ulcers after treatment with prednisone. Case 4 initially showed inflammatory lesions involving the entire colorectal mucosa. Once bloody diarrhea was alleviated, ulcers in the rectosigmoid colon were observed. However, colonoscopy showed inflammatory lesions involving the entire colorectal mucosa again when she was taking prednisone 15 mg/d. Case 5 showed mild inflammatory lesions involving the entire colorectal mucosa with scattered erosions but no ulcers. He was the only patient who did not relapse during nearly 1 year follow up.

Table 1 Demographic data of the five patients

Case	Gender	Age (yr)	Date of admission[1]	Date of initial diagnosis
1	Female	57	Dec 2006	Nov 2006
2	Female	48	Feb 2007	Jun 2006
3	Male	67	Jun 2007	May 2007
4	Female	33	May 2006	May 2004
5	Male	55	Jul 2007	May 2007

[1]Patients were admitted to Division of Gastroenterology, Shanghai Renji Hospital.

The colonoscopic biopsies of 4 patients (cases 1, 2, 3 and 5) and the surgical colon resection specimen of case 1 were examined by 2 experienced pathologists. No vascular inflammatory lesions were found.

Constituents of the particular Chinese medicine compound were: Fllase Asiabell Root Tangshen, Garden Burnet Root, Milkvetch Root, Largehead Atractylodes Rhizome, Lignum et Radix Cudraniae, White Peony Root, Whiteflower Patrinia Herb, Herba Violae, Common Bletilla Tuber, Japanese Pagodatree Flower-bud, Lalang Grass Rhizome, Whipformed Typhonium Rhizome, Folium Isatidis, Hairyvein Agrimonia Herb and Bud, *etc*.

DISCUSSION

ANCA is a group of antibodies directed against certain proteins in the cytoplasm of neutrophils. There are 2 major categories of ANCA that can be determined by immunofluorescence staining. Perinuclear ANCA (pANCA) refers to the more localized perinuclear or nuclear staining pattern; cytoplasmic ANCA (cANCA) refers to the diffuse, granular staining pattern. It has been shown that ANCA is associated with small-vessel vasculitis. MPO-ANCA is related to polyangiitis or allergic granulomatous angiitis, and PR3-ANCA is a specific and sensitive marker of Wegener's granulomatosis[3,4]. Wegener's granulomatosis is a systemic and necrotizing granulomatous vasculitis. The main manifestations of Wegener's granulomatosis are lung nodular infiltration, upper respiratory tract diseases and proteinuria/

Table 2 Common manifestations of disease in the five patients

Case	Bloody diarrhea	Abdominal pain	Relapse	Activity	Colonoscopy	Histological examination	Other colorectal inflammation
1	20 times/d	Yes	Yes	Severe	Entire colorectum erosion, ulcer	Mucosa inflammatory	No
2	4 times/d	Yes	Yes	Moderate	Entire colorectum erosion, ulcer	Mucosa inflammatory	No
3	6 times/d	Yes	Yes	Severe	Entire colorectum erosion, ulcer	Mucosa inflammatory	No
4	7 times/d	Yes	Yes	Severe	Entire colorectum erosion, ulcer	Mucosa inflammatory	No
5	5 times/d	Yes	No	Moderate	Entire colorectum erosion	Mucosa inflammatory	No

Table 3 Treatment of the five patients

Case	Induction of remission	Immunosuppressant		Maintenance of remission
		Azathioprine	Cyclophosphamide	
1	Methylprednisolone 40 mg/d, iv	50 mg/d, po	0.6 g, iv	Colectomy
2	Methylprednisolone 40 mg/d, iv Chinese medicine compound			Chinese medicine compound
3	Methylprednisolone 40mg/d, iv + prednisone 20 mg/d, po Chinese medicine compound	50 mg/d, po	0.6 g, iv	Chinese medicine compound
4	Methylprednisolone 40 mg/d or 80mg/d, iv	50 mg/d, po	0.6 g, iv	Chinese medicine compound
5	Methylprednisolone 40 mg/d, iv			Sulphasalazine 2 g/d

hematuria. Kidney, nasal mucosa or lung biopsies reveal granulomatous lesions. If the disease involves the colon, the patient may have bloody diarrhea. The principle treatment of vasculitis is to use prednisone or cyclophosphamide to induce remission and then use cyclophosphamide or azathioprine for the maintenance of remission. Three to 6 mo therapy is usually needed for inducing remission, but the relapse rate is high. Long-term morbidity and mortality are high[5,6].

The 5 patients reported here did not meet the criteria of Wegener's granulomatosis (case 2 had a nasal polyp but CT scan showed no lung lesion, and biopsies of the nasal polyp and kidney did not show any granuloma). Also, histopathological studies of the 4 patients examined did not reveal any vascular inflammation in either biopsy or surgical specimen of the colon.

It was reported that UC had a high pANCA positivity, which was further identified as MPO-ANCA, LF-ANCA, and BPI-ANCA, *etc*[7,8]. The presence of pANCA combined with anti-Saccharomyces cerevisiae mannan antibodies was recommended as a way to distinguish UC from Crohn's disease[9]. PR3-ANCA belongs to cANCA, which is not a deep concern with UC because of its low positivity. Liu et al reported 5 of 58 UC patients had positive PR3-ANCA, but the significance of this finding was not mentioned[10]. In Elzouki's report, 17 of 141 UC patients were PR3-ANCA positive, but no relationship was found between PR3-ANCA and the severity of UC[11]. In the 5 patients reported here, inflammatory lesions involving the entire colorectum were seen. The probable reason for the difference is that our patients were naive, while corticosteroids could lessen the extent of the lesion. Although corticosteroids could induce remission temporarily, maintenance of remission and recurrence are problems that are hard to solve. What is the effective treatment for UC with positive PR3-ANCA?

Azathioprine was employed in 3 steroid-dependent patients. In the 3 patients, 50 mg/d had been used for more than 3 mo, but bloody diarrhea relapsed when the dose of prednisone was reduced to 30 mg/d. Azathioprine has been recommended for maintenance therapy of both UC and ANCA-associated vasculitis[4,6], but was ineffective in our UC patients. Recurrence of bloody diarrhea suggested that azathioprine was ineffective.

Cyclophosphamide has been recommended for the management of active vasculitis, and it was also reported that it could successfully induce remission in Crohn's disease and indeterminate colitis[12]. Cyclophosphamide had been given in our 3 steroid dependent patients; however it was ineffective. Anti-tumor necrosis factor-α antibody has been reported to be effective for refractory UC patients[13]. However, it is too expensive and could not be afforded by ordinary Chinese patients. In our 3 patients, the Chinese medicine compound successfully induced and maintained remission. (Case 1 had a colectomy and did not have a chance to try the Chinese medicine compound). It is suggested that the Chinese medicine compound might be one of the best treatments for UC with positive PR3-ANCA.

Our preliminary study indicates that UC with positive PR3-ANCA may belong to a subtype of refractory UC. The Chinese medicine compound prescribed is not only more effective than Methylprednisolone but also has the merit of being without noticeable side-effects. However, more evidence is needed. We hope more gastroenterologists can be attracted and more cases will be accumulated. The particular Chinese medicine compound

is worthy of further study and the effective constituents should be identified.

REFERENCES

1 **Shibolet O**, Regushevskaya E, Brezis M, Soares-Weiser K. Cyclosporine A for induction of remission in severe ulcerative colitis. *Cochrane Database Syst Rev* 2005; CD004277
2 **Timmer A**, McDonald JW, Macdonald JK. Azathioprine and 6-mercaptopurine for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2007; CD000478
3 **Geffriaud-Ricouard C**, Noel LH, Chauveau D, Houhou S, Grunfeld JP, Lesavre P. Clinical spectrum associated with ANCA of defined antigen specificities in 98 selected patients. *Clin Nephrol* 1993; **39**: 125-136
4 **Ozaki S**. ANCA-associated vasculitis: diagnostic and therapeutic strategy. *Allergol Int* 2007; **56**: 87-96
5 **Jayne D**, Rasmussen N, Andrassy K, Bacon P, Tervaert JW, Dadoniene J, Ekstrand A, Gaskin G, Gregorini G, de Groot K, Gross W, Hagen EC, Mirapeix E, Pettersson E, Siegert C, Sinico A, Tesar V, Westman K, Pusey C. A randomized trial of maintenance therapy for vasculitis associated with antineutrophil cytoplasmic autoantibodies. *N Engl J Med* 2003; **349**: 36-44
6 **de Groot K**, Jayne D. What is new in the therapy of ANCA-associated vasculitides? Take home messages from the 12th workshop on ANCA and systemic vasculitides. *Clin Nephrol* 2005; **64**: 480-484
7 **Rump JA**, Scholmerich J, Gross V, Roth M, Helfesrieder R, Rautmann A, Ludemann J, Gross WL, Peter HH. A new type of perinuclear anti-neutrophil cytoplasmic antibody (p-ANCA) in active ulcerative colitis but not in Crohn's disease. *Immunobiology* 1990; **181**: 406-413
8 **Cambridge G**, Rampton DS, Stevens TR, McCarthy DA, Kamm M, Leaker B. Anti-neutrophil antibodies in inflammatory bowel disease: prevalence and diagnostic role. *Gut* 1992; **33**: 668-674
9 **Quinton JF**, Sendid B, Reumaux D, Duthilleul P, Cortot A, Grandbastien B, Charrier G, Targan SR, Colombel JF, Poulain D. Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role. *Gut* 1998; **42**: 788-791
10 **Liu X**, Yu T, Zhao M, Tang X, Gu Q, Liu N. [The diagnostic significance of antineutrophil cytoplasmic antibodies in ulcerative colitis] *Zhonghua Neike Zazhi* 1999; **38**: 451-454
11 **Elzouki AN**, Eriksson S, Lofberg R, Nassberger L, Wieslander J, Lindgren S. The prevalence and clinical significance of alpha 1-antitrypsin deficiency (PiZ) and ANCA specificities (proteinase 3, BPI) in patients with ulcerative colitis. *Inflamm Bowel Dis* 1999; **5**: 246-252
12 **Stallmach A**, Wittig BM, Moser C, Fischinger J, Duchmann R, Zeitz M. Safety and efficacy of intravenous pulse cyclophosphamide in acute steroid refractory inflammatory bowel disease. *Gut* 2003; **52**: 377-382
13 **Rutgeerts P**, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, Travers S, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Present D, Sands BE, Colombel JF. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005; **353**: 2462-2476

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

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N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

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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

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[1]Passed away on October 20, 2007
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Contents

Contents

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Contents

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EDITORIAL

Targeted medical therapy of biliary tract cancer: Recent advances and future perspectives

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Abstract

The limited efficacy of cytotoxic therapy for advanced biliary tract and gallbladder cancers emphasizes the need for novel and more effective medical treatment options. A better understanding of the specific biological features of these neoplasms led to the development of new targeted therapies, which take the abundant expression of several growth factors and cognate tyrosine kinase receptors into account. This review will briefly summarize the status and future perspectives of antiangiogenic and growth factor receptor-based pharmacological approaches for the treatment of biliary tract and gallbladder cancers. In view of multiple novel targeted approaches, the rationale for innovative therapies, such as combinations of growth factor (receptor)-targeting agents with cytotoxic drugs or with other novel anticancer drugs will be highlighted.

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Key words: Growth factor receptor; Biliary tract cancer; Small molecule inhibitor; Monoclonal antibody; Innovative cancer treatment; Sorafenib; Bevacizumab; Erlotinib

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INTRODUCTION

Biliary tract cancers (BTC) originate from the intra- or extrahepatic bile duct epithelium[1]. They were first described by Durand-Fardel in 1840[2,3]. The extrahepatic type (cholangiocarcinoma), primarily cancers involving the confluence of the right and left hepatic ducts, accounts for 80%-90%, and the intrahepatic type (cholangiocellular carcinoma) for the remaining 10%-20% of all biliary tract cancers. Hilar BTC as a specific sub-entity was first reported by Klatskin in 1965, hence their designation as Klatskin tumors[4]. BTC have been considered rare malignancies comprising only 3% of gastrointestinal tumors. However, interest in BTC is growing due to a rising worldwide incidence and associated mortality especially in intrahepatic BTC[5-8].

BTC is notoriously difficult to diagnose and is usually fatal because of its late clinical presentation and the lack of effective non-surgical treatment modalities[9]. Surgical resection or liver transplantation remain the only potentially curative therapeutic options. Unfortunately, most patients have unresectable disease at presentation and die within 12 mo. Liver failure and recurrent sepsis, secondary to biliary obstruction, also contribute to the high mortality[10]. Overall survival rate is poor, with less than 5% of BTC patients surviving to 5 years, a rate which has not changed significantly over the past 30 years[11]. Similar to BTC, there is currently no standard chemotherapy regimen for patients with advanced gallbladder cancer.

Therefore, innovative drugs are urgently needed for effective medical treatment of biliary tract and gallbladder cancers. This review will provide a perspective overview of selected agents, which are currently in development, or under consideration or testing for a more effective, targeted treatment of BTC (Table 1; Figure 1)[12-24]. Moreover, we will discuss promising approaches, which

Table 1 Current status of clinical trials with agents that target growth factor receptors and related signaling pathways for treatment of biliary tract and gallbladder cancers

Name	Target	Mechanism	Cotreatment	Status	Clinical trials
Bevacizumab	VEGF	VEGF-neutralizing antibody	Erlotinib	Phase Ⅱ	NCT00350753[17]
			Erlotinib	Phase Ⅱ	NCT00356889[18]
			Radiation	Phase Ⅰ	NCT00426829[21]
			Floxuridine, dexamethasone	Phase Ⅱ	NCT00410956[20]
			Gemcitabine, oxaliplatin	Phase Ⅱ	NCT00361231[19]
Cediranib (AZD2171)	PAN-VEGFR, PDGFR, c-KIT	Tyrosine kinase inhibitor	AZD-0530	Phase Ⅰ	NCT00475956
Cetuximab	EGFR	Monoclonal antibody	Gemcitabine, oxaliplatin	Phase Ⅱ	NCT00552149 (BINGO)[22]
Erlotinib	EGFR	Tyrosine kinase inhibitor		Phase Ⅱ	NCT00033462[12]
			Gemcitabine	Phase Ⅰ b	
			Oxaliplatin, gemcitabine, radiation	Phase Ⅰ	NCT00266097[16]
Lapatinib	EGFR, erbB2	Tyrosine kinase inhibitor		Phase Ⅱ	NCT00107536[14]
Sorafenib	VEGFR, PDGFR, c-Raf, B-Raf	Tyrosine kinase inhibitor	Oxaliplatin,	Phase Ⅱ	NCT00238212[15]
			Capecitabine	Phase Ⅰ / Ⅱ	NCT00634751[23]
			Gemcitabine	Phase Ⅰ / Ⅱ	NCT00661830 (GEMSO)[24]
Bortezomib	Proteasome	Proteasome inhibitor		Phase Ⅱ	NCT00085410[13]
			Docetaxel	Phase Ⅰ	

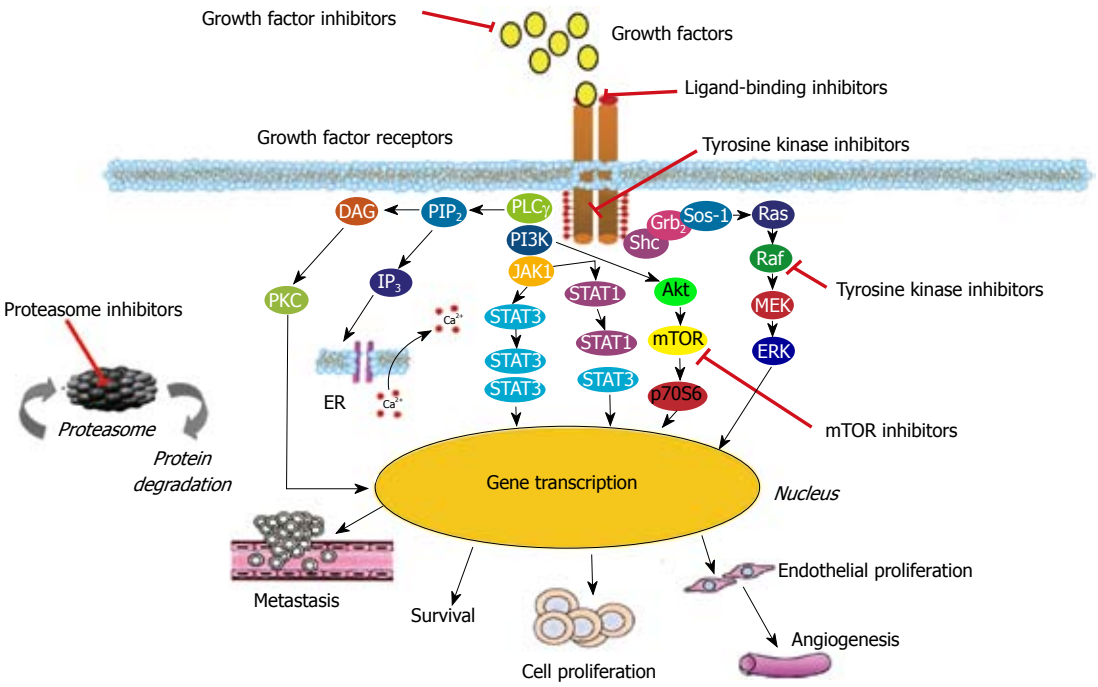

Figure 1 Major growth factor receptor signaling pathways. TK: Tyrosine kinase; P: Phosphorylation; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; PI3K: Phosphatidylinositol-3 kinase; mTOR: Mammalian target of rapamycin; JAK: Janus kinase; STAT: Signal transducer and activator of transcription.

have not yet been tested in BTC or gallbladder cancer, but warrant future evaluation.

ANTIANGIOGENIC TREATMENT STRATEGIES

Angiogenesis plays a central role in tumor growth and progression, and its implications have been extensively investigated and described in the literature for various cancers[25,26]. In the early 1970s, Folkman J[27] was the first to develop the concept of angiogenesis-dependent tumor growth and postulated that the specific blocking of blood flow to the tumor should be a promising strategy for cancer treatment.

Among the angiogenic factors/receptors described so far, the vascular endothelial growth factor (VEGF) and VEGF receptor family including the secreted glycoproteins VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, the placental growth factors (PlGF-1,-2), and their cognate receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk/KDR) play major roles in not only physiological but also in pathological angiogenesis. VEGF-A which binds to both VEGFR-1 and -2 is a key regulator of the development of the vascular system and is commonly

overexpressed in a variety of solid tumors[28].

In addition, elevated levels of circulating VEGF-A are correlated with progression and metastasis of gastrointestinal cancers. A recent study confirmed that elevated VEGF expression correlated with increased metastasis of intrahepatic cholangiocarcinoma[29]. Here upregulated VEGF-C, which plays an important role in the lymph node metastasis of intrahepatic cholangiocarcinoma, was the best independent factor for a poor prognosis[30].

In this vein VEGF protein is overexpressed in cholangiocarcinomas[31], which is paralleled by VEGFR-1, -2 expression in the surrounding endothelial cells[32,33]. Therefore the VEGF/VEGFR system is an attractive target for the treatment of these almost chemoresistant cancers.

ANTIBODY-BASED ANTIANGIOGENIC THERAPY

Anti-VEGF treatment

Bevacizumab is a humanized murine monoclonal anti-VEGF antibody, which when combined with standard cytostatic treatment significantly increased survival in patients with metastatic colorectal cancer as compared to standard treatment alone[34]. This positive phase Ⅲ clinical trial led to approval of bevacizumab for the treatment of advanced colorectal cancer in 2005. Comparable results were obtained in a recent phase Ⅲ clinical trial of bevacizumab in non-small cell lung cancer. This study was interrupted prematurely because of an obvious survival advantage in the antibody treated patients[35].

The first clinical data on the successful treatment of cholangiocarcinoma with bevacizumab were reported in 2006, and described a patient whose metastasis from cholangiocarcinoma resolved after salvage therapy with 3 cycles of bevacizumab (5 mg/kg) combined with cisplatin (75 mg/kg) and high-dose fluorouracil and leucovorin over a period of 2 wk[36].

Currently, several studies using bevacizumab for combination therapy of cholangiocarcinoma are ongoing. An ongoing phase Ⅱ trial explores the combination of systemic bevacizumab with floxuridine and dexamethasone given as a hepatic arterial infusion in patients with unresectable hepatocellular carcinoma or intrahepatic cholangiocarcinoma (NCT00410956). In this trial, patients undergo placement of the hepatic arterial infusion (HAI) pump and a cholecystectomy and receive floxuridine and dexamethasone by HAI continuously for 2 wk. Bevacizumab is concomitantly given for 30-90 min iv at the beginning and end of each cycle. Outcome measure of the study trial is to determine the antitumor efficacy (complete and partial response, stable and progressive disease) as well as toxicity as measured by Common Toxicity Criteria (CTC), which is a standard set by the National Cancer Institute to provide standard language for reporting adverse events which occur in cancer clinical trials[37].

Two other Phase Ⅱ trials are currently determining the safety and efficacy of bevacizumab in combination with the EGFR tyrosine kinase inhibitor erlotinib in patients with metastatic or unresectable gall bladder or bile duct cancers and other advanced upper gastrointestinal carcinomas, which are refractory or intolerant to standard chemotherapy (NCT00350753[17]; NCT00033462[12]). This particular combination was chosen because dual targeting of cancer cells (EGFR) and their nutrient supply (anti-VEGF) will likely have synergistic antitumoral effects[38]. Synergy has been shown at the molecular level, since inhibition of the EGFR suppressed the release of VEGF from tumor cells *in vitro*[39,40]. Moreover, high levels of VEGF have been shown to promote resistance to anti-EGFR treatment in squamous cell carcinoma[41]. Recent clinical trials for refractory non-small-cell lung cancer and advanced renal cell carcinoma proved that this particular combination is highly effective[42-44].

The combination of bevacizumab with radiation therapy is currently being studied in inoperable hepatocellular carcinoma and cholangiocarcinoma in a non-randomized, open label phase I trial (NCT00426829[21]). In rectal cancer patients, bevacizumab was shown to enhance tumor blood flow, to reduce tumor interstitial pressure, and to decrease mean vessel density. These physiologic changes enhanced the efficacy of radiation therapy in a neoadjuvant setting[45]. It is most probable that antiangiogenic preconditioning to normalize the tumor vasculature in primary liver tumors will equally create a "therapeutic window" whereby improved blood flow with enhanced exposure to radiosensitizing oxygen is achieved.

Anti-PlGF treatment

The use of a neutralizing anti-PlGF monoclonal antibody in VEGF-inhibitor resistant tumors is an attractive alternative antiangiogenic strategy. A proof of concept study has been performed in an animal study with melanoma or pancreatic adenocarcinoma bearing mice[46]. The antibody specifically inhibits the binding of PlGF to its receptor VEGFR-1, present on tumor-associated endothelial cells and macrophages. The underlying idea of using this approach was derived from gene inactivation studies showing that endogenous PlGF is redundant in vascular development and physiological vessel maintenance, but an important contributor to the "angiogenic switch" in solid tumor growth. This led to the hypothesis that unlike VEGF inhibitors, PlGF inhibition might reduce pathological angiogenesis, without disturbing physiological blood vessel homeostasis and reduce concomitant unwanted side effects. Hence, anti-PlGF treatment could perhaps substitute for anti-VEGF therapy in the future. Moreover, as PlGF levels increase in the circulation of cancer patients receiving anti-VEGF treatment[47-49], anti-PlGF should also counteract this potential downside of anti-VEGF therapy. Accordingly, anti-PlGF-treatment inhibits angiogenesis, lymphangiogenesis, tumor growth and motility in anti-VEGF-resistant tumor bearing

mice. Here, it blocks the so-called rescue-angiogenesis, a major problem in current antiangiogenic approaches, and showed excellent treatment tolerability. In addition, anti-PlGF treatment may permit long-term treatment of cancers in children, pregnant women, or patients at risk for thrombotic, cardiac or other complications for whom the adverse effects of other VEGF/VEGFR-inhibitors may be excessive and prohibitive.

ANTIANGIOGENIC THERAPY WITH SMALL MOLECULE INHIBITORS

Several agents which inhibit the tyrosine kinase activity of angiogenic growth factor receptors like the VEGFR or PDGFR, have been synthesized by combinatorial chemistry. These tyrosine kinase inhibitors are small molecules which occupy the ATP binding site of the tyrosine kinase domain of the intracellular portion of the receptor. Because of their effects on downstream signaling, these inhibitors interfere with a number of key biologic functions associated with VEGFR activation. Although drugs that are targeted to specific VEGFR kinases have shown clinical efficacy, the redundancy in the angiogenesis pathways necessitates broad spectrum inhibitors that address multiple (VEGFR) targets[50].

AZD2171

AZD2171 is a highly potent small molecule with pan-VEGFR-tyrosine kinase inhibiting activity (50% inhibitory concentration of < 0.002 μmol/L for VEGFR-2 and 0.005 μmol/L for VEGFR-1, respectively). AZD2171 also inhibits VEGFR-3, PDGFR-β and c-Kit at nanomolar concentrations[51]. The antineoplastic potency of AZD2171 has been demonstrated in several tumors, including lung, hepatocellular, colorectal and prostate cancer and in all cases the antitumor effect was associated with strong inhibition of VEGF signaling and angiogenesis[52-54]. A phase I dose-finding study was conducted in 83 patients with advanced solid tumors[52]. The study was divided into parts A and B, with 36 patients on a dose-escalation schema, in which 3 to 8 patients received a single oral dose of AZD2171 ranging from 0.5-60 mg. After a wash-out period of 2 to 7 d, the patients continued with daily treatment at the same dose level. AZD2171 was generally well tolerated at < 45 mg/d with common side effects of fatigue, nausea, diarrhea, and vomiting. In part B, an additional 47 patients were enrolled at 20, 30 or 45 mg orally daily. All patients had liver metastases and six patients had NSCLC. The major toxicities and side-effects included hypertension, headache, diarrhea, and voice hoarseness. Three patients in the 60 mg cohort each experienced one serious adverse event (grade 4 cerebral hemorrhage, grade 4 hypoglycemia, and grade 3 hypertension). Of the 83 patients enrolled, two partial responses were observed, while stable disease was seen in 23 patients.

At present AZD2171 is being evaluated in three different studies (Horizon Ⅰ-Ⅲ) in patients with advanced colorectal cancer. In Horizon Ⅲ, which is a phase Ⅱ/Ⅲ study, AZD2171 is tested as a combination partner for FOLFOX compared to a combination of FOLFOX and bevacizumab in patients with previously untreated metastatic colorectal cancer. AZD2171 has shown encouraging signs of antitumor activity in a clinical development program, which has included over 700 patients to date. Based on the generally encouraging findings, AZD2171 is currently also being investigated in cholangiocarcinoma. A pending phase I trial (NCT00475956) explores the effects of AZD2171 in combination with AZD0530, a dual-specific inhibitor of Src and Abl. Src and Abl are protein tyrosine kinases which are overexpressed in malignancies such as chronic myeloid leukemia (CML), where AZD0530 has already been proved to be an effective anticancer agent[55]. The idea for using this particular combination for the treatment of cholangiocarcinoma may have arisen from observations that the Abl- and Src-inhibitor imatinib (Gleevec) showed apoptosis-inducing and growth-reducing effects in cholangiocarcinoma cells *in vitro*[56]. However, imatinib also inhibits other tyrosine kinases, such as c-kit and PDGFR-β. Thus it is not clear, whether the effects of imatinib on cholangiocarcinomas are related to Src-inhibition. This is doubtful as s-src expression, which is highly correlated with the indices of early stage hepatocellular carcinoma phenotype, is not likely to be involved in the cholangiocarcinoma phenotype, as no s-src activation could be detected in cholangiocarcinoma[57].

STRATEGIES TARGETING THE EGFR

The central role of the epidermal growth factor receptor (EGFR) in the proliferation of tumor epithelia and its overexpression in several solid tumors have provided the rationale for targeting this key signaling network. EGFR blockade with monoclonal antibodies and tyrosine kinase inhibitors has already translated into clinical benefit in gastrointestinal tumors, including primary liver cancer[58,59].

Over the past few years, three EGFR-specific agents have received regulatory approval: (1) The monoclonal anti-EGFR antibody cetuximab for metastatic colorectal cancer, and squamous cell carcinoma of the head and neck; (2) The tyrosine kinase inhibitor erlotinib for advanced or metastatic pancreatic cancer and NSCLC; and (3) The EGFR tyrosine kinase inhibitor gefitinib for advanced or metastatic NSCLC. However, the general FDA approval for NSCLC treatment with gefitinib was recently withdrawn after it failed to demonstrate a survival benefit either alone or with chemotherapy in three phase Ⅲ trials[58,60].

Several reports indicate that the EGFR is frequently (over-)expressed in cholangiocarcinoma. Additionally, sustained EGFR activation due to defective receptor internalization has been reported for cholangiocarcinoma cells[61]. Of note, bile acids activate EGFR-signaling *via* a TGF-alpha-dependent mechanism, thereby contributing to the growth characteristics of cholangiocytes and cholangiocarcinoma cells[62]. Clinicopathologically,

EGFR overexpression was shown to be associated with macroscopic tumor type, lymph node metastasis, tumor stage, lymphatic vessel invasion, and perineural invasion in extrahepatic cholangiocarcinoma. High levels of EGFR expression and activation increased the risk for tumor recurrence in intrahepatic cholangiocarcinoma[29]. EGFR-inhibitors inhibited cholangiocarcinoma cell growth *in vitro* and *in vivo*[33,61,63].

These encouraging preliminary findings on the general suitability of anti-EGFR-based-approaches for the treatment of cholangiocarcinoma spawned several clinical trials. In a cohort composed of 24 chemotherapy-refractory patients and 18 chemotherapy-naive patients administered oral erlotinib (150 mg/d) as monotherapy, the progression free survival at 6 mo was determined[64,65]. Seventeen percent of the patients achieved this primary end point, while disease control was obtained in 50% of patients with a median duration of 5.1 mo. Seven percent of the patients showed a partial response of 4 to 14 mo duration. The results suggest an astonishing therapeutic benefit for EGFR blockade with erlotinib in patients with advanced biliary cancer, however, this has to be confirmed in future larger controlled trials and in trials which use erlotinib in combination with other targeted agents.

An ongoing multicenter phase Ⅱ trial in patients with advanced BTC (BINGO; NCT00552149) evaluates the efficacy of the EGFR-antibody cetuximab (Huether *et al*, 2006), combined with gemcitabine-oxaliplatin chemotherapy (GEMOX). Patients will be randomized 1:1 to receive GEMOX (1000 mg/m² gemcitabine; 100 mg/m² oxaliplatin) alone or GEMOX + cetuximab (500 mg/m²) every other week. The BINGO trial also comprises ancillary basic research and functional imaging studies, in order to identify markers that predict treatment efficacy of bile duct cancer. The primary outcome measure of the study is progression-free survival at 4 mo. Secondary outcome measures are the feasibility and toxicity of the treatments, and an evaluation of the degree and duration of objective tumor response or tumor control in a time frame of one year.

A third study used cetuximab in combination with GEMOX in a small number of nine GEMOX resistant patients with advanced, metastatic and unresectable intrahepatic cholangiocarcinoma[66]. Patients received cetuximab 400 mg/m² on day 1, then 250 mg/m² weekly, combined with gemcitabine 1000 mg/m² on day 1 and oxaliplatin 85 mg/m² on day 2, every 3 wk. Results of the study were encouraging. Cetuximab was well tolerated and provided good palliative effects in advanced cholangiocarcinoma. Moreover, adding cetuximab bypassed tumor resistance to GEMOX[67].

Taken together, anti-EGFR-based therapies for treating BTC appear to have their greatest potential when given in combination either with conventional cytostatics or with other targeted agents. The rationale for using combination therapies is the existence of multilevel receptor cross-stimulation or of redundant signaling pathways which lead to neoplasia. Blocking only one of these pathways allows others to act as salvage or escape mechanisms for cancer cells. Preclinical evidence of synergistic antitumor activity achievable by combining targeted agents that block multiple signaling pathways has recently emerged[68-70]. The multi-target approach can be accomplished by using either combinations of selective agents or single agents, which address various targets[71].

IGF/IGFR-BASED STRATEGIES

Activation of the insulin-like growth factor (IGF) receptor 1 (IGF-1R) by IGF-Ⅰ and IGF-Ⅱ plays a pivotal role in tumor cell proliferation and spread, by promoting cell cycle progression, preventing apoptosis, and by regulating and maintaining the metastatic tumor phenotype[70,72-75]. A wide variety of tumors show abnormal or enhanced expression of IGFs and IGF-1R, which leads to auto- and paracrine growth stimulation, and which has been correlated with enhanced proliferation, tumor de-differentiation, disease stage, development of metastases and reduced patient survival. Enhanced expression of IGF-1R has also been demonstrated in BTC, and the IGF/IGFR system was shown to be centrally involved in proliferation and suppression of apoptosis of cholangiocarcinoma cells[76], making the IGF/IGFR-signaling system an attractive target for the treatment of BTC. Thus IGF-1R blocking antibodies, IGF-1R antisense oligonucleotides, or IGF-1R siRNA have all been shown to effectively interfere with IGF-1R mediated signaling *in vitro* and with tumor growth and spread *in vivo*[76-80].

We and others validated the selective IGF-1R tyrosine kinase inhibitor NVP-AEW541 as a promising novel agent for the therapy of several cancers[81-85]. Moreover, we showed that a combination of IGF-1R inhibitors together with the multi-kinase inhibitor sorafenib, offer additive antitumoral efficacy for cholangiocarcinoma *in vitro*[86]. The antineoplastic properties of NVP-AEW541 and related compounds such as NVP-ADW742 have been demonstrated in preclinical studies on Ewing's sarcoma-bearing mice, fibrosarcoma, breast cancer and musculoskeletal sarcoma[81-83].

Specific IGFR-antibodies potently suppressed prostate and breast cancer cell growth *in vitro*[87]. The clinically most advanced anti-IGFR antibody is CP-751871, which is currently being tested in three phase Ⅱ trials for advanced breast cancer, NSCLC and prostate cancer (www.clinical-trials.gov). Importantly, the preliminary clinical data indicate that IGFR-inhibition is well-tolerated[88-90]. Safety is important, since IGFR-based inhibition has long been regarded as a high-risk intervention, because of the high homology of the IGF-1R receptor with the related insulin-receptor, and the fear that IGF-1R tyrosine kinase inhibitors may lead to insulin resistance and overt diabetes[91]. However, the current *in vivo* data do not support this assumption, resulting in a growing interest in anti-IGFR-based therapies[92].

Crosstalk between the signaling of the IGF/IGFR

system and other growth factor receptors will likely attenuate the antineoplastic effect of monotherapeutic approaches, necessitating combinations of IGF/IGFR-targeting therapies with other therapies to enhance efficacy[93,94]. This can be achieved by dual-targeting the IGF-1R and the EGFR, since the EGFR is activated by the IGF/IGFR-system leading to mito-oncogenic EGFR-tyrosine kinase activity without ligand stimulation of the EGFR[95]. In this line IGFR- combined with EGFR-inhibition can over-additively enhance the antineoplastic effect of the respective monotherapies in gastrointestinal cancers[96-98].

DUAL-TARGETING SMALL MOLECULE INHIBITORS

The use of dual-targeting small molecule inhibitors, simultaneously blocking less related kinases such as VEGFR and EGFR tyrosine kinases, may also be promising for the future treatment of BTC. These agents inhibit both tumor cell proliferation/survival by blocking mito-oncogenic EGFR signaling of the tumor cells and angiogenesis by inhibiting endothelial VEGFRs. Recent *in vivo* studies of non-cholangiocarcinoma models (colon, prostate, NSCLC) demonstrated that the dual-targeting tyrosine kinase inhibitor NVP-AEE788 displayed significant antineoplastic efficacy[99-101]. NVP-AEE788 was recently also shown to be a potent inhibitor of cholangiocarcinoma cell growth[33], further emphasizing the possible suitability of EGFR/VEGFR-dual targeting agents for the treatment of cholangiocarcinoma.

ZD6474 (Zactima) is another EGFR/VEGFR tyrosine kinase inhibitor with potent antineoplastic properties in phase Ⅱ/Ⅲ trials on NSCLC and thyroid cancer. In these trials response rates of 30% in patients with locally advanced medullary thyroid cancer[102] as well as significant prolongation in the progression-free survival of NSCLC patients[103,104] were observed.

Clinical studies on BTC using these dual target kinase inhibitors have not yet been conducted. Nevertheless, the idea of simultaneously inhibiting these two growth factor receptor systems is currently under clinical investigation using a combination of EGFR-inhibiting erlotinib together with VEGF-neutralizing bevacizumab (see before). Indeed, the University of Colorado together with Astra Zeneca only recently started a phase I trial (NCT00551096) to determine the highest dose of Zactima that can be safely given as a single agent or in combination with gemcitabine and capecitabine in advanced solid tumors. This study is explicitly planned with an expanded cohort of patients with biliary cancers (BTC and gallbladder cancer), who will be treated at the highest determined dose in further studies.

OTHER STRATEGIES

Targeting the AKT/mTOR pathway

The activated PI3K/AKT/mTOR pathway has emerged as a novel contributor to BTC development[105]. PI3K associates with the intracellular domain of several growth factor receptors. Upon receptor activation, PI3K triggers the generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), which provokes the subsequent activation of AKT, a serine/threonine kinase that activates multiple cellular target proteins, such as the mammalian target of rapamycin (mTOR) subfamily. mTOR is a serine-threonine kinase that downregulates apoptosis, and *via* stimulation of cell cycle progression enhances proliferation and cell growth. Specifically, mTOR is involved in the activation of mRNA-translation into proteins, which are necessary for cell cycle progression from G1 to S-phase, including the E4-binding protein (E4-BP1), and $p70^{S6}$ kinase[106]. In nontransformed cells the PI3K/AKT/mTOR pathway is controlled by the phosphatase and tensin homolog deleted on chromosome ten (PTEN), a tumor suppressor which inhibits this pathway by reversing PI3K and subsequent AKT activation. Mutation or silencing of the PTEN gene leads to activation of the mTOR pathway and promotion of carcinogenesis.

AKT-inhibition

The tricyclic nucleoside VQD-002 (triciribine phosphate monohydrate, TCN-P, Vioquest Pharmaceuticals) is a small molecule inhibitor of AKT signaling. Identified by the Moffitt Cancer Center through screening the NCI diversity set, VQD-002 was shown to be highly selective for Akt without affecting the activation of other related kinases, such as PI3K, PKC, phosphoinositide-dependent kinase-1, serum and glucocorticoid-inducible kinase, PKA, STAT-3 or ERK1/2. Accordingly, AKT-inhibition by VQD-002 resulted in suppression of cell growth and induction of apoptosis in human cancer cells and in tumor xenograft mouse models, with high selectivity for those tumors with aberrant Akt[107]. An ongoing phase Ⅰ/Ⅱa trial (NCT00363454) on metastatic solid tumors overexpressing AKT, such as pancreatic, breast, ovarian and colorectal cancer is promising, as preliminary results indicate that VQD-002 was well tolerated and prolonged the stable disease period of patients (http:www.vioquestpharm.com). VQD-002 is already earmarked for combination with the EGFR antagonist erlotinib, since preclinical studies showed that coadministration of VQD-002 can help to overcome resistance to EGFR-antibody therapy in breast cancer patients with PTEN-deficiency[108].

mTOR-inhibition

The natural antibiotic rapamycin (sirolimus) is a potent inhibitor of mTOR[109]. Recently, three analogues of rapamycin with superior pharmacokinetic and biological properties have emerged. The cell cycle inhibitor-779 (CCI-779, temsirolimus) is a soluble ester analogue. RAD001 [40-O-(2-hydroxyethyl)-rapamycin, everolimus] is a derivative of rapamycin with high oral bioavailability, and AP23573 is a non-pro-drug analogue of rapamycin. These agents have been successfully tested for their antineoplastic potency and/or tolerability in various malignancies in early clinical trials (e.g. CCI-779 in renal, breast and lung cancers),

or are currently being studied in open clinical trials for the treatment of colorectal, endometrial, and brain tumors (RAD001, everolimus)[110-112]. AP23573 has been successfully tested in a phase Ⅱ trial in sarcomas[113], and two phase I studies in patients with refractory or advanced solid tumors showed partial responses and disease stabilization in individual patients[114]. In preclinical investigations, the antiproliferative, antimigratory and anti-invasive potency of rapamycin in cholangiocarcinoma cells has recently been described[115]. Activated mTOR was also demonstrated to be a negative prognostic factor for patients with BTC, and patients with activated mTOR are likely to benefit from targeted therapy with mTOR inhibitors in the future[116]. However, so far no trials exploring mTOR-inhibitors for BTC have been initiated.

Targeting the Ras/Raf/MARK pathway

The proliferative Ras/Raf/MEK/ERK pathway is one the key signaling cascades that underlies the development and maintenance of cancers. This pathway transduces extracellular signals from the various growth factor receptor tyrosine kinases (e.g. EGFR, IGFR, VEGFR and PDGFR) to the nucleus with a series of specific phosphorylation events, resulting in the expression of proteins for cell cycle progression, apoptosis resistance, extracellular matrix remodeling, cellular motility, angiogenesis or drug resistance[117]. Dysregulation of this crucial pathway occurs due to oncogenic transformation of Ras and Raf isoforms, or to overexpression and/or overactivation (*via* phosphorylation) of the Ras and Raf genes[118,119]. Activating B-Raf mutations are relatively common in cholangiocarcinomas and disruption of the Raf/MEK/ERK (MAPK) kinase pathway, either by B-Raf or Ras mutations, is detected in more than 60% of all BTC, which is therefore one of the most frequent defects in cholangiocellular carcinogenesis[120].

Sorafenib

The bi-aryl urea derivative sorafenib (Nexavar™) is an oral multi-kinase inhibitor, which targets kinases of wild-type B-Raf, mutantV559E B-Raf and C-Raf, and importantly receptor tyrosine kinases involved in angiogenesis, including VEGFR-2, and -3, and PDGFR[121]. Sorafenib has been approved by the FDA for the treatment of advanced renal cell carcinoma and of inoperable hepatocellular cancer.

The effect of sorafenib on several molecular targets in addition to the Raf isoforms makes it difficult to determine which of its targets contributes most to its antitumor activity in a given tumor type. For instance, a recent HCC trial suggested that inhibition of the Raf/MEK/ERK pathway was central to sorafenib's mode of antitumor action[122], whereas in other cancers, such as renal cell carcinoma or NSCLC, the antineoplastic activity was attributed mainly to its antiangiogenic activity[121,123].

Sorafenib alone or in combination with conventional cytostatics (5-fluorouracil, gemcitabine, doxorubicin) or IGF-1R inhibition induces a potent growth suppression of cholangiocarcinoma cells *in vitro*[86]. Antitumor efficacy was even higher when sorafenib was combined with the histone deacetylase inhibitor MS-275[124,125]. These encouraging findings have resulted in an ongoing phase Ⅱ trial which evaluates sorafenib monotherapy in patients with unresectable or metastatic gallbladder cancer or BTC (NCT00238212). In an intermediate evaluation of this study, sorafenib was well tolerated, but as a single agent it did not lead to a clinically significant response rate in these patients, while its impact on survival was comparable to commonly used chemotherapy regimens. These promising results of sorafenib monotherapy will likely facilitate novel therapeutic strategies which will combine multikinase inhibition with conventional cytostatic therapy or with unrelated pathway inhibitors, such as histone deacetylase or proteasome inhibitors (see below) for enhanced and well tolerated medical treatment of advanced BTC[126].

Targeting the proteasome

Another interesting therapeutic approach for innovative cancer treatment is the inhibition of the 26S proteasome, which is a large protease that is present in both the nucleus and the cytoplasm of eukaryotic cells. The proteasome functions as an identifier and proteolytic graveyard for proteins branded for destruction by the ubiquitin system. The so-called ubiquitin-proteasome pathway (UPP) is the major non-lysosomal proteolytic system in eukaryotic cells and triggers degradation of proteins involved in cell cycle progression, apoptosis, nuclear factor kappa B (NF-κB) activation, and angiogenesis. UPP also degrades mutant, damaged, and misfolded proteins[127]. Since these signaling pathways are critical for cell survival and proliferation, especially in cancer cells, inhibition of the proteasome has emerged as an attractive target for cancer therapy.

Bortezomib

Bortezomib (Velcade™) is a proteasome inhibitor, which blocks multi-ubiquitinated protein degradation by reversibly and competitively inhibiting the active site threonine residue of the 26S proteasome[128]. Antineoplastic activity of bortezomib has already been shown in several *in vitro* and *in vivo* studies[129,130]. Only recently we and others showed the potent apoptosis inducing and growth inhibiting features of bortezomib in cholangiocarcinoma cells[125,131]. Bortezomib is the first proteasome inhibitor to be approved for cancer therapy and based on the results of a phase Ⅱ trial[127] has recently been approved by the FDA for the treatment of mantle cell lymphoma[132,133]. Other cancers, including neuroendocrine tumors, RCC, NSCLC, or metastatic sarcomas have also been evaluated in recent phase Ⅱ clinical trials. In some of these studies a significant antineoplastic effect with bortezomib monotherapy was observed, while in other studies no or only marginal responses were found[134-136]. However, in the latter cases further investigation on the role of bortezomib in combination with other antitumoral drugs was recommended, since proteasome

inhibition will likely sensitize cancer cells to other therapeutic agents. Combinations with encouraging results have been reported in two studies of lung cancer and lymphoma[137,138]. In another phase I trial, bortezomib was tested in combination with the cytotoxic agent docetaxel in advanced solid tumors, including cholangiocarcinoma, where it showed generally good tolerability[13]. A phase Ⅱ trial exploring bortezomib as first-line systemic therapy of patients with unresectable or metastatic adenocarcinoma of the bile duct or gallbladder is currently ongoing (NCT00085410). A comparable study in HCC was recently reported to have resulted in disease stabilization in some patients, with generally good tolerability. Here it was again suggested that the focus should next be on combinations of bortezomib with HCC-relevant cytostatics such as doxorubicin[139]. In the *in vitro* studies on cholangiocellular carcinoma cells we found that bortezomib shows over-additive antitumoral effects when combined with multikinase inhibitors like sorafenib or histone deacetylase inhibitors, such as MS-275[125].

CONCLUSION

Targeted-therapies, which specifically inhibit growth factor receptors and their related signaling pathways, are promising approaches for the innovative medical treatment of biliary tract and gallbladder cancers. In particular, antiangiogenic strategies as well as combination treatments with cytostatics have proved particularly efficient, as they leave fewer mechanisms of escape for the tumor cells. Combinations of these targeted drugs are especially intriguing, and in the future multi-kinase inhibitors such as sorafenib will be combined with other growth factor receptor inhibitors, proteasome inhibitors, histone deacetylase inhibitors, farnesyltransferase inhibitors or cytostatics to effectively control advanced biliary tract or gallbladder cancers. The advantage of such novel combination therapies is their higher tumor cell specificity and higher efficacy, combined with acceptable toxicity and side effects. These novel combination treatments will widen the therapeutic spectrum for biliary tract and gallbladder cancers; the results of (ongoing) clinical studies are eagerly awaited.

REFERENCES

1 **de Groen PC**, Gores GJ, LaRusso NF, Gunderson LL, Nagorney DM. Biliary tract cancers. *N Engl J Med* 1999; **341**: 1368-1378
2 **Renshaw K**. Malignant neoplasms of the extrahepatic biliary ducts. *Ann Surg* 1922; **76**: 205-221
3 **Goldzieher M**, von Bókay Z. Der primäre Leberkrebs. *Virchows Arch* 1911; **203**: 75-131
4 **Klatskin G**. Adenocarcinoma of the hepatic duct at its bifurcation within the porta hepatis: an unusual tumor with distinctive clinical and pathological features. *Am J Med* 1965; **38**: 241-256
5 **Khan SA**, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 2002; **37**: 806-813
6 **Patel T**. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001; **33**: 1353-1357
7 **Patel T**. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer* 2002; **2**: 10
8 **Taylor-Robinson SD**, Toledano MB, Arora S, Keegan TJ, Hargreaves S, Beck A, Khan SA, Elliott P, Thomas HC. Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968-1998. *Gut* 2001; **48**: 816-820
9 **Ishak KG**, Anthony PP, Sobin LH. Histological typing of tumours of the liver. World Health Organization International Histological Typing of Tumours. 2nd ed. Berlin: Springer-Verlag, 2007
10 **Carriaga MT**, Henson DE. Liver, gallbladder, extrahepatic bile ducts, and pancreas. *Cancer* 1995; **75**: 171-190
11 **Shaib Y**, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 115-125
12 **Dragovich T**, Huberman M, Von Hoff DD, Rowinsky EK, Nadler P, Wood D, Hamilton M, Hage G, Wolf J, Patnaik A. Erlotinib plus gemcitabine in patients with unresectable pancreatic cancer and other solid tumors: phase IB trial. *Cancer Chemother Pharmacol* 2007; **60**: 295-303
13 **Messersmith WA**, Baker SD, Lassiter L, Sullivan RA, Dinh K, Almuete VI, Wright JJ, Donehower RC, Carducci MA, Armstrong DK. Phase I trial of bortezomib in combination with docetaxel in patients with advanced solid tumors. *Clin Cancer Res* 2006; **12**: 1270-1275
14 **Lapatinib in treating patients with unresectable liver or biliary tract cancer**. ClinicalTrials.gov Identifier: NCT00107536. Available from: URL: http://clinicaltrials.gov/show/NCT00107536
15 **Sorafenib in treating patients with unresectable or metastatic gallbladder cancer or cholangiocarcinoma**. ClinicalTrials.gov Identifier: NCT00238212. Available from: URL: http://clinicaltrials.gov/show/NCT00238212
16 **Oxaliplatin, gemcitabine, erlotinib, and radiation therapy in treating patients with unresectable and/or metastatic pancreatic cancer or biliary tract cancer**. ClinicalTrials.gov Identifier: NCT00266097. Available from: URL: http://clinicaltrials.gov/show/NCT00266097
17 **Avastin and tarceva for upper gastrointestinal cancers**. ClinicalTrials.gov Identifier: NCT00350753. Available from: URL: http://clinicaltrials.gov/show/NCT00350753
18 **Bevacizumab and erlotinib in treating patients with metastatic or unresectable biliary tumors.** ClinicalTrials.gov Identifier: NCT00356889. Available from: URL: http://clinicaltrials.gov/show/NCT00356889
19 **Gemcitabine, oxaliplatin in combination with bevacizumab in biliary tract and gallbladder cancer**. ClinicalTrials.gov Identifier: NCT00361231. Available from: URL: http://clinicaltrials.gov/show/NCT00361231
20 **Floxuridine and dexamethasone as a hepatic arterial infusion and bevacizumab in treating patients with primary liver cancer that cannot be removed by surgery**. ClinicalTrials.gov Identifier: NCT00410956. Available from: URL: http://clinicaltrials.gov/show/NCT00410956
21 **Proton therapy and bevacizumab for primary liver tumors**. ClinicalTrials.gov Identifier: NCT00426829. Available from: URL: http://clinicaltrials.gov/show/NCT00426829
22 **Biliary cancers: egfr inhibitor, gemcitabine and oxaliplatin**. ClinicalTrials.gov Identifier: NCT00552149. Available from: URL: http://clinicaltrials.gov/show/NCT00552149
23 **CO07204-Phase I/II of oxaliplatin, capecitabine & sorafenib for Advanced Pancreatic & Biliary Carcinoma**. ClinicalTrials.gov Identifier: NCT00634751. Available from: URL: http://clinicaltrials.gov/show/NCT00634751
24 **Gemcitabine and Sorafenib in Advanced Biliary Tract Cancer (GEMSO)**. ClinicalTrials.gov Identifier: NCT00661830. Available from: URL: http://clinicaltrials.gov/show/NCT00661830

25 **Carmeliet P**. Angiogenesis in health and disease. *Nat Med* 2003; **9**: 653-660
26 **Folkman J**. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002; **29**: 15-18
27 **Folkman J**. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; **285**: 1182-1186
28 **Shibuya M**. Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis. *Angiogenesis* 2006; **9**: 225-230; discussion 231
29 **Yoshikawa D**, Ojima H, Iwasaki M, Hiraoka N, Kosuge T, Kasai S, Hirohashi S, Shibata T. Clinicopathological and prognostic significance of EGFR, VEGF, and HER2 expression in cholangiocarcinoma. *Br J Cancer* 2008; **98**: 418-425
30 **Park BK**, Paik YH, Park JY, Park KH, Bang S, Park SW, Chung JB, Park YN, Song SY. The clinicopathologic significance of the expression of vascular endothelial growth factor-C in intrahepatic cholangiocarcinoma. *Am J Clin Oncol* 2006; **29**: 138-142
31 **Hida Y**, Morita T, Fujita M, Miyasaka Y, Horita S, Fujioka Y, Nagashima K, Katoh H. Vascular endothelial growth factor expression is an independent negative predictor in extrahepatic biliary tract carcinomas. *Anticancer Res* 1999; **19**: 2257-2260
32 **Benckert C**, Jonas S, Cramer T, Von Marschall Z, Schafer G, Peters M, Wagner K, Radke C, Wiedenmann B, Neuhaus P, Hocker M, Rosewicz S. Transforming growth factor beta 1 stimulates vascular endothelial growth factor gene transcription in human cholangiocellular carcinoma cells. *Cancer Res* 2003; **63**: 1083-1092
33 **Wiedmann M**, Feisthammel J, Bluthner T, Tannapfel A, Kamenz T, Kluge A, Mossner J, Caca K. Novel targeted approaches to treating biliary tract cancer: the dual epidermal growth factor receptor and ErbB-2 tyrosine kinase inhibitor NVP-AEE788 is more efficient than the epidermal growth factor receptor inhibitors gefitinib and erlotinib. *Anticancer Drugs* 2006; **17**: 783-795
34 **Hurwitz H**, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004; **350**: 2335-2342
35 **Sandler A**, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, Lilenbaum R, Johnson DH. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006; **355**: 2542-2550
36 **Tai CJ**, Chiou HY, Wu CH, Pan S, Liu JD. Rapid resolution of liver metastasis from cholangiocarcinoma after bevacizumab with cisplatin and high-dose fluorouracil plus leucovorin. *Onkologie* 2006; **29**: 179-180
37 **Müller RP**, Seegenschmiedt MH, Höffken K, Junginger T, Sauer H. Common Toxicity Criteria (CTC): Dokumentation von Nebenwirkungen in der Onkologie. *Dtsch Ärztebl* 1999; **96**: A489-A495
38 **Greten TF**. [Molecular therapy for HCC?] *Z Gastroenterol* 2006; **44**: 205-206
39 **Riedel F**, Gotte K, Li M, Hormann K, Grandis JR. EGFR antisense treatment of human HNSCC cell lines down-regulates VEGF expression and endothelial cell migration. *Int J Oncol* 2002; **21**: 11-16
40 **Ciardiello F**, Caputo R, Bianco R, Damiano V, Fontanini G, Cuccato S, De Placido S, Bianco AR, Tortora G. Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 2001; **7**: 1459-1465
41 **Viloria-Petit A**, Crombet T, Jothy S, Hicklin D, Bohlen P, Schlaeppi JM, Rak J, Kerbel RS. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. *Cancer Res* 2001; **61**: 5090-5101
42 **Gridelli C**, Maione P, Rossi A, De Marinis F. The role of bevacizumab in the treatment of non-small cell lung cancer: current indications and future developments. *Oncologist* 2007; **12**: 1183-1193
43 **Hainsworth JD**, Sosman JA, Spigel DR, Edwards DL, Baughman C, Greco A. Treatment of metastatic renal cell carcinoma with a combination of bevacizumab and erlotinib. *J Clin Oncol* 2005; **23**: 7889-7896
44 **Herbst RS**, Johnson DH, Mininberg E, Carbone DP, Henderson T, Kim ES, Blumenschein G Jr, Lee JJ, Liu DD, Truong MT, Hong WK, Tran H, Tsao A, Xie D, Ramies DA, Mass R, Seshagiri S, Eberhard DA, Kelley SK, Sandler A. Phase I/II trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. *J Clin Oncol* 2005; **23**: 2544-2555
45 **Czito BG**, Bendell JC, Willett CG, Morse MA, Blobe GC, Tyler DS, Thomas J, Ludwig KA, Mantyh CR, Ashton J, Yu D, Hurwitz HI. Bevacizumab, oxaliplatin, and capecitabine with radiation therapy in rectal cancer: Phase I trial results. *Int J Radiat Oncol Biol Phys* 2007; **68**: 472-478
46 **Fischer C**, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, Chorianopoulos E, Liesenborghs L, Koch M, De Mol M, Autiero M, Wyns S, Plaisance S, Moons L, van Rooijen N, Giacca M, Stassen JM, Dewerchin M, Collen D, Carmeliet P. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 2007; **131**: 463-475
47 **Willett CG**, Boucher Y, Duda DG, di Tomaso E, Munn LL, Tong RT, Kozin SV, Petit L, Jain RK, Chung DC, Sahani DV, Kalva SP, Cohen KS, Scadden DT, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Shellito PC, Mino-Kenudson M, Lauwers GY. Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. *J Clin Oncol* 2005; **23**: 8136-8139
48 **Motzer RJ**, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI. Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *J Clin Oncol* 2006; **24**: 16-24
49 **Rosen LS**, Kurzrock R, Mulay M, Van Vugt A, Purdom M, Ng C, Silverman J, Koutsoukos A, Sun YN, Bass MB, Xu RY, Polverino A, Wiezorek JS, Chang DD, Benjamin R, Herbst RS. Safety, pharmacokinetics, and efficacy of AMG 706, an oral multikinase inhibitor, in patients with advanced solid tumors. *J Clin Oncol* 2007; **25**: 2369-2376
50 **Cabebe E**, Wakelee H. Role of anti-angiogenesis agents in treating NSCLC: focus on bevacizumab and VEGFR tyrosine kinase inhibitors. *Curr Treat Options Oncol* 2007; **8**: 15-27
51 **Zhu AX**. Development of sorafenib and other molecularly targeted agents in hepatocellular carcinoma. *Cancer* 2008; **112**: 250-259
52 **Drevs J**, Siegert P, Medinger M, Mross K, Strecker R, Zirrgiebel U, Harder J, Blum H, Robertson J, Jurgensmeier JM, Puchalski TA, Young H, Saunders O, Unger C. Phase I clinical study of AZD2171, an oral vascular endothelial growth factor signaling inhibitor, in patients with advanced solid tumors. *J Clin Oncol* 2007; **25**: 3045-3054
53 **Aragon-Ching JB**, Dahut WL. The role of angiogenesis inhibitors in prostate cancer. *Cancer J* 2008; **14**: 20-25
54 **Wedge SR**, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, Smith NR, James NH, Dukes M, Curwen JO, Chester R, Jackson JA, Boffey SJ, Kilburn LL, Barnett S, Richmond GH, Wadsworth PF, Walker M, Bigley AL, Taylor ST, Cooper L, Beck S, Jurgensmeier JM, Ogilvie DJ.

AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. *Cancer Res* 2005; **65**: 4389-4400

55 **Nowak D**, Boehrer S, Hochmuth S, Trepohl B, Hofmann W, Hoelzer D, Hofmann WK, Mitrou PS, Ruthardt M, Chow KU. Src kinase inhibitors induce apoptosis and mediate cell cycle arrest in lymphoma cells. *Anticancer Drugs* 2007; **18**: 981-995

56 **Chiorean MV**, Guicciardi ME, Yoon JH, Bronk SF, Kaufmanns SH, Gores GJ. Imatinib mesylate induces apoptosis in human cholangiocarcinoma cells. *Liver Int* 2004; **24**: 687-695

57 **Ito Y**, Kawakatsu H, Takeda T, Sakon M, Nagano H, Sakai T, Miyoshi E, Noda K, Tsujimoto M, Wakasa K, Monden M, Matsuura N. Activation of c-Src gene product in hepatocellular carcinoma is highly correlated with the indices of early stage phenotype. *J Hepatol* 2001; **35**: 68-73

58 **Rocha-Lima CM**, Soares HP, Raez LE, Singal R. EGFR targeting of solid tumors. *Cancer Control* 2007; **14**: 295-304

59 **Sangro B**, Mazzollini G, Prieto J. Future therapies for hepatocellular carcinoma. *Eur J Gastroenterol Hepatol* 2005; **17**: 515-521

60 **Thatcher N**, Chang A, Parikh P, Rodrigues Pereira J, Ciuleanu T, von Pawel J, Thongprasert S, Tan EH, Pemberton K, Archer V, Carroll K. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet* 2005; **366**: 1527-1537

61 **Yoon JH**, Gwak GY, Lee HS, Bronk SF, Werneburg NW, Gores GJ. Enhanced epidermal growth factor receptor activation in human cholangiocarcinoma cells. *J Hepatol* 2004; **41**: 808-814

62 **Werneburg NW**, Yoon JH, Higuchi H, Gores GJ. Bile acids activate EGF receptor via a TGF-alpha-dependent mechanism in human cholangiocyte cell lines. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G31-G36

63 **Jimeno A**, Rubio-Viqueira B, Amador ML, Oppenheimer D, Bouraoud N, Kulesza P, Sebastiani V, Maitra A, Hidalgo M. Epidermal growth factor receptor dynamics influences response to epidermal growth factor receptor targeted agents. *Cancer Res* 2005; **65**: 3003-3010

64 **Philip PA**, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G, Donehower RC, Fitch T, Picus J, Erlichman C. Phase II study of erlotinib in patients with advanced biliary cancer. *J Clin Oncol* 2006; **24**: 3069-3074

65 **Leone F**, Pignochino Y, Cavalloni G, Aglietta M. Targeting of epidermal growth factor receptor in patients affected by biliary tract carcinoma. *J Clin Oncol* 2007; **25**: 1145; author reply 1145-1145; author reply 1146

66 **Paule B**, Bralet M, Herelle M, Rage E, Ducreux M, Guettier C, Adam R. Cetuximab plus gemcitabine/oxaliplatin (GEMOX) for patients with unresectable/recurrent intrahepatic cholangiocarcinoma refractory to GEMOX. *J Clin Oncol* 2007; **24** Suppl 18: 14084

67 **Paule B**, Herelle MO, Rage E, Ducreux M, Adam R, Guettier C, Bralet MP. Cetuximab plus gemcitabine-oxaliplatin (GEMOX) in patients with refractory advanced intrahepatic cholangiocarcinomas. *Oncology* 2007; **72**: 105-110

68 **Ganslmayer M**, Ocker M, Kraemer G, Zopf S, Hahn EG, Schuppan D, Herold C. The combination of tamoxifen and 9cis retinoic acid exerts overadditive anti-tumoral efficacy in rat hepatocellular carcinoma. *J Hepatol* 2004; **40**: 952-956

69 **Ciardiello F**, Troiani T, Bianco R, Orditura M, Morgillo F, Martinelli E, Morelli MP, Cascone T, Tortora G. Interaction between the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF) pathways: a rational approach for multi-target anticancer therapy. *Ann Oncol* 2006; **17** Suppl 7: vii109-vii114

70 **Sachdev D**, Yee D. Disrupting insulin-like growth factor signaling as a potential cancer therapy. *Mol Cancer Ther* 2007; **6**: 1-12

71 **Maione P**, Gridelli C, Troiani T, Ciardiello F. Combining targeted therapies and drugs with multiple targets in the treatment of NSCLC. *Oncologist* 2006; **11**: 274-284

72 **Zhang H**, Yee D. The therapeutic potential of agents targeting the type I insulin-like growth factor receptor. *Expert Opin Investig Drugs* 2004; **13**: 1569-1577

73 **Wang Z**, Ruan YB, Guan Y, Liu SH. Expression of IGF-II in early experimental hepatocellular carcinomas and its significance in early diagnosis. *World J Gastroenterol* 2003; **9**: 267-270

74 **Wang Y**, Sun Y. Insulin-like growth factor receptor-1 as an anti-cancer target: blocking transformation and inducing apoptosis. *Curr Cancer Drug Targets* 2002; **2**: 191-207

75 **Hofmann F**, Garcia-Echeverria C. Blocking the insulin-like growth factor-I receptor as a strategy for targeting cancer. *Drug Discov Today* 2005; **10**: 1041-1047

76 **Alvaro D**, Barbaro B, Franchitto A, Onori P, Glaser SS, Alpini G, Francis H, Marucci L, Sterpetti P, Ginanni-Corradini S, Onetti Muda A, Dostal DE, De Santis A, Attili AF, Benedetti A, Gaudio E. Estrogens and insulin-like growth factor 1 modulate neoplastic cell growth in human cholangiocarcinoma. *Am J Pathol* 2006; **169**: 877-888

77 **Scotlandi K**, Benini S, Nanni P, Lollini PL, Nicoletti G, Landuzzi L, Serra M, Manara MC, Picci P, Baldini N. Blockage of insulin-like growth factor-I receptor inhibits the growth of Ewing's sarcoma in athymic mice. *Cancer Res* 1998; **58**: 4127-4131

78 **Shapiro DN**, Jones BG, Shapiro LH, Dias P, Houghton PJ. Antisense-mediated reduction in insulin-like growth factor-I receptor expression suppresses the malignant phenotype of a human alveolar rhabdomyosarcoma. *J Clin Invest* 1994; **94**: 1235-1242

79 **Salisbury AJ**, Macaulay VM. Development of molecular agents for IGF receptor targeting. *Horm Metab Res* 2003; **35**: 843-849

80 **Ellouk-Achard S**, Djenabi S, De Oliveira GA, Desauty G, Duc HT, Zohair M, Trojan J, Claude JR, Sarasin A, Lafarge-Frayssinet C. Induction of apoptosis in rat hepatocarcinoma cells by expression of IGF-I antisense c-DNA. *J Hepatol* 1998; **29**: 807-818

81 **Tanno B**, Mancini C, Vitali R, Mancuso M, McDowell HP, Dominici C, Raschella G. Down-regulation of insulin-like growth factor I receptor activity by NVP-AEW541 has an antitumor effect on neuroblastoma cells in vitro and in vivo. *Clin Cancer Res* 2006; **12**: 6772-6780

82 **Höpfner M**, Sutter AP, Huether A, Baradari V, Scherubl H. Tyrosine kinase of insulin-like growth factor receptor as target for novel treatment and prevention strategies of colorectal cancer. *World J Gastroenterol* 2006; **12**: 5635-5643

83 **Höpfner M**, Huether A, Sutter AP, Baradari V, Schuppan D, Scherubl H. Blockade of IGF-1 receptor tyrosine kinase has antineoplastic effects in hepatocellular carcinoma cells. *Biochem Pharmacol* 2006; **71**: 1435-1448

84 **Garcia-Echeverria C**, Pearson MA, Marti A, Meyer T, Mestan J, Zimmermann J, Gao J, Brueggen J, Capraro HG, Cozens R, Evans DB, Fabbro D, Furet P, Porta DG, Liebetanz J, Martiny-Baron G, Ruetz S, Hofmann F. In vivo antitumor activity of NVP-AEW541-A novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell* 2004; **5**: 231-239

85 **Scotlandi K**, Manara MC, Nicoletti G, Lollini PL, Lukas S, Benini S, Croci S, Perdichizzi S, Zambelli D, Serra M, Garcia-Echeverria C, Hofmann F, Picci P. Antitumor activity of the insulin-like growth factor-I receptor kinase inhibitor NVP-AEW541 in musculoskeletal tumors. *Cancer Res* 2005; **65**: 3868-3876

86 **Huether A**, Höpfner M, Baradari V, Schuppan D, Scherubl H. Sorafenib alone or as combination therapy for growth control of cholangiocarcinoma. *Biochem Pharmacol* 2007; **73**: 1308-1317

87 **Feng Y**, Zhu Z, Xiao X, Choudhry V, Barrett JC, Dimitrov

DS. Novel human monoclonal antibodies to insulin-like growth factor (IGF)-II that potently inhibit the IGF receptor type I signal transduction function. *Mol Cancer Ther* 2006; **5**: 114-120

88 **Manara MC**, Landuzzi L, Nanni P, Nicoletti G, Zambelli D, Lollini PL, Nanni C, Hofmann F, Garcia-Echeverria C, Picci P, Scotlandi K. Preclinical in vivo study of new insulin-like growth factor-I receptor--specific inhibitor in Ewing's sarcoma. *Clin Cancer Res* 2007; **13**: 1322-1330

89 **Hofmann F**, Brueggen J, Capraro H-G, Cozens R, Evans DB, Fabbro D, Ferrari S, Furet P, Garcia-Echeverria C, Geiger T, Porta DG, Liebetanz J, Maira SM, Marti A, Martiny-Baron G, Mestan J, Meyer T, Ruetz S, Stoltz B, Zimmermann J, Peterson MA. In vitro and in vivo profiling of selective and potent IGF-IR kinase inhibitors. *Proc AACR* 2003; **44**: 3798

90 **Burtrum D**, Zhu Z, Lu D, Anderson DM, Prewett M, Pereira DS, Bassi R, Abdullah R, Hooper AT, Koo H, Jimenez X, Johnson D, Apblett R, Kussie P, Bohlen P, Witte L, Hicklin DJ, Ludwig DL. A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth in vivo. *Cancer Res* 2003; **63**: 8912-8921

91 **Garber K**. IGF-1: old growth factor shines as new drug target. *J Natl Cancer Inst* 2005; **97**: 790-792

92 **Leary A**, Johnston SR. Small molecule signal transduction inhibitors for the treatment of solid tumors. *Cancer Invest* 2007; **25**: 347-365

93 **Desbois-Mouthon C**, Cacheux W, Blivet-Van Eggelpoel MJ, Barbu V, Fartoux L, Poupon R, Housset C, Rosmorduc O. Impact of IGF-1R/EGFR cross-talks on hepatoma cell sensitivity to gefitinib. *Int J Cancer* 2006; **119**: 2557-2566

94 **Tao Y**, Pinzi V, Bourhis J, Deutsch E. Mechanisms of disease: signaling of the insulin-like growth factor 1 receptor pathway--therapeutic perspectives in cancer. *Nat Clin Pract Oncol* 2007; **4**: 591-602

95 **Gilmore AP**, Valentijn AJ, Wang P, Ranger AM, Bundred N, O'Hare MJ, Wakeling A, Korsmeyer SJ, Streuli CH. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. *J Biol Chem* 2002; **277**: 27643-27650

96 **Höpfner M**, Sutter AP, Huether A, Baradari V, Scherubl H. Tyrosine kinase of insulin-like growth factor receptor as target for novel treatment and prevention strategies of colorectal cancer. *World J Gastroenterol* 2006; **12**: 5635-5643

97 **Huether A**, Höpfner M, Baradari V, Schuppan D, Scherubl H. EGFR blockade by cetuximab alone or as combination therapy for growth control of hepatocellular cancer. *Biochem Pharmacol* 2005; **70**: 1568-1578

98 **Huether A**, Hopfner M, Sutter AP, Schuppan D, Scherubl H. Erlotinib induces cell cycle arrest and apoptosis in hepatocellular cancer cells and enhances chemosensitivity towards cytostatics. *J Hepatol* 2005; **43**: 661-669

99 **Heymach JV**. ZD6474-clinical experience to date. *Br J Cancer* 2005; **92** Suppl 1: S14-S20

100 **Busby JE**, Kim SJ, Yazici S, Nakamura T, Kim JS, He J, Maya M, Wang X, Do KA, Fan D, Fidler IJ. Therapy of multidrug resistant human prostate tumors in the prostate of nude mice by simultaneous targeting of the epidermal growth factor receptor and vascular endothelial growth factor receptor on tumor-associated endothelial cells. *Prostate* 2006; **66**: 1788-1798

101 **Younes MN**, Park YW, Yazici YD, Gu M, Santillan AA, Nong X, Kim S, Jasser SA, El-Naggar AK, Myers JN. Concomitant inhibition of epidermal growth factor and vascular endothelial growth factor receptor tyrosine kinases reduces growth and metastasis of human salivary adenoid cystic carcinoma in an orthotopic nude mouse model. *Mol Cancer Ther* 2006; **5**: 2696-2705

102 **Lakhani VT**, You YN, Wells SA. The multiple endocrine neoplasia syndromes. *Annu Rev Med* 2007; **58**: 253-265

103 **Natale RB**, Bodkin D, Govindan R, Sleckman B, Rizvi N, Capo A, Germonpré P, Stockman P, Kennedy S, Ranson M. ZD6474 versus gefitinib in patients with advanced NSCLC: Final results from a two-part, double-blind, randomized phase II trial. *J Clin Oncol* 2006; **24** (18S): 7000

104 **Wells S**, You YN, Lakhani V, Hou J, Langmuir P, Headley D, Skinner M, Morse M, Burch W, Schlumberger M. A phase II trial of ZD6474 in patients with hereditary metastatic medullary thyroid cancer. *J Clin Oncol* 2006; **24** (20S): 5533

105 **Schmitz KJ**, Lang H, Wohlschlaeger J, Sotiropoulos GC, Reis H, Schmid KW, Baba HA. AKT and ERK1/2 signaling in intrahepatic cholangiocarcinoma. *World J Gastroenterol* 2007; **13**: 6470-6477

106 **Duran I**, Salazar R, Casanovas O, Arrazubi V, Vilar E, Siu LL, Yao J, Tabernero J. New drug development in digestive neuroendocrine tumors. *Ann Oncol* 2007; **18**: 1307-1313

107 **Yang L**, Dan HC, Sun M, Liu Q, Kaneko S, Sun XM, Feldman RI, Nicosia SV, Sebti SM, Cheng JQ. Discovery of a small molecule Akt inhibitor with antitumor activity in cancer cells overexpressing Akt. *Proc Amer Assoc Cancer Res* (meeting abstract) 2004; **45** (suppl): 893

108 **Yu D**, Esteva F, Lu CH, Wyszomierski S, Sahin A, Mills G, Hung MC, Hortobagyi G. Strategies for overcoming trastuzumab resistance caused by PTEN deficiency. Proceedings of the 99th Annual Meeting of the American Association for Cancer Research. *Pro AACR* 2008: 675

109 **Tsang CK**, Qi H, Liu LF, Zheng XF. Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discov Today* 2007; **12**: 112-124

110 **Dudkin L**, Dilling MB, Cheshire PJ, Harwood FC, Hollingshead M, Arbuck SG, Travis R, Sausville EA, Houghton PJ. Biochemical correlates of mTOR inhibition by the rapamycin ester CCI-779 and tumor growth inhibition. *Clin Cancer Res* 2001; **7**: 1758-1764

111 **Easton JB**, Houghton PJ. mTOR and cancer therapy. *Oncogene* 2006; **25**: 6436-6446

112 **Wullschleger S**, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell* 2006; **124**: 471-484

113 **Okuno S**. Mammalian target of rapamycin inhibitors in sarcomas. *Curr Opin Oncol* 2006; **18**: 360-362

114 **Smolewski P**. Recent developments in targeting the mammalian target of rapamycin (mTOR) kinase pathway. *Anticancer Drugs* 2006; **17**: 487-494

115 **Sawada T**, Okada T, Kubota K. Rapamycin inhibits the growth of cholangiocarcinoma cells in vitro. *J Clin Oncol* 2007; **25** (Suppl 18): 15153

116 **Herberger B**, Puhalla H, Lehnert M, Wrba F, Novak S, Brandstetter A, Gruenberger B, Gruenberger T, Pirker R, Filipits M. Activated mammalian target of rapamycin is an adverse prognostic factor in patients with biliary tract adenocarcinoma. *Clin Cancer Res* 2007; **13**: 4795-4799

117 **Sridhar SS**, Hedley D, Siu LL. Raf kinase as a target for anticancer therapeutics. *Mol Cancer Ther* 2005; **4**: 677-685

118 **Fukushima T**, Suzuki S, Mashiko M, Ohtake T, Endo Y, Takebayashi Y, Sekikawa K, Hagiwara K, Takenoshita S. BRAF mutations in papillary carcinomas of the thyroid. *Oncogene* 2003; **22**: 6455-6457

119 **Davies H**, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. *Nature* 2002; **417**: 949-954

120 **Tannapfel A**, Sommerer F, Benicke M, Katalinic A, Uhlmann D, Witzigmann H, Hauss J, Wittekind C. Mutations of the BRAF gene in cholangiocarcinoma but not in hepatocellular carcinoma. *Gut* 2003; **52**: 706-712

121 **Wilhelm SM**, Carter C, Tang L, Wilkie D, McNabola A,

Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post LE, Bollag G, Trail PA. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004; **64**: 7099-7109

122 **Gollob JA**, Wilhelm S, Carter C, Kelley SL. Role of Raf kinase in cancer: therapeutic potential of targeting the Raf/MEK/ERK signal transduction pathway. *Semin Oncol* 2006; **33**: 392-406

123 **Liu L**, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, Wilhelm S, Lynch M, Carter C. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* 2006; **66**: 11851-11858

124 **Höpfner M**, Baradari V, Huether A, Scherübl H. Growth inhibition of human cholangiocarcinoma by sorafenib-based mono- and combination treatment. *FASEB J* 2008; **22**: 1136-1139

125 **Baradari V**, Hopfner M, Huether A, Schuppan D, Scherubl H. Histone deacetylase inhibitor MS-275 alone or combined with bortezomib or sorafenib exhibits strong antiproliferative action in human cholangiocarcinoma cells. *World J Gastroenterol* 2007; **13**: 4458-4466

126 **El-Khoueiry AB**, Rankin C, Lenz HJ, Philip P, Rivkin SE, Blanke CD. SWOG 0514: a phase II study of sorafenib (BAY 43-9006) as single agent in patients (pts) with unresectable or metastatic gallbladder cancer or cholangiocarcinomas. *J Clin Oncol* 2007; **25** (20S): 4639

127 **Roccaro AM**, Hideshima T, Richardson PG, Russo D, Ribatti D, Vacca A, Dammacco F, Anderson KC. Bortezomib as an antitumor agent. *Curr Pharm Biotechnol* 2006; **7**: 441-448

128 **Mitsiades CS**, Mitsiades N, Hideshima T, Richardson PG, Anderson KC. Proteasome inhibitors as therapeutics. *Essays Biochem* 2005; **41**: 205-218

129 **Schwartz R**, Davidson T. Pharmacology, pharmacokinetics, and practical applications of bortezomib. *Oncology* (Williston Park) 2004; **18**: 14-21

130 **Brignole C**, Marimpietri D, Pastorino F, Nico B, Di Paolo D, Cioni M, Piccardi F, Cilli M, Pezzolo A, Corrias MV, Pistoia V, Ribatti D, Pagnan G, Ponzoni M. Effect of bortezomib on human neuroblastoma cell growth, apoptosis, and angiogenesis. *J Natl Cancer Inst* 2006; **98**: 1142-1157

131 **Ustundag Y**, Bronk SF, Gores GJ. Proteasome inhibition-induces endoplasmic reticulum dysfunction and cell death of human cholangiocarcinoma cells. *World J Gastroenterol* 2007; **13**: 851-857

132 **Fisher RI**, Bernstein SH, Kahl BS, Djulbegovic B, Robertson MJ, de Vos S, Epner E, Krishnan A, Leonard JP, Lonial S, Stadtmauer EA, O'Connor OA, Shi H, Boral AL, Goy A. Multicenter phase II study of bortezomib in patients with relapsed or refractory mantle cell lymphoma. *J Clin Oncol* 2006; **24**: 4867-4874

133 **Kane RC**, Dagher R, Farrell A, Ko CW, Sridhara R, Justice R, Pazdur R. Bortezomib for the treatment of mantle cell lymphoma. *Clin Cancer Res* 2007; **13**: 5291-5294

134 **Maki RG**, Kraft AS, Scheu K, Yamada J, Wadler S, Antonescu CR, Wright JJ, Schwartz GK. A multicenter Phase II study of bortezomib in recurrent or metastatic sarcomas. *Cancer* 2005; **103**: 1431-1438

135 **Kondagunta GV**, Drucker B, Schwartz L, Bacik J, Marion S, Russo P, Mazumdar M, Motzer RJ. Phase II trial of bortezomib for patients with advanced renal cell carcinoma. *J Clin Oncol* 2004; **22**: 3720-3725

136 **Shah MH**, Young D, Kindler HL, Webb I, Kleiber B, Wright J, Grever M. Phase II study of the proteasome inhibitor bortezomib (PS-341) in patients with metastatic neuroendocrine tumors. *Clin Cancer Res* 2004; **10**: 6111-6118

137 **Fanucchi MP**, Fossella FV, Belt R, Natale R, Fidias P, Carbone DP, Govindan R, Raez LE, Robert F, Ribeiro M, Akerley W, Kelly K, Limentani SA, Crawford J, Reimers HJ, Axelrod R, Kashala O, Sheng S, Schiller JH. Randomized phase II study of bortezomib alone and bortezomib in combination with docetaxel in previously treated advanced non-small-cell lung cancer. *J Clin Oncol* 2006; **24**: 5025-5033

138 **O'Connor OA**. Marked clinical activity of the proteasome inhibitor bortezomib in patients with follicular and mantle-cell lymphoma. *Clin Lymphoma Myeloma* 2005; **6**: 191-199

139 **Hegewisch-Becker S**, Sterneck M, Schubert U, Rogiers X, Guerciolini R, Pierce JE, Hossfeld DK. Phase I/II trial of botrezomib in patients with unresectable hepatocellular carcinoma. ASCO annual meeting proceedings. *J Clin Oncol* 2004; **22** Suppl 15: 4089

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REVIEW

Role of ErbB family receptor tyrosine kinases in intrahepatic cholangiocarcinoma

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Abstract

Aberrant expression and signaling of epidermal growth factor receptor (ErbB) family receptor tyrosine kinases, most notably that of ErbB2 and ErbB1, have been implicated in the molecular pathogenesis of intrahepatic cholangiocarcinoma. Constitutive overexpression of ErbB2 and/or ErbB1 in malignant cholangiocytes has raised interest in the possibility that agents which selectively target these receptors could potentially be effective in cholangiocarcinoma therapy. However, current experience with such ErbB-directed therapies have at best produced only modest responses in patients with biliary tract cancers. This review provides a comprehensive and critical analysis of both preclinical and clinical studies aimed at assessing the role of altered ErbB2 and/or ErbB1 expression, genetic modifications, and dysregulated signaling on cholangiocarcinoma development and progression. Specific limitations in experimental approaches that have been used to assess human cholangiocarcinoma specimens for ErbB2 and/or ErbB1 overexpression and gene amplification are discussed. In addition, current rodent models of intrahepatic cholangiocarcinogenesis associated with constitutive ErbB2 overexpression are reviewed. Select interactive relationships between ErbB2 or ErbB1 with other relevant molecular signaling pathways associated with intrahepatic cholangiocarcinoma development and progression are also detailed, including those linking ErbB receptors to bile acid, cyclooxygenase-2, interleukin-6/gp130, transmembrane mucins, hepatocyte growth factor/Met, and vascular endothelial growth factor signaling. Lastly, various factors that can limit therapeutic efficacy of ErbB-targeted agents against cholangiocarcinoma are considered.

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Key words: Cholangiocarcinoma; ErbB activation; Bile acids; Cyclooxygenase-2; ErbB targeted therapies

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INTRODUCTION

Intrahepatic cholangiocarcinoma, also known as peripheral cholangiocarcinoma, is a primary epithelial cancer that arises within liver and which exhibits differentiation markers of biliary epithelial cells or cholangiocytes[1-3]. This rare, but highly malignant hepatobiliary cancer accounts for approximately 10%-15% of all primary liver cancer[3,4]. More than 90% of intrahepatic cholangiocarcinomas are classified histologically as well-to-moderately differentiated tubular adenocarcinomas, although other rare histological variants, including papillary, adenosquamous, and intestinal-type carcinoma also occur[1,2,5-7]. Typically, a desmoplastic reaction of variable degrees is a common histological feature and in some cases may be the most prominent characteristic of the tumor[1,2].

Morphologically, intrahepatic cholangiocarcinomas have been further classified as either having a mass-forming, periductular infiltrating, or intraductal growth pattern[1,2,8-10]. Of these morphological types, the intraductal growing cholangiocarcinoma is the least common, but has a more favorable prognosis than either

the mass-forming or periductular infiltrating types. Some tumors may also manifest a combination of growth patterns (i.e. mass-forming and periductular infiltrating), thereby precluding an absolute morphology-based classification system based solely on a single type of growth pattern.

Intrahepatic cholangiocarcinoma typically carries a very poor prognosis and the challenges posed by this cancer are formidable. Most notably, early diagnosis of intrahepatic cholangiocarcinoma is problematic, with a vast majority of patients being diagnosed at first presentation with advanced malignant disease. Thus, treatment options are limited and prospects for long-term survival are for the most part dismal. Current epidemiological data have further indicated a global increase over the past two to three decades in the age-adjusted incidence and mortality rates of intrahepatic cholangiocarcinoma[4,11-14], whereas the age-adjusted incidence and mortality rates for extrahepatic cholangiocarcinoma have been reported to be declining over a comparable time period[4,12]. It is of further interest that the most significant rise in intrahepatic cholangiocarcinoma incidence was noted among the older ($\geqslant$ 65 years of age) rather than younger age groups analyzed[4,12].

It has recently been reported that only about 10% of patients with intrahepatic cholangiocarcinoma have been found to have a known established risk factor, such as primary sclerosing cholangitis, hepatolithiasis, infestation with the liver flukes *Opisthorchis viverrini* or *Clonorchis sinensis*, and choledochal cysts[15]. Thus, a vast majority of patients presenting with intrahepatic cholangiocarcinoma do not have a history of these well-recognized risk factors and the cause for the rising incidence, particularly among older age groups, of this often fatal hepatobiliary malignancy remains unclear. However, in addition to the more well-established risk factors listed above, a number of chronic liver diseases, including alcoholic liver disease, hepatitis C and B, human immunodeficiency virus infection, unspecified cirrhosis, and diabetes have also been recently reported to be associated with the development of intrahepatic cholangiocarcinoma[12-14,16,17].

Common features of the few well-established risk factors and the more recently analyzed pre-existing chronic liver conditions seemingly predisposing for intrahepatic cholangiocarcinoma include chronic inflammation and bile duct cell injury often combined with cholestasis and altered bile composition. Molecular perturbations brought about by the mileau of cholangitis and cholestasis have been linked to the initiation, promotion and/or progression stages of cholangiocarcinogenesis[1,18-20]. Among the pathways affected and demonstrated to be playing a role in the molecular pathogenesis of intrahepatic cholangiocarcinogenesis are those mediated by the ErbB family of receptor tyrosine kinases, most notably involving the dysregulation of ErbB2 (HER2/neu) and/or epidermal growth factor receptor (EGFR) signaling. This review will critically evaluate the role played by the ErbB family receptor tyrosine kinases in the development and progression of intrahepatic cholangiocarcinoma. Specifically, the significance of aberrant ErbB2 and EGFR expression and genetic alterations in relation to the pathogenesis of human intrahepatic cholangiocarcinoma will be assessed. Experimental models linking constitutive overexpression of activated ErbB2 to intrahepatic cholangiocarcinoma development will also be described. In addition, relevant interactive relationships between ErbB2, as well as EGFR, with other key molecular pathways associated with intrahepatic cholangiocarcinoma development and/or progression and the effects of bile acids on ErbB receptor signaling in cholangiocarcinoma cells will be discussed. Lastly, the potential value of EGFR and/or ErbB2 as molecular targets in intrahepatic cholangiocarcinoma therapy will be assessed.

THE ERBB FAMILY OF RECEPTOR TYROSINE KINASES AND THEIR SPECIFIC LIGANDS

The ErbB family of class I receptor tyrosine kinases is comprised of four distinct receptors: EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. Each of these plasma membrane receptors, in turn, is composed of an extracellular ligand-binding domain, a transmembrane lipophilic domain, and a conserved cytoplasmic tyrosine kinase domain[21-24]. All of these receptors, with the exception of ErbB2, bind receptor specific ligands belonging to the EGF-family of growth factors. These EGF-related growth factors have been divided into three groups[21,23]. The first group, which includes epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and amphiregulin, binds specifically to EGFR. A second group, which shows dual specificity by binding to both EGFR and ErbB4, includes heparin-binding EGF, epiregulin, and betacellulin. Neuregulins (NRGs), compose the third group, with NRG1 and NRG2 (heregulins) being ligands for ErbB3 and ErbB4, and NRG3 and NRG4 binding only to ErbB4[21,23,25]. EGF-family growth factors are produced in cells as transmembrane precursors, which can then be shed as soluble active ligands through proteolysis catalyzed by cell surface acting proteases, most notably through the activity of matrix metalloproteinases (MMPs)[22,26,27]. This mechanism of cell surface growth factor shedding plays an important role in regulating ligand availability and receptor activation[22,27].

The binding of EGF-family ligands to the extracellular domain of ErbB receptors induces homo- or heterodimerization of the receptor proteins and activation of the intrinsic tyrosine kinase domain, resulting in the trans-phosphorylation of specific tyrosine residues within the receptor's cytoplasmic tail[21-23]. The phosphorylated residues then serve as docking or recruitment sites for a variety of signaling proteins[22,24,28,29], which in turn, initiate downstream signaling cascades and other molecular activities that

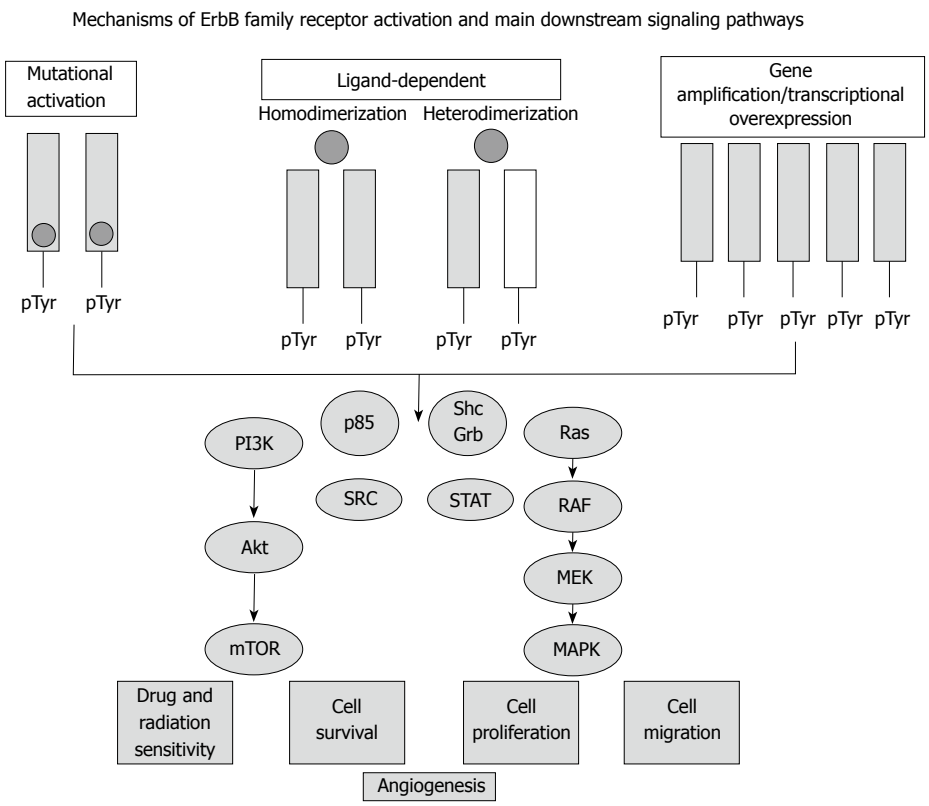

Figure 1 Simplified scheme depicting ErbB receptor tyrosine kinase activation and downstream signaling pathways relevant to intrahepatic cholangiocarcinogenesis.

regulate such fundamental biological responses as cell differentiation, cell proliferation, cell survival, cell migration, and angiogenesis (Figure 1). The type and amplitude of the downstream signaling pathways that are activated and their resulting biological effects are governed by the complexity and diversity of the ErbB network regulating these responses, and as such, are dictated in large part by which ErbB receptors are being expressed, the number of receptors being expressed and their dimerization and tyrosine phosphorylation profiles, the type and concentration of stimulating ligand, receptor stability at the cell surface, and the potential for receptor transactivation and/or cross-talk with other receptor-mediated signaling pathways[22,23,30-33].

ErbB2, because of the unique conformation of its ectodomain compared with the ectodomains of EGFR and ErbB3, is the only member of the ErbB receptor family that does not allow for the binding of any of the known soluble EGF-like ligands[21-23]. The crystal structure of a truncated ErbB2 ectodomain has further revealed a conformation that resembles that of an activated state poised to interact with other ErbB receptors[21,34]. This unique structure helps to explain why ErbB2 has an enhanced capacity for heterodimerization and is the preferred dimerization partner for all other ErbB receptors[23]. Moreover, by having a fixed conformation that resembles the ligand-activated state, it is not surprising that ErbB2 exhibits a constitutively high basal kinase activity and is closely linked to human oncogenesis[23,35]. It is also relevant that ErbB2 overexpression can result in ligand-independent homodimerization and that EGFR levels may be increased in tumors induced in association with the constitutive overexpression of ErbB2[23,36].

ErbB2-containing heterodimers (i.e. ErbB2/EGFR or ErbB2/ErbB3) are associated with a more robust signaling than that generated by homodimers[23]. In this context, it is notable that ErbB2/ErbB3 heterodimers are most potent in terms of stimulating cell mitogenesis and neoplastic transformation[21,23]. The dominant signaling capacity of ErbB2/ErbB3 heterodimers appears to be paradoxical since ErbB2 is a ligandless receptor for EGF family growth factors, while ErbB3 has an impaired kinase activity[21]. However, an allosteric mechanism for the activation of the kinase domain of ErbB3 by its interaction with ErbB2 has been predicted[24]. Furthermore, the potent signaling exhibited by ErbB2/ErbB3 heterodimers relates to their capacity to strongly activate both the *ras-raf*-MEK-p42/44 mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K)-Akt pathways[23]. Activation of the *ras-raf*-MEK-MAPK (ERK) pathway involves the recruitment of select adaptor proteins (Grb-2 or Shc) to the receptor, and is a key pathway for driving cellular proliferation[22,23]. The PI3K-Akt pathway, which regulates cell survival and anti-apoptotic signals is activated by recruitment of the p85 adaptor subunit of PI3K to the receptor[22,23]. Specifically, ErbB3 (and ErbB4) activates PI3K through select p85 docking sites on the cytoplasmic tyrosine kinase domain. Additional factors contributing to potent signaling capacity of the ErbB2/ErbB3 heterodimers is their increased stability at the cell surface and their ability to evade downregulation mechanisms, thereby leading to prolonged signaling[23]. ErbB2 also acts to decrease the rate of ligand dissociation from ErbB2-containing

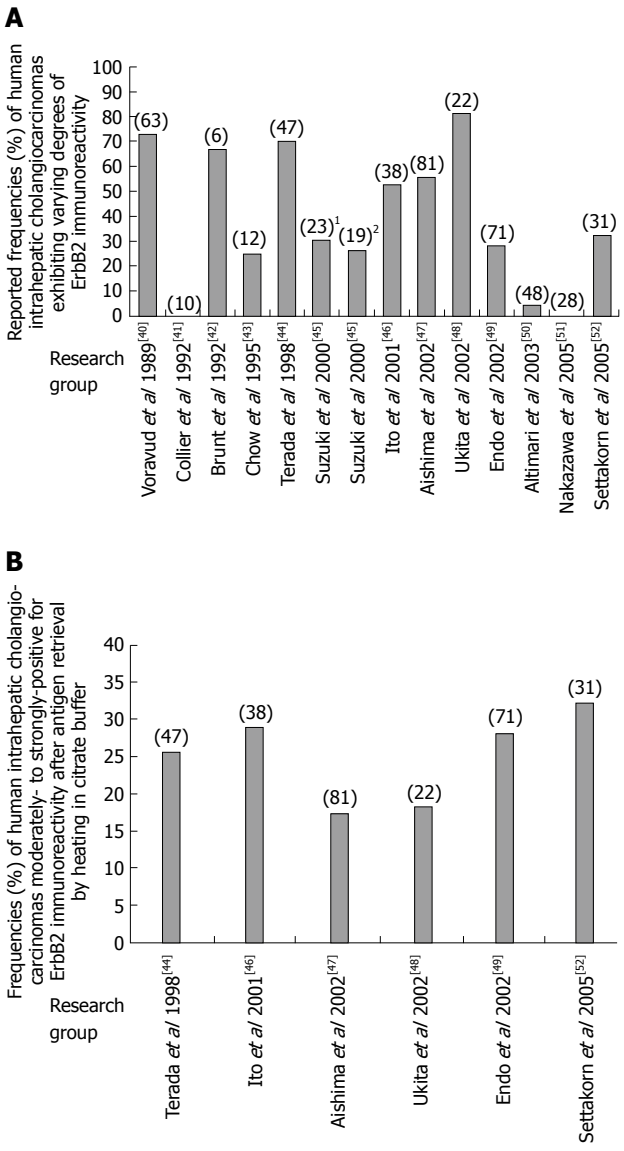

Figure 2 ErbB2 expression in human intrahepatic cholangiocarcinoma. A: Differences in overall reported frequencies of archival human intrahepatic cholangiocarcinoma specimens scored as being immunohistochemically-positive for ErbB2 expression using non-uniform experimental methods and criteria; B: Range of reported frequencies of archival human intrahepatic cholangiocarcinomas scored as being moderately-to-strongly positive (+2 to +3) for ErbB2 immunoreactivity following antigen unmasking by heating of tissue specimens in citric acid buffer, pH 6.0. Number in () = number of cases analyzed; number in [] = reference number; In A, [1]refers to Japanese cases analyzed; [2]refers to Thai cases analyzed.

heterodimeric complexes[23].

In addition to activating the intracellular *ras-raf*-MEK-MAPK and PI3K-Akt cascades, signaling through ErbB family receptors also activates other key cell regulatory molecules affecting cell differentiation, cell cycle progression, and malignant transformation and/or progression, including transcription factors such as *c-myc, c-Jun, c-fos,* Ets family members, signal transduction and activator of transcription (STAT) proteins, and nuclear factor-κB (NF-κB)[21-23,37-39]. Cyclin D1 is an important cell cycle regulator downstream of ErbB receptor signaling, which promotes G1/S phase cell cycle progression[23,30]. Moreover, preferential binding of phospholipase Cγ to the tyrosine kinase domain of the EGFR receptor mediates the generation of lipid second messengers diacylglycerol and inositol 1, 4, 5-triphosphate[21].

ErbB2 and/or EGFR together with EGF-like peptides are frequently found to be overexpressed in various epithelial cancers of both the human and experimental animal models. Mechanisms associated with the aberrant constitutive activation of ErbB2 or EGFR in malignant tumors include not only those associated with a persistent paracrine or autocrine production of growth factor ligands within the tumor, but also may occur as a result of receptor gene amplification and/or transcription-mediated protein overexpression, or by mutational activation. As ErbB2, and to a lesser extent EGFR, have been the most studied of the ErbB family receptor members in intrahepatic cholangiocarcinoma, much of the remainder of this review will focus on establishing their relevance to the molecular pathogenesis of intrahepatic cholangiocarcinoma and their potential as molecular targets for therapy against this lethal cancer.

ABERRANT ERBB2 EXPRESSION IN HUMAN INTRAHEPATIC CHOLANGIOCARCINOMA

The human ErbB2 receptor tyrosine kinase is a 185 kDa transmembrane glycoprotein encoded by the *c-erbB-2* proto-oncogene localized to chromosome 17q. Since 1989, several independent studies have been published describing the results of immunohistochemical analyses of ErbB2 oncoprotein expression in cancerous epithelium of human intrahepatic cholangiocarcinomas relative to that of normal bile ducts in adult liver[40-52]. In each of these studies, ErbB2 immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue samples from human intrahepatic cholangiocarcinomas of either Eastern and/or Western origin, which were mostly obtained from surgical files, but in some studies, also included specimens from autopsy files[44,48]. ErbB2 immunostaining was either not detected or only weakly detected in normal intrahepatic bile duct epithelium in adult and in fetal human livers[40,43,44,47,49]. On the other hand, reported incidences of intrahepatic cholangiocarcinoma cases overexpressing immunoreactive ErbB2 in their cancerous epithelium were found to vary considerably. Figure 2A highlights differences in the published frequencies (%) of analyzed cases of human intrahepatic cholangiocarcinomas that showed positive 1+ (weak) to ⩾ 2+ (moderate to strong) immunoreactivity for ErbB2. The obvious disparities among these published values are likely due, at least in part, to one or more of the following possibilities: (1) differences in tissue processing procedures used to optimize antigen availability, (2) use of different ErbB2 antibody preparations, (3) variations in the criteria or methods used to score and quantify positive cases of ErbB2 overexpression, and (4) in some studies, having too small of a sampling size of

intrahepatic cholangiocarcinoma specimens in the study to be meaningful[41,42].

Notable differences among the experimental approaches used in these various independent studies included the fact that antigen retrieval procedures involving heating tissue specimens in citrate buffer, pH 6.0, were employed in only some[44,46-50,52], but not all of the published studies. Moreover, in some of these studies, mouse monoclonal ErbB2 antibody preparations were used[41-46,48,49] whereas others were performed with rabbit polyclonal anti-ErbB2 antibodies[40,47,50-52]. Furthermore, while in some studies no distinctions were made between plasma membrane and cytoplasmic immunostaining when scoring tumors ostensibly positive for ErbB2 overexpression[40,47], others more appropriately relied largely on evidence of positive membranous immunostaining when classifying positive cases[43,44,48-52]. Also, the methods used in these studies to assess human intrahepatic cholangiocarcinomas for ErbB2 overexpression have been for the most part semi-quantitative, with no standard or uniform guidelines having been adopted to date for quantifying or validating ErbB2 overexpression in human cholangiocarcinogenesis. In this context, the results shown in Figure 2B are somewhat revealing, since they demonstrate that a greater degree of conformity among the reported frequency of expression values shown in Figure 2A can be achieved when the data are reevaluated in terms of (1) being derived only from those studies in which antigen unmasking was accomplished through heating of the tissue specimens in citric acid buffer, pH 6.0, and (2) when only those tumors scored as being moderately-to-strongly-positive ($\geqslant$ 2+) in their immunostaining reactions are used in calculating the overall frequencies of ErbB2-overexpressing tumors. It also follows that before further progress can be made in using immunohistochemistry to reliably and reproducibly assess cases of human intrahepatic cholangiocarcinoma for ErbB2 overexpression, there is a real need to establish rigorous clinical practice guidelines specific for this cancer, as were recently recommended by the American Society of Clinical Oncology and the College of American Pathologists for ErbB2 testing and validation in human breast cancer[53]. Less subjective and more analytical methods, such as the use of computer-assisted image analysis for quantifying immunostaining reactions are also needed to permit more objective measurements of ErbB2 immunoreactivity (as well as that of other marker proteins) in fixed tissue sections from human intrahepatic cholangiocarcinomas and related risk conditions. In this regard, utilizing microdensitometry measurements of immunostaining reactions, Endo *et al*[49] had demonstrated a strong positive correlation between levels of plasma membrane ErbB2 immunoreactivity and that of cytoplasmic cyclooxygenase-2 (COX-2) in human intrahepatic cholangiocarcinogenesis. The data generated from this quantitative immunohistochemical study are consistent with a growing body of experimental evidence that supports a strong positive relationship between ErbB2 receptor expression and signaling and COX-2 protein up-regulation and increased activity in the pathogenesis of intrahepatic cholangiocarcinoma (see below).

Not surprisingly, and likely for the same reasons described above for ErbB2, comparable immunohistochemical studies aimed at assessing EGFR overexpression in archival specimens of human intrahepatic cholangiocarcinomas from surgical files also yielded disparate results. For example, in five different published studies, the frequencies of intrahepatic cholangiocarcinomas exhibiting 2+ to 3+ immunostaining for EGFR were reported to be 10.7%[51], 21.1%[46], 21.6%[54], 47%[55], and 81%[50], respectively. In the positive tumors, EGFR immunostaining, with varying degrees of heterogeneity, was largely localized to the plasma membranes of the cancerous epithelial cells. Ito *et al*[46] further observed that 39.5% of their analyzed cases (n = 38) of human intrahepatic cholangiocarcinoma showed 2+-positive cytoplasmic immunostaining for ErbB3, with 10.5% of the total tumors analyzed also found to be 2+-positive for cytoplasmic ErbB4 immunostaining. In addition, these investigators reported that 21.1% of the cases of intrahepatic cholangiocarcinomas included in their study exhibited positive immunostaining for all four ErbB family members, while 36.8% of the tumors analyzed were found to exhibit positive immunostaining reactions for three of the four type I receptors. However, the purely descriptive nature of this study makes it impossible to predict specific receptor dimerization preferences that may be operative in these analyzed tumors.

While uncommon, activating mutations in the tyrosine kinase domain of ErbB2 have been recently described for a subset of lung cancer patients[56]. Likewise, a novel H878Y missense mutation in the tyrosine kinase domain of ErbB2 has also been recently demonstrated in 11% (2/18) of examined cases of human hepatocellular carcinoma[57]. However, in this same study, none of 22 analyzed cases of biliary cancer were found to harbor the *erbB2* H878Y mutation. All hepatocellular carcinoma and biliary cancer samples analyzed in this particular study were further observed to be negative for gain-of-function somatic mutations affecting the catalytic domain of the EGFR gene. In contrast, Leone *et al*[58] recently described somatic mutations in the tyrosine kinase domain of EGFR in a subgroup of patients with either cholangiocarcinoma or gallbladder carcinoma. Gwak *et al*[59] also recently reported that 13.6% (3/22) of examined cases of human cholangiocarcinoma exhibited EGFR mutations in the kinase domain. While still limited in scope, these collective findings suggest that mutations in the tyrosine kinase domain may be playing a role (albeit limited) in sustaining the activation of ErbB receptors, most particularly EGFR, in a subset of human biliary tract cancers, including some intrahepatic cholangiocarcinomas. Thus, identifying such activating mutations could conceivably contribute towards predicting a positive response of a few select biliary tract

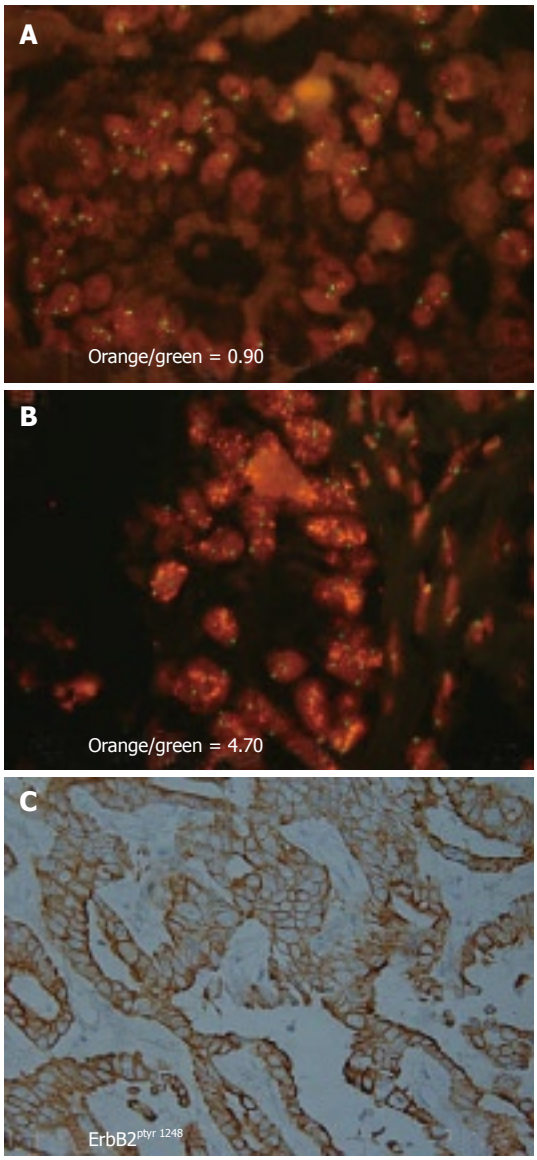

Figure 3 Representative photomicrographs demonstrating *c-erbB2* gene amplification and a corresponding strong positive immunoreactivity for activated ErbB2 oncoprotein in the neoplastic cholangiocytes of a human intrahepatic cholangiocarcinoma. A and B: Sections of two different intrahepatic cholangiocarcinomas with neoplastic cholangiocytes exhibiting FISH nuclear signals for *c-erbB2* (orange dots) and for CEP17 (centromeric region of chromosome 17; green dots). Orange/green values indicate *c-erbB2*/CEP17 signal ratio for each cholangiocarcinoma. An orange/green ratio of > 2.0 = a tumor with *c-erbB2* amplification (B), whereas a ratio value at or approximating 1.0 = a tumor without *c-erbB2* amplification (A). The neoplastic cholangiocytes in the intrahepatic cholangiocarcinomas depicted in A (immunohistochemical staining not shown) and in B (with corresponding immunohistochemical staining shown in C) were each found to exhibit strong positive immunoreactivity at the plasma membrane for activated ErbB2 oncoprotein, using an antibody directed against phosphotyrosine 1248 of ErbB2. Tyrosine 1248 is the main autophosphorylation site in the carboxy terminal domain of ErbB2 linked to downstream signaling through the *ras-raf*-MEK-p42/44 MAPK signal transduction pathway. Thus, activation of ErbB2 *via* autophosphorylation at tyrosine 1248 in neoplastic cholangiocytes of intrahepatic cholangiocarcinomas can be demonstrated either in the presence or absence of *c-erbB-2* amplification. (A, B × 330, C × 132).

cancers to ErbB targeted therapies (see below).

Presently, there have only been limited efforts to investigate *c-erbB2* gene amplification in human intrahepatic cholangiocarcinomas. Moreover, as has been the case with immunohistochemistry, current studies that utilized either fluorescence *in situ* hybridization (FISH), exemplified in Figure 3, or chromogenic *in situ* hybridization (CISH) analysis to evaluate *c-erbB2* amplification in cases of human cholangiocarcinoma have also produced variable and conflicting results. Shiraishi *et al*[60], employing two-color FISH using probes specific for *c-erbB2* and chromosome 17 centromere, did not detect evidence of *c-erbB2* amplification in six analyzed cases of human biliary tract cancers, including two peripheral and three hilar cholangiocarcinomas that showed a gain in 17q. In contrast, Ukita *et al*[48] employing FISH to evaluate gene amplification using only a *c-erbB2* probe reported increased *c-erbB2* signals in all 22 of their analyzed cases of intrahepatic cholangiocarcinoma. In this study, putative *c-erbB2* amplification was detected in the form of cluster signals in most of the tumors. On the other hand, there was a notable discordance between the immunohistochemical findings for ErbB2 overexpression and the FISH data for *c-erbB2* amplification. Furthermore, since Ukita *et al*[48] did not assess for changes in chromosome 17 copy number, it is not possible to determine from their results the extent to which aneuploidy or chromosome 17 polysomy alone may have contributed to their findings. More recently, Altimari *et al*[50] using CISH detected putative *c-erbB2* amplification in 4% of cases of human intrahepatic cholangiocarcinoma that were also found to be moderately to strongly immunoreactive for ErbB2 oncoprotein. However, this study also did not include an internal control for chromosome 17 copy number. On the other hand, Nakazawa *et al*[51] using two color FISH with probes for *c-erbB2* and chromosome 17 centromere reported no evidence of *c-erb2* amplification in 28 analyzed cases of human intrahepatic cholangiocarcinomas. Contrary to the findings of others (see Figure 2) Nakazawa *et al*[51] also reported that none of their analyzed cases of intrahepatic cholangiocarcinoma were immunohistochemically positive for ErbB2 overexpression.

The limited scope of these reported findings together with the fact that experimental variations and non-standardized criteria were used in assessing *c-erbB2* amplification in human intrahepatic cholangiocarcinoma clearly indicates the need for further studies in order to more definitively assess the extent to which gene amplification may be contributing to ErbB2 overexpression in these tumors. Universal and reliable standards need to be adopted for assessing *c-erbB2* amplification in biliary tract malignancies, including intrahepatic cholangiocarcinoma, leading to a more rigorously controlled assessment of *c-erbB2* gene amplification in relation to protein overexpression. It is also important to be able to more definitively assess if *c-erbB2* amplification might provide a more reliable and less variable indicator than immunohistochemistry for predicting favorable therapeutic responses of patients with intrahepatic cholangiocarcinomas to target-based treatments with ErbB2 inhibitors.

Another important deficiency in our current

understanding of ErbB2 and EGFR overexpression in human intrahepatic cholangiocarcinomas is the noticeable lack of mRNA data. *In situ* hybridization (ISH) has been used to investigate *c-erbB2* mRNA expression in the cancerous epithelium of archival formalin-fixed, paraffin-embedded specimens of human cholangiocarcinoma[48], but the staining pattern that was observed was seen to be diffusely intranuclear and to a much lesser extent cytoplasmic, suggesting some possibility of artifact. Regardless, these reported ISH results are descriptive and not quantitative, and have not been validated by more definitive analytical methods, such as real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR). Utilizing laser microbeam microdissection and cDNA microarray analysis, Obama *et al*[61] demonstrated a more than five-fold increase in *c-erbB2* expression in 3 (14%) and more than a two-fold increase in 6 (28%) of 21 cases of human intrahepatic cholangiocarcinomas analyzed. However, it was not evident if the microarray data for *c-erbB2* was validated by real-time RT-PCR. Here it may also be relevant that Endo *et al*[49], utilizing computer-assisted image analysis to evaluate plasma membrane ErbB2 immunostaining, have reported strongly-positive immunoreactivity for plasma membrane ErbB2 to be most prominent in the cancerous epithelium of well differentiated intrahepatic cholangiocarcinomas, whereas most of the tumors analyzed by Obama *et al*[61] were classified as being moderately to poorly differentiated. Settakorn *et al*[52], on the other hand, observed a low level of ErbB2 immunoreactivity in all (n = 9) of their analyzed archival cases of low grade human intrahepatic cholangiocarcinoma and strongly-positive ErbB2 immunoreactivity in 10 of 22 cases of the higher grade tumors. The fact that different commercial antibodies (monoclonal *versus* polyclonal) were used by Endo *et al*[49] and Settakorn *et al*[52], respectively, may account, at least in part, for the discrepancies in their reported immunohistochemical findings. Nevertheless, it is evident from the limited and conflicting results described above that further studies are needed to more clearly define the association between tumor grade and *c-erbB2* mRNA and protein expression in human intrahepatic cholangiocarcinoma.

A number of immunohistochemical studies have demonstrated increased expression of ErbB2 oncoprotein in variable percentages of noncancerous biliary proliferative disorders associated with human cholangiocarcinogenesis, including hepatolithiasis and PSC[44,47,49]. These findings suggest that ErbB2 overexpression associated with risk conditions characterized by cholangitis and partial biliary obstruction may represent a relatively early event linked to human intrahepatic cholangiocarcinogenesis. Interestingly, Su *et al*[62] also measured a higher mean level of ErbB2 oncoprotein in bile collected from patients with cholangiocarcinoma than in bile collected from patients with biliary tract infection, biliary stone disease, or normal controls. This study further supports ErbB2 as playing a role in human cholangiocarcinogenesis and suggests that elevated ErbB2 in bile might have some use in identifying individuals with high risk for developing biliary tract cancers. But again, these findings are limited in scope and need to be significantly expanded and substantiated before ErbB2 in bile can be accepted as a possible diagnostic test for predicting cholangiocarcinoma risk. Moreover, in none of the studies indicated in Figure 2, was the activation status (tyrosine phosphorylation) of the ErbB2 or EGFR receptors determined. In this regard, there is a further need to rigorously establish the tyrosine phosphorylation status of ErbB2 expressed in cholangiocarcinoma cells (i.e. see Figure 3C) compared with non-malignant cholangiocytes in liver, together with that of other ErbB receptor family members. Moreover, this should be done in conjunction with determining associated changes in receptor mRNA and protein expression levels. Such a comprehensive analysis of ErbB receptor family expression and activation would be more informative than that reflected in currently published studies, particularly when viewed in terms of contributing towards devising and tailoring specific strategies aimed at targeting ErbB signaling pathways for intrahepatic cholangiocarcinoma therapy.

ERBB2 AND RODENT MODELS OF CHOLANGIOCARCINOGENESIS

Neu is the rat homologue of human ErbB2. Table 1 lists experimental rodent models in which Neu overexpression has been demonstrated to be associated with the molecular pathogenesis of intrahepatic cholangiocarcinoma. Western and Northern blotting, together with immunohistochemistry and ISH have been used to demonstrate Neu protein and mRNA to both be significantly overexpressed in the cancerous epithelium of furan-induced intrahepatic intestinal-type cholangiocarcinomas when compared with hyperplastic bile ducts/ductules in the liver of bile duct-ligated rats and with normal adult rat liver[63,64]. In these tumors, *c-neu* was further determined not to be amplified nor to exhibit evidence of a key activating point mutation within its transmembrane domain[64]. In addition, Neu oncoprotein overexpressed in furan-induced rat cholangiocarcinomas was tyrosine phosphorylated, indicative of a constitutively activated signaling state. Here, it would seem that constitutive overexpression of activated Neu in furan-induced rat intrahepatic cholangiocarcinomas is the result of an altered transcriptional event rather than to mechanisms involving gene amplification or mutation. Similarly, Neu protein was further demonstrated by immunohistochemistry to be overexpressed in cancerous epithelium of intestinal-type cholangiocarcinoma induced in the livers of rats treated with thioacetamide when compared with normal cholangiocytes[65].

Hepatic cirrhosis and early formed cholangiofibrotic precursor lesions precede the development of intrahepatic cholangiocarcinoma in both furan- and thioacetamide-treated rats. Here it is interesting that Neu

Table 1 Rodent models of intrahepatic cholangiocarcinoma constitutively overexpressing ErbB2/Neu in cancerous epithelium

Model	Tumor			ErbB2/Neu			Tyrosine phosphorylation	Ref.
	Development time	Incidence (%)	Classification	c-erbB-2/neu	mRNA	Protein		
Furan rat model	> 1 yr	70-100	Intestinal-type cholangiocarcinoma	Wild-type/ non-amplified	Increased	Increased	Increased	[63,64]
Thioacetamide rat model	16-22 wk	100	Intestinal-type cholangiocarcinoma	NA	NA	Increased	NA	[65]
P53 deficiency/CCl_4 mouse model	≥ 16 wk	40	Ductal cholangiocarcinoma	NA	NA	Increased	NA	[66]
Rat BDEneu orthotopic cell transplantation model	4 wk	100	Ductal cholangiocarcinoma	Mutated	Increased	Increased	Increased	[68,69]

NA: Not assessed.

overexpression has also been observed in metaplastic/dysplastic glands within these early cholangiofibrotic lesions, as well as in the later developed invasive cholangiocarcinomas, compared with negative or marginal levels of Neu detected in normal or hyperplastic bile ductular epithelium[64,65]. More recently, CCl_4-induced hepatic fibrosis was shown to be associated with the promotion of intrahepatic cholangiocarcinoma in p53-deficient mice[66], with strong Neu immunostaining having also been seen in the cancerous epithelium of a majority of the mass-forming liver tumors, as well as in some metastatic lesions. Here too, Neu expression was detected in early intrahepatic cholangiocarcinomas as well, with only weak or undetected Neu immunostaining being observed in normal bile ducts and in small bile duct hyperplasia. Overall, these experimental results are consistent with previously described epidemiological and immunohistochemical findings for human intrahepatic cholangiocarcinogenesis, which support a role for hepatic cirrhosis as a potential risk factor in the pathogenesis of intrahepatic cholangiocarcinoma development[13,16]. They further suggest Neu overexpression to be a factor in both the early and later stages of intrahepatic cholangiocarcinogenesis.

More direct evidence for the involvement of Neu in the development of biliary tract malignancies, including intrahepatic cholangiocarcinoma comes from genetic-based animal and cell models. Transgenic mice (*BK5.erbB2* mice) generated to constitutively overexpress wild-type rat *c-neu* developed gallbladder adenocarcinoma at a 100% incidence and intrahepatic cholangiocarcinomas at 30% incidence by 8 mo of age[36]. No mutations in either *K-ras* or *p53* were detected in 16 of the murine gallbladder adenocarcinomas analyzed, with *p53* being found to be elevated in only one of these tumors. On the other hand, Neu and EGFR protein (but not ErbB3 or ErbB4) were found to be increased and hyperphosphorylated on tyrosine residues in gallbladder tissue from the *BK5.erbB2* mice. Increased heterodimer formation between Neu and EGFR and increased p42/44 MAPK activation were also observed in gallbladder from these transgenic mice when compared with that from control mice not expressing the rat *c-neu* transgene, further implicating increased Neu signaling in development of biliary tract cancers. Consistent with these transgenic mouse data, it has recently been demonstrated that when non-tumorigenic immortalized adult rat cholangiocytes (BDE1 cells)[67] were stably transfected with a constitutively expressed mutant rat *neu* gene, they became highly tumorigenic[68,69]. These malignant transformants, designated as BDEneu cells, overexpressed activated Neu[68,69], as well as EGFR (Sirica, A.E., unpublished data), and exhibited elevated levels of phospho-p42/44 MAPK and phospho-Akt when compared with both non-tumorigenic parent BDE1 cells and non-tumorigenic control BDEneo cells that were derived by stably transfecting BDE1 cells to only express the gene for neomycin resistance[68]. BDEneu cells transplanted *via* bile duct inoculation into the livers of isogenic rats rapidly formed large moderately differentiated mass-forming intrahepatic cholangiocarcinomas that recapitulated clinical, cellular, and molecular features of advanced human disease[68,69] (Figure 4). Notably, BDEneu tumors were found to be highly invasive and metastatic, to produce bile duct obstruction at the hepatic hilus, and to retain strong immunoreactivity in their cancerous epithelium for Neu constitutively phosphorylated at tyrosine 1248. Tyrosine 1248 is known to be a major autophosphorylation site at the cytoplasmic tyrosine kinase domain of Neu oncoprotein that reflects the activation status of the receptor and couples Neu to the *ras-raf*-MEK-p42/44 MAPK signal transduction pathway[70,71].

The C611B cell line is another novel rat cholangiocarcinoma cell line that constitutively overexpresses Neu when compared with hyperplastic rat intrahepatic bile ducts/ductules induced in bile duct-ligated rats[72-75]. This cell line was established from a transplantable mucin-producing cholangiocarcinoma expressing wild-type *neu* that was derived from the furan rat cholangiocarcinogenesis model[72]. Like BDEneu cells, C611B cells express Neu protein phosphorylated at tyrosine 1248, as well as activated Akt and p42/44 MAPK (Figure 5A). Inoculation of C611B cells into the inguinal fat pads or livers of isogenic rats also yields a 100% incidence of mass-forming adenocarcinomas that closely resemble in their histopathology well differentiated mucin-producing tubular intrahepatic cholangiocarcinomas of the human[72]. As exemplified by the RT-PCR data shown in Figure 5B, C611B

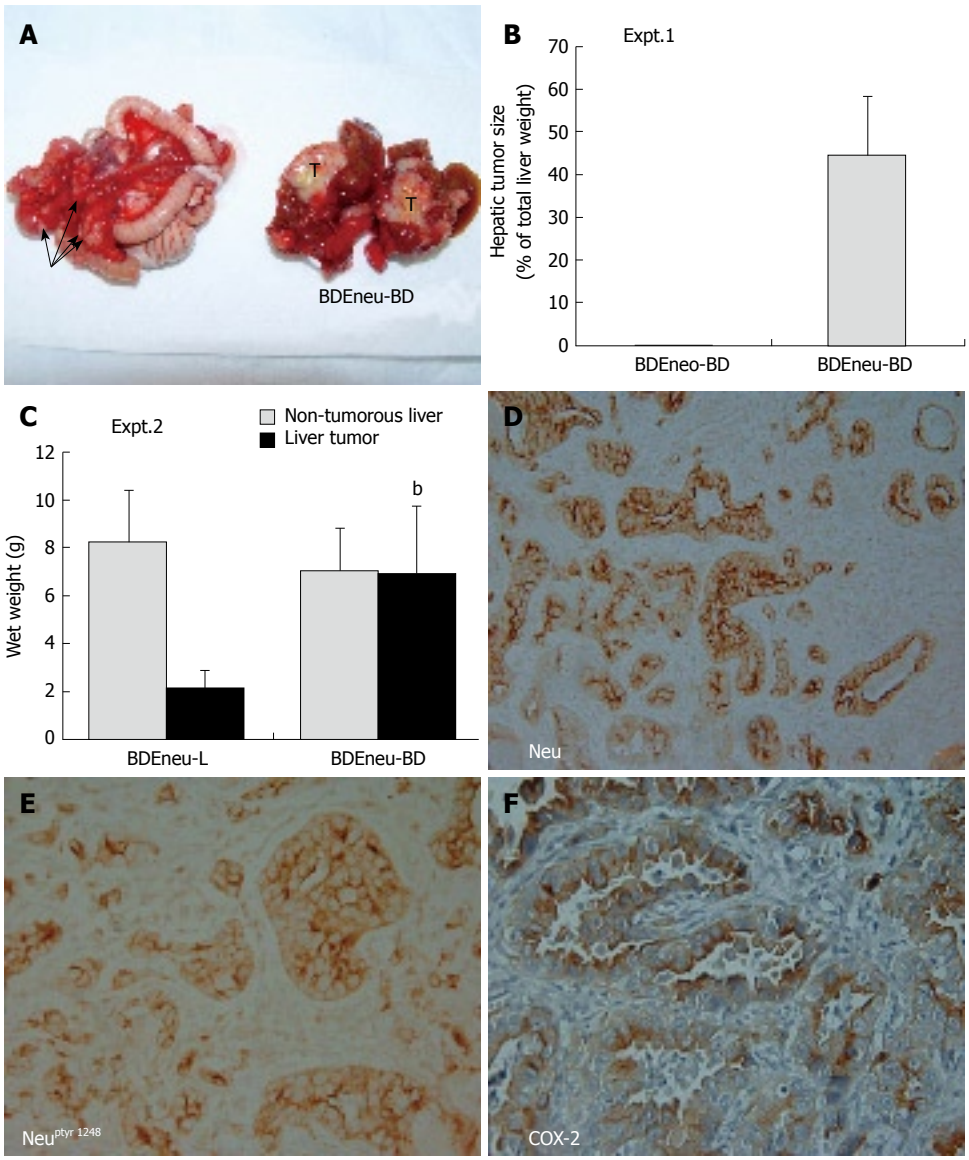

Figure 4 Rat BDEneu cell transplantation model of intrahepatic cholangiocarcinoma progression. A: Gross pathology of a bisected invasive hepatic tumor (T) formed in the liver of an isogenic Fischer 344 rat at 3 wk after bile duct inoculation of 4 x 10^6 tumorigenic rat BDEneu cholangiocytes overexpressing mutationally-activated Neu, together with associated peritoneal metastases (arrows); B: Expt 1. mean tumor size ± SD (as a % of total liver weight) of hepatic tumors that formed at a 100% incidence in isogenic rat livers at 3 wk after bile duct inoculation of 4 x 10^6 BDEneu cells (BDEneu-BD), compared with no tumors formed in rats comparably inoculated with control non-tumorigenic BDEneo cells (BDEneo-BD) transfected to stably express the neomycin resistance gene only, in the absence of oncogenic *neu* $n = 6$; C: Expt 2. Differences in mean wet weight ± SD of hepatic tumors (relative to that of corresponding non-tumorous liver) formed at 3 wk after bile duct inoculation of 4 x 10^6 BDEneu cells (BDEneu-BD) *versus* the mean wet weight of hepatic tumors that formed over the same time period following inoculation of the BDEneu cells directly into liver (BDEneu-L). $^bP \leqslant 0.001$ (BDEneu-BD tumor *versus* BDEneu-L tumor). $n = 4$; D: Representative low power photomicrograph demonstrating neoplastic cholangiocytes within a rapidly growing BDEneu-BD intrahepatic cholangiocarcinoma to be strongly immunoreactive for plasma membrane Neu; E: Photomicrograph showing malignant cholangiocytes within a BDEneu-BD intrahepatic cholangiocarcinoma to exhibit strong uniformly positive immunoreactivity for activated Neu phosphorylated at tyrosine 1248; F: Photomicrograph of a BDEneu-BD tumor demonstrating positive cytoplasmic immunostaining for COX-2 in the cholangiocytes of the neoplastic ducts. Positive immunostaining in D-F is indicated by brown reaction product. (See References 68 and 69 for a more complete description of rat BDEneu model). (D × 66, E, F × 132).

cholangiocarcinomas simultaneously express mRNA for ErbB growth factor ligands (i.e. TGF-α, heregulin) and for three of the four ErbB family receptors, with the exception of ErbB4, which is also not detected in normal rat liver[76]. These data, although limited, suggest that ligand-dependent activation of ErbB family receptors are likely operative in these tumors, and that cognate ligands, such as TGF-α, may be playing an important role in this process[26]. EGFR immunoreactivity has also been detected in the cancerous epithelium of 100% of analyzed specimens of rat cholangiocarcinomas induced by thioacetamide[55], further suggesting that signaling through EGFR may also be a relevant contributing factor to the genesis and progression of intrahepatic cholangiocarcinoma[77]. However, additional mechanism-based studies are needed to more fully substantiate this likely possibility.

It is surprising that there is a noticeable lack of published data on the use of well established hamster models of cholangiocarcinogenesis, such as those

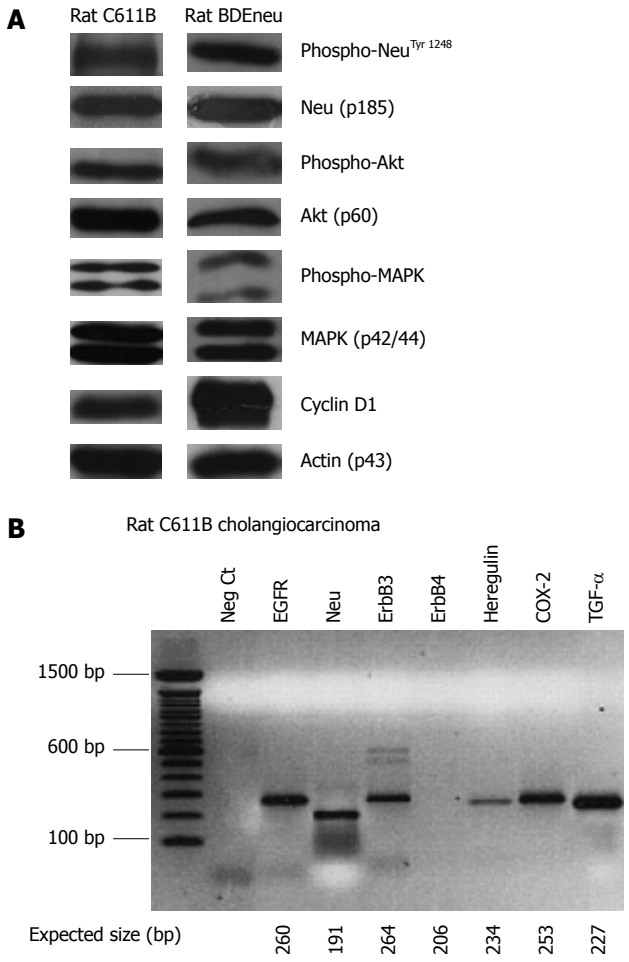

Figure 5 Western blot (A) and RT-PCR (B) analyses. A: Relevant protein and phosphorylation profiles associated with aberrant Neu expression in rat C611B and BDEneu cholangiocarcinoma cell lines. C611B overexpress the wild-type *c-neu* gene[64], whereas BDEneu cells overexpress mutationally-activated *neu* oncogene[68,69]. Note that both cell lines are positive for phospho-Neu, phospho-Akt, and phospho-p42/44 MAPK, as well as show prominent expression of cyclin D1; B: RT-PCR analysis of *c-erbB* family receptor together with heregulin, TGF-α, and COX-2 mRNAs detected in malignant neoplastic ducts obtained by laser capture microdissection from a hepatic tumor formed in an isogenic Fischer 344 male rat following cell transplantation of rat C611B cholangiocarcinoma cells directly into liver. Note that as expected, among the ErbB family receptors, only ErbB4 mRNA is not detected in the tumor ducts. Moreover, it is also significant that mRNA signals for both the EGFR ligand TGF-α and for COX-2, which is known to be up-regulated in response to EGFR and/or Neu signaling, are each shown to be prominently detected in C611B cholangiocarcinoma.

which combine tumor initiating doses of carcinogenic nitrosamines with *Opisthorchis viverrini* or *Clonorchis sinensis* infection[78-82] as a means to investigate the role of aberrant ErbB family receptor signaling in intrahepatic cholangiocarcinoma development and progression. This may be due to the fact that only very limited hamster-specific immunochemical and molecular reagents are currently available for such studies. Nevertheless, the establishment of novel rodent models as those described above and in Table 1 have proven to be valuable resources for investigating the role of ErbB-family receptors in intrahepatic cholangiocarcinogenesis. Clearly more studies are needed in this regard, but from the standpoint of clinical relevance, it is also apparent that some recently developed rodent models of intrahepatic cholangicarcinoma development and progression have the potential of serving as powerful preclinical platforms for rapidly testing novel ErbB targeted treatment strategies for cholangiocarcinoma prevention and therapy.

INTERACTIVE RELATIONSHIPS BETWEEN ERBB FAMILY RECEPTORS AND OTHER MOLECULAR PATHWAYS

The interplay between the EGFR and/or ErbB2 receptor tyrosine kinases with other diverse signaling pathways provides multiple functional links by which intrahepatic cholangiocarcinoma growth and/or progression may be promoted or potentiated. Furthermore, such interactive molecular relationships can have important implications with respect to devising novel molecular targeting strategies for cholangiocarcinoma therapy or chemoprevention[1]. EGFR and/or ErbB2 have been linked to other molecular pathways implicated in intrahepatic cholangiocarcinoma growth, apoptosis resistance, angiogenesis and/or invasion and metastasis. Each of these depicted interactions will be discussed separately, although it is important to keep in mind that they are likely functioning in concert.

Bile acids

Secondary bile acids, such as deoxycholic acid and its conjugated forms, which have been reported to be increased in the serum of cholangiocarcinoma patients[83], may be acting to promote cholangiocarcinogenesis in the biliary tract[20,84,85] through a mechanism involving bile acid-mediated activation of EGFR, leading to up-regulation of COX-2 and protein turnover inhibition of the potent antiapoptotic protein, myeloid cell leukemia 1 (Mcl-1). Werneburg *et al*[26] have demonstrated that bile acids like deoxycholic acid can act to activate EGFR in cultured human H-69 cholangiocyte and KMBC cholangiocarcinoma cell lines by a TGF-α-dependent mechanism mediated by bile acid stimulation of matrix metalloproteinase activity catalyzing TGF-α membrane release. Yoon *et al*[86] have further shown deoxycholic acid and conjugated forms, such as taurochenodeoxycholate, to significantly induce COX-2 expression in cultured human KMBC cholangiocarcinoma cells *via* EGFR transactivation and subsequent activation of p42/44 MAPK, p38 MAPK, and c-jun-N-terminal kinase (JNK). In addition, deoxycholic acid was found to prolong Mcl-1 protein turnover in cultured KMBC cholangiocarcinoma cells by an EGFR/Raf-1-dependent mechanism[87]. Comparable mechanisms have been reported for bile-acid mediated induction of COX-2 and Mcl-1 in human and rat hepatic stellate cells[88], suggesting that bile acids might also be playing a role in enhancing the survival and proliferation of myofibroblastic tumor stromal cells associated with the desmoplastic reaction typically characteristic of intrahepatic cholangiocarcinoma. It may also be of interest that deoxycholic acid, as well as

taurodeoxycholic, and taurochenodeoxycholic bile acids have recently been reported to regulate the expression of MUC4, a modulator of ErbB2 signaling (see below), in human esophageal carcinoma cells by a promoter-dependent mechanism involving activation of the PI3K pathway[89], and in the case of taurodeoxycholic and taurochenodeoxycholic bile acids, have also been found to be mediated by hepatocyte nuclear factor-1α[90].

Cox-2

Cox-2, the inducible form of prostaglandin endoperoxidase, has been reported to be commonly up-regulated in the malignant neoplastic epithelium of intrahepatic cholangiocarcinomas of both the human[49,91,92] and of experimental rodent models[68,69,73-75] (Figures 4F and 5B). Accumulating evidence over the past several years has further supported COX-2-derived prostaglandin signaling as playing an important role in cholangiocarcinogenesis as a mediator of mitogenesis, anti-apoptosis, and angiogenesis[93,94]. Moreover, a close relationship between COX-2-derived prostaglandin signaling and that of EGFR or ErbB2 overexpression and activation has been established in animal and cell models of biliary tract carcinogenesis[36,68,74].

Vadlamudi *et al*[95] were the first to demonstrate the regulation of COX-2 expression by heterodimeric ErbB2 signaling. Furthermore, the presence of a nuclear ErbB2 has been identified, which appears to act as a transcriptional regulator of COX-2[96]. Benoit *et al*[97] have further demonstrated that COX-2, through the production of its major enzymatic product prostaglandin E_2 (PGE_2), positively regulates ErbB2 expression, supporting a model in which ErbB2 and COX-2 regulate each other in a positive loop. Cross-talk between COX-2- derived PGE_2 and EGFR has also been demonstrated in human cholangio- and hepatocellular carcinoma cells[33,98], and EGFR kinase inhibitors were found to decrease COX-2 expression levels in cultured human cholangiocarcinoma cells[77], suggesting a similar positive regulatory loop for EGFR and COX-2. In addition, the recent findings of Zhang *et al*[99] have suggested that in human cholangiocarcinoma cells, PGE_2-enhanced phosphorylation of MAPK/ERK is, at least in part, mediated through EP1 receptors and EGFR phosphorylation that is associated with increased intracellular calcium concentration.

As previously noted in this review, a positive correlation between immunohistochemically-determined ErbB2 expression and COX-2 up-regulation has been described in human cholangiocarcinogenesis[49]. COX-2 up-regulation has also been determined to closely parallel constitutive *c-neu* protein and mRNA overexpression in cholangiocarcinoma cells and tumors derived from the furan rat model of intrahepatic cholangiocarcinogenesis[73,74]. Of particular interest are the findings of Kiguchi *et al*[36] who observed a prominent up-regulation of COX-2 protein and mRNA in gallbladder tissues and tumors from *BK5.erbB2* transgenic mice constitutively overexpressing wild-type rat *c-neu,* thereby adding more convincing support for the role of Neu (ErbB2) in COX-2 expression in biliary tract carcinogenesis. Consistent with the findings of Kiguchi *et al*[36], Lai *et al*[68] more recently reported COX-2 protein and mRNA, together with a concomitant overproduction of PGE_2, to be significantly induced in rat BDE1 cholangiocytes upon *in vitro* malignant neoplastic transformation of these cells with mutationally-activated rat *neu* oncogene. Neoplastic epithelium of tumors overexpressing mutationally-activated Neu formed from orthotopically transplanted *neu*-transformed rat cholangiocytes (BDEneu cells) were also found to be positively immunoreactive for cytoplasmic COX-2[68] (Figure 4F).

Interleukin-6 (IL-6)/gp130

The inflammatory cytokine IL-6 has been demonstrated to be frequently overexpressed in malignant neoplastic cells of human well differentiated intrahepatic cholangiocarcinomas[100] and to be increased in the serum and bile of cholangiocarcinoma patients[101-103]. IL-6 has been further shown to function as an autocrine growth factor for human cholangiocarcinoma cell lines[100,104,105], and to up-regulate Mcl-1 in malignant human cholangiocyte cell lines[106,107].

Gp130 is the signal transducing element of the IL-6 receptor. Current data support a mechanism whereby sustained IL-6-induced phosphorylation (activation) of gp130, leading to enhanced activation of STAT-3, contributes to cholangiocarcinoma cell mitogenesis and enhanced Mcl-1 up-regulation and resistance to apoptosis[106-109]. Isomoto *et al*[108] have further reported that sustained IL-6/STAT-3 signaling in human cholangiocarcinoma cells is due to epigenetic silencing of suppressor of cytokine signaling 3 (SOCS-3). Here, it is also noteworthy that COX-2-derived PGE_2 was recently found by Han *et al*[109] to induce IL-6 production through activation of the EP4 receptor and subsequent phosphorylation of gp130/STAT-3 in SG231 human cholangiocarcinoma cells, demonstrating the potential for cross-talk between the COX-2/PGE_2 and IL-6/gp130 pathways in cholangiocarcinoma. In this same study, it was also found that PGE_2 signaling in human CCLP1 human cholangiocarcinoma cells could activate STAT-3 through EP1 receptor-mediated intracellular activation of *c-Src* and EGFR[109].

Qiu *et al*[110], utilizing human prostate carcinoma cells, were the first to demonstrate that ErbB2 forms a complex with the gp130 subunit of the IL-6 receptor, thereby serving as a functional component of IL-6 receptor signaling activating the p42/44 MAPK pathway. Grant *et al*[111] subsequently reported gp130 to be constitutively associated with ErbB2 and ErbB3 in human breast cancer cells and found EGF to induce tyrosine phosphorylation of gp130 in these cells, further supporting a functional interaction between gp130 and the ErbB receptor family. Interestingly, IL-6 overexpression was recently reported to also decrease EGFR promoter methylation in human cholangiocarcinoma cells, leading to enhanced EGFR protein expression[112].

MUC1 and MUC4

Aberrant overexpression of the transmembrane mucins MUC1 and MUC 4 has been frequently observed in human intrahepatic cholangiocarcinomas[55,113-116], and both MUC1 and MUC4 have been reported as being independent prognostic factors for predicting poor outcomes in patients with mass-forming intrahepatic cholangiocarcinoma[113-115]. MUC1 overexpression has also been detected in rat intrahepatic cholangiocarcinoma[55], and more recently, MUC1 mRNA was demonstrated to be significantly increased in the highly tumorigenic rat BDEneu cholangiocarcinoma cell line[68] over that of non-tumorigenic parent BDE1 cholangiocytes, as well as of that expressed in a spontaneously transformed rat cholangiocyte cell line, designated as BDEsp, which was less tumorigenic than the BDEneu cell line[69]. MUC4 has also been cited as being overexpressed, as well as co-localized with Neu protein, in gallbladder adenocarcinoma formed in *BK5.erbB2* transgenic mice overexpressing the wild-type rat *c-neu* transgene[117].

The transmembrane mucins MUC1 and MUC4 interact by different mechanisms with ErbB family receptors and each can function to potentiate ErbB-dependent signal transduction[118-120]. The cytoplasmic tail of MUC1 harbors several tyrosine residues that when phosphorylated may provide critical docking sites for initiating downstream cytoplasmic signaling pathways relevant to cancer development and progression[118]. Notably, the MUC1 cytoplasmic tail has been shown to interact with all four members of the ErbB family of receptor tyrosine kinases[118,119]. EGFR-mediated phosphorylation of the MUC1 cytoplasmic tail at its YEKV motif has further been demonstrated to enhance its affinity for β-catenin, potentially favoring decreased cell adhesion and in the case of tumor cells, increased invasiveness[118,119]. Heregulin was also reported to enhance the formation of MUC1-ErbB2 complexes in tumor cells and to induce the binding of the MUC1 cytoplasmic domain with γ-catenin and targeting of the MUC1 cytoplasmic tail-γ-catenin complex to the nucleolus[121]. This latter finding also supports cross-talk between the ErbB2 receptor tyrosine kinase and MUC1. However, the role of a MUC1 cytoplasmic tail-γ-catenin complex in the nucleolus still needs to be elucidated, particularly in relation to malignant neoplastic cell transformation and progression.

MUC1's cytoplasmic tail, through its interaction with ErbB family receptor tyrosine kinases, has been demonstrated to potentiate ErbB signaling mediated through Grb2-Sos-activation of the Ras-p42/44 MAPK signaling cascade[118,119]. More recently, MUC1 has also been reported to modulate TGF-α-dependent cancer progression[122], as well as to regulate EGFR stability upon activation[123], thereby suggesting a possible alternative pathway whereby cancer development and progression may be promoted *via* a mechanism based on MUC1-mediated inhibition of EGFR degradation. MUC1's C-terminal subunit has further been reported to be translocated to mitochondria by a mechanism in which heregulin-induced tyrosine phosphorylation of ErbB receptors results in the activation of *c-Src*[124]. This interaction, in turn, stimulates binding of the MUC1 cytoplasmic domain to the molecular chaperone HSP90, thus forming a complex to transport MUC1 oncopeptide to the mitochondrial outer membrane, where it may then act to facilitate neoplastic development by blocking activation of the intrinsic apoptotic pathway[124].

In contrast to MUC1, MUC4 has been shown to function as a novel intramembrane ligand and modulator for ErbB2 receptor tyrosine kinase[118-120]. Specifically, it has been proposed that EGF-like domains on MUC4 interact with the cognate receptor portion of ErbB2[119]. However, the actual mechanism whereby MUC4 can signal through ErbB2 is complex and still not completely understood. Caraway and his associates[118,125] first reported that MUC4 alone was capable of inducing tyrosine phosphorylation of ErbB2 and up-regulation of $p27^{kip}$ (a cyclin-dependent kinase inhibitor), but did not produce activation of MAPKs or PI3K-Akt. On the other hand, these researchers found that in the presence of NRG1, MUC4 potentiated ErbB2/ErbB3 phosphorylation, as well as enhanced p42/44 MAPK and Akt activation and $p27^{kip}$ repression[118,125]. More recently, it was reported by Funes *et al*[126] that MUC4 potentiates NRG1 signaling by making more ErbB2/ErbB3 receptors available at the plasma membrane for interaction with the NRG1 ligand, without affecting the total quantity of receptors expressed by the cell. Interestingly, in this study, without NRG1, no evidence of MUC4-stimulated ErbB2 receptor tyrosine phosphorylation was detected. The basis for this difference with earlier reported results[118,125] is unclear, but it seems that MUC4 may be acting to stabilize the ErbB2/ErbB3-NRG1 complex at the plasma membrane by suppressing its internalization[119,126].

MUC 4 has also been reported to suppress apoptosis in cells in the presence or absence of NRG treatment[118]. Indeed, based on the cumulative data for MUC4 interaction with ErbB2, Carraway *et al*[118] have proposed that in polarized untransformed epithelium, the MUC4-ErbB2 complex in the absence of NRG1 receptor signaling would promote cell differentiation. In contrast, in unpolarized cancer cells, MUC4 and NRG1 could function simultaneously to augment ErbB2/ErbB3 signaling, leading to downstream activation of both the Ras-p42/44 MAPK and PI3K-Akt signaling pathways and transcriptional regulation of cyclin D1, promoting cancer cell growth, survival, and/or progression[119].

HGF/Met

Met, the heterodimeric tyrosine kinase receptor for hepatocyte growth factor (HGF), its cognate ligand, is commonly overexpressed in the neoplastic epithelium of intrahepatic cholangiocarcinomas of both human and rodent origin[47,64-66,72,127]. Positive immunoreactivity for Met was determined to be most prominent in well differentiated human cholangiocarcinomas and to be low in poorly differentiated tumors[47,127]. Rat models of intrahepatic cholangiocarcinoma overexpressing

Neu were further found to concomitantly overexpress Met[64,65,72]. In this context, it is noteworthy that like Neu, constitutive overexpression of tyrosine phosphorylated Met in the cancerous epithelium of furan-induced rat intrahepatic cholangiocarcinomas was determined not to be the result of gene amplification, but to correlate with increased Met mRNA expression[64]. Constitutively-activated Met has also been detected in intrahepatic cholangiocarcinomas formed in liver of p53-/- mice following CCl_4 treatment[66].

Typically, in epithelial cancers, Met activation occurs mainly through a paracrine mechanism with stromal cell-secreted HGF, although evidence supporting the existence of a HGF/Met autocrine growth control circuit in cholangiocarcinoma cell lines of both human[105] and rat[73,128] origin has been described. However, cross-talk between EGFR and Met has been demonstrated to also occur in malignant epithelial cell lines in the absence of HGF, but in the presence of TGF-α or EGF[31]. EGFR signaling has also been implicated in HGF-induced hepatocyte proliferation[129], and downstream signaling from HGF/Met has been demonstrated to synergize with ErbB2 to enhance malignant progression[130]. Furthermore, evidence has been provided indicating that EGFR can transactivate the Met receptor tyrosine kinase in human hepatocellular and pancreatic carcinoma cells[131]. PGE_2 generated by COX-2 has also been shown in human colon and hepatocellular carcinoma cells to transactivate the Met receptor in a manner dependent on functional EGFR[32,33]. Moreover, TGF-α and PGE_2, factors which are overexpressed in cholangiocarcinoma cells[74,132,133], may function as inducers of HGF production by tumor stromal fibroblasts[134]. Overall, these findings support interactive functional relationships between ErbB family receptors, HGF/Met, and COX-2 that are likely contributing in a major way towards regulating intrahepatic cholangiocarcinoma growth and progression.

VEGF

Moderate to strong expression of the angiogenic factor, vascular endothelial growth factor (VEGF or VEGF-A), as well as of the lymphangiogenic factor VEGF-C, has been detected in the cancerous epithelium of a significant percentage of human intrahepatic cholangiocarcinomas[135-139] and in human cholangiocarcinoma cell lines[135,140]. Increased VEGF expression has been reported to be associated with a significant vascularization of human intrahepatic cholangiocarcinomas, as assessed by microvessel density[135,136]. In comparison, hypovascularity of cholangiocarcinoma may be related to a down-regulation of VEGF together with an up-regulation of the angiogenesis inhibitor thrombospondin-1[136,141,142], although further studies are needed to validate the likelihood of this being a primary mechanism underlying the limited angiogenesis observed in cases of intrahepatic cholangiocarcinoma.

Cultured rat BDE1 cholangiocytes stably transfected with constitutively expressed rat neu oncogene overexpress VEGF[68]. Moreover, ErbB homo- and heterodimers have been demonstrated to increase VEGF expression in other tumorigenic cell types, with EGFR/ErbB2 and ErbB2/ErbB3 heterodimers determined to be the most potent inducers of VEGF mRNA expression when compared with various other paired combinations, such as EGFR/ErbB3 or ErbB2/ErbB4[143]. Interestingly, in both mouse and human cell models, thrombospondin-1 mRNA and protein were down-regulated *in vitro* and in tumors overexpressing specific paired combinations of ErbB receptors, compared with cells stably transfected to overexpress only single ErbB receptors[144].

ErbB2-mediated up-regulation of VEGF has been shown to involve activation of PI3K-Akt, mTOR and p70S6 kinase[145], as well as to be linked to increased expression of hypoxia inducible factor-1α (HIF-1α), an activator of VEGF transcription, *via* a PI3K-Akt-dependent mechanism[146]. In addition, targeting STAT3 was found to block HIF-1 and VEGF expression induced by ErbB2 and other oncogenic growth signaling pathways[147]. Moreover, EGFR tyrosine kinase inhibitors have been shown to decrease VEGF expression in cancerous epithelial cells by both HIF-1-dependent and HIF-1-independent mechanisms[148]. PGE_2 has also been reported to up-regulate VEGF in cancer cells *via* transactivation of EGFR[149]. Thus, aberrant ErbB receptor signaling in malignant cells, including cholangiocarcinoma cells, has important implications not only for promoting cancer cell growth and progression, but also for regulation of the tumor microenvironment.

THERAPEUTIC TARGETING OF ERBB RECEPTOR TYROSINE KINASES IN CHOLANGIOCARCINOMA CELLS

Conventional chemotherapy, which does not distinguish between cancer cells and normal healthy cells is of limited benefit in the treatment of unresectable or metastatic intrahepatic cholangiocarcinoma[183]. However, in recent years, the emergence of target-based cancer therapies has provided the option of developing and testing novel treatment strategies that have the potential of improving therapeutic efficacy against cancers like intrahepatic cholangiocarcinoma, which are typically refractory to conventional cancer chemotherapeutic modalities. Since ErbB receptor tyrosine kinases, most notably EGFR and/or ErbB2, are frequently overexpressed and/or constitutively activated in human cancers, including intrahepatic cholangiocarcinoma, agents that selectively target ErbB receptor family members have generated considerable interest among clinicians and researchers alike, with monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors (TKIs) having advanced the furthest in clinical development[155]. Table 2 lists mAbs and TKIs targeting ErbB family receptors that are currently approved and/or in phase clinical trials for the treatment of various

Table 2 Selected anti-cancer agents targeting ErbB family receptors

Agent	Class/Type	Target	Route of administration	Development stage	Ref.
Trastuzumab (Herceptin)	Recombinant humanized mAb	Extracellular domain of ErbB2	Intravenous infusion	Approved (ErbB2-positive breast cancer)	[150-152]
Pertuzumab (Omnitarg, $2C_4$)	Recombinant humanized mAb	Dimerization domain of ErbB2	Intravenous infusion	Phase Ⅱ/Ⅲ	[151,153,154]
Cetuximab (Erbitux, C225)	Recombinant human/mouse chimeric mAb	Extracellular domain of EGFR	Intravenous infusion	Approved (EGFR-positive metastatic colorectal cancer and squamous cell carcinoma head and neck cancer)	[151,155-157]
Panitumumab (ABX-EGF, Vectibix)	Fully human mAb	Extracellular domain of EGFR	Intravenous infusion	Approved (EGFR-positive metastatic colorectal cancer)	[157-159]
Matuzumab (EMI-72000)	Recombinant humanized mAb	Extracellular domain of EGFR	Intravenous infusion	Phase Ⅰ/Ⅱ	[157,159,160]
MDX-447	Humanized bispecific mAb	Extracellular domain of EGFR and high affinity IgG receptor CD64	Intravenous infusion	Phase Ⅰ/Ⅱ	[157,161]
Gefitinib (Iressa)	Anilinoquinazoline/Reversible TKI	EGFR tyrosine kinase	Oral	Limited approval (NSCLC)	[157,162-164]
Erlotinib (Tarceva)	Anilinoquinazoline/Reversible TKI	EGFR tyrosine kinase	Oral	Approved (NSCLC and pancreatic cancer)	[153,155,157,165,166]
Lapatinib (Tykerb, GW572016)	Thiazolylquinazoline/Reversible TKI	EGFR and ErbB2 tyrosine kinases	Oral	Approved (ErbB2-positive advanced metastatic breast cancer)	[157,166-169]
PKI-166	Pyrrolopyrimidine/Reversible TKI	EGFR and ErbB2 tyrosine kinases	Oral	Phase Ⅰ	[153,155,157,166,170]
BMS-599626	Pyrrolotriazine/Reversible TKI	EGFR and ErbB2 tyrosine kinases	Oral	Phase Ⅰ	[171,172]
EKB-569 (Pelitinib)	Cyanoquinoline/Irreversible TKI	EGFR tyrosine kinase	Oral	Phase Ⅰ/Ⅱ	[157,166,173]
BIBW-2992	Anilinoquinazoline/Irreversible TKI	EGFR and ErbB2 tyrosine kinases	Oral	Phase Ⅰ/Ⅱ	[168,174]
CI-1033 (Canertinib)	Anilinoquinazoline/Irreversible TKI	Pan-ErbB tyrosine kinases	Oral	Phase Ⅰ/Ⅱ	[153,157,164,168]
HKI-272	Cyanoquinoline/Irreversible TKI	Pan-ErbB tyrosine kinases	Oral	Phase Ⅰ/Ⅱ	[153,164,168,175]

mAb: Monoclonal antibody; TKI: Tyrosine kinase inhibitor; EGFR: Epidermal growth factor receptor; NSCLC: Non-small cell lung cancer.

human solid cancers.

Recent preclinical studies, summarized in Table 3, have demonstrated that select TKIs targeting either EGFR or ErbB2, as well as those producing dual inhibition of EGFR and of ErbB2, are capable of effectively suppressing cellular growth and inducing significant apoptosis in human and rodent biliary cancer cell lines *in vitro*, and in some studies were also found to significantly inhibit tumor growth in athymic nude mice that had been subcutaneously xenografted with select human biliary tract carcinoma cell lines. Orally active TKIs were also shown to exhibit both chemopreventive and therapeutic effects in the *BK5.erbB2* transgenic mouse model of gallbladder carcinoma[182]. These cumulative preclinical results provide a "proof of principal" for EGFR and/or ErbB2 targeting as being a promising strategy for the chemoprevention and/or adjuvant therapy of biliary tract cancers. Most importantly, select preclinical studies further indicated that dual targeting of EGFR and ErbB2 with TKIs, such as lapatinib[180] or NVP-AEE788[181], as well as treatments with EGFR or ErbB2 inhibitors administered in combination with other small drug inhibitors, such as the COX-2 inhibitor celecoxib[75], or the MEK inhibitor CI-1040[179], were found to be significantly more potent than corresponding treatments with the single targeting agents alone in suppressing cholangiocarcinoma or gallbladder carcinoma cell growth *in vitro* and/or the tumorigenic growth of biliary tract cancer xenografts *in vivo*. Consistent with previously described results obtained with other carcinoma cell types[169,184], growth suppression and induced apoptosis of cultured rat and human cholangiocarcinoma cell lines produced by *in vitro* treatments with the dual EGFR/ErbB2-TKI lapatinib or by single agent TKIs combined to simultaneously inhibit EGFR and ErbB2 tyrosine phosphorylation were also observed to be correlated with prominent dose-dependent inhibition of both p42/44 MAPK and Akt activation[180] (Figure 6). On the other hand, while the dual EGFR/ErbB2 inhibitor NVP-AEE788 was reported to suppress tumorigenic growth in nude mice subcutaneously implanted with human biliary tract cancer cell lines by mechanisms involving downstream blocking of p42/44 MAPK signaling, induction of apoptosis, and inhibition of angiogenesis, treatment with this agent had no apparent effect on reducing Akt activation in the tumor xenografts[181]. In contrast, the EGFR-TKI gefitinib, was determined to effectively

Table 3 Preclinical biological effects of ErbB RTK inhibitors alone or combined with other target-based treatments for biliary tract cancer cells

Agent	Target	Experimental condition	Biliary cancer cell line/ tumor	Biological effects	Ref.
Gefitinib	EGFR	Cell culture	HAG-1 human gallbladder adenocarcinoma cell line	Dose-dependent *in vitro* cell growth inhibition by arresting cells in G0/G1, followed by progressive cell apoptosis; inhibition of EGFR phosphorylation and of Erk1/2 and Akt activation; decreased cyclin D1 mRNA and induced accumulation of p27 protein, a negative cell cycle regulator	[176]
Gefitinib + Ionizing radiation	EGFR	Cell culture	HuCCT1 human intrahepatic cholangiocarcinoma cell line; TFK-1 human bile duct carcinoma cell line	Gefitinib induced increase in radiosensitivity of HuCCT1 and TFK-1 cells	[177]
Cetuximab + erlotinib	EGFR	Cell culture and subcutaneous tumor xenografts in athymic nude mice	HuCCT1 cell line	Combined treatment with cetuximab blunted erlotinib-induced EGFR up-regulation and regulated in HuCCT1 growth inhibition and apoptosis *in vitro* and HuCCT1 tumor growth arrest *in vivo*	[178]
Gefitinib + CI-1040	EGFR + MEK-Erk1/2	Cell culture and subcutaneous tumor xenografts in athymic nude mice	HuCCT1 cell line	Drug combination significantly more effective than single agent treatments in suppressing both *in vitro* and *in vivo* tumor cell growth; combination treatment dramatically decreased phosphorylation levels of EGFR and Erk1/2 in cultured cells and in xenografted tumors, whereas HuCCT1 cells were found to be resistant to treatments with gefitinib or CI-1040 alone	[179]
Lapatinib	EGFR/ ErbB2	Cell culture	Rat C611B and human HuCCT1 cholangiocarcinoma cell lines	Lapatinib was demonstrated to be a potent inhibitor of C611B and HuCCT1 cholangiocarcinoma cell growth *in vitro* by a mechanism involving inhibition of EGFR and ErbB2 activation, suppression of p42/44 MAPK and Akt phosphorylation, and induction of apoptosis	[180]
NVP-AEE788	EGFR/ ErbB2 and VEGFR-2	Cell culture and subcutaneous tumor xenografts in athymic nude mice	EGI-1, TFK-1, CC-SW-1, CC-LP-1 and SK-ChA-1 human extrahepatic bile duct cancer cell lines; MZ-ChA-1 and MZ-CA-2 human gallbladder adenocarcinoma cell lines	NVP-AEE788 more efficacious than the EGFR RTK inhibitors gefitinib and erlotinib in suppressing *in vitro* cell growth; EGI-1 tumors in mice treated with NVP-AEE788 had significantly reduced volume and mass compared with those in placebo-treated mice, while erlotinib was without effect in inhibiting *in vivo* tumor growth; main mechanisms of NVP-AEE788 drug action were suppression of Erk1/2 phosphorylation, induced apoptosis, and inhibition of tumor angiogenesis	[181]
Emodin + Celecoxib	ErbB2 +COX-2	Cell culture	C611B rat intrahepatic cholangiocarcinoma cell line	Emodin and celecoxib combined to synergistically suppress anchorage-dependent and anchorage-independent cell growth *in vitro* through a mechanism involving enhanced inhibition of ErbB2 activation, decreased phospho-Akt, and enhanced caspase-9 and -3 activation, resulting in significantly increased apoptosis	[75]
Gefitinib or GW2974	EGFR EGFR/ ErbB2	BK5.erbB2 transgenic mice constitutively expressing wild-type rat ErbB2	Gallbladder adenocarcinoma	Both agents produce significant chemopreventative and therapeutic effects in reducing gallbladder adenocarcinoma incidence, which was associated with prominent decreases in both the phosphorylation and protein levels of EGFR and ErbB2, with significantly decreased Erk1/2 acitivity and with a reduction in COX-2 protein levels in BK5.erbB2 mouse gallbladders	[182]

RTK: Receptor tyrosine kinase; EGFR: Epidermal growth factor receptor; VEGFR-2: Vascular endothelial growth factor receptor-2; COX-2: Cyclooxygenase-2.

block EGFR tyrosine phosphorylation and associated downstream activation of Akt, but had a minimal effect on inhibiting p42/44 MAP kinase activation in cultured human HuCCT1 cholangiocarcinoma cells[179]. This TKI also failed to block p42/44 MAP kinase phosphorylation and tumor growth *in vivo* in athymic nude mice subcutaneously transplanted with HuCCT1 cells. The fact that the HuCCT1 cholangiocarcinoma cell line expresses a mutant *Kras* may explain why upstream inhibition of EGFR by gefitinib was ineffective in blocking p42/44 MAPK activation in these cells[179]. However, gefitinib given in combination with the MEK inhibitor CI-1040 was associated with a profound inhibition of HuCCT1 tumor growth that appeared to be linked to either a greater p42/44 MAP kinase inhibition or to simultaneous inhibition of Akt and p42/44 MAPK signaling[179]. In comparison, gefitinib alone suppressed both the autophosphorylation of p42/44 MAPK and to a lesser degree that of Akt in the HAG1 human gallbladder carcinoma cell line[176].

Interestingly, the EGFR-TKI erlotinib was found to up-regulate EGFR in HuCCT1 cholangiocarcinoma cells, but combined treatment with the anti-EGFR antibody cetuximab or blockage of erlotinib-induced EGFR synthesis by a small interfering RNA abrogated this TKI effect, and resulted in an inhibition of cell proliferation and increased apoptosis in the HuCCT1 cells[178]. Moreover, in this study, cetuximab was shown to be equally effective when administered alone or in combination with erlotinib in partially suppressing

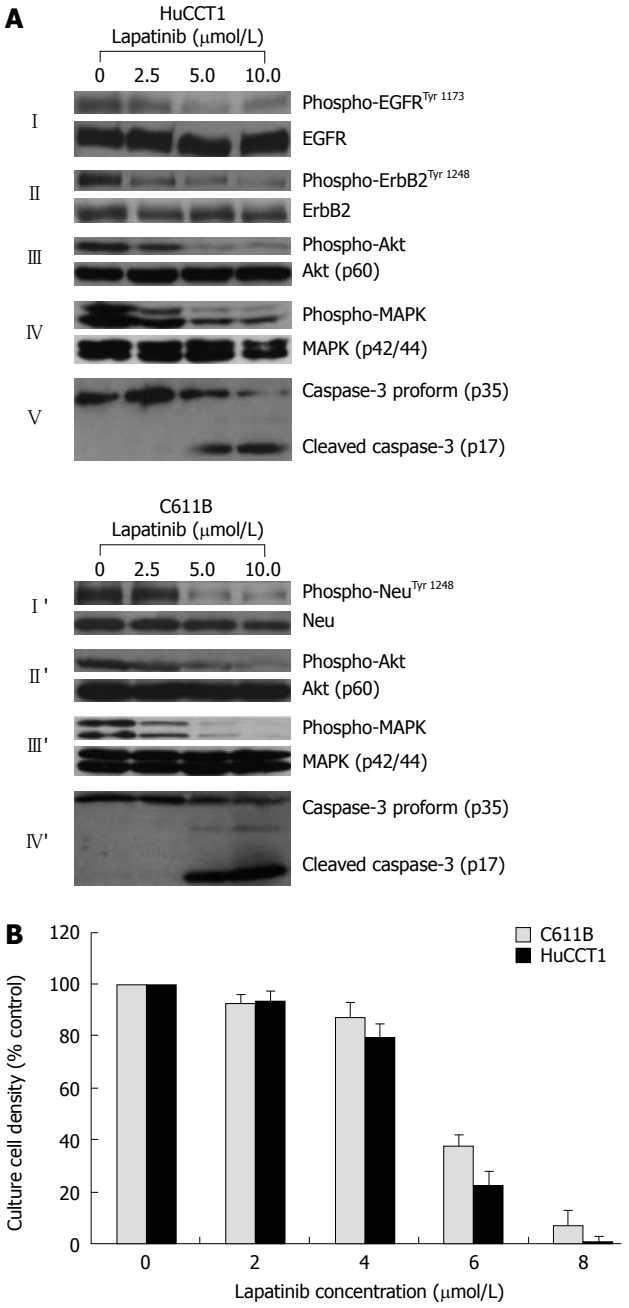

Figure 6 Dose-dependent inhibition test. A: Western blots demonstrating dose-dependent suppression by the dual ErbB1 (EGFR)/ErbB2 (Neu) inhibitor lapatinib of phospho-EGFR$^{Tyr\ 1173}$ and/or of phospho-ErbB2/Neu$^{Tyr\ 1248}$, together with concomitant downstream inhibition of phospho-Akt and of phospho-p42/44 MAPK expressed in cultured human HuCCT1 and in rat C611B cholangiocarcinoma cells, respectively. Note that the lapatinib treatment *in vitro* did not affect corresponding total protein levels, but in both cell lines induced a dose-dependent activation of the apoptotic enzyme, caspase-3; B: Dose response curves for lapatinib on suppressing anchorage-dependent growth of cultured rat C611B and human HuCCT1 cholangiocarcinoma cells. Each value represents the mean ± SD (n = 3). Note the close relationship between the lapatinib concentrations required to induce prominent suppression of EGFR and/or ErbB-2/Neu signaling ($\geqslant$ 5 μmol/L) and those associated with marked inhibition of *in vitro* cholangiocarcinoma cell growth ($\geqslant$ 6 μmol/L). Lapatinib-induced suppression of C611B and HuCCT1 growth *in vitro* correlated with both a down-regulation of cyclin D1 protein expression and with a prominent apoptosis being induced in these respective cultured cholangiocarcinoma cell lines (data not shown).

tumorigenic growth in HuCCT1 bearing mice, suggesting that antibody-induced down-regulation of EGFR may provide an effective strategy for circumventing resistance to specific EGFR-TKI inhibitors in cholangiocarcinoma and other EGFR-expressing cancer cell types.

In the *BK5.erbB2* transgenic mouse model, decreased gallbladder carcinoma incidence produced by chemopreventive or therapeutic treatments involving single targeting of EGFR with gefitinib or dual targeting of EGFR/ErbB2 with GW2974 were each demonstrated to not only reduce tyrosine phosphorylation levels, but also total protein levels of EGFR and ErbB2 in the gallbladders of responsive mice[182]. Of further note, these targeted treatments were also associated with decreases in COX-2 protein level and of MAPK activity in gallbladders from both the gefitinib and GW2974-treated *BK5.erbB2* mice, with GW2974 showing a greater inhibitory effect than gefitinib on the development of gallbladder carcinoma and also a strong reduction in the tyrosine phosphorylated forms of both EGFR and ErbB2. Interestingly, gallbladder adenocarcinomas refractory to the gefitinib treatment were reported to exhibit sustained activation of EGFR and ErbB2.

ErbB2-TKI emodin in combination with the COX-2 inhibitor celecoxib was further demonstrated to act synergistically to suppress anchorage-dependent and-independent growth of rat C611B cholangiocarcinoma cells overexpressing activated wild-type ErbB2 through a mechanism involving enhanced Akt inactivation leading to increased apoptosis[75]. From a clinical standpoint, targeted treatments, including the use of select EGFR- and/or ErbB2-TKIs combined with other agents that act to suppress the phosphorylation of Akt are likely to prove to be more effective than single agent treatments for the therapy of biliary tract cancers. In this context, it is noteworthy that both the EGFR-TKI gefitinib and the Akt inhibitor LY294002 were each determined in a preclinical study to increase the radiosensitivity of human HuCCT1 and TFK1 biliary carcinoma cell lines by suppressing Akt activation[177]. Thus, preclinical testing of various paradigms utilizing select targeting of aberrant EGFR and/or ErbB2 signaling affecting MAPK and/or Akt activation, in combination with other classes of agents that act to suppress Akt activation, would seem to be warranted as a potential strategy for developing new adjuvant therapies for biliary tract carcinomas, including cholangiocarcinoma.

As reflected in Table 4, there have been to date only a limited number of published clinical studies on EGFR and/or ErbB2 targeting in human biliary tract cancers. Unfortunately, while the various treatments described in these studies were determined in most cases to be well tolerated, the anti-tumor effects produced by such treatments in which ErbB receptor targeting was included as part of the therapeutic regimen, was at best modest, and mostly without effect in the vast majority of biliary tract cancer patients who presented with unresected or metastatic disease. Moreover, the extent of EGFR expression was reported not to be significantly associated with outcome[185]. In one case report, a patient with metastatic cholangiocarcinoma was described as having a response to cetuximab-based therapies; however, the patient's tumors were determined

Table 4 Outcomes of ErbB-targeted therapies in patients with advanced biliary cancer

Assessable patients	Tumor	ErbB status	ErbB inhibitor	Target	Administration	Response	Ref.
40	Unresected or metastatic biliary tract cancers (gallbladder, intra- and extrahepatic bile duct)	29/36 (81%) assessable tumor samples positive for EGFR expression	Erlotinib	EGFR	Single agent	PR-3 patients; SD-17 patients; median time to disease-progression of 2.6 mo	[185]
1	Metastatic cholangiocarcinoma	Negative EGFR expression in tumor	Cetuximab	EGFR	In combination with 5'flurouracil, folic acid, and radiotherapy	PR in intra-chemotherapeutic state	[186]
1	Unresected cholangiocarcinoma with peritoneal carcinomatosis	Positive EGFR expression in tumor	Cetuximab	EGFR	In combination with gemcitabine	PR with 30% reduction in hepatic mass and disappearance of peritoneal carcinomatosis as shown by computed tomography	[187]
9	Unresected cholangiocarcinoma with disease progression after at least 3 cycles of gemcitabine-oxaliplatin	9/9 (100%) tumor samples positive for EGFR expression, with all being negative for membranous ErbB2	Cetuximab	EGFR	In combination with gemcitabine-oxaliplatin	After 6 mo, CR-1 patient; PR-1 patient; SD-1 patient; progressive disease-6 patients; all patients relapsed, with a median time to disease progression of 4 mo	[188]
17	Unresected advanced biliary tract cancers (gallbladder, bile duct)	Not reported	Lapatinib	EGFR/ ErbB2	Single agent	No observed responses; 5 patients with SD; cohort closed due to no noted lapatinib activity	[189]
6 with biliary tract cancers out of a total of 34 with various types of solid tumors	Advanced cholangiocarcinoma (5) or gallbladder cancer (1)	Not investigated	Lapatinib	EGFR/ ErbB2	In combination with oxaliplatin/ 5-flurouracil/ leucovorin	PR-1 patient with cholangiocarcinoma and 1 patient with gallbladder cancer	[190]

CR: Complete response; PR: Partial response; SD: Stable disease.

by immunohistochemistry not to express EGFR[186]. Also, while immunohistochemistry was used in some of these clinical therapeutic studies to confirm a positive EGFR status[185,187,188] of the tumors prior to treatment, unlike some of the preclinical *in vivo* studies described above[179,181,182] (Table 3), no effort was made in any of the studies listed in Table 4 to investigate the effects of the ErbB-target agents being tested on markers of biological activity (i.e. suppression of EGFR tyrosine phosphorylation; inhibition of p42/44 MAPK and/or Akt activation, suppression of cyclin D1) in tumors of responsive *versus* non-responsive patients.

In view of the promising preclinical results obtained with ErbB-targeting agents against human and rodent biliary tract cancer cells, including cholangiocarcinoma cells *in vitro* as well as *in vivo* in tumor xenograft and transgenic models, it is disappointing that the current clinical experience with ErbB-directed therapies have resulted in only very limited anti-tumor activity having been demonstrated in select patients with biliary tract cancers. However, these (albeit limited) clinical findings are also quite consistent with an increasing body of data from different clinical trials demonstrating at best, only modest therapeutic responses of various other epithelial tumor types to single-drug treatments with ErbB inhibitors. In addition, the discrepancies between current preclinical and clinical data point out a real need to establish "patient-like" *in vivo* models of cholangiocarcinoma progression that would more closely mimic the advanced human disease, and which could better serve to more accurately predict the clinical efficacy of ErbB-directed therapies. In this context, it is noteworthy that very recent preliminary studies in the author's laboratory have demonstrated that when the dual EGFR/ErbB2-TKI lapatinib was administered to rats by gavage over a three week period, beginning at 2 d after bile duct inoculation of oncogenic *neu*-transformed rat cholangiocytes (BDEneu cells), this treatment produced an approximately 70% inhibition in mean intrahepatic tumor wet weight compared with that of tumors formed in the livers of vehicle control-treated rats. In contrast, when the lapatinib treatment was delayed and given to rats with more advanced tumors that resulted in bile duct obstruction, it was without any anti-tumor effect (Sirica AE, Zhang Z, and Campbell DJ, unpublished data). As already indicated above, a similar lack of clinical response was reported by Ramanathan *et al*[189] in a Phase Ⅱ study evaluating the anti-tumor efficacy of lapatinib as a single agent treatment in patients with advanced biliary tree cancer. Thus, the rat model of intrahepatic cholangiocarcinoma progression based on bile duct inoculation of highly tumorigenic BDEneu cholangiocytes[68,69] would appear to closely reproduce certain relevant clinical (i.e. bile duct obstruction, icteric liver, elevated serum bilirubin), pathologic (moderately differentiated invasive ductal cholangiocarcinoma with desmoplasia, peritoneal carcinomatosis), and key molecular features (i.e. overexpression of tyrosine

Table 5 Factors affecting ErbB-targeted therapies for intrahepatic cholangiocarcinoma and other biliary tract cancers

Factors
Patient selection and sampling size
Suboptimal drug dosing and/or scheduling
Tumor microenvironment and bioavailability
Intratumoral heterogeneity in receptor expression and activation
Receptor dynamic effects
Mutational effects
Different mechanisms of acquired resistance
Constitutive overexpression of ErbB family ligands
Co-activation of multiple receptor tyrosine kinases resulting in signaling redundancy and interplay
Lack of uniform biomarkers to effectively predict therapeutic response
Co-morbid disease and toxicity

phosphorylated p185^{neu}, COX-2, and MUC1) of the progressive human disease, as well as permit for a rapid preclinical assessment within a three-to-four week period of innovative molecular targeting strategies that include specific targeting of ErbB2 (and EGFR) regulated signaling pathways. Validating such target-based ErbB-targeting strategies in this "patient-like" model may prove to be most useful in developing new paradigms for adjuvant therapies for intrahepatic cholangiocarcinoma. However, more definitive basic and translational studies are now required to determine the significance of this preclinical rat model of intrahepatic cholangiocarcinoma progression for predicting the clinical efficacy of ErbB-directed therapies against the early *versus* advanced human malignant disease.

FACTORS AFFECTING THERAPEUTIC RESPONSES TO ERBB-TARGET AGENTS

Table 5 lists various factors that should be considered when devising strategies for ErbB-target-based therapies against intrahepatic cholangiocarcinoma. Obviously, patient selection is important and there is a real need now for clinical trials aimed at identifying biological markers that would better predict which of those with this malignant disease would best benefit from such ErbB-directed therapies. It also seems apparent from the limited clinical data that is currently available that single agent testing in cholangiocarcinoma patients with advanced disease that is refractory to conventional therapeutic modalities may not be the best way to assess the therapeutic value of agents that target ErbB receptor signaling pathways. Here again, it can be restated that preclinical models of intrahepatic cholangiocarcinoma that closely recapitulate clinical and molecular features of human disease progression are needed to test novel therapeutic paradigms that combine ErbB-targeted therapies with other target-based therapies in an effort to maximize the inhibition of redundant signaling pathways that aberrantly regulate malignant tumor growth and survival. In addition, preclinical testing of paradigms that combine ErbB target-based therapies with current conventional modalities could further lead to more rational approaches to developing new strategies for adjuvant management of tumor cell resistance, recurrence or progression. Because this cancer is relatively rare, it is also apparent that for the testing of ErbB-targeted therapies against intrahepatic cholangiocarcinoma to be statistically meaningful and rigorously controlled, multi-center trials set in well established treatment centers for hepatobiliary cancer located in both Eastern and Western countries would be needed to achieve appropriate patient selection. Furthermore, well controlled preclinical and multi-center clinical trials should also be directed towards evaluating the chemopreventative effects of ErbB-directed targeted treatments alone and/or in combination with other therapies in select patient populations with known high risk premalignant conditions for intrahepatic cholangiocarcinoma.

Data on optimum dosing and scheduling of ErbB-targeted agents alone and in combination with other target-based or conventional treatments against intrahepatic cholangiocarcinoma are severely lacking and need to be developed, both preclinically and clinically. Tumor microenvironment and bioavailability is also an important factor in determining the potential therapeutic effectiveness of ErbB-targeted therapies against intrahepatic cholangiocarcinoma. For example, intrahepatic cholangiocarcinomas are often hypovascularized[136,141,142,191], thereby limiting the possibility of ErbB-targeted agents of reaching the malignant cells in sufficient concentrations to be therapeutically effective. In addition, hypoxic tumor microenvironments inducing HIF-1α[192,193] and tumor extracellular matrix components, like laminin 5[194], may alter the effectiveness of ErbB directed antineoplastic therapies. Moreover, intratumoral heterogeneity of expression for tyrosine kinase growth factor receptors (i.e. EGFR and ErbB2) indicate that targeting of single ErbB family receptor tyrosine kinase receptors in adenocarcinoma types, including intrahepatic cholangiocarcinoma, may not in itself be sufficient to elicit a strong therapeutic response[195]. ErbB receptor dynamics have also been shown to influence response to ErbB-targeted agents. For example, as described above, exposure of HuCCT1 human cholangiocarcinoma cells to erlotinib was shown to induce EGFR protein and mRNA expression in these cells, leading to resistance to EGFR tyrosine kinase inhibition[178]. However, cetuximab or blockage of EGFR synthesis in HuCCT1 cells by small interfering RNA (siRNA) was found to abrogate erlotinib-induced EGFR up-regulation in these cells, suggesting that resistance and sensitivity to EGFR-targeted agents are dynamic events, and that combinations of more than one type of ErbB-directed therapy may be needed to elicit a positive therapeutic outcome.

Mutational events play a key role in mediating resistance or sensitivity to cell-based ErbB-targeted therapies[196-202]. The efficacy of EGFR-TKIs, such as gefitinib or erlotinib, has been linked to activating

mutations in lung cancer cell EGFR[199,201], whereas secondary mutations, such as EGFR T790M mutation, can also lead to resistance to EGFR-TKI therapy[197,201] and to lapatinib-mediated inhibition of receptor autophosphorylation[202]. *Kras* mutation has also been demonstrated to be an important predictor of resistance to therapy with ErbB-TKIs in some solid tumors[197,199,200]. In addition, tumor cells with ErbB2 mutations were reported to be resistant to EGFR-TKI treatment, but sensitive to ErbB2 inhibitors, both *in vitro* and *in vivo*[198,199].

In addition to secondary mutations, various other mechanisms of acquired resistance to ErbB-targeted therapies have also been described, including EGFR ubiquitination[203], signaling interplay between the EGFR and IGF-1 receptors[204], steric hindrance of ErbB2 by MUC4[205], and flexibility of the ErbB receptor family signaling system in the face of inhibition of a single member, such as EGFR[206]. Moreover, it has been hypothesized that co-activation of multiple receptor tyrosine kinases, (i.e. EGFR and/or ErbB2, Met, PDGFR, IL-6R) in solid malignant tumors results in redundant inputs that drive downstream signaling, therefore limiting the effectiveness of therapies that target just a single receptor tyrosine kinase[207]. For example, Met/*c-Src* signaling has been recently shown to mediate EGFR tyrosine phosphorylation and cell growth in cultured SUM229 breast cancer cells in the presence of EGFR-TKIs[208].

Presently, there is no uniform set of biomarkers that can be used to accurately predict a therapeutic response in solid tumors, including intrahepatic cholangiocarcinoma, to ErbB-directed therapies. TGF-α expression has recently been reported to drive constitutive EGFR pathway activation and sensitivity to gefitinib in human pancreatic cancer cell lines[209]. Elevated TGF-α also appeared to predict for a partial response to lapatinib in patients with advanced EGFR and/or ErbB2 solid malignancies[210]. However, Ishikawa *et al*[211] have also presented data to suggest that increased levels of TGF-α and of amphiregulin in serum may serve as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancer. More recently, Jimeno *et al*[212], utilizing global gene expression profiling together with gene set enrichment analysis, defined an EGFR pathway-based signature that was predictive of a therapeutic response to erlotinib and cetuximab in a subset of xenografted human pancreatic cancers. Further studies are needed, however, to determine if this approach can be effective in a clinical setting as a means of defining biomarkers of ErbB-directed therapies.

Impaired liver function and cirrhosis, together with older age, genetic background, co-morbitities, such as cardiovascular disease, and immune-status all need to also be considered as factors that may affect the potential use and/or effectiveness of ErbB target-based treatments for intrahepatic cholangiocarcinoma therapy. Presently, only a very few clinical trials have investigated cardiotoxicities of the various ErbB target agents, using defined cardiac endpoints, such as left ventricular function[213], although it is now well established that herceptin when administered in combination with anthracyclines, can induce significant cardiotoxicity[153,213]. On the otherhand, treatments with small drug TKI, like lapatinib, erlotinib, and gefitinib, or with the anti-EGFR mAbs cetuximab and panitumumab, have been reported to be associated with low cardiotoxicity[213]. Skin rash is a classic adverse effect of ErbB-targeted therapy and diarrhea is relatively common in patients treated with EGFR antibodies, or with TKIs, including lapatinib, erlotinib, and gefitinib[153,213,214]. Interstitial lung disease, elevated hepatic transaminases, and nephritic syndrome have also been rarely reported to occur with small drug EGFR-TKIs[214], and a high prevalence of hypersensity reactions to cetuximab, including anaphylaxis, has been reported in some areas of the United States[215]. Thus, ErbB target-based therapies are not without risk, although the current clinical experience has indicated that such therapies are generally well tolerated.

FINAL REMARKS

While it now seems apparent that aberrant EGFR and/or ErbB2 expression and signaling is associated with the molecular pathogenesis of intrahepatic cholangiocarcinoma, there is still a significant gap in our knowledge as how to best exploit such alterations in terms of targeted therapies that can then be successfully translated into positive clinical outcomes. More definitive approaches, including cDNA microarray analysis, quantitative immunohistochemistry, and possibly proteomics, need to be evaluated in terms of their potential for profiling individual patient samples (cholangiocarcinoma tumor tissue and cytological specimens and corresponding serum and/or bile samples) for select molecular biomarkers that would be predictive of a therapeutic response to ErbB targeted therapies. In addition, there is a need to develop more effective drug delivery systems for these agents, in order to maximize their bioavailability. Preclinical animal platforms for rapidly testing target-based treatments of intrahepatic cholangiocarcinoma that closely mimic key clinicopathological and molecular features of the human disease also need to be more fully developed and assessed for their ability to more effectively predict therapeutic responses in human clinical trials. Furthermore, based on the complex interactive growth factor receptor signaling and microenvironment properties of solid tumors like intrahepatic cholangiocarcinoma, it seems evident that novel therapeutic strategies involving multiple targeted therapeutics aimed at both cancer and tumor stromal cell targets should be rigorously explored as a means of achieving more effective molecular therapies for this devastating cancer.

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REFERENCES

1 **Sirica AE**. Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. *Hepatology* 2005; **41**: 5-15
2 **Malhi H**, Gores GJ. Cholangiocarcinoma: modern advances in understanding a deadly old disease. *J Hepatol* 2006; **45**: 856-867
3 **Shimoda M**, Kubota K. Multi-disciplinary treatment for cholangiocellular carcinoma. *World J Gastroenterol* 2007; **13**: 1500-1504
4 **Shaib Y**, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 115-125
5 **Nakajima T**, Kondo Y, Miyazaki M, Okui K. A histopathologic study of 102 cases of intrahepatic cholangiocarcinoma: histologic classification and modes of spreading. *Hum Pathol* 1988; **19**: 1228-1234
6 **Bae JY**, Park YN, Nakanuma Y, Lee WJ, Kim JY, Park C. Intestinal type cholangiocarcinoma of intrahepatic large bile duct associated with hepatolithiasis--a new histologic subtype for further investigation. *Hepatogastroenterology* 2002; **49**: 628-630
7 **Nakanuma Y**, Sasaki M, Ishikawa A, Tsui W, Chen TC, Huang SF. Biliary papillary neoplasm of the liver. *Histol Histopathol* 2002; **17**: 851-861
8 **Suh KS**, Chang SH, Lee HJ, Roh HR, Kim SH, Lee KU. Clinical outcomes and apomucin expression of intrahepatic cholangiocarcinoma according to gross morphology. *J Am Coll Surg* 2002; **195**: 782-789
9 **Yamasaki S**. Intrahepatic cholangiocarcinoma: macroscopic type and stage classification. *J Hepatobiliary Pancreat Surg* 2003; **10**: 288-291
10 **Sasaki A**, Kawano K, Aramaki M, Ohno T, Tahara K, Kitano S. Correlation between tumor size and mode of spread in mass-forming intrahepatic cholangiocarcinoma. *Hepatogastroenterology* 2004; **51**: 224-228
11 **Patel T**. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001; **33**: 1353-1357
12 **Khan SA**, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. *Lancet* 2005; **366**: 1303-1314
13 **Shaib YH**, El-Serag HB, Davila JA, Morgan R, McGlynn KA. Risk factors of intrahepatic cholangiocarcinoma in the United States: a case-control study. *Gastroenterology* 2005; **128**: 620-626
14 **McGlynn KA**, Tarone RE, El-Serag HB. A comparison of trends in the incidence of hepatocellular carcinoma and intrahepatic cholangiocarcinoma in the United States. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1198-1203
15 **Ben-Menachem T**. Risk factors for cholangiocarcinoma. *Eur J Gastroenterol Hepatol* 2007; **19**: 615-617
16 **Welzel TM**, Mellemkjaer L, Gloria G, Sakoda LC, Hsing AW, El Ghormli L, Olsen JH, McGlynn KA. Risk factors for intrahepatic cholangiocarcinoma in a low-risk population: a nationwide case-control study. *Int J Cancer* 2007; **120**: 638-641
17 **Patel T**, Singh P. Cholangiocarcinoma: emerging approaches to a challenging cancer. *Curr Opin Gastroenterol* 2007; **23**: 317-323
18 **Gores GJ**. Cholangiocarcinoma: current concepts and insights. *Hepatology* 2003; **37**: 961-969
19 **Lazaridis KN**, Gores GJ. Cholangiocarcinoma. *Gastroenterology* 2005; **128**: 1655-1667
20 **Fava G**, Marzioni M, Benedetti A, Glaser S, DeMorrow S, Francis H, Alpini G. Molecular pathology of biliary tract cancers. *Cancer Lett* 2007; **250**: 155-167
21 **Roskoski R Jr**. The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem Biophys Res Commun* 2004; **319**: 1-11
22 **Hynes NE**, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005; **5**: 341-354
23 **Normanno N**, Bianco C, Strizzi L, Mancino M, Maiello MR, De Luca A, Caponigro F, Salomon DS. The ErbB receptors and their ligands in cancer: an overview. *Curr Drug Targets* 2005; **6**: 243-257
24 **Linggi B**, Carpenter G. ErbB receptors: new insights on mechanisms and biology. *Trends Cell Biol* 2006; **16**: 649-656
25 **Hobbs SS**, Coffing SL, Le AT, Cameron EM, Williams EE, Andrew M, Blommel EN, Hammer RP, Chang H, Riese DJ 2nd. Neuregulin isoforms exhibit distinct patterns of ErbB family receptor activation. *Oncogene* 2002; **21**: 8442-8452
26 **Werneburg NW**, Yoon JH, Higuchi H, Gores GJ. Bile acids activate EGF receptor via a TGF-alpha-dependent mechanism in human cholangiocyte cell lines. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G31-G36
27 **Ii M**, Yamamoto H, Adachi Y, Maruyama Y, Shinomura Y. Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. *Exp Biol Med* (Maywood) 2006; **231**: 20-27
28 **Fuller SJ**, Sivarajah K, Sugden PH. ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium. *J Mol Cell Cardiol* 2008; **44**: 831-854
29 **Wolf-Yadlin A**, Kumar N, Zhang Y, Hautaniemi S, Zaman M, Kim HD, Grantcharova V, Lauffenburger DA, White FM. Effects of HER2 overexpression on cell signaling networks governing proliferation and migration. *Mol Syst Biol* 2006; **2**: 54
30 **Harari D**, Yarden Y. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. *Oncogene* 2000; **19**: 6102-6114
31 **Jo M**, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. *J Biol Chem* 2000; **275**: 8806-8811
32 **Pai R**, Nakamura T, Moon WS, Tarnawski AS. Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. *FASEB J* 2003; **17**: 1640-1647
33 **Han C**, Michalopoulos GK, Wu T. Prostaglandin E2 receptor EP1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. *J Cell Physiol* 2006; **207**: 261-270
34 **Garrett TP**, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Kofler M, Jorissen RN, Nice EC, Burgess AW, Ward CW. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell* 2003; **11**: 495-505
35 **Michalopoulos GK**, Khan Z. Liver regeneration, growth factors, and amphiregulin. *Gastroenterology* 2005; **128**: 503-506
36 **Kiguchi K**, Carbajal S, Chan K, Beltran L, Ruffino L, Shen J, Matsumoto T, Yoshimi N, DiGiovanni J. Constitutive expression of ErbB-2 in gallbladder epithelium results in development of adenocarcinoma. *Cancer Res* 2001; **61**: 6971-6976
37 **Zhou BP**, Hung MC. Dysregulation of cellular signaling by HER2/neu in breast cancer. *Semin Oncol* 2003; **30**: 38-48
38 **Bhat-Nakshatri P**, Sweeney CJ, Nakshatri H. Identification of signal transduction pathways involved in constitutive NF-kappaB activation in breast cancer cells. *Oncogene* 2002; **21**: 2066-2078
39 **Makino K**, Day CP, Wang SC, Li YM, Hung MC. Upregulation of IKKalpha/IKKbeta by integrin-linked kinase is required for HER2/neu-induced NF-kappaB antiapoptotic pathway. *Oncogene* 2004; **23**: 3883-3887

40 **Voravud N**, Foster CS, Gilbertson JA, Sikora K, Waxman J. Oncogene expression in cholangiocarcinoma and in normal hepatic development. *Hum Pathol* 1989; **20**: 1163-1168
41 **Collier JD**, Guo K, Mathew J, May FE, Bennett MK, Corbett IP, Bassendine MF, Burt AD. c-erbB-2 oncogene expression in hepatocellular carcinoma and cholangiocarcinoma. *J Hepatol* 1992; **14**: 377-380
42 **Brunt EM**, Swanson PE. Immunoreactivity for c-erbB-2 oncopeptide in benign and malignant diseases of the liver. *Am J Clin Pathol* 1992; **97**: S53-S61
43 **Chow NH**, Huang SM, Chan SH, Mo LR, Hwang MH, Su WC. Significance of c-erbB-2 expression in normal and neoplastic epithelium of biliary tract. *Anticancer Res* 1995; **15**: 1055-1059
44 **Terada T**, Ashida K, Endo K, Horie S, Maeta H, Matsunaga Y, Takashima K, Ohta T, Kitamura Y. c-erbB-2 protein is expressed in hepatolithiasis and cholangiocarcinoma. *Histopathology* 1998; **33**: 325-331
45 **Suzuki H**, Isaji S, Pairojkul C, Uttaravichien T. Comparative clinicopathological study of resected intrahepatic cholangiocarcinoma in northeast Thailand and Japan. *J Hepatobiliary Pancreat Surg* 2000; **7**: 206-211
46 **Ito Y**, Takeda T, Sasaki Y, Sakon M, Yamada T, Ishiguro S, Imaoka S, Tsujimoto M, Higashiyama S, Monden M, Matsuura N. Expression and clinical significance of the erbB family in intrahepatic cholangiocellular carcinoma. *Pathol Res Pract* 2001; **197**: 95-100
47 **Aishima SI**, Taguchi KI, Sugimachi K, Shimada M, Sugimachi K, Tsuneyoshi M. c-erbB-2 and c-Met expression relates to cholangiocarcinogenesis and progression of intrahepatic cholangiocarcinoma. *Histopathology* 2002; **40**: 269-278
48 **Ukita Y**, Kato M, Terada T. Gene amplification and mRNA and protein overexpression of c-erbB-2 (HER-2/neu) in human intrahepatic cholangiocarcinoma as detected by fluorescence in situ hybridization, in situ hybridization, and immunohistochemistry. *J Hepatol* 2002; **36**: 780-785
49 **Endo K**, Yoon BI, Pairojkul C, Demetris AJ, Sirica AE. ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. *Hepatology* 2002; **36**: 439-450
50 **Altimari A**, Fiorentino M, Gabusi E, Gruppioni E, Corti B, D'Errico A, Grigioni WF. Investigation of ErbB1 and ErbB2 expression for therapeutic targeting in primary liver tumours. *Dig Liver Dis* 2003; **35**: 332-338
51 **Nakazawa K**, Dobashi Y, Suzuki S, Fujii H, Takeda Y, Ooi A. Amplification and overexpression of c-erbB-2, epidermal growth factor receptor, and c-met in biliary tract cancers. *J Pathol* 2005; **206**: 356-365
52 **Settakorn J**, Kaewpila N, Burns GF, Leong AS. FAT, E-cadherin, beta catenin, HER 2/neu, Ki67 immunoexpression, and histological grade in intrahepatic cholangiocarcinoma. *J Clin Pathol* 2005; **58**: 1249-1254
53 **Wolff AC**, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, Hayes DF. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007; **25**: 118-145
54 **Nonomura A**, Ohta G, Nakanuma Y, Izumi R, Mizukami Y, Matsubara F, Hayashi M, Watanabe K, Takayanagi N. Simultaneous detection of epidermal growth factor receptor (EGF-R), epidermal growth factor (EGF) and ras p21 in cholangiocarcinoma by an immunocytochemical method. *Liver* 1988; **8**: 157-166
55 **Jan YY**, Yeh TS, Yeh JN, Yang HR, Chen MF. Expression of epidermal growth factor receptor, apomucins, matrix metalloproteinases, and p53 in rat and human cholangiocarcinoma: appraisal of an animal model of cholangiocarcinoma. *Ann Surg* 2004; **240**: 89-94
56 **Buttitta F**, Barassi F, Fresu G, Felicioni L, Chella A, Paolizzi D, Lattanzio G, Salvatore S, Camplese PP, Rosini S, Iarussi T, Mucilli F, Sacco R, Mezzetti A, Marchetti A. Mutational analysis of the HER2 gene in lung tumors from Caucasian patients: mutations are mainly present in adenocarcinomas with bronchioloalveolar features. *Int J Cancer* 2006; **119**: 2586-2591
57 **Bekaii-Saab T**, Williams N, Plass C, Calero MV, Eng C. A novel mutation in the tyrosine kinase domain of ERBB2 in hepatocellular carcinoma. *BMC Cancer* 2006; **6**: 278
58 **Leone F**, Cavalloni G, Pignochino Y, Sarotto I, Ferraris R, Piacibello W, Venesio T, Capussotti L, Risio M, Aglietta M. Somatic mutations of epidermal growth factor receptor in bile duct and gallbladder carcinoma. *Clin Cancer Res* 2006; **12**: 1680-1685
59 **Gwak GY**, Yoon JH, Shin CM, Ahn YJ, Chung JK, Kim YA, Kim TY, Lee HS. Detection of response-predicting mutations in the kinase domain of the epidermal growth factor receptor gene in cholangiocarcinomas. *J Cancer Res Clin Oncol* 2005; **131**: 649-652
60 **Shiraishi K**, Kusano N, Okita S, Oga A, Okita K, Sasaki K. Genetic aberrations detected by comparative genomic hybridization in biliary tract cancers. *Oncology* 1999; **57**: 42-49
61 **Obama K**, Ura K, Li M, Katagiri T, Tsunoda T, Nomura A, Satoh S, Nakamura Y, Furukawa Y. Genome-wide analysis of gene expression in human intrahepatic cholangiocarcinoma. *Hepatology* 2005; **41**: 1339-1348
62 **Su WC**, Shiesh SC, Liu HS, Chen CY, Chow NH, Lin XZ. Expression of oncogene products HER2/Neu and Ras and fibrosis-related growth factors bFGF, TGF-beta, and PDGF in bile from biliary malignancies and inflammatory disorders. *Dig Dis Sci* 2001; **46**: 1387-1392
63 **Sirica AE**, Radaeva S, Caran N. NEU overexpression in the furan rat model of cholangiocarcinogenesis compared with biliary ductal cell hyperplasia. *Am J Pathol* 1997; **151**: 1685-1694
64 **Radaeva S**, Ferreira-Gonzalez A, Sirica AE. Overexpression of C-NEU and C-MET during rat liver cholangiocarcinogenesis: A link between biliary intestinal metaplasia and mucin-producing cholangiocarcinoma. *Hepatology* 1999; **29**: 1453-1462
65 **Yeh CN**, Maitra A, Lee KF, Jan YY, Chen MF. Thioacetamide-induced intestinal-type cholangiocarcinoma in rat: an animal model recapitulating the multi-stage progression of human cholangiocarcinoma. *Carcinogenesis* 2004; **25**: 631-636
66 **Farazi PA**, Zeisberg M, Glickman J, Zhang Y, Kalluri R, DePinho RA. Chronic bile duct injury associated with fibrotic matrix microenvironment provokes cholangiocarcinoma in p53-deficient mice. *Cancer Res* 2006; **66**: 6622-6627
67 **Yang L**, Faris RA, Hixson DC. Long-term culture and characteristics of normal rat liver bile duct epithelial cells. *Gastroenterology* 1993; **104**: 840-852
68 **Lai GH**, Zhang Z, Shen XN, Ward DJ, Dewitt JL, Holt SE, Rozich RA, Hixson DC, Sirica AE. erbB-2/neu transformed rat cholangiocytes recapitulate key cellular and molecular features of human bile duct cancer. *Gastroenterology* 2005; **129**: 2047-2057
69 **Sirica AE**, Zhang Z, Lai GH, Asano T, Shen XN, Ward DJ, Mahatme A, Dewitt JL. A novel "patient-like" model of cholangiocarcinoma progression based on bile duct inoculation of tumorigenic rat cholangiocyte cell lines. *Hepatology* 2008; **47**: 1178-1190
70 **Kwon YK**, Bhattacharyya A, Alberta JA, Giannobile WV, Cheon K, Stiles CD, Pomeroy SL. Activation of ErbB2 during wallerian degeneration of sciatic nerve. *J Neurosci* 1997; **17**: 8293-8299
71 **Cicenas J**, Urban P, Küng W, Vuaroqueaux V, Labuhn M, Wight E, Eppenberger U, Eppenberger-Castori S. Phosphorylation of tyrosine 1248-ERBB2 measured by

chemiluminescence-linked immunoassay is an independent predictor of poor prognosis in primary breast cancer patients. *Eur J Cancer* 2006; **42**: 636-645

72 **Lai GH**, Sirica AE. Establishment of a novel rat cholangiocarcinoma cell culture model. *Carcinogenesis* 1999; **20**: 2335-2340

73 **Sirica AE**, Lai GH, Zhang Z. Biliary cancer growth factor pathways, cyclo-oxygenase-2 and potential therapeutic strategies. *J Gastroenterol Hepatol* 2001; **16**: 363-372

74 **Sirica AE**, Lai GH, Endo K, Zhang Z, Yoon BI. Cyclooxygenase-2 and ERBB-2 in cholangiocarcinoma: potential therapeutic targets. *Semin Liver Dis* 2002; **22**: 303-313

75 **Lai GH**, Zhang Z, Sirica AE. Celecoxib acts in a cyclooxygenase-2-independent manner and in synergy with emodin to suppress rat cholangiocarcinoma growth in vitro through a mechanism involving enhanced Akt inactivation and increased activation of caspases-9 and -3. *Mol Cancer Ther* 2003; **2**: 265-271

76 **Carver RS**, Stevenson MC, Scheving LA, Russell WE. Diverse expression of ErbB receptor proteins during rat liver development and regeneration. *Gastroenterology* 2002; **123**: 2017-2027

77 **Yoon JH**, Gwak GY, Lee HS, Bronk SF, Werneburg NW, Gores GJ. Enhanced epidermal growth factor receptor activation in human cholangiocarcinoma cells. *J Hepatol* 2004; **41**: 808-814

78 **Lee JH**, Rim HJ, Bak UB. Effect of Clonorchis sinensis infection and dimethylnitrosamine administration on the induction of cholangiocarcinoma in Syrian golden hamsters. *Korean J Parasitol* 1993; **31**: 21-30

79 **Thamavit W**, Pairojkul C, Tiwawech D, Shirai T, Ito N. Strong promoting effect of Opisthorchis viverrini infection on dimethylnitrosamine-initiated hamster liver. *Cancer Lett* 1994; **78**: 121-125

80 **Lee JH**, Rim HJ, Sell S. Heterogeneity of the "oval-cell" response in the hamster liver during cholangiocarcinogenesis following Clonorchis sinensis infection and dimethylnitrosamine treatment. *J Hepatol* 1997; **26**: 1313-1323

81 **Chaimuangraj S**, Thamavit W, Tsuda H, Moore MA. Experimental investigation of opisthorchiasis-associated cholangiocarcinoma induction in the Syrian hamster - pointers for control of the human disease. *Asian Pac J Cancer Prev* 2003; **4**: 87-93

82 **Loilome W**, Yongvanit P, Wongkham C, Tepsiri N, Sripa B, Sithithaworn P, Hanai S, Miwa M. Altered gene expression in Opisthorchis viverrini-associated cholangiocarcinoma in hamster model. *Mol Carcinog* 2006; **45**: 279-287

83 **Changbumrung S**, Tungtrongchitr R, Migasena P, Chamroenngan S. Serum unconjugated primary and secondary bile acids in patients with cholangiocarcinoma and hepatocellular carcinoma. *J Med Assoc Thai* 1990; **73**: 81-90

84 **Kinami Y**, Ashida Y, Gotoda H, Seto K, Kojima Y, Takashima S. Promoting effects of bile acid load on the occurrence of cholangiocarcinoma induced by diisopropanolnitrosamine in hamsters. *Oncology* 1993; **50**: 46-51

85 **Kinami Y**, Miyakoshi M, Fujikawa K. Bile acid load on the DNA distribution pattern of bile ductules and cholangiocarcinoma induced by diisopropanolnitrosamine in hamsters. *Oncology* 1998; **55**: 77-86

86 **Yoon JH**, Higuchi H, Werneburg NW, Kaufmann SH, Gores GJ. Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. *Gastroenterology* 2002; **122**: 985-993

87 **Yoon JH**, Werneburg NW, Higuchi H, Canbay AE, Kaufmann SH, Akgul C, Edwards SW, Gores GJ. Bile acids inhibit Mcl-1 protein turnover via an epidermal growth factor receptor/Raf-1-dependent mechanism. *Cancer Res* 2002; **62**: 6500-6505

88 **Kim KM**, Yoon JH, Gwak GY, Kim W, Lee SH, Jang JJ, Lee HS. Bile acid-mediated induction of cyclooxygenase-2 and Mcl-1 in hepatic stellate cells. *Biochem Biophys Res Commun* 2006; **342**: 1108-1113

89 **Mariette C**, Perrais M, Leteurtre E, Jonckheere N, Hémon B, Pigny P, Batra S, Aubert JP, Triboulet JP, Van Seuningen I. Transcriptional regulation of human mucin MUC4 by bile acids in oesophageal cancer cells is promoter-dependent and involves activation of the phosphatidylinositol 3-kinase signalling pathway. *Biochem J* 2004; **377**: 701-708

90 **Piessen G**, Jonckheere N, Vincent A, Hémon B, Ducourouble MP, Copin MC, Mariette C, Van Seuningen I. Regulation of the human mucin MUC4 by taurodeoxycholic and taurochenodeoxycholic bile acids in oesophageal cancer cells is mediated by hepatocyte nuclear factor 1alpha. *Biochem J* 2007; **402**: 81-91

91 **Chariyalertsak S**, Sirikulchayanonta V, Mayer D, Kopp-Schneider A, Förstenberger G, Marks F, Möller-Decker K. Aberrant cyclooxygenase isozyme expression in human intrahepatic cholangiocarcinoma. *Gut* 2001; **48**: 80-86

92 **Hayashi N**, Yamamoto H, Hiraoka N, Dono K, Ito Y, Okami J, Kondo M, Nagano H, Umeshita K, Sakon M, Matsuura N, Nakamori S, Monden M. Differential expression of cyclooxygenase-2 (COX-2) in human bile duct epithelial cells and bile duct neoplasm. *Hepatology* 2001; **34**: 638-650

93 **Wu T**. Cyclooxygenase-2 and prostaglandin signaling in cholangiocarcinoma. *Biochim Biophys Acta* 2005; **1755**: 135-150

94 **Zhi YH**, Liu RS, Song MM, Tian Y, Long J, Tu W, Guo RX. Cyclooxygenase-2 promotes angiogenesis by increasing vascular endothelial growth factor and predicts prognosis in gallbladder carcinoma. *World J Gastroenterol* 2005; **11**: 3724-3728

95 **Vadlamudi R**, Mandal M, Adam L, Steinbach G, Mendelsohn J, Kumar R. Regulation of cyclooxygenase-2 pathway by HER2 receptor. *Oncogene* 1999; **18**: 305-314

96 **Wang SC**, Lien HC, Xia W, Chen IF, Lo HW, Wang Z, Ali-Seyed M, Lee DF, Bartholomeusz G, Ou-Yang F, Giri DK, Hung MC. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. *Cancer Cell* 2004; **6**: 251-261

97 **Benoit V**, Relic B, Leval Xd X, Chariot A, Merville MP, Bours V. Regulation of HER-2 oncogene expression by cyclooxygenase-2 and prostaglandin E2. *Oncogene* 2004; **23**: 1631-1635

98 **Han C**, Wu T. Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. *J Biol Chem* 2005; **280**: 24053-24063

99 **Zhang L**, Jiang L, Sun Q, Peng T, Lou K, Liu N, Leng J. Prostaglandin E2 enhances mitogen-activated protein kinase/Erk pathway in human cholangiocarcinoma cells: involvement of EP1 receptor, calcium and EGF receptors signaling. *Mol Cell Biochem* 2007; **305**: 19-26

100 **Sugawara H**, Yasoshima M, Katayanagi K, Kono N, Watanabe Y, Harada K, Nakanuma Y. Relationship between interleukin-6 and proliferation and differentiation in cholangiocarcinoma. *Histopathology* 1998; **33**: 145-153

101 **Goydos JS**, Brumfield AM, Frezza E, Booth A, Lotze MT, Carty SE. Marked elevation of serum interleukin-6 in patients with cholangiocarcinoma: validation of utility as a clinical marker. *Ann Surg* 1998; **227**: 398-404

102 **Cheon YK**, Cho YD, Moon JH, Jang JY, Kim YS, Kim YS, Lee MS, Lee JS, Shim CS. Diagnostic utility of interleukin-6 (IL-6) for primary bile duct cancer and changes in serum IL-6 levels following photodynamic therapy. *Am J Gastroenterol* 2007; **102**: 2164-2170

103 **Rosen HR**, Winkle PJ, Kendall BJ, Diehl DL. Biliary interleukin-6 and tumor necrosis factor-alpha in patients undergoing endoscopic retrograde cholangiopancreatography. *Dig Dis Sci* 1997; **42**: 1290-1294

104 **Okada K**, Shimizu Y, Nambu S, Higuchi K, Watanabe A. Interleukin-6 functions as an autocrine growth factor in a cholangiocarcinoma cell line. *J Gastroenterol Hepatol* 1994; **9**: 462-467

105 **Yokomuro S**, Tsuji H, Lunz JG 3rd, Sakamoto T, Ezure T, Murase N, Demetris AJ. Growth control of human biliary epithelial cells by interleukin 6, hepatocyte growth factor, transforming growth factor beta1, and activin A: comparison of a cholangiocarcinoma cell line with primary cultures of non-neoplastic biliary epithelial cells. *Hepatology* 2000; **32**: 26-35

106 **Isomoto H**, Kobayashi S, Werneburg NW, Bronk SF, Guicciardi ME, Frank DA, Gores GJ. Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells. *Hepatology* 2005; **42**: 1329-1338

107 **Meng F**, Yamagiwa Y, Ueno Y, Patel T. Over-expression of interleukin-6 enhances cell survival and transformed cell growth in human malignant cholangiocytes. *J Hepatol* 2006; **44**: 1055-1065

108 **Isomoto H**, Mott JL, Kobayashi S, Werneburg NW, Bronk SF, Haan S, Gores GJ. Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. *Gastroenterology* 2007; **132**: 384-396

109 **Han C**, Demetris AJ, Stolz DB, Xu L, Lim K, Wu T. Modulation of Stat3 activation by the cytosolic phospholipase A2alpha and cyclooxygenase-2-controlled prostaglandin E2 signaling pathway. *J Biol Chem* 2006; **281**: 24831-24846

110 **Qiu Y**, Ravi L, Kung HJ. Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells. *Nature* 1998; **393**: 83-85

111 **Grant SL**, Hammacher A, Douglas AM, Goss GA, Mansfield RK, Heath JK, Begley CG. An unexpected biochemical and functional interaction between gp130 and the EGF receptor family in breast cancer cells. *Oncogene* 2002; **21**: 460-474

112 **Wehbe H**, Henson R, Meng F, Mize-Berge J, Patel T. Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. *Cancer Res* 2006; **66**: 10517-10524

113 **Higashi M**, Yonezawa S, Ho JJ, Tanaka S, Irimura T, Kim YS, Sato E. Expression of MUC1 and MUC2 mucin antigens in intrahepatic bile duct tumors: its relationship with a new morphological classification of cholangiocarcinoma. *Hepatology* 1999; **30**: 1347-1355

114 **Matsumura N**, Yamamoto M, Aruga A, Takasaki K, Nakano M. Correlation between expression of MUC1 core protein and outcome after surgery in mass-forming intrahepatic cholangiocarcinoma. *Cancer* 2002; **94**: 1770-1776

115 **Shibahara H**, Tamada S, Higashi M, Goto M, Batra SK, Hollingsworth MA, Imai K, Yonezawa S. MUC4 is a novel prognostic factor of intrahepatic cholangiocarcinoma-mass forming type. *Hepatology* 2004; **39**: 220-229

116 **Sasaki M**, Ikeda H, Nakanuma Y. Expression profiles of MUC mucins and trefoil factor family (TFF) peptides in the intrahepatic biliary system: physiological distribution and pathological significance. *Prog Histochem Cytochem* 2007; **42**: 61-110

117 **Thomas MB**. Biological characteristics of cancers in the gallbladder and biliary tract and targeted therapy. *Crit Rev Oncol Hematol* 2007; **61**: 44-51

118 **Carraway KL**, Ramsauer VP, Haq B, Carothers Carraway CA. Cell signaling through membrane mucins. *Bioessays* 2003; **25**: 66-71

119 **Singh PK**, Hollingsworth MA. Cell surface-associated mucins in signal transduction. *Trends Cell Biol* 2006; **16**: 467-476

120 **Singh AP**, Chaturvedi P, Batra SK. Emerging roles of MUC4 in cancer: a novel target for diagnosis and therapy. *Cancer Res* 2007; **67**: 433-436

121 **Li Y**, Yu WH, Ren J, Chen W, Huang L, Kharbanda S, Loda M, Kufe D. Heregulin targets gamma-catenin to the nucleolus by a mechanism dependent on the DF3/MUC1 oncoprotein. *Mol Cancer Res* 2003; **1**: 765-775

122 **Pochampalli MR**, Bitler BG, Schroeder JA. Transforming growth factor alpha dependent cancer progression is modulated by Muc1. *Cancer Res* 2007; **67**: 6591-6598

123 **Pochampalli MR**, el Bejjani RM, Schroeder JA. MUC1 is a novel regulator of ErbB1 receptor trafficking. *Oncogene* 2007; **26**: 1693-1701

124 **Ren J**, Bharti A, Raina D, Chen W, Ahmad R, Kufe D. MUC1 oncoprotein is targeted to mitochondria by heregulin-induced activation of c-Src and the molecular chaperone HSP90. *Oncogene* 2006; **25**: 20-31

125 **Jepson S**, Komatsu M, Haq B, Arango ME, Huang D, Carraway CA, Carraway KL. Muc4/sialomucin complex, the intramembrane ErbB2 ligand, induces specific phosphorylation of ErbB2 and enhances expression of p27(kip), but does not activate mitogen-activated kinase or protein kinaseB/Akt pathways. *Oncogene* 2002; **21**: 7524-7532

126 **Funes M**, Miller JK, Lai C, Carraway KL 3rd, Sweeney C. The mucin Muc4 potentiates neuregulin signaling by increasing the cell-surface populations of ErbB2 and ErbB3. *J Biol Chem* 2006; **281**: 19310-19319

127 **Terada T**, Nakanuma Y, Sirica AE. Immunohistochemical demonstration of MET overexpression in human intrahepatic cholangiocarcinoma and in hepatolithiasis. *Hum Pathol* 1998; **29**: 175-180

128 **Lai GH**, Radaeva S, Nakamura T, Sirica AE. Unique epithelial cell production of hepatocyte growth factor/scatter factor by putative precancerous intestinal metaplasias and associated "intestinal-type" biliary cancer chemically induced in rat liver. *Hepatology* 2000; **31**: 1257-1265

129 **Scheving LA**, Stevenson MC, Taylormoore JM, Traxler P, Russell WE. Integral role of the EGF receptor in HGF-mediated hepatocyte proliferation. *Biochem Biophys Res Commun* 2002; **290**: 197-203

130 **Khoury H**, Naujokas MA, Zuo D, Sangwan V, Frigault MM, Petkiewicz S, Dankort DL, Muller WJ, Park M. HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion. *Mol Biol Cell* 2005; **16**: 550-561

131 **Fischer OM**, Giordano S, Comoglio PM, Ullrich A. Reactive oxygen species mediate Met receptor transactivation by G protein-coupled receptors and the epidermal growth factor receptor in human carcinoma cells. *J Biol Chem* 2004; **279**: 28970-28978

132 **Elmore LW**, Sirica AE. "Intestinal-type" of adenocarcinoma preferentially induced in right/caudate liver lobes of rats treated with furan. *Cancer Res* 1993; **53**: 254-259

133 **Zhang Z**, Lai GH, Sirica AE. Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. *Hepatology* 2004; **39**: 1028-1037

134 **Matsumoto K**, Nakamura T. Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. *Int J Cancer* 2006; **119**: 477-483

135 **Benckert C**, Jonas S, Cramer T, von Marschall Z, Schäfer G, Peters M, Wagner K, Radke C, Wiedenmann B, Neuhaus P, Höcker M, Rosewicz S. Transforming growth factor beta 1 stimulates vascular endothelial growth factor gene transcription in human cholangiocellular carcinoma cells. *Cancer Res* 2003; **63**: 1083-1092

136 **Tang D**, Nagano H, Yamamoto H, Wada H, Nakamura M, Kondo M, Ota H, Yoshioka S, Kato H, Damdinsuren B, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Dono K, Wakasa K, Monden M. Angiogenesis in cholangiocellular carcinoma: expression of vascular endothelial growth factor, angiopoietin-1/2, thrombospondin-1 and clinicopathological significance. *Oncol Rep* 2006; **15**: 525-532

137 **Yoshikawa D**, Ojima H, Iwasaki M, Hiraoka N, Kosuge T, Kasai S, Hirohashi S, Shibata T. Clinicopathological and prognostic significance of EGFR, VEGF, and HER2 expression in cholangiocarcinoma. *Br J Cancer* 2008; **98**:

418-425
138 **Park BK**, Paik YH, Park JY, Park KH, Bang S, Park SW, Chung JB, Park YN, Song SY. The clinicopathologic significance of the expression of vascular endothelial growth factor-C in intrahepatic cholangiocarcinoma. *Am J Clin Oncol* 2006; **29**: 138-142
139 **Aishima S**, Nishihara Y, Iguchi T, Taguchi K, Taketomi A, Maehara Y, Tsuneyoshi M. Lymphatic spread is related to VEGF-C expression and D2-40-positive myofibroblasts in intrahepatic cholangiocarcinoma. *Mod Pathol* 2008; **21**: 256-264
140 **Ogasawara S**, Yano H, Higaki K, Takayama A, Akiba J, Shiota K, Kojiro M. Expression of angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in human biliary tract carcinoma cell lines. *Hepatol Res* 2001; **20**: 97-113
141 **Kawahara N**, Ono M, Taguchi K, Okamoto M, Shimada M, Takenaka K, Hayashi K, Mosher DF, Sugimachi K, Tsuneyoshi M, Kuwano M. Enhanced expression of thrombospondin-1 and hypovascularity in human cholangiocarcinoma. *Hepatology* 1998; **28**: 1512-1517
142 **Aishima S**, Taguchi K, Sugimachi K, Asayama Y, Nishi H, Shimada M, Sugimachi K, Tsuneyoshi M. The role of thymidine phosphorylase and thrombospondin-1 in angiogenesis and progression of intrahepatic cholangiocarcinoma. *Int J Surg Pathol* 2002; **10**: 47-56
143 **Yen L**, Benlimame N, Nie ZR, Xiao D, Wang T, Al Moustafa AE, Esumi H, Milanini J, Hynes NE, Pages G, Alaoui-Jamali MA. Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. *Mol Biol Cell* 2002; **13**: 4029-4044
144 **Alaoui-Jamali MA**, Song DJ, Benlimame N, Yen L, Deng X, Hernandez-Perez M, Wang T. Regulation of multiple tumor microenvironment markers by overexpression of single or paired combinations of ErbB receptors. *Cancer Res* 2003; **63**: 3764-3774
145 **Klos KS**, Wyszomierski SL, Sun M, Tan M, Zhou X, Li P, Yang W, Yin G, Hittelman WN, Yu D. ErbB2 increases vascular endothelial growth factor protein synthesis via activation of mammalian target of rapamycin/p70S6K leading to increased angiogenesis and spontaneous metastasis of human breast cancer cells. *Cancer Res* 2006; **66**: 2028-2037
146 **Laughner E**, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol* 2001; **21**: 3995-4004
147 **Xu Q**, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL, Cheng JQ, Jove R, Yu H. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. *Oncogene* 2005; **24**: 5552-5560
148 **Pore N**, Jiang Z, Gupta A, Cerniglia G, Kao GD, Maity A. EGFR tyrosine kinase inhibitors decrease VEGF expression by both hypoxia-inducible factor (HIF)-1-independent and HIF-1-dependent mechanisms. *Cancer Res* 2006; **66**: 3197-3204
149 **Ding YB**, Shi RH, Tong JD, Li XY, Zhang GX, Xiao WM, Yang JG, Bao Y, Wu J, Yan ZG, Wang XH. PGE2 up-regulates vascular endothelial growth factor expression in MKN28 gastric cancer cells via epidermal growth factor receptor signaling system. *Exp Oncol* 2005; **27**: 108-113
150 **Vogel CL**, Franco SX. Clinical experience with trastuzumab (herceptin). *Breast J* 2003; **9**: 452-462
151 **Esteva FJ**. Monoclonal antibodies, small molecules, and vaccines in the treatment of breast cancer. *Oncologist* 2004; **9** Suppl 3: 4-9
152 **Zhang H**, Berezov A, Wang Q, Zhang G, Drebin J, Murali R, Greene MI. ErbB receptors: from oncogenes to targeted cancer therapies. *J Clin Invest* 2007; **117**: 2051-2058
153 **Rabindran SK**. Antitumor activity of HER-2 inhibitors. *Cancer Lett* 2005; **227**: 9-23
154 **Engel RH**, Kaklamani VG. HER2-positive breast cancer: current and future treatment strategies. *Drugs* 2007; **67**: 1329-1341
155 **Nautiyal J**, Rishi AK, Majumdar AP. Emerging therapies in gastrointestinal cancers. *World J Gastroenterol* 2006; **12**: 7440-7450
156 **Harari PM**. Epidermal growth factor receptor inhibition strategies in oncology. *Endocr Relat Cancer* 2004; **11**: 689-708
157 **Rocha-Lima CM**, Soares HP, Raez LE, Singal R. EGFR targeting of solid tumors. *Cancer Control* 2007; **14**: 295-304
158 **Giusti RM**, Shastri KA, Cohen MH, Keegan P, Pazdur R. FDA drug approval summary: panitumumab (Vectibix). *Oncologist* 2007; **12**: 577-583
159 **Socinski MA**. Antibodies to the epidermal growth factor receptor in non small cell lung cancer: current status of matuzumab and panitumumab. *Clin Cancer Res* 2007; **13**: s4597-s4601
160 **Seiden MV**, Burris HA, Matulonis U, Hall JB, Armstrong DK, Speyer J, Weber JD, Muggia F. A phase II trial of EMD72000 (matuzumab), a humanized anti-EGFR monoclonal antibody, in patients with platinum-resistant ovarian and primary peritoneal malignancies. *Gynecol Oncol* 2007; **104**: 727-731
161 **Fury MG**, Lipton A, Smith KM, Winston CB, Pfister DG. A phase-I trial of the epidermal growth factor receptor directed bispecific antibody MDX-447 without and with recombinant human granulocyte-colony stimulating factor in patients with advanced solid tumors. *Cancer Immunol Immunother* 2008; **57**: 155-163
162 **Arora A**, Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther* 2005; **315**: 971-979
163 **Pérol M**, Arpin D. [Tyrosine kinase inhibitors in the management of non-small cell lung cancer] *Rev Mal Respir* 2007; **24**: 6S188-6S197
164 **Sharma SV**, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer* 2007; **7**: 169-181
165 **Bonomi P**. Erlotinib: a new therapeutic approach for non-small cell lung cancer. *Expert Opin Investig Drugs* 2003; **12**: 1395-1401
166 **Nyati MK**, Morgan MA, Feng FY, Lawrence TS. Integration of EGFR inhibitors with radiochemotherapy. *Nat Rev Cancer* 2006; **6**: 876-885
167 **Nelson MH**, Dolder CR. Lapatinib: a novel dual tyrosine kinase inhibitor with activity in solid tumors. *Ann Pharmacother* 2006; **40**: 261-269
168 **Reid A**, Vidal L, Shaw H, de Bono J. Dual inhibition of ErbB1 (EGFR/HER1) and ErbB2 (HER2/neu). *Eur J Cancer* 2007; **43**: 481-489
169 **Montemurro F**, Valabrega G, Aglietta M. Lapatinib: a dual inhibitor of EGFR and HER2 tyrosine kinase activity. *Expert Opin Biol Ther* 2007; **7**: 257-268
170 **Hoekstra R**, Dumez H, Eskens FA, van der Gaast A, Planting AS, de Heus G, Sizer KC, Ravera C, Vaidyanathan S, Bucana C, Fidler IJ, van Oosterom AT, Verweij J. Phase I and pharmacologic study of PKI166, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. *Clin Cancer Res* 2005; **11**: 6908-6915
171 **Garland LL**, Pegram M, Song S, Mendelson D, Parker KE, Martell RE, Gordon MS. Phase I study of BMS-599626, an oral pan-HER tyrosine kinase inhibitor, in patients with advanced solid tumors. *J Clin Oncol* (meeting abstract) 2005; 23 Suppl **16**: 3152
172 **Albanell J**, Gascón P. Small molecules with EGFR-TK inhibitor activity. *Curr Drug Targets* 2005; **6**: 259-274
173 **Britten CD**. Targeting ErbB receptor signaling: a pan-ErbB approach to cancer. *Mol Cancer Ther* 2004; **3**: 1335-1342
174 **Mom CH**, Eskens FA, Gietema JA, Nooter K, De Jonge MJ, Amelsberg A, Huisman H, Stopfer P, De Vries EG, Verweij J. Phase 1 study with BIBW 2992, an irreversible dual tyrosine

kinase inhibitor of epidermal growth factor receptor 1 (EGFR) and 2 (HER2) in a 2 week on 2 week off schedule. *J Clin Oncol* (meeting abstract) 2006; **24** Suppl: 3025

175 **Rabindran SK**, Discafani CM, Rosfjord EC, Baxter M, Floyd MB, Golas J, Hallett WA, Johnson BD, Nilakantan R, Overbeek E, Reich MF, Shen R, Shi X, Tsou HR, Wang YF, Wissner A. Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase. *Cancer Res* 2004; **64**: 3958-3965

176 **Ariyama H**, Qin B, Baba E, Tanaka R, Mitsugi K, Harada M, Nakano S. Gefitinib, a selective EGFR tyrosine kinase inhibitor, induces apoptosis through activation of Bax in human gallbladder adenocarcinoma cells. *J Cell Biochem* 2006; **97**: 724-734

177 **Miyata H**, Sasaki T, Kuwahara K, Serikawa M, Chayama K. The effects of ZD1839 (Iressa), a highly selective EGFR tyrosine kinase inhibitor, as a radiosensitiser in bile duct carcinoma cell lines. *Int J Oncol* 2006; **28**: 915-921

178 **Jimeno A**, Rubio-Viqueira B, Amador ML, Oppenheimer D, Bouraoud N, Kulesza P, Sebastiani V, Maitra A, Hidalgo M. Epidermal growth factor receptor dynamics influences response to epidermal growth factor receptor targeted agents. *Cancer Res* 2005; **65**: 3003-3010

179 **Hidalgo M**, Amador ML, Jimeno A, Mezzadra H, Patel P, Chan A, Nielsen ME, Maitra A, Altiok S. Assessment of gefitinib- and CI-1040-mediated changes in epidermal growth factor receptor signaling in HuCCT-1 human cholangiocarcinoma by serial fine needle aspiration. *Mol Cancer Ther* 2006; **5**: 1895-1903

180 **Zhang Z**, Sirica AE. Simultaneous inhibition of ErbB1 and ErbB2 signaling significantly enhances the growth suppression of rat and human cholangiocarcinoma cell lines. *FASEB J* 2007; **21**: A71-A72

181 **Wiedmann M**, Fiesthammel J, Blöthner T, Tannapfel A, Kamenz T, Kluge A, Mossner J, Caca K. Novel targeted approaches to treating biliary tract cancer: the dual epidermal growth factor receptor and ErbB-2 tyrosine kinase inhibitor NVP-AEE788 is more efficient than the epidermal growth factor receptor inhibitors gefitinib and erlotinib. *Anticancer Drugs* 2006; **17**: 783-795

182 **Kiguchi K**, Ruffino L, Kawamoto T, Ajiki T, Digiovanni J. Chemopreventive and therapeutic efficacy of orally active tyrosine kinase inhibitors in a transgenic mouse model of gallbladder carcinoma. *Clin Cancer Res* 2005; **11**: 5572-5580

183 **Alberts SR**, Gores GJ, Kim GP, Roberts LR, Kendrick ML, Rosen CB, Chari ST, Martenson JA. Treatment options for hepatobiliary and pancreatic cancer. *Mayo Clin Proc* 2007; **82**: 628-637

184 **Xia W**, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, Owens G, Alligood KJ, Spector NL. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 2002; **21**: 6255-6263

185 **Philip PA**, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G, Donehower RC, Fitch T, Picus J, Erlichman C. Phase II study of erlotinib in patients with advanced biliary cancer. *J Clin Oncol* 2006; **24**: 3069-3074

186 **Huang TW**, Wang CH, Hsieh CB. Effects of the anti-epidermal growth factor receptor antibody cetuximab on cholangiocarcinoma of the liver. *Onkologie* 2007; **30**: 129-131

187 **Sprinzl MF**, Schimanski CC, Moehler M, Schadmand-Fischer S, Galle PR, Kanzler S. Gemcitabine in combination with EGF-Receptor antibody (Cetuximab) as a treatment of cholangiocarcinoma: a case report. *BMC Cancer* 2006; **6**: 190

188 **Paule B**, Herelle MO, Rage E, Ducreux M, Adam R, Guettier C, Bralet MP. Cetuximab plus gemcitabine-oxaliplatin (GEMOX) in patients with refractory advanced intrahepatic cholangiocarcinomas. *Oncology* 2007; **72**: 105-110

189 **Ramanathan RK**, Belani CP, Singh DA, Tanaka M, Lenz HJ, Yen Y, Kindler HL, Iqbal S, Longmate J, Gandara DR. Phase II study of lapatinib, a dual inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase 1 and 2 (Her2/Neu) in patients (pts) with advanced biliary tree cancer (BTC) or hepatocellular cancer (HCC). A California Consortium (CCC-P) Trial. *J Clin Oncol* (meeting abstract) 2006; **24** Suppl: 4010

190 **Siegel-Lakhai WS**, Beijnen JH, Vervenne WL, Boot H, Keessen M, Versola M, Koch KM, Smith DA, Pandite L, Richel DJ, Schellens JH. Phase I pharmacokinetic study of the safety and tolerability of lapatinib (GW572016) in combination with oxaliplatin/fluorouracil/leucovorin (FOLFOX4) in patients with solid tumors. *Clin Cancer Res* 2007; **13**: 4495-4502

191 **Miura F**, Okazumi S, Takayama W, Asano T, Makino H, Shuto K, Ochiai T. Hemodynamics of intrahepatic cholangiocarcinoma: evaluation with single-level dynamic CT during hepatic arteriography. *Abdom Imaging* 2004; **29**: 467-471

192 **Lu Y**, Liang K, Li X, Fan Z. Responses of cancer cells with wild-type or tyrosine kinase domain-mutated epidermal growth factor receptor (EGFR) to EGFR-targeted therapy are linked to downregulation of hypoxia-inducible factor-1alpha. *Mol Cancer* 2007; **6**: 63

193 **Franovic A**, Gunaratnam L, Smith K, Robert I, Patten D, Lee S. Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. *Proc Natl Acad Sci USA* 2007; **104**: 13092-13097

194 **Giannelli G**, Azzariti A, Fransvea E, Porcelli L, Antonaci S, Paradiso A. Laminin-5 offsets the efficacy of gefitinib ('Iressa') in hepatocellular carcinoma cells. *Br J Cancer* 2004; **91**: 1964-1969

195 **Kuwai T**, Nakamura T, Kim SJ, Sasaki T, Kitadai Y, Langley RR, Fan D, Hamilton SR, Fidler IJ. Intratumoral heterogeneity for expression of tyrosine kinase growth factor receptors in human colon cancer surgical specimens and orthotopic tumors. *Am J Pathol* 2008; **172**: 358-366

196 **Ono M**, Kuwano M. Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. *Clin Cancer Res* 2006; **12**: 7242-7251

197 **Pao W**. Defining clinically relevant molecular subsets of lung cancer. *Cancer Chemother Pharmacol* 2006; **58** Suppl 1: s11-s15

198 **Wang SE**, Narasanna A, Perez-Torres M, Xiang B, Wu FY, Yang S, Carpenter G, Gazdar AF, Muthuswamy SK, Arteaga CL. HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. *Cancer Cell* 2006; **10**: 25-38

199 **Mitsudomi T**, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. *Cancer Sci* 2007; **98**: 1817-1824

200 **Massarelli E**, Varella-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD, Bekele BN, Herbst RS, Wistuba II. KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. *Clin Cancer Res* 2007; **13**: 2890-2896

201 **Yu Z**, Boggon TJ, Kobayashi S, Jin C, Ma PC, Dowlati A, Kern JA, Tenen DG, Halmos B. Resistance to an irreversible epidermal growth factor receptor (EGFR) inhibitor in EGFR-mutant lung cancer reveals novel treatment strategies. *Cancer Res* 2007; **67**: 10417-10427

202 **Gilmer TM**, Cable L, Alligood K, Rusnak D, Spehar G, Gallagher KT, Woldu E, Carter HL, Truesdale AT, Shewchuk L, Wood ER. Impact of common epidermal growth factor receptor and HER2 variants on receptor activity and inhibition by lapatinib. *Cancer Res* 2008; **68**: 571-579

203 **Lu Y**, Li X, Liang K, Luwor R, Siddik ZH, Mills GB, Mendelsohn J, Fan Z. Epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired

resistance escaping treatment by the anti-EGFR monoclonal antibody cetuximab. *Cancer Res* 2007; **67**: 8240-8247

204 **Jones HE**, Gee JM, Hutcheson IR, Knowlden JM, Barrow D, Nicholson RI. Growth factor receptor interplay and resistance in cancer. *Endocr Relat Cancer* 2006; **13** Suppl 1: S45-S51

205 **Nahta R**, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol* 2006; **3**: 269-280

206 **Rajput A**, Koterba AP, Kreisberg JI, Foster JM, Willson JK, Brattain MG. A novel mechanism of resistance to epidermal growth factor receptor antagonism in vivo. *Cancer Res* 2007; **67**: 665-673

207 **Stommel JM**, Kimmelman AC, Ying H, Nabioullin R, Ponugoti AH, Wiedemeyer R, Stegh AH, Bradner JE, Ligon KL, Brennan C, Chin L, DePinho RA. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science* 2007; **318**: 287-290

208 **Mueller KL**, Hunter LA, Ethier SP, Boerner JL. Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. *Cancer Res* 2008; **68**: 3314-3322

209 **Pino MS**, Shrader M, Baker CH, Cognetti F, Xiong HQ, Abbruzzese JL, McConkey DJ. Transforming growth factor alpha expression drives constitutive epidermal growth factor receptor pathway activation and sensitivity to gefitinib (Iressa) in human pancreatic cancer cell lines. *Cancer Res* 2006; **66**: 3802-3812

210 **Spector NL**, Xia W, Burris H 3rd, Hurwitz H, Dees EC, Dowlati A, O'Neil B, Overmoyer B, Marcom PK, Blackwell KL, Smith DA, Koch KM, Stead A, Mangum S, Ellis MJ, Liu L, Man AK, Bremer TM, Harris J, Bacus S. Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. *J Clin Oncol* 2005; **23**: 2502-2512

211 **Ishikawa N**, Daigo Y, Takano A, Taniwaki M, Kato T, Hayama S, Murakami H, Takeshima Y, Inai K, Nishimura H, Tsuchiya E, Kohno N, Nakamura Y. Increases of amphiregulin and transforming growth factor-alpha in serum as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancers. *Cancer Res* 2005; **65**: 9176-9184

212 **Jimeno A**, Tan AC, Coffa J, Rajeshkumar NV, Kulesza P, Rubio-Viqueira B, Wheelhouse J, Diosdado B, Messersmith WA, Iacobuzio-Donahue C, Maitra A, Varella-Garcia M, Hirsch FR, Meijer GA, Hidalgo M. Coordinated epidermal growth factor receptor pathway gene overexpression predicts epidermal growth factor receptor inhibitor sensitivity in pancreatic cancer. *Cancer Res* 2008; **68**: 2841-2849

213 **Force T**, Krause DS, Van Etten RA. Molecular mechanisms of cardiotoxicity of tyrosine kinase inhibition. *Nat Rev Cancer* 2007; **7**: 332-344

214 **Johnston JB**, Navaratnam S, Pitz MW, Maniate JM, Wiechec E, Baust H, Gingerich J, Skliris GP, Murphy LC, Los M. Targeting the EGFR pathway for cancer therapy. *Curr Med Chem* 2006; **13**: 3483-3492

215 **Chung CH**, Mirakhur B, Chan E, Le QT, Berlin J, Morse M, Murphy BA, Satinover SM, Hosen J, Mauro D, Slebos RJ, Zhou Q, Gold D, Hatley T, Hicklin DJ, Platts-Mills TA. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *N Engl J Med* 2008; **358**: 1109-1117

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TOPIC HIGHLIGHT

Carlos J Pirola, PhD, FAHA, *Series Editor*

Aquaporins: Their role in cholestatic liver disease

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Abstract

This review focuses on current knowledge on hepatocyte aquaporins (AQPs) and their significance in bile formation and cholestasis. Canalicular bile secretion results from a combined interaction of several solute transporters and AQP water channels that facilitate water flow in response to the osmotic gradients created. During choleresis, hepatocytes rapidly increase their canalicular membrane water permeability by modulating the abundance of AQP8. The question was raised as to whether the opposite process, i.e. a decreased canalicular AQP8 expression would contribute to the development of cholestasis. Studies in several experimental models of cholestasis, such as extrahepatic obstructive cholestasis, estrogen-induced cholestasis, and sepsis-induced cholestasis demonstrated that the protein expression of hepatocyte AQP8 was impaired. In addition, biophysical studies in canalicular plasma membranes revealed decreased water permeability associated with AQP8 protein downregulation. The combined alteration in hepatocyte solute transporters and AQP8 would hamper the efficient coupling of osmotic gradients and canalicular water flow. Thus cholestasis may result from a mutual occurrence of impaired solute transport and decreased water permeability.

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Key words: Aquaporin; Cholestasis; Estrogen; Hepatocyte; Obstructive cholestasis; Sepsis; Water transport

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INTRODUCTION

Bile secretion is the main function of the exocrine liver, and the maintenance of normal bile formation and delivery into the intestinal lumen is essential for physiological processes such as digestion and absorption of dietary lipids and elimination of endo- and xenobiotics. Cholestasis is a pathologic condition defined as an impairment of normal bile formation, bile flow obstruction or both[1]. There have been major advances in the understanding of the molecular mechanisms underlying bile secretion and cholestasis, and much of this work has been focused on the study of solute membrane transporters[2,3]. However, considering that bile is composed of more than 95% water, less attention has been paid to the molecular basis and regulatory mechanisms of water transport in hepatocytes during bile formation. The cloning and functional characterization of a family of proteins that works as membrane water channels, named aquaporins (AQPs)[4], challenged the former concepts of water transport and contributed to the better understanding of bile physiology. The aim of this work is to give a concise overview of the current knowledge and recent advances in the role of AQPs during bile formation as well as the significance of AQPs in the development of bile secretory failure.

AQUAPORINS OVERVIEW-GENERAL STRUCTURE AND FUNCTION

AQPs are small integral proteins which belong to a family of homologous tetrameric proteins widely

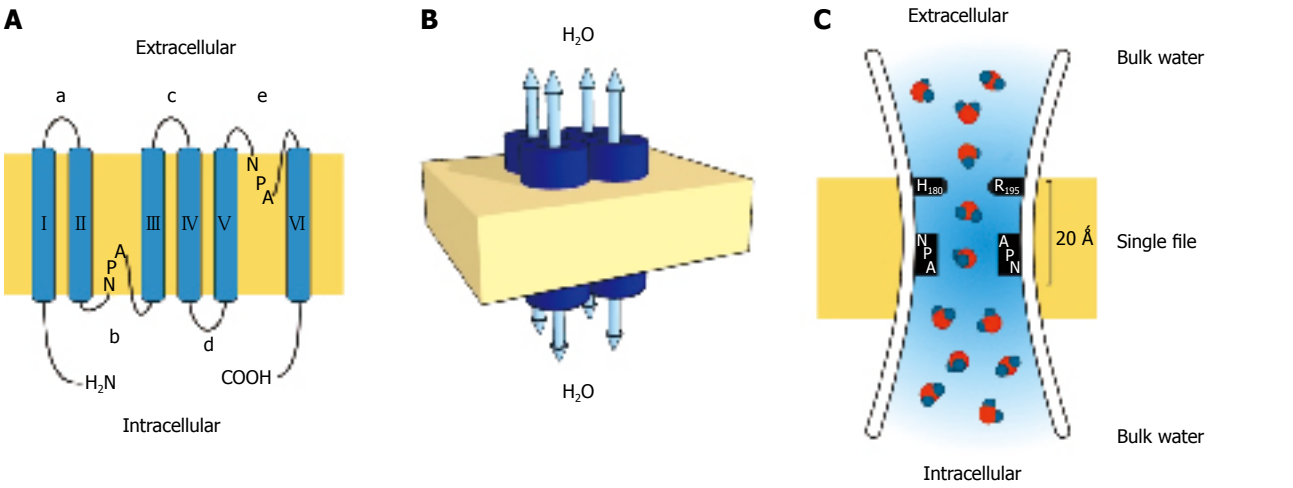

Figure 1 Topology, organization and functioning of the aquaporin water channel molecule. A: Each AQP monomer consist of six transmembrane domains (I -VI) connected by five loops (a-e) with two NPA boxes shaping the water pore, and the amino and carboxy termini oriented toward the cytoplasm; B: Aquaporins are arranged in tetramers. The water pore does not reside in the center of the molecule, but is formed by connecting loops b and e in each subunit that functions as a unique water pore allowing bidirectional water movement; C: The hourglass model for aquaporin structure. The channel consist of an extracellular and intracellular vestibule containing water in bulk solution joined in the center by a central constriction 20 Å in length where water molecules pass in single file. The a/R constriction delimited by arginine in the position 195 (R195) and histidine in the position 180 (H180) provides fixed positive charges which prevent proton passage. The second constriction is bounded by two asparagine residues from the highly conserved NPA motif. The single water molecule passes through the constriction with no resistance as it forms transient hydrogen bonds with the nearby asparagines.

distributed in mammals, plants, and lower organisms[4]. The first AQP was purified from human erythrocytes and was initially named CHIP28. Following expression studies in Xenopus oocytes the protein was functionally identified as a water channel and renamed AQP1[5]. The discovery of AQPs triggered an immense number of studies which advanced the current understanding of water permeation across biological membranes. At least 13 AQP isoforms have been identified from mammalian tissues (AQP0-AQP12)[4,6,7]. While AQPs function primarily as water-transporting channels, some of these proteins also exhibit permeability to certain small solutes such as glycerol, ammonia[8], hydrogen peroxide[9] and some gases such as carbon dioxide and nitric oxide[10].

AQP1 was the first member of the AQP water channel family to be identified[5]. The functional unit of AQP1 is a homotetramer, but in contrast to ion channels where the permeation site is placed in the center of the tetramer, each AQP subunit contains a distinct aqueous pore. The monomers, composed of approximately 270 amino acids, possess six transmembrane alpha-helix regions connected by five loops, with the amino- and carboxy-termini oriented towards the cytosol. Among the five connecting loops, two enclose the strictly conserved three-amino acid motif (asparagine-proline-alanine, NPA), which overlap in the center of the pore and are responsible for the water channel selectivity (Figure 1A and B).

Cryoelectron microscopy in combination with atomic force microscopy and X-ray analysis confirmed the so-called "hourglass model". It consists of a wide extracellular and intracellular vestibule, joined in the center by a narrow region of approximately 20 Å in length that shapes the filter responsible for water selectivity[11]. In the vestibules water exists in bulk solution, while in the center of the channel water transits in single file. Actually it has been identified as a two-stage filter in the central region of the pore: an outer barrier termed the aromatic/arginine constriction (ar/R), and the central constriction or NPA region. Both stages of the filter add to the remarkable efficiency and selectivity of the AQPs[12] (Figure 1C).

The filter selectivity appears to be based on size exclusion, as the central constriction of the filter is slightly wider than the water molecule (~2.8 Å) and represents a steric limit for bigger molecules through the channel. The ar/R constriction is composed of a highly conserved arginine residue in the 195 position and a nearby histidine residue in the 180 position. This filter provides supplementary criteria for solute selection, as the conserved residues have a strong positive charge that repels protonated water. In the NPA region, water-water interactions are distorted so water molecules remain isolated from their solvation shell in the bulk, a process essential for the filter selectivity[13]. As water molecules approximate to the constriction, the oxygen atom rotates towards the asparagine residues from the NPA motifs (asparagines 76 and 192) and creates new hydrogen bonds between the oxygen and the asparagine residues. The dipole reorientation breaks the hydrogen bonds among water molecules and avoids the passage of protonated water[13,14].

The hourglass model also explains the reversible inhibition caused by mercurial compounds. Mercury reacts with sulfhydryl groups from cysteine residues. Among the 4 cysteine residues present in AQP1, only that located in the 189 position reacts with mercurial compounds producing water transport inhibition[15]. This residue is located in the E loop, next to the NPA motif inside the pore[13].

HEPATOCYTE AQPS: EXPRESSION AND SUBCELLULAR LOCALIZATION

There has been much interest in the localization and

Table 1 Cellular and subcellular localization and permeability characteristics of hepatic AQPs

Cell type	Aquaporin	Subcellular localization	Permeability	Ref.
Hepatocytes	AQP0	ICV	Water	[16]
	AQP8	ICV-CPM-Mitochondria-SER	Water/NH_3/H_2O_2	[9,16-20,28,29,32]
	AQP9	BLM	Water/glycerol/urea/certain small uncharged molecules	[16,21,22,30,31]
	AQP11	ND	ND	[6]
Cholangiocytes	AQP1	APM-BLM-ICV	Water	[23,25,36]
	AQP4	BLM	Water	[26]
Gallbladder epithelia	AQP1	APM-BLM	Water	[27]
	AQP8	APM	Water/NH_3/H_2O_2	
Peribiliary vascular endothelia	AQP1	APM-BLM	Water	[24]

ICV: Intracellular vesicles; CPM: Canalicular plasma membrane; BLM: Basolateral plasma membrane; APM: Apical membrane; SER: smooth endoplasmic reticulum; NH_3: Ammonia; H_2O_2: Hydrogen peroxide; ND: Not determined.

physiological role of AQPs in the liver. We and others have recently shown that rat hepatocytes express mRNA and protein for AQP0[16], AQP8[16-20], AQP9[16,21,22] and AQP11[6]. In addition, other cells of the hepatobiliary tract such as cholangiocytes, gallbladder epithelia and peribiliary vascular endothelia also express AQPs[23-27]. The cellular and subcellular distribution and permeability characteristics of AQPs in the hepatobiliary tract are summarized in Table 1. We will focus essentially on the hepatocyte AQPs that play a role in bile formation and secretory failure, i.e. AQP8 and AQP9.

Typically, AQP8 has a varied subcellular localization, which probably correlates with broad physiological roles in the hepatocyte. Accordingly, it was estimated that under basal (unstimulated) conditions most of the hepatocyte AQP8 (75%) reside in intracellular structures, while the remainder reside in the plasma membrane[19]. Detailed biochemical, confocal immunofluorescence and immunoelectron microscopy analyses revealed that the intracellular AQP8 is mostly located in transport vesicles[17,19], smooth endoplasmic reticulum[28] and in the inner membrane of some mitochondria[29]. The protein expression in the plasma membrane is specifically located in the canalicular domain[16,17] and it is regulated by cAMP. Based on immunoblot analysis it was found that hepatocyte AQP8 can be present in two forms of different molecular weight: a N-glycosylated protein of about 34 kDa, and a non-glycosylated protein of 28 kDa[19,29]. According to this, it can be assumed that hepatocytes exhibit two intracellular subpopulations of AQP8 with different physiological functions. A 34 kDa AQP8 in transport vesicles and canalicular plasma membrane domains involved in the formation and regulation of bile (see below); and a 28 kDa AQP8, located in the inner mitochondrial membrane with still uncharacterized functions[29]. Regarding AQP8 distribution in the liver, there is evidence that it has a differential lobular localization. Accordingly, immunohistochemical studies showed staining predominantly in the hepatocytes surrounding the central vein of rat liver[16]. Different results were shown in mouse hepatic lobules, where AQP8 seems to be predominantly distributed in the periportal and midlobular hepatocytes with some immunostaining in the pericentral region[28].

AQP9 is a water channel of approximately 32 kDa that allows the passage of water and a wide variety of neutral solutes such as urea, glycerol, purines and pyrimidines[30,31]. Immunolocalization studies performed in rodent liver revealed that AQP9 is exclusively restricted to the hepatocyte sinusoidal plasma membrane domain[21], with an expression pattern strongest around the perivenous zone[32].

Hepatocytes also express AQP0, formerly named major intrinsic protein[16]. AQP0 is mainly localized to intracellular vesicular compartments, but it is not responsive to cAMP and so far, there is no evidence that its trafficking is regulated[14]. The function of AQP0 in hepatocytes has not yet been determined.

The last member to be identified was AQP11[6]. However, the study showed protein levels in total liver membranes, thus the AQP11 cellular and subcellular localization remains to be elucidated.

REGULATION OF HEPATOCYTE AQP TRAFFICKING

Certain epithelia adjust their transport capacity in the short term (i.e. min) by rapid insertion of specific transporters in the secretory membrane. Therefore, the epithelial secretory or absorptive activity can be regulated by handling the number of transport molecules in the plasma membrane. AQP2 is the vasopressin-regulated water channel of the kidney collecting duct[33]. Accordingly, the vasopressin-induced exocytic insertion and endocytic retrieval into and out of the plasma membrane represents a rapid mechanism to regulate its water membrane permeability[34]. Likewise, AQP5 is the main AQP expressed in the acinar cells of the salivary gland. While the water channel is normally sequestered in intracellular vesicles, it redistributes to the apical plasma membrane upon stimulation by muscarinic agonists[35]. Furthermore, it was demonstrated in cholangiocytes that AQP1 is located in intracellular vesicles and undergoes secretin-induced exocytic insertion into the apical membrane[25,36]. Thus, fluid-transporting epithelia can regulate the rate of water transport across cell membranes by rapid relocalization of AQP molecules.

By using isolated rat hepatocytes, it was found that the water channel AQP8 is localized largely in intracellular vesicles and can be redistributed to the plasma membrane in a mechanism stimulated by cAMP. This was the first evidence that hepatocytes were able to regulate their membrane water permeability[19]. Furthermore, confocal immunofluorescence microscopy and functional studies in polarized isolated rat hepatocyte couplets showed that the insertion of AQP8 occurs specifically in the hepatocyte canalicular membrane domain, a mechanism that facilitates the osmotically-driven canalicular water secretion in response to a choleretic stimulus[16]. The microtubule blocker colchicine specifically inhibits the dibutiryl cAMP effect on both AQP8 translocation to plasma membrane and water transport, suggesting that the hormone-dependent AQP8 trafficking relies on microtubules[19].

Glucagon is a choleretic hormone, and its actions in hepatocytes are mostly mediated by cAMP-dependent protein kinase A (PKA). We found that glucagon induces the translocation of intracellular AQP8-containing vesicles to the canalicular domain in hepatocytes, and that this mechanism is dependent on the activation of PKA and the integrity of the microtubular network[37]. Because AQP8 lacks consensus PKA phosphorylation sites[38], we suggested that unidentified protein mediators might be involved in the vesicle trafficking induced by glucagon. The understanding of the precise mechanisms by which glucagon stimulates AQP8 translocation requires further investigation. Because AQP1 also lacks phosphorylation sites for PKA, the mechanisms involved in AQP8 translocation are probably similar to those involved in the secretin-mediated AQP1 trafficking in cholangiocytes[25,36,39].

It has been shown that glucagon is able to induce phosphatidylinositol-3-kinase (PI3K) activation in rat hepatocytes[40]. PI3K mediates several signaling transduction pathways in hepatocytes, including some involved in the regulation of vesicle trafficking and in the process of bile formation[41-44]. In fact, we showed that PI3K is involved in the hepatocyte trafficking of AQP8 stimulated by glucagon[45]. Thus our studies indicate that there is a dual requirement of PKA and PI3K for glucagon-induced AQP8 trafficking. The cross-talk between PKA and PI3K signaling pathways has already been suggested for the regulated translocation of Bsep to the hepatocyte canalicular membrane[46]. This may reflect the need for a cooperative action between PKA and PI3K on a single downstream effector. Consistent with this, it has been reported that in rat hepatocytes, cAMP can activate protein kinase B/Akt, a downstream PI3K effector[44,46]. Interestingly, it has recently been shown that cAMP-PKA mediated phosphorylation of the p85 regulatory subunit of PI3K which was suggested to be an important point of convergence of cAMP-PKA and PI3K signaling pathways[47]. Thus, the glucagon-induced AQP8 trafficking in hepatocytes seems to involve both the cAMP/PKA and PI3K signaling pathways in a cooperative manner.

Therefore, during active choleresis, hepatocytes rapidly increase their canalicular membrane water permeability by vesicle trafficking and thus modulate the abundance of AQP8 in the membrane.

PHYSIOLOGICAL SIGNIFICANCE OF HEPATOCYTE AQPS: BILE FORMATION

Hepatocytes are highly polarized epithelial cells characterized by two definite plasma membrane domains: a basolateral domain in contact with the sinusoidal blood and a bile canalicular domain, defining a sealed apical compartment. The asymmetric distribution of protein transporters to the apical and basolateral membrane domains is the basis of vectorial flux of solutes and water from blood into the bile canaliculi and therefore for the generation of bile[1-3]. Canalicular bile formation is an osmotic secretory process resulting from the inflow of water into the biliary space in response to osmotic gradients created by the active secretion of solutes. The excretion of bile salts via the bile salt transporter Bsep, glutathione via the organic anion transporter Mrp2, and HCO3- *via* the Cl-/HCO3- exchanger AE2 are known to be the major driving forces for water movement from the sinusoidal blood to the bile canaliculus[2]. While the generation of bile flow depends on the molecular and functional canalicular expression of the aforementioned solute transporters, the molecular route for water movement has been largely disregarded albeit the majority of canalicular bile is water.

Theoretically, water can flow through the hepatocyte epithelial barrier either across tight junctions between adjacent hepatocytes (paracellular route) or across hepatocyte plasma membranes (transcellular route). The paracellular route was traditionally proposed as the major pathway for water movement. Nonetheless, the experimental data supporting this view remained limited and largely indirect[48]. Experimental evidence supporting the transcellular pathway came from AQP inhibitory experiments in polarized rat hepatocyte couplets. Under choleretic stimuli, the AQP blockers prevented osmotically-driven water transport into the bile canaliculus[16]. Direct osmotic water permeability assessment by stopped-flow spectrophotometry in canalicular and sinusoidal plasma membrane vesicles revealed the presence of both lipid (non-channel) and AQP-mediated pathways for sinusoidal and canalicular water movement[49]. The study demonstrated that the canalicular plasma membrane domain has lower water permeability than the sinusoidal membrane, and thus it is rate limiting for transcellular water transport in hepatocytes. However, upon cAMP stimulus the intracellular AQP8 inserts to the canalicular domain and so this membrane becomes highly water permeable. Approximate estimations of transcellular hepatocyte water permeability suggest it to be similar to rat kidney proximal tubule, in which water flow seems to be largely transcellular[49]. Therefore, the transcellular pathway via water channels seems to account for most of the water entering the bile canaliculus.

As stated above, hepatocytes express AQPs in intracellular compartments as well as in the basolateral and canalicular plasma membrane domain. In the canalicular membrane, AQP8 was shown to be localized in lipid microdomains ("rafts") enriched with cholesterol and sphingolipids[50,51]. These rafts are thought to promote the assembly of specific proteins into definite regions of the plasma membrane. Because other canalicular transporters such as AE2 and Mrp2 are also localized in membrane microdomains[51], it seems plausible that in the apical membrane, AQP8 is clustered with functionally associated solute transporters, which would generate the driving force necessary for osmotic water transport mediated by AQP8.

The hormone glucagon is known to modulate canalicular bile formation[52]. Although the actual osmotic driving force involved in glucagon-induced choleresis is currently unknown, the solute gradients are thought to be created by active HCO3-excretion mediated by the canalicular transporter AE2[52]. In line with this, it was shown that glucagon (via cAMP) is able to stimulate the microtubule-dependent vesicle insertion of AE2 to hepatocyte plasma membrane[52]. This mechanism, in association with an increased activity of the exchanger, may account for the bicarbonate-rich choleresis induced by glucagon. Furthermore, recent immunofluorescence studies carried out in the hepatoma-derived hybrid cell line WIF-B, showed that AQP8 and AE2 are packaged in the same vesicle population, possibly conforming to a functional bile secretory unit[53]. Thus our findings provide evidence that AQP8 may improve the efficient coupling of canalicular water transport to the HCO3- secreted by AE2 during glucagon-stimulated hepatocyte bile formation.

Further evidence for the role of AQP8 in bile secretion comes from ontogenic expression studies during mice liver development. It was shown that at the time of weaning, there is a rapid increase in AQP8 mRNA and protein expression when the hepatobiliary transport systems complete their maturation, suggesting that AQP8 is necessary for canalicular bile formation[28].

Although the mentioned studies support a role for AQP8 in canalicular water secretion, conclusive evidence should come from studies performed in hepatocytes lacking AQP8 expression. With regard to this, experimental evidence against a role for AQP8 in canalicular bile formation came from data obtained from AQP8 knockout mice[54]. This study revealed that AQP8-null mice challenged with a high-fat diet did not show a significantly different phenotype when compared to their wild-type counterpart. The lack of dietary fat misprocessing could suggest that the excretion of bile salts required for proper lipid digestion was at least preserved. However, direct studies on bile formation in AQP8-null mice are mandatory, in order to determine if these animals develop cholestasis. On the other hand, as hepatocytes express several members of the AQP water channel family, the normal AQP8-null mice phenotype could result from a compensatory overexpression or functional modification of other genes.

Functional studies from our laboratory provided further evidence supporting the notion that canalicular water transport during bile secretion is AQP8-mediated. Indeed, we found that AQP8 gene suppression by RNA interference is able to inhibit osmotically-driven and cAMP-induced canalicular water secretion in the human hepatocyte cell line HepG2[55].

Therefore, while according to our model, AQP8 modulates the canalicular, rate limiting water flow, AQP9 would contribute to the sinusoidal uptake. In agreement with this, functional studies in rat hepatocyte basolateral membrane indicate that sinusoidal water transport is AQP-mediated[49]. As bile secretion requires the transcellular movement of water to the bile canaliculi and AQP9 is the only sinusoidal water channel described so far, it is logical to believe that water moves from the sinusoidal blood, at least in part, through AQP9.

PATHOPHYSIOLOGICAL SIGNIFICANCE OF HEPATOCYTE AQPS: CHOLESTASIS

Bile secretion failure is a consequence of several pathologic conditions with the risk of producing severe liver injury and systemic disease. There have been major advances in the understanding of the molecular pathogenesis of bile secretory failure[1-3]. It is well known that hepatocyte canalicular bile secretion results from the coordinated interaction of several solute membrane transport systems together with, as detailed above, AQP water channels. Hence, it is conceivable that defective AQP membrane expression may lead to alterations in normal bile physiology. The significance of liver AQPs in bile secretory failure are summarized in Table 2.

Extrahepatic cholestasis is a pathologic condition caused by a mechanical obstruction of the biliary tree secondary to a wide variety of acute and chronic conditions including gallstones, pancreatic carcinoma and cholangiosarcoma[56]. If uncorrected, the obstruction may lead to hepatocyte damage, secondary biliary cirrhosis and portal hypertension. The experimental model of bile duct ligation (BDL) in the rat has been extensively used to assess modifications in the molecular expression of hepatocyte membrane transporters in obstructive cholestasis. In a recent study, we examined the effect of BDL on the protein expression and subcellular localization of the hepatocyte water channel AQP8[57]. Biochemical and immunohistochemical studies determined that BDL-induced extrahepatic cholestasis caused downregulation of hepatocyte AQP8 at the protein level (Figure 2). In opposition, the AQP8 mRNA steady-state levels in BDL were increased, possibly as a compensatory mechanism in response to AQP8 protein reduction. The fact that AQP8 protein downregulation was not associated with reduced levels of the mRNA may indicate the involvement of posttranscriptional regulatory mechanisms. Additionally, the AQP8 translocation to the hepatocyte plasma membrane in BDL was found to be impaired. Hence it was concluded that the defective hepatocyte AQP8 functional expression as well as impaired translocation

Table 2 Molecular and functional expression of AQPs in cholestasis

Experimental model	AQP8			AQP9			Ref.
	Protein	mRNA	CPM P_f	Protein	mRNA	BLM P_f	
Obstructive cholestasis							
BDL	↓↓	↑	ND	↓↓↓	↓	↓	[57,58]
Intracellular cholestasis							
EE-induced cholestasis	↓↓↓	↑	↓	↔	ND	ND	[59]
LPS-induced cholestasis	↓↓↓	↑	↓	↔	ND	ND	[64]
CLP-induced cholestasis	↓↓↓	ND	ND	↔	ND	ND	[66]

Arrows depict significant protein and mRNA changes in treated rats compared with controls: ↑, increased; ↓, decreased; ↔, without change. ND: Not determined; P_f: Osmotic membrane water permeability; CPM: Canalicular plasma membrane; BLM: Basolateral plasma membrane; BDL: Bile duct ligation; EE: 17α-ethinylestradiol; LPS: Lipopolysaccharide; CLP: Cecal ligation and puncture.

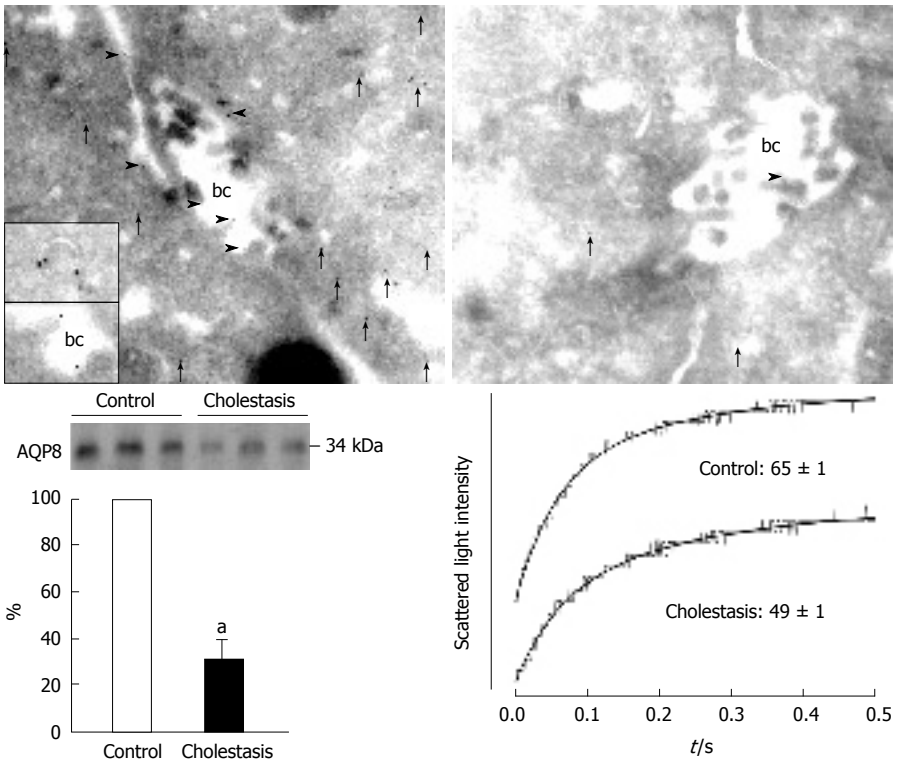

Figure 2 Functional expression of AQP8 in normal and cholestatic liver. Superior panel: Immunogold electron microscopy for AQP8 in liver from control (left) and 7-d BDL rats (right). Arrowheads indicate AQP8 in bile canalicular (bc) membranes. Arrows indicate AQP8 in the pericanalicular cytoplasm. The upper inset shows an AQP8-containing vesicle, and the lower insert shows AQP8 in the tip of a microvillus and in the intermicrovillar plasma membrane region. (Original magnification X 60 000) Modified and reproduced with permission[57]. Inferior panel: On the left, anti-AQP8 immunoblot of the canalicular plasma membranes from normal and LPS-induced cholestatic liver. On the right, water permeability assessment of canalicular membranes from LPS-induced cholestatic liver. Typical tracings of a time course of scattered light intensity (osmotic water transport), along with single exponential fits in canalicular plasma membrane vesicles in response to a 250 mosM hypertonic sucrose gradient. Calculated P_f values (μm/s) are shown under each curve. Data are mean ± SE from 3 independent vesicle preparations. $^aP < 0.05$ *vs* conrtol. Modified and reproduced with permission[64].

may contribute to the secretory dysfunction caused by obstructive cholestasis.

In addition, very recent work suggested a potential involvement of sinusoidal AQP9 in the pathogenesis of obstructive cholestasis. Detailed biochemical studies demonstrated that in BDL there is a decrease in AQP9 protein in basolateral membranes with a simultaneous intracellular increase of the protein[58]. In addition, functional studies performed in sinusoidal hepatocyte membrane found a close correspondence between the AQP9 decreased membrane protein levels and impaired osmotic water permeability. It was concluded that downregulation of AQP9 in the hepatocyte basolateral plasma membrane affects sinusoidal water uptake during bile formation, thus contributing along with AQP8 downregulation to the bile flow dysfunction in obstructive cholestasis.

Estrogens are known to cause intrahepatic cholestasis in susceptible women. The most common clinical features of this disorder are oral contraceptive-induced cholestasis and cholestasis associated with pregnancy or postmenopausal replacement therapy[2]. Experimental cholestasis induced by 17α-ethinylestradiol (EE) has been widely used to investigate *in vivo* alterations in the expression of hepatocyte membrane transporters in this pathological condition. In a recent work, we found that the protein expression of hepatocyte AQP8 is downregulated in estrogen-induced cholestasis possibly by posttranscriptional mechanisms, without significant changes in the sinusoidal AQP9[59]. In fact, complementary studies in primary cultured rat hepatocytes with protease inhibitors indicated that estrogen-induced AQP8 downregulation was mediated by increased lysosomal degradation. Of note, the canalicular AQP8 downregulation was correlated with a 22% reduction in the canalicular membrane water permeability. Previous reports have estimated that under basal conditions the AQP-mediated water

pathway contributes to approximately 30% of the total canalicular water transport[49]. For that reason, the 22% water permeability decrease caused by estrogens may be enough to impair the efficient canalicular coupling between osmotic solutes and water transport during bile formation. On the other hand, it is worth mentioning that the contribution of AQP8 in acute cholestasis appears to be less significant. A recent study showed that experimental acute cholestasis in rats caused by the estrogen metabolite estradiol-17-*d*-glucuronide ($E_2$17G) failed to cause endocytic internalization of canalicular AQP8[60], in contrast to that observed for Bsep and Mrp2[61,62]. Therefore the rapid retrieval of solute transporters, but not that of AQP8, seems to be the main cause of acute cholestasis induced by $E_2$17G.

It is well known that sepsis, a systemic inflammatory response secondary to bacterial infection, is frequently associated with intrahepatic cholestasis[62]. Lipopolysaccharides (LPS) are endotoxins released into the circulation from bacterial sites of infection and are responsible for the macrophage secretion of proinflammatory cytokines, primarily tumor necrosis factor α (TNF α), interleukin 1-β and interleukin-6. These cytokines are the principal mediators of bile secretory failure[63]. In a study performed using a rodent model of endotoxemia, we demonstrated that LPS reduced the functional expression of hepatocyte canalicular AQP8[64]. As shown in Figure 2, a decrease in canalicular AQP8 protein expression of approximately 70% was associated with a 25% decrease in water canalicular permeability measured by stopped-flow spectrophotometry. This result is in good agreement with the above-mentioned studies of estrogen-induced cholestasis. Thus, LPS-induced cholestasis may ultimately be caused by impaired transient osmotic gradients generated by defective canalicular expression of the solute transporters Bsep and Mrp2[65], together with reduced canalicular water permeability secondary to defective AQP8 expression. The impairment in AQP8 expression was found to be posttranscriptional and mediated by the cytokine TNFα. Indeed, the passive immunization *in vivo* with anti-TNFα antibody prevented LPS-induced cholestasis and AQP8 protein downregulation. These results were confirmed in cultured rat hepatocytes treated with recombinant TNFα. Complementary *in vitro* studies using lysosome and proteasome inhibitors showed that AQP8 degradation was mediated *via* both lysosomal and proteasomal pathways. It was concluded that LPS induces posttranscriptional AQP8 downregulation and an associated decrease in canalicular membrane water permeability, a mechanism that is likely to contribute to the molecular pathogenesis of LPS-induced cholestasis.

As clinical sepsis is commonly polymicrobial, the above-mentioned results were further confirmed in an animal model of peritoneal sepsis characterized by a focus of infection with mixed intestinal flora instead of an endotoxic challenge. Thus liver AQP8 expression was studied in rats with sepsis induced by cecal ligation and puncture (CLP)[66]. In agreement with the endotoxic model, immunoblotting and immunohistochemical studies revealed a significant decrease in AQP8 protein level in canalicular membranes, without any significant reduction in AQP9 expression. These results are in agreement with the findings in LPS-treated rats, and further support the notion that the defective expression of hepatocyte AQP8 contributes to the development of bile secretory dysfunction in sepsis.

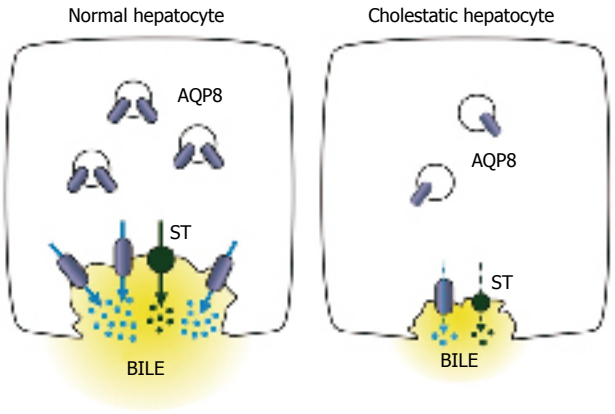

Figure 3 Proposed contribution of hepatocyte AQP8 to the development of cholestasis. On the left a normal hepatocyte is illustrated with AQP8 expressed at the canalicular membrane domain and in intracellular vesicles. Bile is formed by the active secretion of solute transporters (ST) such as Bsep and Mrp2, which generate the osmotic driving forces for water transport through canalicular AQP8. On the right, a cholestatic hepatocyte is illustrated with decreased expression and functioning of ST. AQP8 is downregulated at the canalicular domain, which impairs the water osmotic permeability thus contributing to decreased bile formation.

Based on the cumulative evidence described above, a schematic model for the hepatocyte AQP8 contribution to the development of bile secretory failure is depicted in Figure 3.

CONCLUSION

In conclusion, this review summarized recent progress in research and current available data on the expression and pathophysiological significance of AQP water channels in the hepatocyte. It has long been established that canalicular bile secretion is the result of a combined interaction of several solute transporters. However, in the last few years further insight has been provided on the molecular basis of water movement during bile secretion. The functional expression of AQP8 is impaired in several experimental models of cholestasis such as extrahepatic obstructive cholestasis, estrogen-induced cholestasis and sepsis-induced cholestasis. A combined alteration in solute transporters and AQP8 would hamper the efficient coupling of osmotic gradients and canalicular water flow. Therefore, the common association of impaired solute transport together with decreased water permeability would ultimately lead to bile secretory failure. Nevertheless, more research is needed to expand the current knowledge underlying AQP expression regulation and water transport in cholestasis.

REFERENCES

1 **Zollner G**, Trauner M. Mechanisms of cholestasis. *Clin Liver*

Dis 2008; **12**: 1-26, vii
2 **Arrese M**, Trauner M. Molecular aspects of bile formation and cholestasis. *Trends Mol Med* 2003; **9**: 558-564
3 **Roma MG**, Crocenzi FA, Sanchez Pozzi EA. Hepatocellular transport in acquired cholestasis: new insights into functional, regulatory and therapeutic aspects. *Clin Sci* (Lond) 2008; **114**: 567-588
4 **King LS**, Kozono D, Agre P. From structure to disease: the evolving tale of aquaporin biology. *Nat Rev Mol Cell Biol* 2004; **5**: 687-698
5 **Preston GM**, Carroll TP, Guggino WB, Agre P. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. *Science* 1992; **256**: 385-387
6 **Gorelick DA**, Praetorius J, Tsunenari T, Nielsen S, Agre P. Aquaporin-11: a channel protein lacking apparent transport function expressed in brain. *BMC Biochem* 2006; **7**: 14
7 **Itoh T**, Rai T, Kuwahara M, Ko SB, Uchida S, Sasaki S, Ishibashi K. Identification of a novel aquaporin, AQP12, expressed in pancreatic acinar cells. *Biochem Biophys Res Commun* 2005; **330**: 832-838
8 **Saparov SM**, Liu K, Agre P, Pohl P. Fast and selective ammonia transport by aquaporin-8. *J Biol Chem* 2007; **282**: 5296-5301
9 **Bienert GP**, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, Jahn TP. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem* 2007; **282**: 1183-1192
10 **Wu B**, Beitz E. Aquaporins with selectivity for unconventional permeants. *Cell Mol Life Sci* 2007; **64**: 2413-2421
11 **Jung JS**, Preston GM, Smith BL, Guggino WB, Agre P. Molecular structure of the water channel through aquaporin CHIP. The hourglass model. *J Biol Chem* 1994; **269**: 14648-14654
12 **Beitz E**, Wu B, Holm LM, Schultz JE, Zeuthen T. Point mutations in the aromatic/arginine region in aquaporin 1 allow passage of urea, glycerol, ammonia, and protons. *Proc Natl Acad Sci USA* 2006; **103**: 269-274
13 **de Groot BL**, Engel A, Grubmuller H. A refined structure of human aquaporin-1. *FEBS Lett* 2001; **504**: 206-211
14 **Murata K**, Mitsuoka K, Hirai T, Walz T, Agre P, Heymann JB, Engel A, Fujiyoshi Y. Structural determinants of water permeation through aquaporin-1. *Nature* 2000; **407**: 599-605
15 **Savage DF**, Stroud RM. Structural basis of aquaporin inhibition by mercury. *J Mol Biol* 2007; **368**: 607-617
16 **Huebert RC**, Splinter PL, Garcia F, Marinelli RA, LaRusso NF. Expression and localization of aquaporin water channels in rat hepatocytes. Evidence for a role in canalicular bile secretion. *J Biol Chem* 2002; **277**: 22710-22717
17 **Calamita G**, Mazzone A, Bizzoca A, Cavalier A, Cassano G, Thomas D, Svelto M. Expression and immunolocalization of the aquaporin-8 water channel in rat gastrointestinal tract. *Eur J Cell Biol* 2001; **80**: 711-719
18 **Elkjaer ML**, Nejsum LN, Gresz V, Kwon TH, Jensen UB, Frokiaer J, Nielsen S. Immunolocalization of aquaporin-8 in rat kidney, gastrointestinal tract, testis, and airways. *Am J Physiol Renal Physiol* 2001; **281**: F1047-F1057
19 **Garcia F**, Kierbel A, Larocca MC, Gradilone SA, Splinter P, LaRusso NF, Marinelli RA. The water channel aquaporin-8 is mainly intracellular in rat hepatocytes, and its plasma membrane insertion is stimulated by cyclic AMP. *J Biol Chem* 2001; **276**: 12147-12152
20 **Tani T**, Koyama Y, Nihei K, Hatakeyama S, Ohshiro K, Yoshida Y, Yaoita E, Sakai Y, Hatakeyama K, Yamamoto T. Immunolocalization of aquaporin-8 in rat digestive organs and testis. *Arch Histol Cytol* 2001; **64**: 159-168
21 **Elkjaer M**, Vajda Z, Nejsum LN, Kwon T, Jensen UB, Amiry-Moghaddam M, Frokiaer J, Nielsen S. Immunolocalization of AQP9 in liver, epididymis, testis, spleen, and brain. *Biochem Biophys Res Commun* 2000; **276**: 1118-1128
22 **Nicchia GP**, Frigeri A, Nico B, Ribatti D, Svelto M. Tissue distribution and membrane localization of aquaporin-9 water channel: evidence for sex-linked differences in liver. *J Histochem Cytochem* 2001; **49**: 1547-1556
23 **Roberts SK**, Yano M, Ueno Y, Pham L, Alpini G, Agre P, LaRusso NF. Cholangiocytes express the aquaporin CHIP and transport water via a channel-mediated mechanism. *Proc Natl Acad Sci USA* 1994; **91**: 13009-13013
24 **Nielsen S**, Smith BL, Christensen EI, Agre P. Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capillary endothelia. *Proc Natl Acad Sci USA* 1993; **90**: 7275-7279
25 **Marinelli RA**, Tietz PS, Pham LD, Rueckert L, Agre P, LaRusso NF. Secretin induces the apical insertion of aquaporin-1 water channels in rat cholangiocytes. *Am J Physiol* 1999; **276**: G280-G286
26 **Marinelli RA**, Pham LD, Tietz PS, LaRusso NF. Expression of aquaporin-4 water channels in rat cholangiocytes. *Hepatology* 2000; **31**: 1313-1317
27 **Calamita G**, Ferri D, Bazzini C, Mazzone A, Botta G, Liquori GE, Paulmichl M, Portincasa P, Meyer G, Svelto M. Expression and subcellular localization of the AQP8 and AQP1 water channels in the mouse gall-bladder epithelium. *Biol Cell* 2005; **97**: 415-423
28 **Ferri D**, Mazzone A, Liquori GE, Cassano G, Svelto M, Calamita G. Ontogeny, distribution, and possible functional implications of an unusual aquaporin, AQP8, in mouse liver. *Hepatology* 2003; **38**: 947-957
29 **Calamita G**, Ferri D, Gena P, Liquori GE, Cavalier A, Thomas D, Svelto M. The inner mitochondrial membrane has aquaporin-8 water channels and is highly permeable to water. *J Biol Chem* 2005; **280**: 17149-17153
30 **Tsukaguchi H**, Shayakul C, Berger UV, Mackenzie B, Devidas S, Guggino WB, van Hoek AN, Hediger MA. Molecular characterization of a broad selectivity neutral solute channel. *J Biol Chem* 1998; **273**: 24737-24743
31 **Tsukaguchi H**, Weremowicz S, Morton CC, Hediger MA. Functional and molecular characterization of the human neutral solute channel aquaporin-9. *Am J Physiol* 1999; **277**: F685-F696
32 **Carbrey JM**, Gorelick-Feldman DA, Kozono D, Praetorius J, Nielsen S, Agre P. Aquaglyceroporin AQP9: solute permeation and metabolic control of expression in liver. *Proc Natl Acad Sci USA* 2003; **100**: 2945-2950
33 **Nielsen S**, DiGiovanni SR, Christensen EI, Knepper MA, Harris HW. Cellular and subcellular immunolocalization of vasopressin-regulated water channel in rat kidney. *Proc Natl Acad Sci USA* 1993; **90**: 11663-11667
34 **Nielsen S**, Chou CL, Marples D, Christensen EI, Kishore BK, Knepper MA. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *Proc Natl Acad Sci USA* 1995; **92**: 1013-1017
35 **Ishikawa Y**, Yuan Z, Inoue N, Skowronski MT, Nakae Y, Shono M, Cho G, Yasui M, Agre P, Nielsen S. Identification of AQP5 in lipid rafts and its translocation to apical membranes by activation of M3 mAChRs in interlobular ducts of rat parotid gland. *Am J Physiol Cell Physiol* 2005; **289**: C1303-C1311
36 **Marinelli RA**, Pham L, Agre P, LaRusso NF. Secretin promotes osmotic water transport in rat cholangiocytes by increasing aquaporin-1 water channels in plasma membrane. Evidence for a secretin-induced vesicular translocation of aquaporin-1. *J Biol Chem* 1997; **272**: 12984-12988
37 **Gradilone SA**, Garcia F, Huebert RC, Tietz PS, Larocca MC, Kierbel A, Carreras FI, Larusso NF, Marinelli RA. Glucagon induces the plasma membrane insertion of functional aquaporin-8 water channels in isolated rat hepatocytes. *Hepatology* 2003; **37**: 1435-1441
38 **Ishibashi K**, Kuwahara M, Kageyama Y, Tohsaka A, Marumo F, Sasaki S. Cloning and functional expression of a second new aquaporin abundantly expressed in testis. *Biochem Biophys Res Commun* 1997; **237**: 714-718
39 **Tietz PS**, McNiven MA, Splinter PL, Huang BQ, Larusso NF. Cytoskeletal and motor proteins facilitate trafficking of

AQP1-containing vesicles in cholangiocytes. *Biol Cell* 2006; **98**: 43-52
40 **Zhao AZ**, Shinohara MM, Huang D, Shimizu M, Eldar-Finkelman H, Krebs EG, Beavo JA, Bornfeldt KE. Leptin induces insulin-like signaling that antagonizes cAMP elevation by glucagon in hepatocytes. *J Biol Chem* 2000; **275**: 11348-11354
41 **Blommaart EF**, Krause U, Schellens JP, Vreeling-Sindelarova H, Meijer AJ. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur J Biochem* 1997; **243**: 240-246
42 **Folli F**, Alvaro D, Gigliozzi A, Bassotti C, Kahn CR, Pontiroli AE, Capocaccia L, Jezequel AM, Benedetti A. Regulation of endocytic-transcytotic pathways and bile secretion by phosphatidylinositol 3-kinase in rats. *Gastroenterology* 1997; **113**: 954-965
43 **Misra S**, Ujhazy P, Varticovski L, Arias IM. Phosphoinositide 3-kinase lipid products regulate ATP-dependent transport by sister of P-glycoprotein and multidrug resistance associated protein 2 in bile canalicular membrane vesicles. *Proc Natl Acad Sci USA* 1999; **96**: 5814-5819
44 **Webster CR**, Anwer MS. Role of the PI3K/PKB signaling pathway in cAMP-mediated translocation of rat liver Ntcp. *Am J Physiol* 1999; **277**: G1165-G1172
45 **Gradilone SA**, Carreras FI, Lehmann GL, Marinelli RA. Phosphoinositide 3-kinase is involved in the glucagon-induced translocation of aquaporin-8 to hepatocyte plasma membrane. *Biol Cell* 2005; **97**: 831-836
46 **Kagawa T**, Varticovski L, Sai Y, Arias IM. Mechanism by which cAMP activates PI3-kinase and increases bile acid secretion in WIF-B9 cells. *Am J Physiol Cell Physiol* 2002; **283**: C1655-C1666
47 **Cosentino C**, Di Domenico M, Porcellini A, Cuozzo C, De Gregorio G, Santillo MR, Agnese S, Di Stasio R, Feliciello A, Migliaccio A, Avvedimento EV. p85 regulatory subunit of PI3K mediates cAMP-PKA and estrogens biological effects on growth and survival. *Oncogene* 2007; **26**: 2095-2103
48 **Masyuk AI**, Marinelli RA, LaRusso NF. Water transport by epithelia of the digestive tract. *Gastroenterology* 2002; **122**: 545-562
49 **Marinelli RA**, Tietz PS, Caride AJ, Huang BQ, LaRusso NF. Water transporting properties of hepatocyte basolateral and canalicular plasma membrane domains. *J Biol Chem* 2003; **278**: 43157-43162
50 **Mazzone A**, Tietz P, Jefferson J, Pagano R, LaRusso NF. Isolation and characterization of lipid microdomains from apical and basolateral plasma membranes of rat hepatocytes. *Hepatology* 2006; **43**: 287-296
51 **Tietz P**, Jefferson J, Pagano R, Larusso NF. Membrane microdomains in hepatocytes: potential target areas for proteins involved in canalicular bile secretion. *J Lipid Res* 2005; **46**: 1426-1432
52 **Banales JM**, Prieto J, Medina JF. Cholangiocyte anion exchange and biliary bicarbonate excretion. *World J Gastroenterol* 2006; **12**: 3496-3511
53 **Gradilone SA**, Tietz PS, Splinter PL, Marinelli RA, LaRusso NF. Expression and subcellular localization of aquaporin water channels in the polarized hepatocyte cell line, WIF-B. *BMC Physiol* 2005; **5**: 13
54 **Yang B**, Song Y, Zhao D, Verkman AS. Phenotype analysis of aquaporin-8 null mice. *Am J Physiol Cell Physiol* 2005; **288**: C1161-C1170
55 **Hofmann AF**. Cholestatic liver disease: pathophysiology and therapeutic options. *Liver* 2002; **22** Suppl 2: 14-19
56 **Larocca MC**, Soria LR, Espelt MV, Lehmann GL, Marinelli RA. The knockdown of hepatocyte aquaporin-8 by rna interference induces defective bile canalicular water transport. *Am J Physiol Gastrointest Liver Physiol* 2009; **296:** G93-G100
57 **Carreras FI**, Gradilone SA, Mazzone A, Garcia F, Huang BQ, Ochoa JE, Tietz PS, Larusso NF, Calamita G, Marinelli RA. Rat hepatocyte aquaporin-8 water channels are down-regulated in extrahepatic cholestasis. *Hepatology* 2003; **37**: 1026-1033
58 **Calamita G**, Ferri D, Gena P, Carreras FI, Liquori GE, Portincasa P, Marinelli RA, Svelto M. Altered expression and distribution of aquaporin-9 in the liver of rat with obstructive extrahepatic cholestasis. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G682-C690
59 **Carreras FI**, Lehmann GL, Ferri D, Tioni MF, Calamita G, Marinelli RA. Defective hepatocyte aquaporin-8 expression and reduced canalicular membrane water permeability in estrogen-induced cholestasis. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G905-G912
60 **Mottino AD**, Carreras FI, Gradilone SA, Marinelli RA, Vore M. Canalicular membrane localization of hepatocyte aquaporin-8 is preserved in estradiol-17beta-D-glucuronide-induced cholestasis. *J Hepatol* 2006; **44**: 232-233
61 **Crocenzi FA**, Mottino AD, Cao J, Veggi LM, Pozzi EJ, Vore M, Coleman R, Roma MG. Estradiol-17beta-D-glucuronide induces endocytic internalization of Bsep in rats. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G449-G459
62 **Mottino AD**, Cao J, Veggi LM, Crocenzi F, Roma MG, Vore M. Altered localization and activity of canalicular Mrp2 in estradiol-17beta-D-glucuronide-induced cholestasis. *Hepatology* 2002; **35**: 1409-1419
63 **Geier A**, Fickert P, Trauner M. Mechanisms of disease: mechanisms and clinical implications of cholestasis in sepsis. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 574-585
64 **Lehmann GL**, Carreras FI, Soria LR, Gradilone SA, Marinelli RA. LPS induces the TNF-alpha-mediated downregulation of rat liver aquaporin-8: role in sepsis-associated cholestasis. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G567-G575
65 **Lee JM**, Trauner M, Soroka CJ, Stieger B, Meier PJ, Boyer JL. Expression of the bile salt export pump is maintained after chronic cholestasis in the rat. *Gastroenterology* 2000; **118**: 163-172
66 **Lehmann GL**, Marinelli RA. Peritoneal sepsis downregulates liver expression of Aquaporin-8: a water channel involved in bile secretion. *Liver Int* 2009; **29:** 317-318

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TOPIC HIGHLIGHT

Carlos J Pirola, PhD, FAHA, *Series Editor*

Hepatic drug transporters and nuclear receptors: Regulation by therapeutic agents

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Abstract

The canalicular membrane represents the excretory pole of hepatocytes. Bile is an important route of elimination of potentially toxic endo- and xenobiotics (including drugs and toxins), mediated by the major canalicular transporters: multidrug resistance protein 1 (MDR1, ABCB1), also known as P-glycoprotein, multidrug resistance-associated protein 2 (MRP2, ABCC2), and the breast cancer resistance protein (BCRP, ABCG2). Their activities depend on regulation of expression and proper localization at the canalicular membrane, as regulated by transcriptional and post-transcriptional events, respectively. At transcriptional level, specific nuclear receptors (NR)s modulated by ligands, co-activators and co-repressors, mediate the physiological requirements of these transporters. This complex system is also responsible for alterations occurring in specific liver pathologies. We briefly describe the major Class Ⅱ NRs, pregnane X receptor (PXR) and constitutive androstane receptor (CAR), and their role in regulating expression of multidrug resistance proteins. Several therapeutic agents regulate the expression of relevant drug transporters through activation/inactivation of these NRs. We provide some representative examples of the action of therapeutic agents modulating liver drug transporters, which in addition, involve CAR or PXR as mediators.

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Key words: Drug transport; Biliary secretion; ABC proteins; Multidrug resistance proteins; Nuclear receptors; Constitutive androstane receptor; Pregnane X receptor; Therapeutic agents

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INTRODUCTION

Hepatocytes are polarized cells and represent 80% of the liver mass. The basolateral and canalicular membranes differ in their composition and functions and are separated by tight junctions that seal off the bile canaliculi. The basolateral membrane is in contact with the sinusoidal blood. The canalicular membrane represents the excretory pole of hepatocytes. Bile formation is largely dependent on active transport of solutes such as bile acids, glutathione and bicarbonate through the canalicular membrane followed by the passive movement of water. Canalicular excretion is the rate-limiting step of bile formation since biliary constituents are secreted into bile against concentration gradients. The canalicular primary bile is further modified by absorptive and secretory processes along the biliary tree. Considerable species specific differences in bile formation exist, including the contribution of ductular bile and bile acid composition[1]. In mammals, bile is essential for solubilization and digestion of dietary lipids.

The sinusoidal uptake and canalicular excretion of most biliary constituents is mediated by several transport systems expressed at the two polar surface domains of liver cells. Basolateral transport systems are responsible for the translocation of molecules across the sinusoidal membrane, whereas active canalicular transport systems are in charge of the biliary excretion. Numerous transport proteins involved in basolateral transport have been identified including the Na^+-taurocholate co-transporting

polypeptide (NTCP, SLC10A1), organic anion transporting polypeptides (OATPs: SLCO family), multidrug resistance-associated proteins 1, 3 and 4 (MRP1, 3 and 4; ABCC1, 3 and 4), and organic anion and cation transporters (OATs, OCTs: SLC22A family). Canalicular transport of osmotically active solutes, contributing to bile formation, is mediated by MRP2 (ABCC2), the bile salt export pump (BSEP, ABCB11), and the organic anion 2 (AE2, SLC4A2), which are involved in biliary excretion of glutathione and glucuronide conjugates, monoanionic bile salts, and bicarbonate, respectively[1-4]. Biliary elimination of drugs is mediated by the multidrug resistance protein 1 (MDR1, ABCB1), also known as P-glycoprotein, MRP2, and the breast cancer resistance protein (BCRP, ABCG2)[5-11]. Though they all belong to the superfamily ABC, in contrast to the majority of its members, BCRP is present as a monomer and consists of only one ATP-binding site and 6 transmembrane regions.

In this review we focus our attention only on the hepatic drug transporters and their regulation by nuclear receptors activated by compounds habitually used as therapeutic agents.

APICAL DRUG TRANSPORTERS AND NUCLEAR RECEPTORS

Drug transporters are constitutively expressed in several organs playing an important role in the efflux of xenobiotics and their metabolites; the apical membrane of epithelial secretory tissues, and particularly the liver, being the most relevant sites. Substrates recognized by P-glycoprotein, MRP2 and BCRP represent a wide spectrum of endo- and xenobiotics, including contaminants and therapeutic drugs, either neutral, cationic or anionic, and of hydrophobic or hydrophilic nature. P-glycoprotein is a member of the ABC superfamily of transporters originally described in cancer cell lines, conferring resistance to therapeutic agents. It was the first ABC transporter identified in canalicular membranes of normal hepatocytes. MDR1 functions as an efflux pump for a wide range of amphiphilic, bulky type Ⅱ cationic drugs together with other hydrophobic compounds, including endogenous and exogenous metabolites or toxins, steroid hormones, hydrophobic peptides and even glycolipids[12]. MRP2 mediates the biliary elimination of different organic anions, including glutathione-S-conjugates (e.g. of leukotriene C4), glucuronides (e.g. of bilirubin and estrogens), and oxidized glutathione[2,13]. MRP2 also mediates the canalicular transport of glucuronidated and sulfated bile salts[14]. In addition, MRP2 was found to transfer reduced glutathione, though with very low affinity. MRP1 but not MRP2 was the first member of the superfamily of ABC (ATP Binding Cassette) transporters dependent on ATP hydrolysis, and was initially identified in a human lung cancer cell line[15]. BCRP that was initially found to confer resistance to breast cancer treatment was more recently found to be expressed normally in epithelial tissues and transport sulfated metabolites of drugs with high specificity[16-18].

Expression of these canalicular drug transporters are subject to transcriptional and post-transcriptional regulation in response to endogenous and exogenous compounds and different pathologic situations. The short-term changes in transporter activity and expression are generated at post-transcriptional level. An intensive regulation mediated by second messenger and protein-kinases modulates the recruitment of the transporters from intracellular reservoirs to plasmatic membrane or alters the activity by phosphorylation-dephosphorylation and protein-protein interactions. All these factors act in association to regulate the functional state of drug transporters, which were found to be affected to different degrees by liver disease[1,4,19]. Particular interest has been given recently to proper localization of BSEP and MRP2 in the normal situation, which is disrupted in cholestatic disease as a consequence of their internalization and abnormal localization to subapical membrane. This was observed not only in experimental animals such as the rat, but also in humans[20,21].

At transcriptional level, a wide variety of nuclear receptors (NR)s activated by ligands, co-activators and co-repressors, mediate the physiological requirements of these transporters. This complex system is also responsible for alterations occurring in specific liver pathologies. The biotransformation systems (phase Ⅰ and Ⅱ reactions) that act in coordination with efflux proteins are also regulated by this same network of NRs[22,23]. Transcriptional regulation of drug transporters by NRs is a complex process involving: (1) ligand binding, (2) the association of NRs with regulatory sites in the genome through their DNA sites, (3) co-regulator recruitment, (4) the regulation of polymerase Ⅱ binding and activity at target promoters, and (5) the ending or attenuation of NR-dependent signaling[24-26].

Co-activators and co-repressors represent key factors in modulation of NR activity. Co-activators are implicated in chromatin relaxation due to their intrinsic histone acetyltransferase or methyltransferase activity. After binding to the activated (agonist-bound) NR, co-activators contribute to the full activation of expression of the target genes. Several co-activators were shown to cooperate with nuclear receptors: the p160 family (SRC-1, TIF-2, GRIP, ACTR), p/CIP and CBP/p300. On the other hand, co-repressors, such as NcoR and SMRT, preferentially bind to inactivated receptors (absence of ligand, antagonist-bound, reverse agonist-bound) and recruit various forms of histone deacetylases, thus leading to chromatin condensation and ultimately, to repression of the target gene expression[27].

As anticipated above, NRs comprise a superfamily of transcription factors activated by ligands which can both activate and repress gene expression. According to their dimerization and DNA binding properties, NRs can be classified into four groups. Class Ⅰ comprises the classical receptors of steroid hormones (estradiol, testosterone, progesterone, cortisol). These form homodimers before binding to response elements in the promoter regions of target genes. NRs belonging to Class Ⅱ, such as pregnane X receptor (PXR), constitutive androstane

receptor (CAR), and farnesoid X receptor (FXR), form heterodimers with the retinoid X receptor (RXR), prior to interacting with target genes. Since RXR is the obligated partner in the heterodimer formation, its low availability may result in a trans-repressive effect. Receptors with no ligand can exist, and have been found to bind DNA as homodimers. They belong to Class Ⅲ (e.g. RXR, and the nuclear hepatic factor 4, HNF4). Class IV consists of NRs that act as monomers, like the liver receptor LRH1[28].

We will focus on class Ⅱ receptors since they represent the best characterized. More specifically, we will briefly describe those receptors involved in regulating drug transporters, i.e. PXR and CAR. Originally, these NRs were identified as sensors able to respond to a wide variety of environmental xenobiotics to promote detoxification by phase I *CYP450* genes[29]. Lehmann *et al*[30] showed that hPXR receptor binds to the rifampicin/dexamethasone response element in the *CYP3A4* promoter region as a heterodimer with the 9-cis-retinoic acid receptor (RXR). They also reported that hPXR is activated by many *CYP3A4* inducers, including several steroids, lovastatin, clotrimazole, rifampicin and phenobarbital. Increasingly at present, data reveals the involvement of NRs in the regulation of Phase I and Ⅱ enzymes, along with the proteins effluxing their metabolites[31].

PXR

In 1998, Kliewer *et al*[32] identified a new member of the nuclear hormone receptor family activated primarily by pregnanes: PXR (NR1I2). It was principally cloned from mouse liver and later from rabbit, rat and human. PXR is predominantly expressed in liver and intestine, and to a lower extent, in lung and kidney[32,33]. PXR dimerizes with RXRα immediately after its activation by ligand binding. It was originally believed to be localized mainly at the nucleus, but later it was found that it is present at the cytoplasm, interacting with a protein complex and that, after activation, it translocates to the nucleus to regulate gene transcription[34]. One relevant feature of this receptor is that it recognizes a wide variety of xenobiotics such as ligands, dexamethasone, rifampicin, spironolactone, and pregnenolone 16α-carbonitrile being among the best characterized. It can also bind some specific bile acids such as litocholic, 3-ketolitocholic, cholic and deoxycholic acids[32-34]. PXR regulates genes involved in phase I metabolism (e.g. *CYP3A*) and several genes associated with drug transport such as *MDR1*, *OATP2*, *MRP2*, and *MRP3*[35-37]. PXR is remarkably divergent between species, with the rabbit, rat and human receptors sharing only approximately 80% of the amino acid identity in their ligand-binding domains. This feature is reflected by marked pharmacological differences in PXR activation profiles. PXRs from different species are differentially activated by specific compounds, thus correlating well with species-specific induction of *CYP3A* gene expression. For example, the hypocholesterolemic drug SR12813, the macrolide antibiotic rifampicin and the antidiabetic drug troglitazone are effective activators of the human and rabbit PXR but have modest activity on the rat and mouse PXR. On the contrary, pregnane 16α-carbonitrile is a more potent activator of the rat and mouse than the human and rabbit receptor[33]. In addition, PXR polymorphism has been described and it is assumed to contribute to the observed interindividual variability of gene expression and atypical responses to drugs or altered sensitivity to carcinogens[38,39].

CAR

Also known as NR1I3, this NR was identified in 1994 as a receptor interacting with a subset of retinoic acid response elements[40]. It was originally defined as a constitutively activated receptor since it forms a heterodimer with RXR and binds to retinoic acid response element in the absence of ligand[41]. It was demonstrated more recently that CAR activation is a multistep process. The initial step is translocation to the nucleus and interaction with RXRα, a process that can be independent of ligand binding[37,42]. It is known that CAR participates in regulation of transcription of drug transporter genes such as *MRPs* (*MRP2*, *3*, and *4*) and *Oatp2*[23,43,44].

CAR is found mainly in liver and it is also detected in certain extrahepatic tissues such as the intestine[40,45]. Pathophysiological conditions such as trauma, sepsis, inflammation[46] or drugs[47] can modify CAR expression. *In vivo*, CAR is sequestered in the cytoplasm forming a complex with proteins such as heat shock protein 90 (HSP90) and CAR cytoplasmic retention protein (CCRP)[48]. In addition, phosphatase 2A (PP2A) is recruited to the HSP90-CCRP-CAR complex[49]. Translocation of CAR to the nucleus, most likely dependent on the activity of PP2A, is followed by association with RXR and binding to the phenobarbital responsive enhancer modules (PBREM). Thus, CAR activation can imply direct binding of an agonist, recruitment of co-activators, dissociation of co-repressors, and the subsequent nuclear translocation and heterodimerization with RXRα[50], prior to DNA binding and induction of gene expression[51]. CAR co-activators so far identified are GRIP1/TIF2, PGC-1, SRC-1, Sp1, ASC-2 and SMC-1. CAR transcriptional activity correlates well with its concentration in the nucleus. The blockage of phenobarbital-mediated induction of *CYP2B* gene in rodents by okadaic acid, a protein phosphatase inhibitor, has provided an additional indication of the importance of CAR nuclear accumulation in the increase of transcription rate[52]. Some ligands of CAR like androstenol act as inverse agonists, affecting the protein in such a way that co-repressors instead of co-activators are recruited, and the transcriptional activity of the receptor is decreased[53]. Estrogen derivatives display both agonist and antagonist nature by inducing the recruitment of both SRC-1 and NcoR after binding to CAR[54]. Alternatively, some CAR activators are not ligands *in vitro*. Among others, phenobarbital and bilirubin can modulate CAR activity by indirect activation, promoting the nuclear translocation of the receptor without binding to the ligand domain, although the mechanism is not totally understood[49,55].

MODULATION OF DRUG TRANSPORTERS BY THERAPEUTIC AGENTS: ROLE OF NUCLEAR RECEPTORS

Synthetic drugs, natural products, endogenous substances, and environmental toxicants are chemicals known to modulate the activity of major Class Ⅱ nuclear receptors, CAR and PXR[56,57]. It is widely recognized that CAR and PXR are major determinants in the regulation of an extensive spectrum of genes involved in the metabolism and disposition of xeno- and endobiotics[37,58-60]. Thus, among other factors, drug exposure can influence the activity of these NRs, affecting the metabolism, toxicity and drug-drug interactions of many xenobiotics or endogenous substances. The following paragraphs describe some representative examples of the action of therapeutic agents modulating drug transporters and involving CAR or PXR as mediators.

Pharmaceutical agents that are agonists of PXR and CAR had been used for treatment of human diseases long before their mechanism of action was clarified. Rifampicin, a human PXR agonist, was found to be effective in the treatment of pruritus in cholestatic disorders[61,62]. Furthermore, administration of rifampicin to healthy human volunteers significantly induced UDP-glucuronosyltransferase 1A1 (UGT1A1), involved in bilirubin glucuronidation, and MRP2 expression, leading to reduction in serum bilirubin levels[63]. Certain traditional Chinese herbs are powerful CAR activators and have been used extensively for management of neonatal jaundice[64]. Phenobarbital, in addition to rifampicin, has been empirically used to treat hyperbilirubinemia[65,66] due to its inductive properties on UGTs. These compounds are activators of PXR and CAR and the identification of the UGT locus as a direct target for hPXR and hCAR has relevance in both xenobiotic/endobiotic metabolism and disposition in human disease. Simultaneous induction of biotransformation and transport systems by these same agents was also effective in increasing the disposition of a variety of carcinogens, as well as estrogen and thyroxin[67]. MRP2 is one of the best characterized drug transporters to act in coordination with biotransformation systems to increase drug elimination[22]. This is in part due to its universal capacity to respond to NR activators, which in turn activate a wide spectrum of phase I and Ⅱ reactions. Indeed, Kast *et al*[36] have reported that *MRP2/Mrp2* genes are modulated by PXR, FXR and CAR in human and rodents. Interestingly, these three distinct nuclear receptor signaling pathways converge on a common response element in the 5'-flanking region of these same genes.

Glucocorticoids are also well known inducers of several biotransformation and transport systems. In acute cholestasis, as well as in chronic cholestatic disorders such as primary biliary cirrhosis, the beneficial effects of steroids could be attributed not only to their anti-inflammatory and immune-modulatory actions but also to the effects mediated by alterations in biotransformation enzymes and transporters, these latter systems being regulated by NRs[68]. CAR seems to act as a primary glucocorticoid receptor (GR)-response gene, since the CAR gene promoter harbors a GR response element[69]. In addition, glucocorticoids such as dexamethasone induce CAR nuclear translocation. Glucocorticoids also induce PXR expression and nuclear translocation and thus induce target genes expression like *CYP3A4*, *BSEP* and *MRP2*. These latter findings explain the improvement of liver cholestatic diseases such as that induced experimentally by endotoxin administration[70].

As was demonstrated for the steroids pregnenolone 16α-carbonitrile, 5 β-pregnane-3, 20-dione and dexamethasone[71], spironolactone, widely used as a diuretic, also binds to PXR[72]. Rats treated with spironolactone, exhibit up-regulation of Mrp2 and P-gp in liver[73,74] along with increased phase Ⅱ biotransformation reactions[75,76]. Data on increased expression of Mrp2 (protein and mRNA) are consistent with transcriptional regulation of the target genes and with spironolactone-PXR interaction. The potentiality of spironolactone to counteract alterations in biliary secretory function emerges from studies demonstrating that this steroid was able to prevent the decrease in bile flow and biliary secretion of Mrp2 substrates induced by the cholestatic ethynylestradiol[77]. It is interesting to note that spironolactone also leads to up-regulation of PXR mRNA and protein levels (ML Ruiz, SSM Villanueva, MG Luquita, AD Mottino, and VA Catania, unpublished results), reinforcing a role for this nuclear receptor as a modulator of the action of spironolactone. This finding also suggests that an adaptive response to prolonged treatment with therapeutic drugs may result from changes in expression of the NR gene, and consequently from its availability for binding to the respective ligands. Clearly, the binding of an agonist or antagonist to NRs can directly translate physiological and pathophysiological requirements into alterations of gene expression[1,78,79]. These effects can be additionally modulated by transcriptional or post-transcriptional regulation of the transcription factor itself[80,81].

Acetaminophen is a widely used therapeutic drug which can produce hepatotoxicity when administered at high doses. CAR is a key regulator of acetaminophen metabolism and hepatotoxicity. CAR activators, as well as high doses of acetaminophen, induce expression of key drug metabolizing enzymes in wild-type but not in *Car*-/- mice, and administration of the inverse agonist ligand androstanol after treatment with acetaminophen blocks hepatotoxicity in wild-type but not in *Car*-/- mice[82]. In addition, *Car*-/- mice are resistant to acetaminophen hepatotoxicity. In contrast, mice deficient in Nrf2 are highly susceptible to acetaminophen hepatotoxicity and were unable to increase the hepatic basolateral drug transporters Mrp3 and Mrp4, as detected in wild type animals[83]. These transporters may represent an attractive target to reduce acetaminophen hepatotoxicity. Indeed, pretreatment of rats with acetaminophen was shown to increase Mrp3 expression, and thereby induced a shift from biliary to urinary elimination of acetaminophen glucuronide; the subsequent decreased enterohepatic recirculation was postulated to decrease exposure of the liver to acetaminophen and thereby protect against hepatoxicity[84]. Whether CAR

or PXR, in addition to NrF, are involved in modulation of key drug transporters regulating acetaminophen toxicity, at toxic or subtoxic doses, needs further exploration.

Whereas a number of drugs targeting different NRs, which form heterodimers with RXR, have been approved for treatment of metabolic diseases[85], finding new therapeutic compounds that could modulate drug efflux in a similar way still represents a major challenge. Our increasing understanding of the molecular regulation of transport and detoxification systems, including mediation of NRs, should help significantly.

CONCLUSION

Major drug transporters in the liver, either at the apical or basolateral level, are extensively regulated by therapeutic agents, and likely involve mediation of NRs. Targeting NRs such as CAR and PXR to improve liver diseases, particularly those involving alterations in biliary secretory function, represents a promising perspective. Most of the studies referenced in this current review, which clearly support this possibility, were performed either in rodents or in human cell lines. To what extent the results obtained in these experiments apply to humans is poorly known and needs further exploration.

REFERENCES

1 **Trauner M**, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* 2003; **83**: 633-671
2 **Borst P**, Elferink RO. Mammalian ABC transporters in health and disease. *Annu Rev Biochem* 2002; **71**: 537-592
3 **Crocenzi FA**, Mottino AD, Roma MG. Regulation of synthesis and trafficking of canalicular transporters and its alteration in acquired hepatocellular cholestasis. Experimental therapeutic strategies for its prevention. *Curr Med Chem* 2004; **11**: 501-524
4 **Stieger B**, Meier Y, Meier PJ. The bile salt export pump. *Pflugers Arch* 2007; **453**: 611-620
5 **Paulusma CC**, Bosma PJ, Zaman GJ, Bakker CT, Otter M, Scheffer GL, Scheper RJ, Borst P, Oude Elferink RP. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* 1996; **271**: 1126-1128
6 **Gerk PM**, Vore M. Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. *J Pharmacol Exp Ther* 2002; **302**: 407-415
7 **Matsushima S**, Maeda K, Kondo C, Hirano M, Sasaki M, Suzuki H, Sugiyama Y. Identification of the hepatic efflux transporters of organic anions using double-transfected Madin-Darby canine kidney II cells expressing human organic anion-transporting polypeptide 1B1 (OATP1B1)/multidrug resistance-associated protein 2, OATP1B1/multidrug resistance 1, and OATP1B1/breast cancer resistance protein. *J Pharmacol Exp Ther* 2005; **314**: 1059-1067
8 **Krishnamurthy P**, Schuetz JD. Role of ABCG2/BCRP in biology and medicine. *Annu Rev Pharmacol Toxicol* 2006; **46**: 381-410
9 **Sarkadi B**, Homolya L, Szakacs G, Varadi A. Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. *Physiol Rev* 2006; **86**: 1179-1236
10 **Nies AT**, Keppler D. The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch* 2007; **453**: 643-659
11 **Gradhand U**, Kim RB. Pharmacogenomics of MRP transporters (ABCC1-5) and BCRP (ABCG2). *Drug Metab Rev* 2008; **40**: 317-354
12 **Muller M**, Jansen PL. Molecular aspects of hepatobiliary transport. *Am J Physiol* 1997; **272**: G1285-G1303
13 **Konig J**, Nies AT, Cui Y, Leier I, Keppler D. Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta* 1999; **1461**: 377-394
14 **Akita H**, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H, Sugiyama Y. Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. *Biochim Biophys Acta* 2001; **1511**: 7-16
15 **Cole SP**, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992; **258**: 1650-1654
16 **Suzuki M**, Suzuki H, Sugimoto Y, Sugiyama Y. ABCG2 transports sulfated conjugates of steroids and xenobiotics. *J Biol Chem* 2003; **278**: 22644-22649
17 **Choudhuri S**, Klaassen CD. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. *Int J Toxicol* 2006; **25**: 231-259
18 **Zamek-Gliszczynski MJ**, Nezasa K, Tian X, Kalvass JC, Patel NJ, Raub TJ, Brouwer KL. The important role of Bcrp (Abcg2) in the biliary excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in mice. *Mol Pharmacol* 2006; **70**: 2127-2133
19 **Haussinger D**, Schmitt M, Weiergraber O, Kubitz R. Short-term regulation of canalicular transport. *Semin Liver Dis* 2000; **20**: 307-321
20 **Mottino AD**, Cao J, Veggi LM, Crocenzi F, Roma MG, Vore M. Altered localization and activity of canalicular Mrp2 in estradiol-17beta-D-glucuronide-induced cholestasis. *Hepatology* 2002; **35**: 1409-1419
21 **Kojima H**, Nies AT, Konig J, Hagmann W, Spring H, Uemura M, Fukui H, Keppler D. Changes in the expression and localization of hepatocellular transporters and radixin in primary biliary cirrhosis. *J Hepatol* 2003; **39**: 693-702
22 **Catania VA**, Sanchez Pozzi EJ, Luquita MG, Ruiz ML, Villanueva SS, Jones B, Mottino AD. Co-regulation of expression of phase II metabolizing enzymes and multidrug resistance-associated protein 2. *Ann Hepatol* 2004; **3**: 11-17
23 **Eloranta JJ**, Meier PJ, Kullak-Ublick GA. Coordinate transcriptional regulation of transport and metabolism. *Methods Enzymol* 2005; **400**: 511-530
24 **Glass CK**, Rosenfeld MG. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 2000; **14**: 121-141
25 **Acevedo ML**, Kraus WL. Transcriptional activation by nuclear receptors. *Essays Biochem* 2004; **40**: 73-88
26 **Metivier R**, Reid G, Gannon F. Transcription in four dimensions: nuclear receptor-directed initiation of gene expression. *EMBO Rep* 2006; **7**: 161-167
27 **Nishioka K**, Reinberg D. Transcription. Switching partners in a regulatory tango. *Science* 2001; **294**: 2497-2498
28 **Mangelsdorf DJ**, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: the second decade. *Cell* 1995; **83**: 835-839
29 **Fuhr U**. Induction of drug metabolising enzymes: pharmacokinetic and toxicological consequences in humans. *Clin Pharmacokinet* 2000; **38**: 493-504
30 **Lehmann JM**, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* 1998; **102**: 1016-1023
31 **Roy-Chowdhury J**, Locker J, Roy-Chowdhury N. Nuclear receptors orchestrate detoxification pathways. *Dev Cell* 2003; **4**: 607-608
32 **Kliewer SA**, Moore JT, Wade L, Staudinger JL, Watson MA,

Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T, Lehmann JM. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 1998; **92**: 73-82
33 **Jones SA**, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, Moore JT. The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* 2000; **14**: 27-39
34 **Squires EJ**, Sueyoshi T, Negishi M. Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver. *J Biol Chem* 2004; **279**: 49307-49314
35 **Staudinger J**, Liu Y, Madan A, Habeebu S, Klaassen CD. Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor. *Drug Metab Dispos* 2001; **29**: 1467-1472
36 **Kast HR**, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, Edwards PA. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* 2002; **277**: 2908-2915
37 **Maglich JM**, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT, Kliewer SA. Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* 2002; **62**: 638-646
38 **Zhang J**, Kuehl P, Green ED, Touchman JW, Watkins PB, Daly A, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Wrighton SA, Hancock M, Kim RB, Strom S, Thummel K, Russell CG, Hudson JR Jr, Schuetz EG, Boguski MS. The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics* 2001; **11**: 555-572
39 **Gardner-Stephen D**, Heydel JM, Goyal A, Lu Y, Xie W, Lindblom T, Mackenzie P, Radominska-Pandya A. Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression. *Drug Metab Dispos* 2004; **32**: 340-347
40 **Baes M**, Gulick T, Choi HS, Martinoli MG, Simha D, Moore DD. A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol Cell Biol* 1994; **14**: 1544-1552
41 **Honkakoski P**, Zelko I, Sueyoshi T, Negishi M. The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* 1998; **18**: 5652-5658
42 **Wang H**, LeCluyse EL. Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes. *Clin Pharmacokinet* 2003; **42**: 1331-1357
43 **Geier A**, Wagner M, Dietrich CG, Trauner M. Principles of hepatic organic anion transporter regulation during cholestasis, inflammation and liver regeneration. *Biochim Biophys Acta* 2007; **1773**: 283-308
44 **Urquhart BL**, Tirona RG, Kim RB. Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *J Clin Pharmacol* 2007; **47**: 566-578
45 **Wei P**, Zhang J, Dowhan DH, Han Y, Moore DD. Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J* 2002; **2**: 117-126
46 **Beigneux AP**, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR. Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response. *Biochem Biophys Res Commun* 2002; **293**: 145-149
47 **Gerbal-Chaloin S**, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ, Maurel P. Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J Biol Chem* 2002; **277**: 209-217
48 **Qatanani M**, Moore DD. CAR, the continuously advancing receptor, in drug metabolism and disease. *Curr Drug Metab* 2005; **6**: 329-339
49 **Yoshinari K**, Kobayashi K, Moore R, Kawamoto T, Negishi M. Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital. *FEBS Lett* 2003; **548**: 17-20
50 **Cai Y**, Konishi T, Han G, Campwala KH, French SW, Wan YJ. The role of hepatocyte RXR alpha in xenobiotic-sensing nuclear receptor-mediated pathways. *Eur J Pharm Sci* 2002; **15**: 89-96
51 **Goodwin B**, Moore JT. CAR: detailing new models. *Trends Pharmacol Sci* 2004; **25**: 437-441
52 **Timsit YE**, Negishi M. CAR and PXR: the xenobiotic-sensing receptors. *Steroids* 2007; **72**: 231-246
53 **Forman BM**, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM, Moore DD. Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* 1998; **395**: 612-615
54 **Makinen J**, Reinisalo M, Niemi K, Viitala P, Jyrkkarinne J, Chung H, Pelkonen O, Honkakoski P. Dual action of oestrogens on the mouse constitutive androstane receptor. *Biochem J* 2003; **376**: 465-472
55 **Huang W**, Zhang J, Chua SS, Qatanani M, Han Y, Granata R, Moore DD. Induction of bilirubin clearance by the constitutive androstane receptor (CAR). *Proc Natl Acad Sci USA* 2003; **100**: 4156-4161
56 **Willson TM**, Kliewer SA. PXR, CAR and drug metabolism. *Nat Rev Drug Discov* 2002; **1**: 259-266
57 **Handschin C**, Meyer UA. Induction of drug metabolism: the role of nuclear receptors. *Pharmacol Rev* 2003; **55**: 649-673
58 **Waxman DJ**. P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Arch Biochem Biophys* 1999; **369**: 11-23
59 **Rosenfeld JM**, Vargas R Jr, Xie W, Evans RM. Genetic profiling defines the xenobiotic gene network controlled by the nuclear receptor pregnane X receptor. *Mol Endocrinol* 2003; **17**: 1268-1282
60 **Klaassen CD**, Slitt AL. Regulation of hepatic transporters by xenobiotic receptors. *Curr Drug Metab* 2005; **6**: 309-328
61 **Bachs L**, Pares A, Elena M, Piera C, Rodes J. Comparison of rifampicin with phenobarbitone for treatment of pruritus in biliary cirrhosis. *Lancet* 1989; **1**: 574-576
62 **Prince MI**, Burt AD, Jones DE. Hepatitis and liver dysfunction with rifampicin therapy for pruritus in primary biliary cirrhosis. *Gut* 2002; **50**: 436-439
63 **Marschall HU**, Wagner M, Zollner G, Fickert P, Diczfalusy U, Gumhold J, Silbert D, Fuchsbichler A, Benthin L, Grundstrom R, Gustafsson U, Sahlin S, Einarsson C, Trauner M. Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans. *Gastroenterology* 2005; **129**: 476-485
64 **Huang W**, Zhang J, Moore DD. A traditional herbal medicine enhances bilirubin clearance by activating the nuclear receptor CAR. *J Clin Invest* 2004; **113**: 137-143
65 **Yaffe SJ**, Levy G, Matsuzawa T, Baliah T. Enhancement of glucuronide-conjugating capacity in a hyperbilirubinemic infant due to apparent enzyme induction by phenobarbital. *N Engl J Med* 1966; **275**: 1461-1466
66 **Cancado EL**, Leitao RM, Carrilho FJ, Laudanna AA. Unexpected clinical remission of cholestasis after rifampicin therapy in patients with normal or slightly increased levels of gamma-glutamyl transpeptidase. *Am J Gastroenterol* 1998; **93**: 1510-1517
67 **Xie W**, Yeuh MF, Radominska-Pandya A, Saini SP, Negishi Y, Bottroff BS, Cabrera GY, Tukey RH, Evans RM. Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. *Proc Natl Acad Sci USA* 2003; **100**: 4150-4155
68 **Marschall HU**, Wagner M, Zollner G, Trauner M. Clinical hepatotoxicity. Regulation and treatment with inducers of

transport and cofactors. *Mol Pharm* 2007; **4**: 895-910
69 **Pascussi JM**, Busson-Le Coniat M, Maurel P, Vilarem MJ. Transcriptional analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element. *Mol Endocrinol* 2003; **17**: 42-55
70 **Kubitz R**, Wettstein M, Warskulat U, Haussinger D. Regulation of the multidrug resistance protein 2 in the rat liver by lipopolysaccharide and dexamethasone. *Gastroenterology* 1999; **116**: 401-410
71 **Kliewer SA**, Willson TM. Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *J Lipid Res* 2002; **43**: 359-364
72 **Schuetz EG**, Brimer C, Schuetz JD. Environmental xenobiotics and the antihormones cyproterone acetate and spironolactone use the nuclear hormone pregnenolone X receptor to activate the CYP3A23 hormone response element. *Mol Pharmacol* 1998; **54**: 1113-1117
73 **Ruiz ML**, Villanueva SS, Luquita MG, Sanchez-Pozzi EJ, Crocenzi FA, Pellegrino JM, Ochoa JE, Vore M, Mottino AD, Catania VA. Mechanisms involved in spironolactone-induced choleresis in the rat. Role of multidrug resistance-associated protein 2. *Biochem Pharmacol* 2005; **69**: 531-539
74 **Ghanem CI**, Gomez PC, Arana MC, Perassolo M, Delli Carpini G, Luquita MG, Veggi LM, Catania VA, Bengochea LA, Mottino AD. Induction of rat intestinal P-glycoprotein by spironolactone and its effect on absorption of orally administered digoxin. *J Pharmacol Exp Ther* 2006; **318**: 1146-1152
75 **Catania VA**, Luquita MG, Sanchez Pozzi EJ, Mottino AD. Differential induction of glutathione S-transferase subunits by spironolactone in rat liver, jejunum and colon. *Life Sci* 1998; **63**: 2285-2293
76 **Catania VA**, Luquita MG, Sanchez Pozzi EJ, Ikushiro S, Emi Y, Iyanagi T, Mottino AD. Effect of spironolactone on the expression of rat hepatic UDP-glucuronosyltransferase. *Biochem Pharmacol* 2003; **66**: 171-177
77 **Ruiz ML**, Villanueva SS, Luquita MG, Ikushiro S, Mottino AD, Catania VA. Beneficial effect of spironolactone administration on ethynylestradiol-induced cholestasis in the rat: involvement of up-regulation of multidrug resistance-associated protein 2. *Drug Metab Dispos* 2007; **35**: 2060-2066
78 **Karpen SJ**. Nuclear receptor regulation of hepatic function. *J Hepatol* 2002; **36**: 832-850
79 **Eloranta JJ**, Kullak-Ublick GA. Coordinate transcriptional regulation of bile acid homeostasis and drug metabolism. *Arch Biochem Biophys* 2005; **433**: 397-412
80 **Beigneux AP**, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR. The acute phase response is associated with retinoid X receptor repression in rodent liver. *J Biol Chem* 2000; **275**: 16390-16399
81 **Pascussi JM**, Drocourt L, Fabre JM, Maurel P, Vilarem MJ. Dexamethasone induces pregnane X receptor and retinoid X receptor-alpha expression in human hepatocytes: synergistic increase of CYP3A4 induction by pregnane X receptor activators. *Mol Pharmacol* 2000; **58**: 361-372
82 **Zhang J**, Huang W, Chua SS, Wei P, Moore DD. Modulation of acetaminophen-induced hepatotoxicity by the xenobiotic receptor CAR. *Science* 2002; **298**: 422-424
83 **Aleksunes LM**, Slitt AL, Maher JM, Augustine LM, Goedken MJ, Chan JY, Cherrington NJ, Klaassen CD, Manautou JE. Induction of Mrp3 and Mrp4 transporters during acetaminophen hepatotoxicity is dependent on Nrf2. *Toxicol Appl Pharmacol* 2008; **226**: 74-83
84 **Ghanem CI**, Ruiz ML, Villanueva SS, Luquita MG, Catania VA, Jones B, Bengochea LA, Vore M, Mottino AD. Shift from biliary to urinary elimination of acetaminophen-glucuronide in acetaminophen-pretreated rats. *J Pharmacol Exp Ther* 2005; **315**: 987-995
85 **Shulman AI**, Mangelsdorf DJ. Retinoid x receptor heterodimers in the metabolic syndrome. *N Engl J Med* 2005; **353**: 604-615

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Acute and persisting Th2-like immune response after fractionated colorectal γ-irradiation

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Abstract

AIM: To investigate if an immune imbalance may account for the development and progression of chronic radiation enteritis. We analyzed the Th1/Th2 immune response profile early and 6 mo after fractionated colorectal irradiation.

METHODS: A rat model of fractionated colorectal γ-irradiation (4-Gy fractions, 3 fractions per week) was designed to investigate the effects of cumulative dose on inflammatory mediators (cytokines and chemokines) and immune response (Th1/Th2 profile and immunosuppressive mediator IL-10) during acute (early) response and 6 mo after the end of fractionated irradiation (chronic response). Analyses were performed 1 d after the cumulative doses of 16 Gy and 36 Gy and 1 d, 3 d, and 26 wk after the cumulative dose of 52 Gy.

RESULTS: Without causing histological damage, fractionated radiation induced elevated expression of IL-1β, TNFα, MCP-1, and iNOS in distal colonic mucosa during the early post-irradiation phase. At that time, a Th2 profile was confirmed by expression of both the Th2-specific transcription factor GATA-3 and the chemokine receptor CCR4 and by suppression of the Th1 cytokine IFNγ/IP-10 throughout the irradiation protocol. After 6 mo, despite the 2-fold reduction of iNOS and MCP-1 levels, the Th2 profile persisted, as shown by a 50% reduction in the expression of the Th1 transcription factor T-bet, the chemokine receptor CCXCR3, and the IFNγ/STAT1 pathway. At the same time-point, the immunosuppressive IL-10/STAT3 pathway, known to regulate the Th1/Th2 balance, was expressed, in irradiated rats, at approximately half its level as compared to controls. This suppression was associated with an overexpression of SOCS3, which inhibits the feedback of the Th1 polarization and regulates IL-10 production.

CONCLUSION: Colorectal irradiation induces Th2 polarization, defective IL-10/STAT3 pathway activation and SOCS3 overexpression. These changes, in turn, maintain a immunological imbalance that persists in the long term.

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Key words: Colorectum; Inflammation; Th2 cells; Irradiation, Suppressor of cytokine signaling 3; STAT

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INTRODUCTION

During radiation therapy for pelvic or abdominal cancer, the intestines are a critical dose-limiting organ. Despite precautions in treatment planning (e.g. multiple fields) and delivery, patients develop radiation-induced bowel injury during and after therapy[1]. Radiation enteropathy therefore remains an important obstacle to the radiocurability of abdominal tumors and continues to impair patients' "quality of life". Symptoms of acute bowel toxicity occur among about 80% of these patients, and include vomiting, diarrhea, hemorrhages and ulcerations due to the direct effects of radiation on the intestinal mucosa[2]. Explanations based on recognition that irradiation induces changes in cellular functions have recently replaced the concept attributing the severity of acute intestinal radiation toxicity to disruption of the epithelial barrier and mucosal inflammation.

Acute (early) effects are those observed during the course of radiation therapy: they are usually transient

and cease shortly after its completion. In some patients, persistent acute damage causes consequential effects to appear later on, i.e. after radiation exposure[3]. These delayed effects, called chronic radiation enteritis, concern fewer patients (50%) but are important clinically because of their chronic progressive nature and their significant long-term morbidity. Delayed radiation enteropathy typically presents, from the clinical standpoint, 6 mo to 3 years after irradiation. It is characterized by dysmotility and malabsorption, sometimes developing into fibrosis and eventually, in some cases, bowel obstruction, years or even decades after radiation exposure. A latency period was previously thought to exist between the time of radiation treatment and the onset of radiation-induced damage, but many studies now show that this is not the case. Experimental evidence indicates that the onset of delayed radiation effects is a continuous process that starts immediately at irradiation[4]. The process includes the production of cytokines and reactive oxygen species (ROS), which cause responses in the surrounding tissue, including cell infiltration. These "waves" of response may be interpreted as the result of failed attempts at adaptation and then later as the evidence of dysregulated tissue response. Recent studies show that irradiation induces the synthesis of various cytokines in several tissues, including the intestines[5] and lungs[6]. These cytokines lead to cell infiltration and fibroblast stimulation, thus enhancing collagen synthesis[7]. In addition, cytokine production by immune cells is crucial to immune response to infectious agents and disease prevention.

Many diseases, including inflammatory bowel disease[8], are associated with imbalances between Th1 (T helper cell type 1) and Th2 (T helper cell type 2) cytokines. Because these cell subpopulations tend to function antagonistically towards each another, the persistence of disease susceptibility and resistance depends on the profiles of the cytokines that each type secretes. Several reports show that ionizing radiation induces the preferential differentiation of Th cells into Th2 cells in the spleen[9,10] and more recently, in the intestines[11], where this Th2 dominance is characterized by repression of IFNγ. Th2 cells play a critical role in the pathogenesis of radiation-induced pneumonitis, which precedes lung fibrosis[12].

Th1 and Th2 cells were originally distinguished from each other by their specific cytokine expression profiles. *In vivo* analysis of these polarized Th cells has revealed differential sets of molecular expression, including specific chemokine/chemokine receptors. Typically, the chemokine receptor CXCR3 is expressed exclusively on Th1 lymphocytes, which migrate to the inflammatory sites in response to CXCR3 ligands and interferon-inducible protein (IP)-10[13,14]. Likewise, CCR4 is preferentially expressed on Th2 cells[15]. Specific expression of transcription factors leads to differential expression of polarized Th cells. For example, T-bet, expressed specifically in Th1 cells, mediates IFNγ production, while GATA-binding protein 3 (GATA-3), which suppresses this production, is thought to be the most important factor associated with the development of the Th2 phenotype and inhibition of the Th1 phenotype[16]. Th1 cytokines can activate macrophages, regulate cell-mediated immune responses, and promote tumoricidal activity. Th2 cells, in contrast, promote humoral immunity. Each Th subset mutually inhibits the growth and function of the other one. Members of the suppressor of cytokine signaling (SOCS) family of proteins are described as feedback inhibitors of a broad range of cytokine signaling pathways, regulating the amplitude and duration of the polarization influenced by T-bet or GATA-3[17]. Notably, SOCS3 inhibits the signal transduction pathway implicating IFNγ[18].

Determining Th1/Th2 balance during the radiotherapy protocol and, in particular, its long-term balance requires a longitudinal study. In this study, a rat model of fractionated colorectal-irradiation was designed to investigate the effects of cumulative dose on the inflammatory mediators and the immune response. Our study suggests that the downregulation of Th1-type cells, induced by γ-irradiation, persists for at least 6 mo after the end of the irradiation protocol. This supports the hypothesis that the radiation-induced impairment of inflammation control mechanisms plays a critical role in both acute and late effects.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (Elevage Janvier, France) weighing 200-250 g were housed (three per cage) with food and water *ad libitum*. All experiments were conducted in accordance with the French Ministry of Agriculture regulations for animal experimentation (No. 2001-464, May 2001).

Radiation schedule

Anesthetized rats were sham-irradiated or exposed to a γ-ray source (^{60}Co, 1 Gy/min). The radiation field was confined to the colorectum (field size: 2 by 2.5 cm). Radiation was delivered 3 times a week (4-Gy fractions) for a total dose of 16, 36, or 52 Gy. The cumulative doses of 16 and 36 Gy correspond nearly to one third and two thirds of the total fractionated dose (52 Gy), respectively. Radiation response was assessed on the first day after the cumulative dose of 16 and 36 Gy, and day 1 and day 3 of the cumulative dose of 52 Gy, and 26 wk after it reached 52 Gy.

Tissue isolation

After the rats were anesthetized, distal colon tissue in the irradiation field was excised and rinsed with saline. Whole-tissue samples were collected and fixed with 4% formaldehyde for immunostaining assays, while scraped mucosa layers were snap-frozen and then stored at -80℃ for RNA extraction.

PCR analysis

The mRNA levels of inflammation-related cytokines and chemokines and of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) were measured by real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from the colon mucosa

Table 1 Specifications of the primer sets used for gene expression analysis by RT-PCR

Gene	Forward primer	Reverse primer
IL-1β	CAACAAAAATGCCTCGTGC	TGCTGATGTACCAGTTGGG
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
MCP-1	CAGCCAGATGCAGTTAATGCC	AGCCGACTCATTGGGATCAT
INOS	GATTTTTCACGACACCCT	GGTCCTCTGGTCAAACTC
IL-10	GTTGCCAAGCCT-TGTCAGAAA	TTTCTGGGCCATGGTTCTCT
IFN-γ	CACGCCGCGTCTTGGT	TCTAGGCTTTCAATGAGTGTGCC
Suppressors of cytokine signalling proteins		
SOCS3	CCTCCAGCATCTTTGTC-GGAAGAC	TACTGGTCCAGGAACTCCCGAATG
Transcription factors		
T-Bet	TCCTGTCTCCAGCCGTTTCT	CGCTCACTGCTCGGAACTCT
GATA-3	GGCGGCGAGATGGTACTG	TCTGCCCATTCATTTTATGGTAGA
Chemokine receptors		
CXCR3	AGGTCAGTGAACGTCAAGTGCTAG	CAAAAAGAGGAGGCTGTAGAGGA
CCR4	GCCTCCAAGACAGACTTCCTTG	AGCGTTCGGTTCTAGTTTCCAC
Housekeeping		
HPRT	GCTCGAGATGTCATGAAGGAGA	TCAGCGCTTTAATGTAATCCAGC

samples with the RNeasy kit (Qiagen). RNA purity and integrity were checked by spectrophotometric analysis and agarose gel electrophoresis. In accordance with the manufacturer's instructions, 1 μg of total RNA was reverse-transcribed into cDNA with random hexamers and the SuperScript II RNase H (GibcoBRL) in a 20-μL reaction volume. SYBR chemistry (Applied Biosystems) was used to amplify PCR in the ABI-Prism 7000 detection system (Applied Biosystems), under the following conditions: 50℃ for 2 min, 95℃ for 10 min and 40 cycles at 95℃ for 15 s and 60℃ for 1 min. The primer sequences, designed with Primer Express software (Applied Biosystems), are listed in Table 1.

Immunoblot analysis

Total proteins were obtained by colon homogenization in a cold RIPA buffer (Sigma) containing a standard protease-inhibitor cocktail. Protein concentrations of cytoplasmic and nuclear extracts were measured with a modified Bradford method from Biorad Laboratories (Biorad, France). The samples were then stored at -80℃.

Proteins (25 μg) were boiled in SDS and mercapto-ethanol buffer and then separated on a 120 g/L polyacrylamide gel (NuPAGE gels; Invitrogen, France) and electroblotted. Polyvinylidene difluoride membranes were incubated with a blocking solution (50 g/L skimmed milk in TPBS containing 1 mL/L Tween 20), washed with TPBS and incubated with rabbit polyclonal STAT1 p84/p91 (1/700, Santa Cruz), STAT3 (1/300, Santa Cruz) and SOCS3 (1/200, Santa Cruz) for 1 h at room temperature. After washing, immunodetection was performed with the respective horseradish-linked secondary antibodies (1/1000; Santa Cruz). GAPDH protein was detected similarly with a rabbit anti-GAPDH polyclonal antibody (1/1000, Santa Cruz) to verify uniformity in gel loading. Chemiluminescence was detected according to the manufacturer's protocol (ECL, Biorad). Mean band densities were quantified with a Las 3000 apparatus (Fugifilm) and normalized to the total amount of protein in the control.

Immunostaining

Neutrophils and macrophages: Fixed distal colon specimens were dehydrated, embedded in paraffin and then sectioned into 5 μm thick slices. After dewaxing in xylene and rehydration by exposure to graded ethanols, sections were processed to reveal immunoreactivity to myeloperoxidase (MPO), a marker of neutrophils, or were prepared for macrophage detection. All sections were subjected to an endogenous peroxidase blocking solution (3% H_2O_2). For macrophage staining only, the slides were boiled in 10% citrate buffer for antigen retrieval. To reduce non-specific binding, all slides were pre-incubated with the protein blocker (Dako) before treatment for 1 hour at 26℃ with anti-rat MPO antibody (NCL-MYELOp; 1:300, Novocastra) or at 37℃ with an antibody binding the activated macrophage marker ED1 (MCA341; 1:50, Serotec). Secondary reagents were a secondary antibody, followed by the Vector Elite ABC kit (Dako) for neutrophil detection, and the LSAB 2 system HRP kit (Dako) for macrophages. Whenever necessary, slides were washed with a Tris buffer (50 mmol/L Tris-HCl; 0.3 mol/L NaCl; 0.1% Tween 20; pH 7.6). For both the primary and secondary staining, sections were treated with a NovaRED kit (Vector Laboratories Inc.) for color development, and then counterstained with Mayer's hemalum. For MPO staining, positive cells were counted under a light microscope in a blinded fashion and expressed as the mean number of marked cells per ten random crypts (100 crypts).

Apoptotic cell detection: Apoptotic cells were visualized by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick-end labeling assay (TUNEL) using the In Situ Cell Death Detection kit (Roche Molecular Biochemicals, France) according to the manufacturer's instructions. Briefly, deparaffinized and rehydrated tissue sections were incubated in proteinase K (20 mg/L in 10 mmol/L Tris-HCl, pH 7.6) for 10 min at 37℃. Sections were exposed to the TUNEL reaction mixture at 37℃ for 1 h. After washing in PBS buffer,

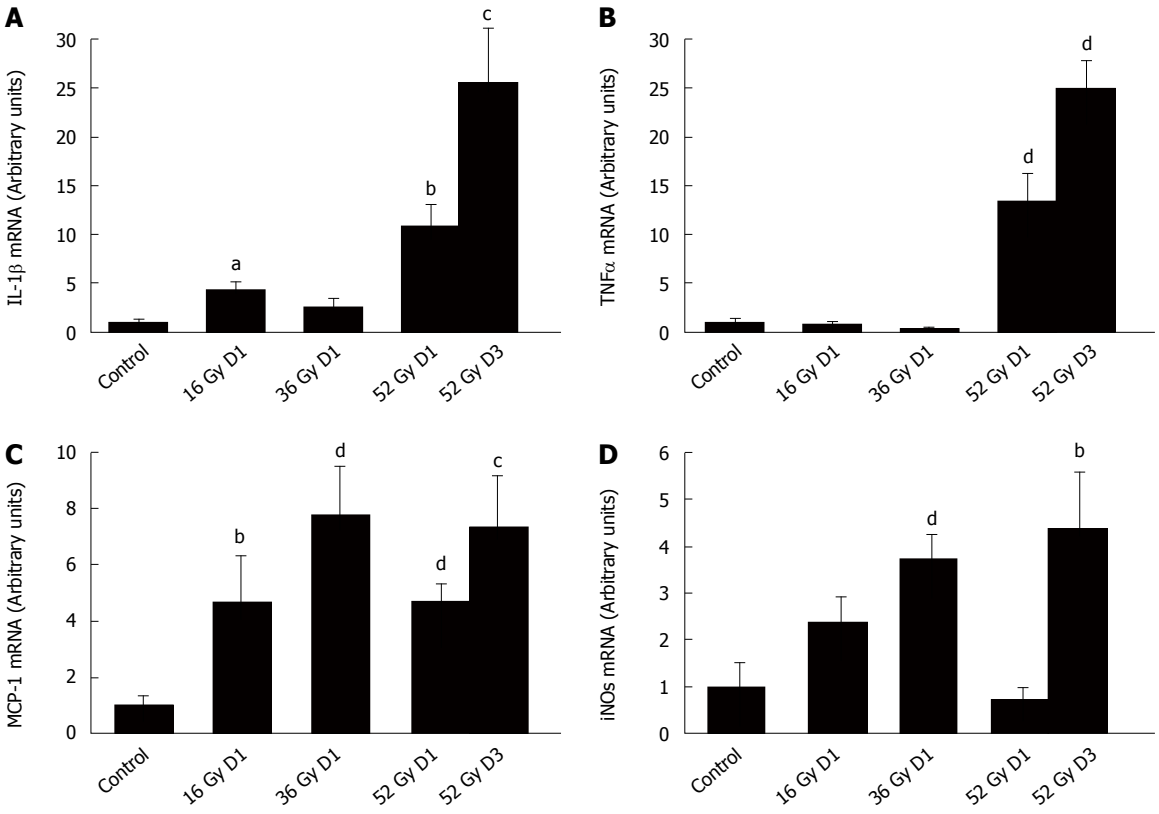

Figure 1 Effects of colorectal fractionated irradiation protocol on inflammatory mediator expression. Levels of IL-1β (A), TNFα (B), MCP-1 (C) and iNOS (D) mRNA were assessed in distal colon mucosa one day after cumulative doses of 16 Gy, 36 Gy, 52 Gy as well as 3 d after 52 Gy. The quantification of target genes was normalized to the reference gene HPRT. Data are mean ± SE, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.005$; $^{d}P < 0.001$ *vs* control.

POD (peroxidase) was added to react for 30 min at 37℃. For both stainings, the slides were treated with a NovaRED™ kit (Vector Laboratories Inc, Burlingame, CA) for colour development and counterstained with Meyer's hemalun.

Result expression and statistical analysis

For the real-time PCR, we used the comparative $\Delta\Delta C_T$-method for the relative mRNA quantification of target genes, normalized to the reference gene HPRT, and the relevant sham-irradiated sample. Specifically, we determined $2^{-\Delta\Delta CT}$, with $\Delta\Delta C_T$ the difference between $\Delta C_{T(\text{irradiated sample})}$ and $\Delta C_{T(\text{sham-irradiated sample})}$ and ΔC_T the difference between the mean $C_{T(\text{gene of interest})}$ and the mean $C_{T(\text{HPRT reference gene})}$. C_T is the threshold cycle of fluorescence intensity. Each experimental group consisted of 6 animals. Values of PCR and MPO-positive cell counts are expressed as the mean ± SE.

All data are expressed as the mean ± SE for 6 animals. Data were analyzed by one-way ANOVA followed by a Bonferroni test to determine the significance of the differences.

RESULTS

Pattern of inflammatory mediators during fractionated γ irradiation

Overexpression of pro-inflammatory cytokines is often a sign of the onset of inflammation and has been characterized as an early effect of intestinal radiation[5]. To test whether fractionated irradiation induces a persistent and progressive inflammatory process, we assessed the expression of inflammatory mediators, relative to the housekeeping gene HPRT, in rat colon mucosa on D1 in irradiated rats in all 3 dose groups (16, 36 and 52 Gy) and on D3 in the 52 Gy group. IL-1β expression increased by a factor of 4.5 ($P < 0.05$) on D1 after colorectal γ-irradiation to 16-Gy exposure, compared with controls. On the other hand, 24 h after the 36-Gy cumulative dose, IL-1β expression did not differ from that in the sham-exposed rats. Finally, after the 52-Gy dose, IL-1β mRNA rose to levels dramatically higher than in controls (11-fold, $P < 0.01$) on D1 and still higher (25-fold, $P < 0.005$) on D3 (Figure 1A). TNFα expression after the total 52-Gy dose was also 13.5 times higher than in controls on D1 ($P < 0.005$) and remained elevated at D3 ($P < 0.001$) (Figure 1B). Similarly, the level of MCP-1, known to recruit and activate monocytes and macrophages in tissue, increased significantly during the fractionated protocol (Figure 1C). In addition, the expression of iNOS, strongly secreted by macrophages, remained high throughout the protocol (Figure 1D).

Histology and assessment of inflammatory cell accumulation

Histological analysis of distal colons showed no distinct difference between the sham-irradiated group and the rats with cumulative doses of 16, 36, and 52 Gy.

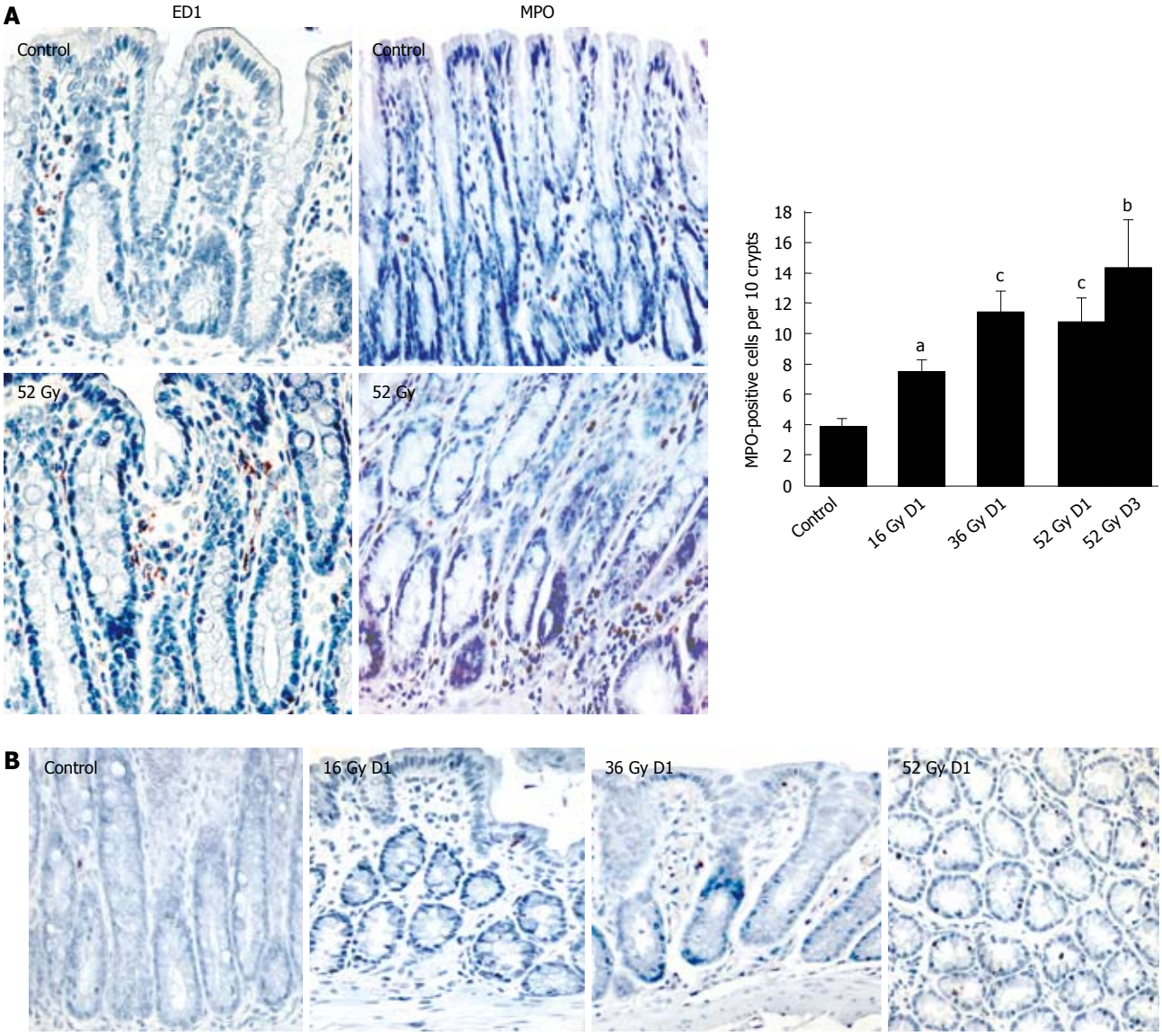

Figure 2 Effects of colorectal fractionated irradiation protocol on inflammatory cells and apoptotic cell presence. Recruitment of ED-1-positive and MPO-positive cells (A) was assessed in distal colon mucosa in the normal distal colon (control) and three days after cumulative doses of 52 Gy. MPO-positive cells were correlated with the average number of neutrophils one day after cumulative doses of 16 Gy, 36 Gy, and one day and three days after 52 Gy. The presence of apoptotic cells was confirmed by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) staining at each timepoint of the fractionated protocol (B). Data are mean ± SE, $^{a}P < 0.01$; $^{b}P < 0.005$; $^{c}P < 0.001$ *vs* control values. (× 20).

Immunostaining of macrophages showed an increase in ED1-positive macrophages in the lamina propria on D1 and D3 after the maximal cumulative dose of 52 Gy (Figure 2A). This macrophage accumulation may be related to the overexpression of TNFα, MCP-1 and iNOS. In addition, the number of MPO-positive neutrophils was nearly twice as high as in controls on D1 after the 16-Gy exposure ($P < 0.01$) and remained elevated throughout the protocol, about 3 times higher than in controls in the 36-Gy group ($P < 0.001$) and in the 52-Gy groups on D1 and D3 (Figure 2A). In addition, TUNEL assay showed no significant modification of the apoptotic cell number during fractionated irradiation (Figure 2B).

Imbalance of Th1/Th2-associated genes during fractionated irradiation

As previously reported[11], abdominal irradiation initiated a Th2-cell immune response characterized by suppression of IFNγ expression. Here too, we observed that fractionated irradiation induced a wave suppressing IFNγ expression, reducing it significantly (3-fold decrease, $P < 0.005$) (Figure 3A). This decrease was associated with reduction of IFN-inducible genes by the end of the protocol, including IFNγ-inducible 10 kDa protein (IP-10) ($P < 0.05$).

We measured Th cell populations present in the mucosa during the fractionated protocol more directly by taking into account the fact that activated Th cells acquire and maintain high levels of specific patterns of chemokine receptors: CXCR3 is thus a marker of Th1 and CCR4 of Th2[15]. Fractionated irradiation modified the chemokine receptor profile (Figure 3B). CXCR3 levels fell by 50% ($P < 0.05$) after a 16-Gy cumulative dose and 80% ($P < 0.01$) after a 36-Gy dose. The

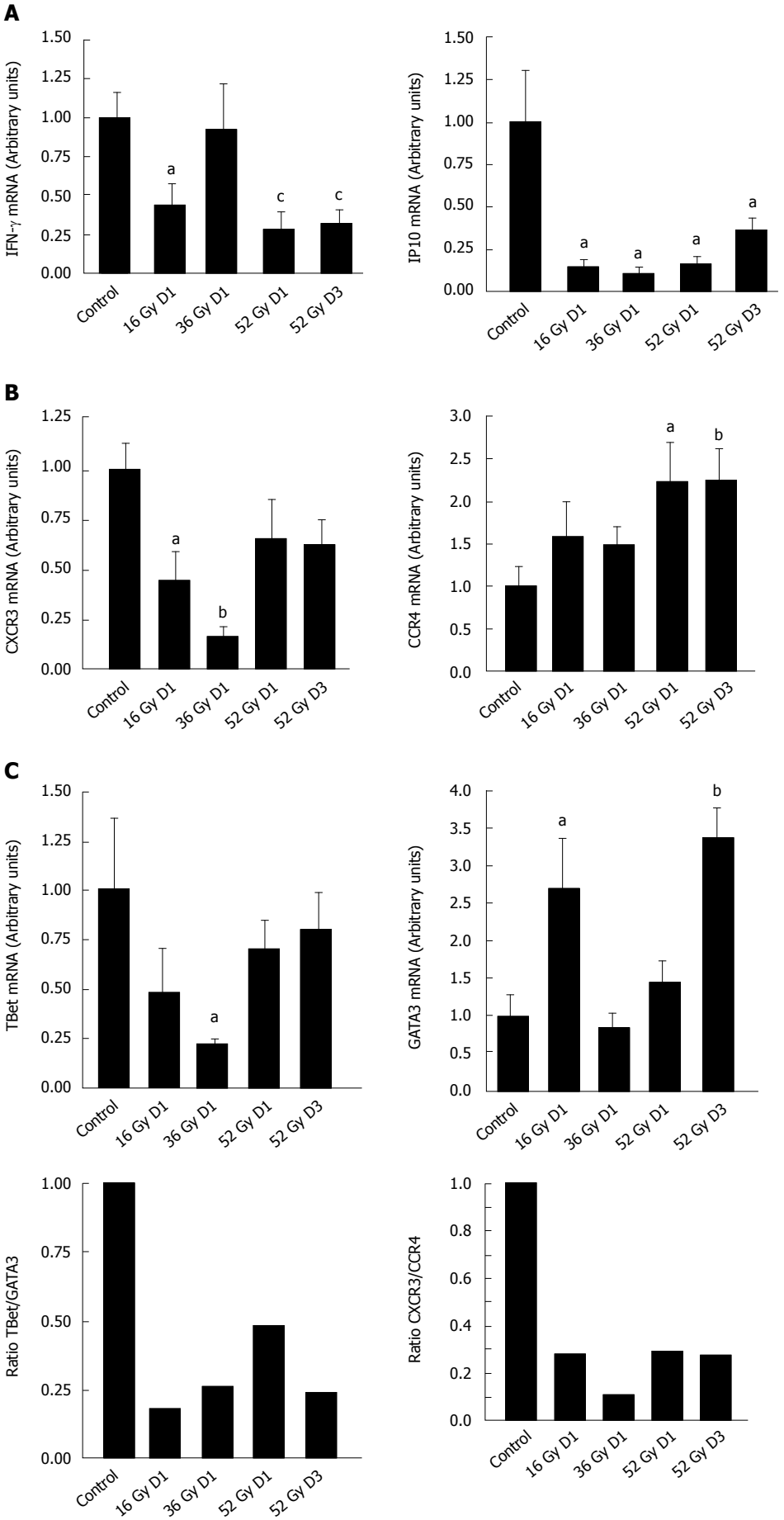

Figure 3 Early Th1/Th2 polarization by fractionated irradiation protocol within 1 and 3 d of irradiation. Colorectal fractionated irradiation protocol induced suppression of Th1 cytokines (IFNγ and IFN-inducible genes (IP-10)) (A). Gene expression of the chemokine receptors CXCR3 and CCR4 (B) and transcription factors T-bet and Gata3 (C) implicated in the Th1/Th2 balance was analyzed one day after cumulative doses of 16 Gy, 36 Gy, 52 Gy and 3 d after 52 Gy in colon mucosa. Data are mean ± SE, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.005$ *vs* control.

CXCR3 level remained low after a cumulative dose of 52 Gy, but CCR4 expression increased significantly ($P < 0.01$). The CXCR3/CCR4 ratio, which serves as an indicator of the Th1/Th2 balance[15], fell about

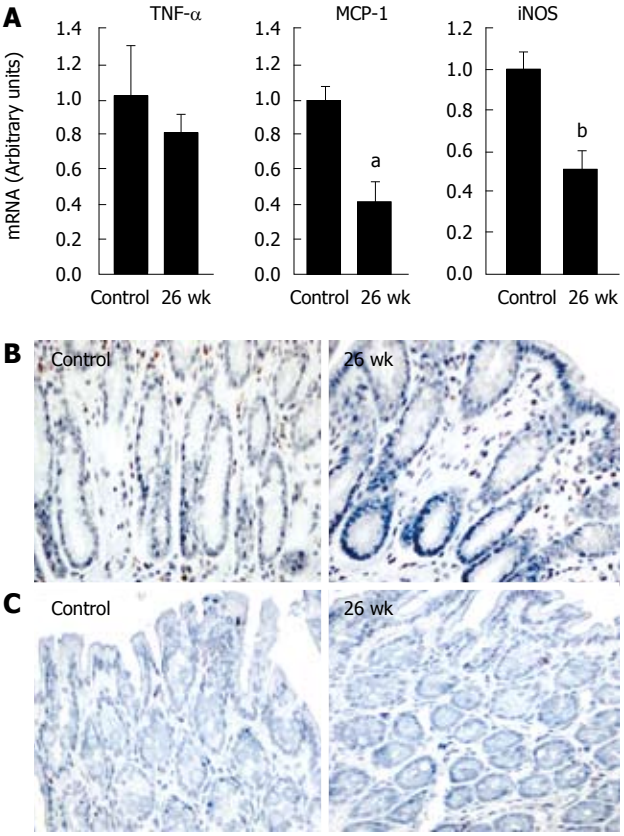

Figure 4 Long-term impairment of inflammatory response. Delayed effects of the radiation schedule on expression of TNFα, MCP-1 and iNOS in colon mucosa 26 wk after the end of cumulative dose of 52 Gy (A). The quantification of target genes was normalized to the reference gene HPRT. Immunostaining for macrophages (B) and apoptotic cells (C) in the colon: Fractionated irradiated group shows no intense ED1-positive macrophages in the lamina propria and no significant modification of the apoptotic cell number 26 wk post-irradiation. Data are mean ± SE, $^{a}P < 0.01$; $^{b}P < 0.005$ *vs* control.

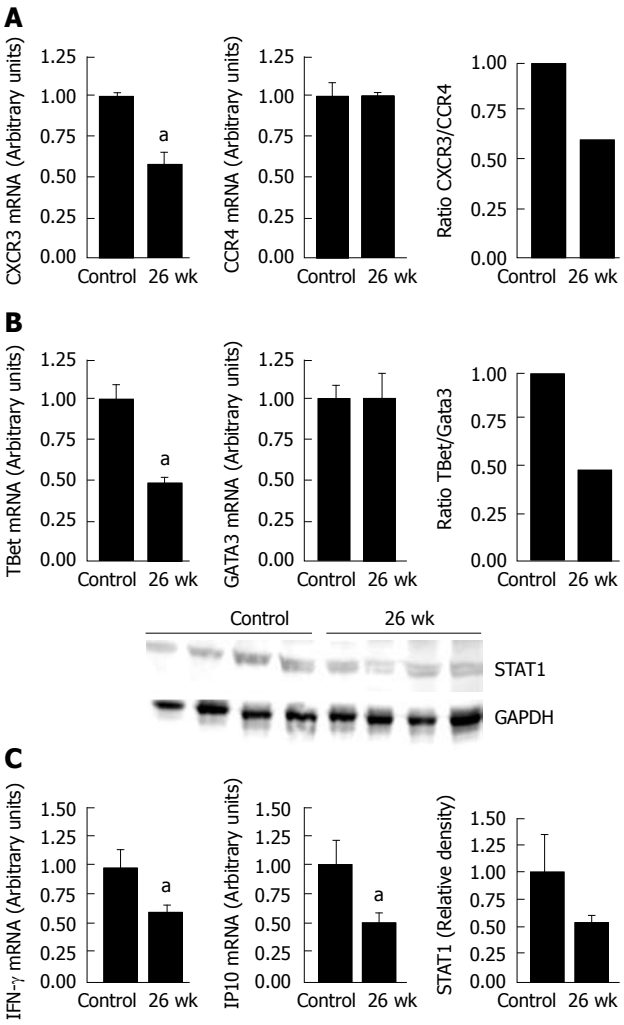

Figure 5 Persistence of Th2 polarization at 6 mo. Expression of chemokine receptors CXCR3 and CCR4 (A) and transcription factors T-bet and Gata3 expression (B) in colon mucosa was assessed 26 wk after the end of cumulative dose of 52Gy. Aspects of immune response that changed long after irradiation included IFNγ and IP-10 expression and downregulation of signal transducer and activator 1 (STAT1) (C). Immunoblot showing STAT1 in total protein extracts from mucosa colon. GAPDH levels are used as internal standards and relative densitometric data are analysed following normalization to the control. Dot plots of four experiments on different samples are shown. Data are the mean ± SE; $^{a}P < 0.05$ *vs* control.

70% during the course of treatment, thus confirming that irradiation induced Th2 dominance. Further confirmation comes from analysis of the transcription factors T-bet and GATA-3, respectively, selectively expressed in Th1 and Th2 cells. The T-bet profile closely resembled that of CXCR3 (Figure 3C). GATA-3 expression increased more than T-bet expression did at every period, and the ratio of T-Bet to GATA-3 ratio fell, decreasing 4-fold by the end of the protocol.

Long-term impairment of inflammatory response

Histological analysis of the colon 26 wk after the last delivered fraction showed no changes. To investigate the inflammatory response, we analyzed the expression of mediators related to macrophage infiltration. No change in TNFα expression was observed during this period. Surprisingly, however, expression of MCP-1 and iNOS decreased significantly, by 60 and 50% respectively. No increase in macrophage infiltration and no significant modification of apoptotic cell umber were observed at 6 mo (Figure 4).

Persistence of the imbalance in the immune response

The fractionated schedule may increase the probability that radiation-induced inflammation will become chronic and/or induce damage at intermediate times post-irradiation. No previous report has prospectively identified changes in the Th1/Th2 balance according to expression of chemokine receptors and transcription factors over time after irradiation. Analysis of the Th1 profile showed a significant 2-fold reduction ($P < 0.05$) in both CXCR3 and T-bet expression compared with the controls 26 wk after the end of the protocol (Figure 5A and B). Levels of CCR4 and GATA3 (both Th2 markers) did not change.

The consequence of the Th1/Th2 imbalance was seen in the significant reduction in IFNγ and IP-10 ($P < 0.05$): expression of both fell by half. Because IFNγ delivers signals through the STAT family of signal transducers, such as STAT1, we sought to determine if reduced IFNγ expression in irradiated mucosa was correlated with a reduction in STAT1. Western blot

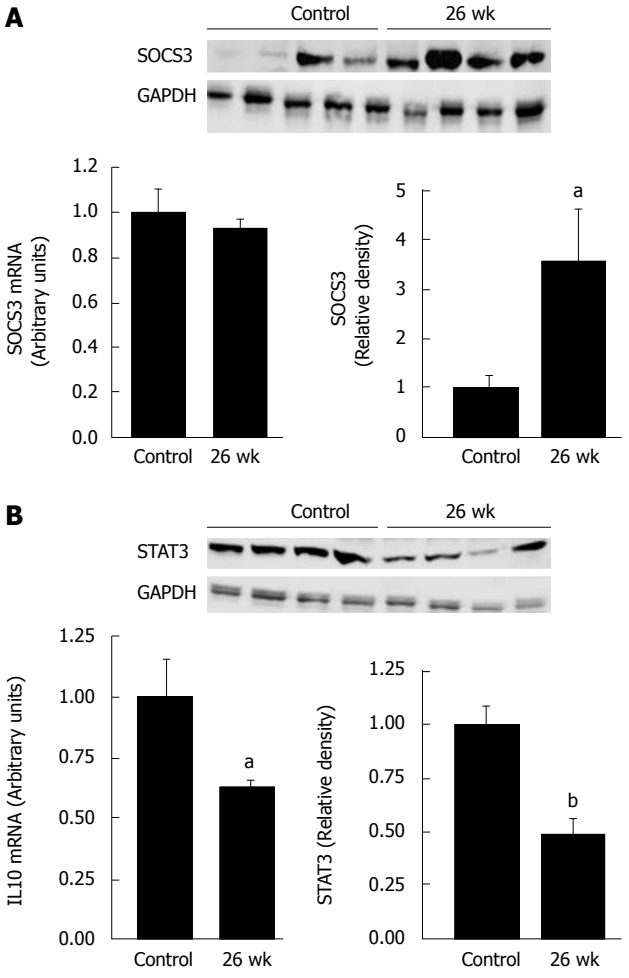

Figure 6 Fractionated irradiation-modified SOCS3, IL-10 and STAT3 expression. SOCS3 mRNA and protein (A), IL-10 mRNA and STAT3 protein (B) were measured in the colonic mucosa 26 wk after the end of cumulative dose of 52 Gy. Western blot analyses are showing SOCS3 and STAT3 in total protein extracts from mucosa colon. GAPDH levels are used as internal standards and relative densitometric data are analysed following normalization to the control. Dot plots of four experiments on different samples are shown. Data are expressed as the mean ± SE; $^{a}P < 0.05$; $^{b}P < 0.001$ *vs* control.

analysis showed that STAT1 immunoreactivity was also lower at 26 wk than in controls (Figure 5C). Taken together, these data indicate that the shift towards Th2 dominance is maintained 26 wk after irradiation.

Irradiation induced expression of SOCS3 and regulated expression of IL-10 and STAT3 in the long term

The relative decrease of STAT1 protein levels prompted us to investigate the possible role of the SOSC proteins, which inhibit STAT signaling. A particularly interesting candidate from this family is SOCS3, known to interfere with the IFNγ/Stat-1 pathway[19,20]. Western blot analysis showed the SOCS3 protein levels remained high at 26 wk after irradiation ($P < 0.05$) (Figure 6A). Because SOCS3 regulates production of the immunoregulatory cytokine IL-10 by modulating STAT3 signaling[21], we assessed IL-10 and STAT3 expression (Figure 6B). IL-10 expression ($P < 0.05$) 26 wk after the end of the protocol was half as much compared to the control rats and was correlated with a similar decrease in STAT3 protein levels ($P < 0.001$). These data show that elevated SOCS3 levels modify expression of IL-10 and STAT3.

DISCUSSION

The pathological process of radiation injury begins immediately after radiation exposure, but its clinical and histological features may not become apparent for weeks, months, or even years after treatment. In this study, we investigated whether fluctuations in cytokine levels after radiation treatment continue over a relatively long period 6 mo after the end of treatment, and whether they predict late effects. Identifying the critical factors could provide useful tools for selecting patients for treatment to prevent late damage or to reduce its severity. If we knew how early cytokine responses modify downstream late effects, we might be able to target cytokines for prevention. The results showed that each dose fraction influenced tissue inflammatory responses and led to long-term persistence of the immune imbalance.

Fractionated radiation produces a series of repeated insults to healthy tissues: these lead to inflammatory events that do not dissipate within 24 h, so that repetitive responses accumulate over the period of radiation therapy[22]. During our fractionated protocol in the rat, we identified inflammatory reactions in the colon induced by a cascade of inflammatory mediators, including IL-1β, TNFα, MCP-1, and iNOS, with levels and latency periods specific for each mediator. In addition, histological observations showed infiltration of inflammatory cells (macrophages and neutrophils). TNFα is known to be a key mediator of pathogenesis in a broad range of infectious and predominantly Th-mediated inflammatory diseases, possibly via macrophage induction of iNOS and thus NO synthesis. However, iNOS has also been shown to enhance induction of TNFα synthesis[23], contributing to local tissue destruction during the chronic inflammation that is one of the direct consequences of inflammatory processes. A previous study found a positive correlation between iNOS activity levels and disease severity in patients with ulcerative colitis, but this association is less clear in Crohn's disease[24]. The involvement of iNOS-derived NO in acute radiation syndrome is suggested by an increase in iNOS gene expression or enzyme activity in the intestines[25]. The present study revealed a biphasic pattern of iNOS expression: early overexpression during the fractionated protocol, followed by delayed suppression, seen at 6 mo. In particularly, iNOS regulates chemokine expression involved in leukocyte (especially neutrophil) trafficking: the absence of iNOS enhances lung inflammatory responses, associated with increased production of MCP-1 by endothelial cells and macrophages[26]. Reports about iNOS regulation of the production of this CC chemokine are contradictory. *In vitro* studies show that inhibition of endogenous NO synthesis by a NOS inhibitor increases MCP-1 levels in endothelial cells[27], whereas, in a rat model of pulmonary granulomatous inflammation, inhibition of NO production reduced MCP-1 expression[28]. Our results are consistent with the hypothesis that iNOS can regulate MCP-1 expression, because increased levels

of iNOS were associated with both increased neutrophil recruitment and MCP-1 expression during the fractionated treatment. Moreover, at a later phase, the expression of both iNOS and MCP-1 was cut in half. At this time, no explanation is given, but additional attention is being placed on macrophages, the essential iNOS producers that are inactivated with regard to inflammatory mediator production. A more general suppression is achieved with IL-10, but exposure to IL-4 or related cytokines initiates a so called "alternatively activated macrophage" or M2 macrophages[29]. These cells have distinct functional properties that integrate them into polarized type 2 responses, tissue remodeling and repair. These cells show diminished capacity to produced iNOS. Furthermore, the maturation stage of the macrophage population may be worth considering in the later phase.

The immunomodulatory activity of iNOS is reported to influence Th cell development and to downregulate the induction of Th1 responses, thereby promoting a Th2 response[30]. Multiple pathways appear to be involved in initiating the type 2 T-cell responses. Gu *et al*[31] reported an impaired Th2 response in MCP-1 knockout mice, thus demonstrating that MCP-1 is necessary for the generation of Th2 cells. A possible feedback loop for Th2 activation might be the production of IL-4 and IL-13 by Th2 cells: these cytokines stimulate MCP-1 production and lead to further recruitment of Th2 cells[13]. Our results corroborate previously published work reporting on the initiation of a Th2 response in the early phase of abdominal radiation[11]. In that study, we identified a Th2 response during the fractionated protocol. It included suppression of the Th1 cytokine profile, that is, of both IFNγ and the IFN-inducible gene IP-10. These are also identified by the specific pattern of chemokine receptors (CXCR3 and CCR4) and the specific transcription factors (T-bet and GATA-3), selectively expressed in Th1 cells and Th2 cells, respectively, that control the differentiation and the functions of these Th cell subsets. The correlation between the suppression of CXCR3 and T-bet observed in the present study also corroborates a previous report[32] that deletion of T-bet reduces CXCR3 expression: CXCR3 is both a direct transcription target of T-bet and the chemokine receptor of IP-10. IFNγ may act upstream of T-bet to drive CXCR3 expression[32]. Consequences of T-bet deletion include the failure of primary $CD4^+$ T cells generated under Th1 polarizing conditions to migrate to the site of inflammation because of defects in several specific mechanisms of the T-cell trafficking pathway. Surprisingly, suppression of CXCR3 and T-bet continued during the later phase, for reasons that we could not determine.

An essential role of STAT1 is the activation of Th1 production of T-bet and IFNγ. Analysis of the expression of IFNγ and STAT1 showed that the persistent suppression of the IFNγ/IP-10 was correlated with low STAT1 protein levels at 6 mo. Taken together, the low ratios of CXCR3/CCR4 and Tbet/Gata3, as well as the defective IFNγ/STAT1 expression, indicate a prolonged Th2 dominance 6 mo after irradiation. Experiments with STAT1 knockout mice have demonstrated the crucial importance of this protein for macrophage activation and NK cytolytic activity *in vivo*: as a consequence, these mice have elevated susceptibility to viral and bacterial infections[33,34]. The differentiation of the IFNγ-producing Th1 cells is crucial for the resistance to intracellular infections, notably by "alternatively activated macrophage" inactivation.

Some reports describe families of cytokines that induce inhibition of the STAT1 signal cascade, such as SOCS. SOCS3 impairs IFNγ-induced STAT1-dependent gene activation[35]. We recently showed that acute irradiation induces SOCS3 overexpression[36]. In this study, immunoblot analysis showed that the protein levels of SOCS3 remained high in the later post-irradiation phase. Berlato *et al*[37] reported that constitutive expression of SOCS3 diminishes the quantities of TNFα and NO produced by the transduced cells in response to LPS stimulation. Crespo *et al*[38] confirmed that SOCS3 inhibits transcription of the iNOS gene, apparently by suppressing interaction between STAT1α and IFNγ. SOCS3 might also block the signaling pathways required to activate the posttranscriptional mechanisms that regulate TNFα synthesis[39]. Thus, the low levels of MCP1 and iNOS and the unchanged amount of TNFα observed 6 mo after irradiation suggest a possible role for SOCS3 in the suppression of these genes.

Previous studies report discovered that IL-10 rapidly induces SOCS3 in a STAT3-dependent manner[40,41]. Because SOSC3 can negatively regulate responses to different activating cytokines and bacterial products, it is speculated that the anti-inflammatory action of IL-10 is due to induction of SOCS3[42]. Here, however, our analysis of the expression of IL-10/STAT3 signaling shows that the irradiation-induced SOCS3 overexpression was instead associated with suppression of IL-10 mRNA and STAT3 protein. Berlato *et al*[37] found previously that constitutive expression of SOCS3 can block the capacity of IL-10 to activate STAT3, and Kinjyo *et al*[43] showed that STAT3 is hyperactivated in SOCS3-deficient T cells during T cell differentiation.

The development and persistence of chronic inflammatory disorders such as inflammatory bowel disease can be induced by defects in IL-10 production or in STAT3 signaling molecules or by overexpression of SOCS3[44]. Accordingly, suppression of T-cell expression of SOCS3 and the identification of this protein intracellular target may be the mechanisms to introduce tolerance and thus prevent the immune dysregulation induced by radiotherapy protocols. These results raise the question whether the presence of Th2 immune cells in the intestines after irradiation (acute and delayed) may result from changes in the dynamics of lymphocyte recirculation, involving the alteration of the microvasculature and the presence of differential chemokines.

COMMENTS

Background

The use of radiation therapy to treat cancer inevitably involves exposure of normal tissues. Gastrointestinal symptoms after pelvic radiotherapy, which affect quality of life, are substantially more common than generally recognised and are frequently poorly managed. Patients may experience symptoms associated with damage to normal tissue during the course of abdominal therapy for a few

weeks after therapy or months or years later. In fact, it is not known what venet occurring at an early stage may predispose to significant changes later on.

Research frontiers

In this article, it is shown that immune mechanism alterations may assume greater importance during the early irradiation. Experimentally carried out in the rat model, fractionated irradiation modified the T helper cell polarization, as shown by Th1 cytokine (IFNγ) repression in the colon. This shift occurred *via* transcriptional regulation of the level of cytokine mediators, *via* modification of specific signal transducers of IFN signalling and a through the secretion of a feed-back inhibitor of Th1 polarization, thus potentiating the Th2 profile.

Innovations and breakthroughs

The major result is that the immunity alteration persisted in long term, i.e. after the end of the radiotherapy protocol. These data raise the question whether the post-irradiation Th2 polarization in the intestine (both acute and delayed) may result from changes in the dynamics of lymphocyte recirculation.

Applications

Although the irradiation-induced immune damage has not yet been studied in great detail, a good understanding of this immunity alteration and the relationship between acute and late effects may motivate clinicians to look more often at methods of decreasing tissue toxicity, thus contributing to ameliorate the patents' quality of life after radiotherapy. In radiotherapy, priority needs to be given to assessing simple methods of preventing bowel toxicity, without compromising the control of the tumor.

Peer review

The paper is technically well done and well written and presents novel data.

REFERENCES

1 **Stone HB**, Coleman CN, Anscher MS, McBride WH. Effects of radiation on normal tissue: consequences and mechanisms. *Lancet Oncol* 2003; **4**: 529-536

2 **Andreyev HJ**. Gastrointestinal problems after pelvic radiotherapy: the past, the present and the future. *Clin Oncol* (R Coll Radiol) 2007; **19**: 790-799

3 **Dorr W**, Hendry JH. Consequential late effects in normal tissues. *Radiother Oncol* 2001; **61**: 223-231

4 **McBride WH**, Chiang CS, Olson JL, Wang CC, Hong JH, Pajonk F, Dougherty GJ, Iwamoto KS, Pervan M, Liao YP. A sense of danger from radiation. *Radiat Res* 2004; **162**: 1-19

5 **Linard C**, Marquette C, Mathieu J, Pennequin A, Clarencon D, Mathe D. Acute induction of inflammatory cytokine expression after gamma-irradiation in the rat: effect of an NF-kappaB inhibitor. *Int J Radiat Oncol Biol Phys* 2004; **58**: 427-434

6 **Hong JH**, Chiang CS, Tsao CY, Lin PY, McBride WH, Wu CJ. Rapid induction of cytokine gene expression in the lung after single and fractionated doses of radiation. *Int J Radiat Biol* 1999; **75**: 1421-1427

7 **Strup-Perrot C**, Mathe D, Linard C, Violot D, Milliat F, Francois A, Bourhis J, Vozenin-Brotons MC. Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs, and TIMPs in late radiation enteritis. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G875-G885

8 **MacDonald TT**, Monteleone G, Pender SL. Recent developments in the immunology of inflammatory bowel disease. *Scand J Immunol* 2000; **51**: 2-9

9 **Park HR**, Jo SK, Paik SG. Factors effecting the Th2-like immune response after gamma-irradiation: low production of IL-12 heterodimer in antigen-presenting cells and small expression of the IL-12 receptor in T cells. *Int J Radiat Biol* 2005; **81**: 221-231

10 **Han SK**, Song JY, Yun YS, Yi SY. Effect of gamma radiation on cytokine expression and cytokine-receptor mediated STAT activation. *Int J Radiat Biol* 2006; **82**: 686-697

11 **Gremy O**, Benderitter M, Linard C. Caffeic acid phenethyl ester modifies the Th1/Th2 balance in ileal mucosa after gamma-irradiation in the rat by modulating the cytokine pattern. *World J Gastroenterol* 2006; **12**: 4996-5004

12 **Westermann W**, Schobl R, Rieber EP, Frank KH. Th2 cells as effectors in postirradiation pulmonary damage preceding fibrosis in the rat. *Int J Radiat Biol* 1999; **75**: 629-638

13 **Moser B**, Loetscher P. Lymphocyte traffic control by chemokines. *Nat Immunol* 2001; **2**: 123-128

14 **Garcia-Lopez MA**, Sanchez-Madrid F, Rodriguez-Frade JM, Mellado M, Acevedo A, Garcia MI, Albar JP, Martinez C, Marazuela M. CXCR3 chemokine receptor distribution in normal and inflamed tissues: expression on activated lymphocytes, endothelial cells, and dendritic cells. *Lab Invest* 2001; **81**: 409-418

15 **Bonecchi R**, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998; **187**: 129-134

16 **Ritz SA**, Cundall MJ, Gajewska BU, Swirski FK, Wiley RE, Alvarez D, Coyle AJ, Stampfli MR, Jordana M. The lung cytokine microenvironment influences molecular events in the lymph nodes during Th1 and Th2 respiratory mucosal sensitization to antigen in vivo. *Clin Exp Immunol* 2004; **138**: 213-220

17 **Langberg CW**, Hauer-Jensen M, Sung CC, Kane CJ. Expression of fibrogenic cytokines in rat small intestine after fractionated irradiation. *Radiother Oncol* 1994; **32**: 29-36

18 **Egwuagu CE**, Yu CR, Zhang M, Mahdi RM, Kim SJ, Gery I. Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. *J Immunol* 2002; **168**: 3181-3187

19 **Federici M**, Giustizieri ML, Scarponi C, Girolomoni G, Albanesi C. Impaired IFN-gamma-dependent inflammatory responses in human keratinocytes overexpressing the suppressor of cytokine signaling 1. *J Immunol* 2002; **169**: 434-442

20 **Schreiber S**, Rosenstiel P, Hampe J, Nikolaus S, Groessner B, Schottelius A, Kuhbacher T, Hamling J, Folsch UR, Seegert D. Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. *Gut* 2002; **51**: 379-385

21 **Kinjyo I**, Inoue H, Hamano S, Fukuyama S, Yoshimura T, Koga K, Takaki H, Himeno K, Takaesu G, Kobayashi T, Yoshimura A. Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor-beta 1. *J Exp Med* 2006; **203**: 1021-1031

22 **Denham JW**, Hauer-Jensen M. The radiotherapeutic injury--a complex 'wound'. *Radiother Oncol* 2002; **63**: 129-145

23 **Huang FP**, Niedbala W, Wei XQ, Xu D, Feng GJ, Robinson JH, Lam C, Liew FY. Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. *Eur J Immunol* 1998; **28**: 4062-4070

24 **Guihot G**, Guimbaud R, Bertrand V, Narcy-Lambare B, Couturier D, Duee PH, Chaussade S, Blachier F. Inducible nitric oxide synthase activity in colon biopsies from inflammatory areas: correlation with inflammation intensity in patients with ulcerative colitis but not with Crohn's disease. *Amino Acids* 2000; **18**: 229-237

25 **Freeman SL**, Hossain M, MacNaughton WK. Radiation-induced acute intestinal inflammation differs following total-body versus abdominopelvic irradiation in the ferret. *Int J Radiat Biol* 2001; **77**: 389-395

26 **Speyer CL**, Neff TA, Warner RL, Guo RF, Sarma JV, Riedemann NC, Murphy ME, Murphy HS, Ward PA. Regulatory effects of iNOS on acute lung inflammatory responses in mice. *Am J Pathol* 2003; **163**: 2319-2328

27 **Desai A**, Miller MJ, Huang X, Warren JS. Nitric oxide modulates MCP-1 expression in endothelial cells: implications for the pathogenesis of pulmonary granulomatous vasculitis. *Inflammation* 2003; **27**: 213-223

28 **Setoguchi K**, Takeya M, Akaike T, Suga M, Hattori R, Maeda H, Ando M, Takahashi K. Expression of inducible nitric oxide synthase and its involvement in pulmonary granulomatous inflammation in rats. *Am J Pathol* 1996; **149**:

2005-2022
29 **Gordon S**. Alternative activation of macrophages. *Nat Rev Immunol* 2003; **3**: 23-35
30 **Lawrence CE**, Paterson JC, Wei XQ, Liew FY, Garside P, Kennedy MW. Nitric oxide mediates intestinal pathology but not immune expulsion during Trichinella spiralis infection in mice. *J Immunol* 2000; **164**: 4229-4234
31 **Gu L**, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 2000; **404**: 407-411
32 **Lord GM**, Rao RM, Choe H, Sullivan BM, Lichtman AH, Luscinskas FW, Glimcher LH. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. *Blood* 2005; **106**: 3432-3439
33 **Meraz MA**, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M, Schreiber RD. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 1996; **84**: 431-442
34 **Lee CK**, Rao DT, Gertner R, Gimeno R, Frey AB, Levy DE. Distinct requirements for IFNs and STAT1 in NK cell function. *J Immunol* 2000; **165**: 3571-3577
35 **Ekchariyawat P**, Pudla S, Limposuwan K, Arjcharoen S, Sirisinha S, Utaisincharoen P. Burkholderia pseudomallei-induced expression of suppressor of cytokine signaling 3 and cytokine-inducible src homology 2-containing protein in mouse macrophages: a possible mechanism for suppression of the response to gamma interferon stimulation. *Infect Immun* 2005; **73**: 7332-7339
36 **Linard C**, Gremy O, Benderitter M. Reduction of peroxisome proliferation-activated receptor gamma expression by gamma-irradiation as a mechanism contributing to inflammatory response in rat colon: modulation by the 5-aminosalicylic acid agonist. *J Pharmacol Exp Ther* 2008; **324**: 911-920
37 **Berlato C**, Cassatella MA, Kinjyo I, Gatto L, Yoshimura A, Bazzoni F. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. *J Immunol* 2002; **168**: 6404-6411
38 **Crespo A**, Filla MB, Murphy WJ. Low responsiveness to IFN-gamma, after pretreatment of mouse macrophages with lipopolysaccharides, develops via diverse regulatory pathways. *Eur J Immunol* 2002; **32**: 710-719
39 **Kontoyiannis D**, Kotlyarov A, Carballo E, Alexopoulou L, Blackshear PJ, Gaestel M, Davis R, Flavell R, Kollias G. Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. *EMBO J* 2001; **20**: 3760-3770
40 **Ding Y**, Chen D, Tarcsafalvi A, Su R, Qin L, Bromberg JS. Suppressor of cytokine signaling 1 inhibits IL-10-mediated immune responses. *J Immunol* 2003; **170**: 1383-1391
41 **Williams L**, Bradley L, Smith A, Foxwell B. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol* 2004; **172**: 567-576
42 **Donnelly RP**, Dickensheets H, Finbloom DS. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *J Interferon Cytokine Res* 1999; **19**: 563-573
43 **Kinjyo I**, Inoue H, Hamano S, Fukuyama S, Yoshimura T, Koga K, Takaki H, Himeno K, Takaesu G, Kobayashi T, Yoshimura A. Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor-beta 1. *J Exp Med* 2006; **203**: 1021-1031
44 **Suzuki A**, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, Akira S, Matsumoto S, Toyonaga A, Sata M, Yoshimura A. CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. *J Exp Med* 2001; **193**: 471-481

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CLINICAL RESEARCH

Web-based system for training and dissemination of a magnification chromoendoscopy classification

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Abstract

AIM: To evaluate the use of web-based technologies to assess the learning curve and reassess reproducibility of a simplified version of a classification for gastric magnification chromoendoscopy (MC).

METHODS: As part of a multicenter trial, a hybrid approach was taken using a CD-ROM, with 20 films of MC lasting 5 s each and an "autorun" file triggering a local HTML frameset referenced to a remote questionnaire through an Internet connection. Three endoscopists were asked to prospectively and independently classify 10 of these films randomly selected with at least 3 d apart. The answers were centrally stored and returned to participants together with adequate feedback with the right answer.

RESULTS: For classification in 3 groups, both intra- [Cohen's kappa (κ) = 0.79-1.00 to 0.89-1.00] and inter-observer agreement increased from 1st (moderate) to 6th observation (κ = 0.94). Also, agreement with reference increased in the last observations (0.90, 1.00 and 1.00, for observers A, B and C, respectively). Validity of 100% was obtained by all observers at their 4th observation. When a 4th (sub)group was considered, inter-observer agreement was almost perfect (κ = 0.92) at 6th observation. The relation with reference clearly improved into κ (0.93-1.00) and sensitivity (75%-100%) at their 6th observations.

CONCLUSION: This MC classification seems to be easily explainable and learnable as shown by excellent intra- and inter-observer agreement, and improved agreement with reference. A web system such as the one used in this study may be useful for endoscopic or other image based diagnostic procedures with respect to definition, education and dissemination.

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Key words: Magnification; Chromoendoscopy; Reproducibility; Learning curve

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INTRODUCTION

The dissemination and teaching of image based medical technologies depend on adequate training. Mostly, medical doctors perform specific training by visiting experts. New information technologies, namely those based on the internet, may circumvent such difficulties at least at early phases of training.

Gastric cancer is the second most lethal cancer in the World. Early stages at diagnosis are related to better prognosis[1]. Minute flat non-invasive neoplastic lesions

(dysplasia)[2] may be found during screening programs (in Japan) or during the follow-up of patients with atrophic chronic gastritis (ACG) or intestinal metaplasia (IM)-the milieu where neoplastic changes develop[3-6]. However, for patients with lesions such as ACG or IM there is not a definite proposal for their management. The difficulty in proposing a guide of practice for the management of patients with atrophic chronic gastritis and intestinal metaplasia may be related to the fact that conventional endoscopy used in most studies shows a low reproducibility and poor relation with histology at diagnosing these diffuse mucosal changes and minute lesions of cancer[7,8]. For the last ten years, several studies considered magnification and high-resolution endoscopy in conjunction with chromoendoscopy for the diagnosis of precancerous[9-14] and neoplastic lesions[15-22], in the gastrointestinal tract. However, mostly authors focused on the validity assessment and rarely were reliability or the learning of each group description defined[16,17]. Furthermore, several classifications were defined and the need for standardization stressed[23]. In fact, aimed at improving the evaluation of patients with precancerous gastric conditions, our own group described a classification for the diagnosis of intestinal metaplasia and minute dysplastic lesions using magnification chromoendoscopy with methylene blue[17].

As part of a multicenter trial, the training of endoscopists and teaching of this classification was planned using a web-based system. This manuscript reports the feasibility of such a system for the learning and dissemination of endoscopic classifications.

MATERIALS AND METHODS

Study design

Three endoscopists (A, B and C), independently and blinded to other endoscopists' answers, were asked to prospectively classify 20 endoscopic videos of magnification chromoendoscopy using a web-based learning system, a hybrid system composed of a CD-ROM and a dynamic website connected to a database, aiming at classifying each video 6 times (1st to 6th time).

Endoscopic videos selection

Endoscopic videos were selected according to a modified version of a magnification chromoendoscopy pattern classification of gastric mucosa[17]. For video selection the records of magnification chromo-endoscopy with methylene blue (1%) using an Olympus Q240Z magnification endoscope (Olympus Corp., Tokyo, Japan), performed in a cohort of patients under follow-up at our institution, were used[6]. Videos were recorded using a S-Video interface wih a digital DVCAM Sony Recorder (DSR-20MDP, Sony, Tokyo, Japan). Endoscopic patterns were obtained using the maximum magnification power possible with this endoscope, defined according to differences in color and homogeneity: Group I definition was when the mucosa showed a regular mucosal pattern and no change in color after staining with methylene blue; Group Ⅱ if the mucosa presented a regular pattern and was stained in blue. Subgroup Ⅱ E, for areas of mucosa with blue irregular marks [initially (Dinis Ribeiro GIE 2003) called Ⅱ A] or blue round and tubular pits (Ⅱ B); and subgroup Ⅱ F when blue villi (formerly Ⅱ C) or blue small pits (Ⅱ D) were described in the observed mucosa; Group Ⅲ was the definition if neither a clear pattern nor a change in color (heterogeneous staining) were noticeable.

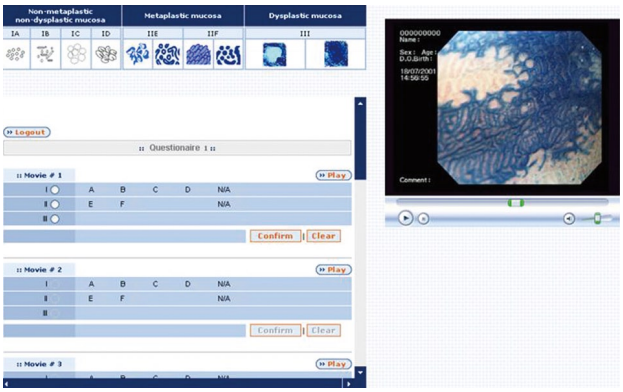

Figure 1 Graphical user interface for movie classification. The right frame is used to play a video clip loaded from the distributed CD; the left frame shows the patterns at the top and a form to retrieve user classifications.

Web-based learning system

The expected download time for each 5 s film (Windows Media Player video clips with 36 Mbytes) of about 120 min (at a 56 Kbits/s connection) and the user's physical location were instrumental in choosing the hybrid system architecture[24,25]. Using an Internet connection, a CD-ROM including all 20 selected endoscopic movies and an "autorun" file used to trigger a local HTML frameset (with two frames) referenced to the remote questionnaire on the classification of each film were developed for this project (on the left)[26]. The right frame was used to play the films stored on the CD-ROM (Figure 1). At the top, a schematic representation of each pattern was always visible.

Each endoscopist was asked to classify 10 videos randomly selected from the 20 videos included in the CD, with a minimum interval of 3 d. Before classifying each video, the user could run the film as many times as necessary before the decision was taken. After deciding, the user had to lock his answer in order to advance to the next question, not allowing subsequent videos to influence previous responses. After each questionnaire, ie for each 10 film sets classified, their answer or classification together with the proposed answer (to be used as reference, see below) was returned to participants. By this time, all videos included in that set could again be seen.

The HTML questionnaire was stored on an Oracle database using a PHP script. The web-server is run on RedHat Linux 7.2 (Enigma), Apache 1.3, PHP 4.0 compiled with GD Graphics Library 1.8 and Oracle 8 DBMS. Answers were centrally stored on an Oracle database using a Hypertext Pre-Processor (PHP) script.

Endoscopists

Endoscopists (M.A., P.A., R.S.) were invited to participate as they belonged to two different centers (POI

Table 1 Reproducibility for the classification in groups (Ⅰ *vs* Ⅱ *vs* Ⅲ) and in subgroups (Ⅰ *vs* ⅡE *vs* ⅡF *vs* Ⅲ) according to number of times observers (A, B and C) classified the films of magnification chromoendoscopy

Number of classification (nth)	Inter-observer agreement (95% CI)	
	Proportion of agreement	Weighted kappa
Classification in groups		
1st observation	0.60 (0.36-0.81)	0.52 (0.26-0.75)
2nd observation	0.85 (0.62-0.97)	0.73 (0.53-0.87)
3rd observation	0.85 (0.62-0.97)	0.71 (0.51-0.86)
4th observation	0.95 (0.75-1.00)	0.97 (0.94-0.99)
5th observation	0.80 (0.56-0.94)	0.71 (0.50-0.86)
6th observation	0.90 (0.68-0.99)	0.94 (0.89-0.98)
Classification in subgroups		
1st observation	0.45 (0.23-0.69)	0.49 (0.23-0.73)
2nd observation	0.55 (0.31-0.77)	0.69 (0.48-0.85)
3rd observation	0.45 (0.23-0.69)	0.66 (0.42-0.83)
4th observation	0.70 (0.46-0.88)	0.90 (0.81-0.96)
5th observation	0.60 (0.36-0.81)	0.66 (0.43-0.83)
6th observation	0.75 (0.51-0.91)	0.92 (0.84-0.96)

in Porto, and CUH in Coimbra) inclined to implement this technology, but with no previous experience of it or without previous participation in the development of the classification.

Statistical analysis

For each image, proposed classification (Group Ⅰ, Group Ⅱ, Subgroup ⅡE or ⅡF, or Group Ⅲ) was considered as if another observer would have classified it, and, also, as a reference classification or gold standard. This allowed us to consider both agreement and validity measures, respectively, in the evaluation of reproducibility and learning curve.

Inter-observer agreement and agreement with the reference agreement were estimated using different measures of agreement[27], simple proportions of agreement (Pa) and proportions of specific agreement, and quadratic weighted Cohen's kappa coefficient (Kc) (estimated by intra-class correlation coefficient)[28-33]. The confidence intervals for proportions of agreements were estimated with binomial distribution[33]. Strength of agreement was considered as follows: 0.01-0.2 slight, 0.21-0.4 fair, 0.41-0.6 moderate, 0.61-0.8 substantial, 0.81-1 almost perfect[34]. No bias was observed [McNemar test ($P = 1.0$); bias index (0.117, $P = 0.2891$)][35,36].

Estimates of sensitivity (Se), specificity (Sp) and validity were also calculated comparing the classification for each film against the proposed classified as reference. For classification in groups, true positives were defined if the observer correctly classified each film as group Ⅲ. For classification in subgroups, diagnostic positivity was considered in cases of Subgroups ⅡF and Ⅲ. These options and the decision to use weighted kappa coefficient were based upon the relation of these patterns with both dysplasia and incomplete intestinal metaplasia, named as being high-risk lesions for adenocarcinoma[4-6].

The learning curve was defined by visual analysis of a plot of both validity and agreement measures. Statistical estimates were performed with r-project v2.1.1, SPSS® and MedCalc®.

RESULTS

Reproducibility

Both classification in groups (Ⅰ *vs* Ⅱ *vs* Ⅲ) and subgroups (Ⅰ *vs* ⅡE *vs* ⅡF *vs* Ⅲ) showed substantial to excellent inter-observer agreement. In fact, at 6th observation, proportion of agreement is 0.90 and 0.75 respectively and weighted kappa is 0.94 and 0.94, respectively (Table 1).

As far as intra-observer agreement is concerned this was substantial in all observers, initially from first to second observation ($\kappa = 0.79$ to 1.00), and excellent from 5th to 6th observation (0.89 to 1) considering the classification in Groups (Ⅰ *vs* Ⅱ *vs* Ⅲ); and for classification in subgroups (Ⅰ *vs* ⅡE *vs* ⅡF *vs* Ⅲ), 0.74-0.85 to 0.75-1.00.

Specific proportions of agreement were also very high varying from 0.43, 0.79 and 0.82 (for groups Ⅲ, Ⅱ and Ⅰ, respectively) to 0.96, 0.92 and 0.92 (Ⅲ, Ⅱ and Ⅰ) at last classification. Concerning specific proportions of agreement, only a slight increase was observed from 0.50 and 0.50 (ⅡE and ⅡF) to 0.64 and 0.60 (at last observation).

Learning curve

An increase was observed in both proportions of agreement and kappa values, as far as agreement with original classification was concerned, from moderate to substantial/excellent in all observers (Table 2). Also inter-observer agreement varied from Kc = 0.52 or 0.49, respectively for groups and subgroups classification, from 1st to 6th classification (Figure 2). Excellent agreement was obtained by the 4th time for all observers irrespective of institution or time between classifications, by the time they had evaluated 80 videos.

Also, concerning validity measures, paired sensitivity and specificity of 100% were achieved at 4th classification for all observers, at 4th time for classification in groups and at 6th for classification in subgroups by observer A. Observers B and C achieved a validity of 0.85 and 0.90 at their 6th classification (Figure 2).

DISCUSSION

The concept of 'learning by doing' in invasive procedures such as endoscopy, even though current and acceptable, may be affected by the continuous research in this field leading to new endoscopes and gastrointestinal mucosal description availability.

In a preliminary form, we have described the feasibility of a hybrid approach of Internet and CD-ROM/DVD technology as a web-based education system[37]. Such desktop virtual reality systems[38] were described in several fields of knowledge[24,25] as recently in endoscopy by de Lange[39].

According to our study, the classification proposed is both easily explainable and learnable. The simplicity of this classification, the fact that it includes in the instrument description the phenomenon itself (i.e. intestinal metaplasia) and the feedback given to each observer at the end of a single classification[40,41] may

Table 2 Agreement with reference and validity measures for the classification in groups (Ⅰ *vs* Ⅱ *vs* Ⅲ) and in subgroups (Ⅰ *vs* ⅡE *vs* ⅡF *vs* Ⅲ) according to number of times observers (A, B and C) classified the films of magnification chromoendoscopy (95% CI)

	Classification in groups Ⅰ *vs* Ⅱ *vs* Ⅲ					Classification in Subgroups Ⅰ *vs* ⅡE *vs* ⅡF *vs* Ⅲ				
	Pa	wK	Se	Sp	V	Pa	wK	Se	Sp	V
Observer A										
1st observation	0.90 (0.68-0.99)	0.66 (0.32-0.85)	0.75 (0.56-0.94)	0.94 (0.83-1.00)	0.90 (0.77-1.00)	0.75 (0.51-0.91)	0.63 (0.29-0.84)	0.75 (0.56-0.94)	0.75 (0.56-0.94)	0.75 (0.56-0.94)
2nd observation	0.95 (0.75-1.00)	0.84 (0.64-0.93)	1.00	0.94 (0.83-1.00)	0.95 (0.85-1.00)	0.70 (0.46-0.88)	0.76 (0.50-0.90)	0.75 (0.56-0.94)	0.67 (0.46-0.88)	0.70 (0.50-0.90)
3rd observation	0.90 (0.68-0.99)	0.79 (0.54-0.91)	1.00	0.94 (0.83-1.00)	0.95 (0.85-1.00)	0.80 (0.56-0.94)	0.79 (0.55-0.91)	1.00	0.75 (0.56-0.94)	0.85 (0.69-1.00)
4th observation	1.00 (0.86-1.00)	1.00	1.00	1.00	1.00	0.90 (0.68-0.99)	0.97 (0.92-0.99)	1.00	0.83 (0.67-1.00)	0.90 (0.85-1.00)
5th observation	0.95 (0.75-1.00)	0.83 (0.63-0.93)	1.00	0.94 (0.83-1.00)	0.95 (0.85-1.00)	0.95 (0.75-1.00)	0.85 (0.66-0.94)	1.00	0.92 (0.79-1.00)	0.95 (0.85-1.00)
6th observation	1.00 (0.86-1.00)	1.00	1.00	1.00	1.00	1.00 (0.86-1.00)	1.00	1.00	1.00	1.00
Observer B										
1st observation	0.90 (0.51-0.91)	0.80 (0.57-0.92)	0.75 (0.56-0.94)	1.00	0.95 (0.85-1.00)	0.65 (0.41-0.85)	0.71 (0.42-0.88)	0.50 (0.28-0.72)	0.83 (0.67-1.00)	0.70 (0.50-0.90)
2nd observation	0.90 (0.68-0.99)	0.77 (0.52-0.90)	0.75 (0.56-0.94)	1.00	0.95 (0.85-1.00)	0.80 (0.56-0.94)	0.78 (0.52-0.90)	0.75 (0.56-0.94)	0.92 (0.79-1.00)	0.85 (0.69-1.00)
3rd observation	0.90 (0.68-0.99)	0.77 (0.52-0.90)	0.75 (0.56-0.94)	1.00	0.95 (0.85-1.00)	0.70 (0.46-0.88)	0.69 (0.37-0.86)	0.63 (0.41-0.84)	0.75 (0.56-0.94)	0.70 (0.50-0.90)
4th observation	0.95 (0.75-1.00)	0.96 (0.90-0.98)	1.00	1.00	1.00	0.65 (0.41-0.85)	0.82 (0.60-0.92)	0.5 (0.28-0.72)	0.75 (0.56-0.94)	0.65 (0.44-0.86)
5th observation	0.90 (0.68-0.99)	0.77 (0.52-0.90)	0.75 (0.56-0.94)	1.00	0.95 (0.85-1.00)	0.65 (0.41-0.85)	0.73 (0.45-0.88)	0.63 (0.41-0.84)	0.92 (0.79-1.00)	0.80 (0.62-0.98)
6th observation	1.00 (0.86-1.00)	1.00	0.75 (0.56-0.94)	1.00	1.00	0.85 (0.62-0.97)	0.95 (0.87-0.98)	0.75 (0.56-0.94)	0.92 (0.79-1.00)	0.85 (0.69-1.00)
Observer C										
1st observation	0.75 (0.51-0.91)	0.80 (0.57-0.92)	0.75 (0.56-0.94)	0.81 (0.64-0.99)	0.80 (0.62-0.98)	0.60 (0.36-0.81)	0.82 (0.60-0.92)	0.75 (0.56-0.94)	0.83 (0.67-1.00)	0.80 (0.62-0.98)
2nd observation	0.95 (0.75-1.00)	0.96 (0.89-1.00)	1.00	1.00	1.00	0.60 (0.36-0.81)	0.85 (0.67-0.94)	0.50 (0.28-0.72)	0.75 (0.56-0.94)	0.65 (0.44-0.86)
3rd observation	0.85 (0.62-0.97)	0.72 (0.42-0.88)	1.00	0.84 (0.67-1.00)	0.85 (0.69-1.00)	0.50 (0.27-0.73)	0.65 (0.31-0.84)	0.38 (0.16-0.59)	0.75 (0.56-0.94)	0.60 (0.38-0.82)
4th observation	0.95 (0.75-1.00)	0.96 (0.89-0.98)	1.00	1.00	1.00	0.70 (0.46-0.88)	0.89 (0.74-0.95)	0.5 (0.28-0.72)	0.92 (0.79-1.00)	0.75 (0.56-0.94)
5th observation	0.85 (0.62-0.97)	0.74 (0.47-0.89)	0.75 (0.56-0.94)	0.94 (0.83-1.00)	0.90 (0.77-1.00)	0.65 (0.41-0.85)	0.73 (0.45-0.88)	0.63 (0.41-0.84)	0.83 (0.67-1.00)	0.75 (0.56-0.94)
6th observation	0.90 (0.68-0.99)	0.92 (0.81-0.97)	1.00	0.94 (0.83-1.00)	0.95 (0.85-1.00)	0.80 (0.56-0.94)	0.93 (0.84-0.97)	0.88 (0.73-1.00)	0.92 (0.79-1.00)	0.90 (0.77-1.00)

Pa: Proportion of agreement; wK: Weighted kappa, Se: Sensitivity; V: Validity.

justify the excellent results.

Learning curves for most procedures concern efficacy and time to achievement of such efficacy. For example, in surgical procedures how fast trainees achieve the ability to get surgery adequately performed without complications[42,43]. Also in endoscopy some reports use colonoscopy models[44] and endoscopic ultrasound fine needle aspiration[45] with similar methodology.

A single report exists on the learning curve for the diagnostic performance of endoscopy. Besides simplification of any visual categorization, Tung and Tagashi defined the need of a steep learning curve for magnifying colonoscopy. They used for that evaluation the evolution of validity measures, that is sensitivity, specificity and global accuracy. However, there is no one particular statistical procedure for learning assessment, to be named in diagnostic technologies outcomes as a measure of reality[46].

Diagnostic procedures are aimed at being both valid, ie to measure what they are supposed to. However, even though most studies concern validity assessment, reliability of a measure should be a condition to be verified before any other quality feature. For dichotomic, nominal or ordinal variables, proportion of agreement (Pa) or Kappa statistics may be used. Pa is easily acceptable and interpretable. However, it is not corrected to the amount of agreement that was expected by chance (Pe)[47]. With the aim of solving this problem, Cohen developed the named Kappa statistics (Kc); a method which takes into consideration the so-called agreement by chance. In ordinal variables, distance from total agreement may be weighted, either linearly or exponentially[48]. Therefore, kappa is a global index of agreement and easy to calculate. However, some concern has been raised by others[48,49]. Cohen's Kappa varies with the distribution of cases for each category, namely as far as the total number of cases or prevalence[50] and if unbalanced marginal totals is present. Additionally, bias can influence kappa's interpretation[51]. Therefore, some authors recommend the estimation of both prevalence

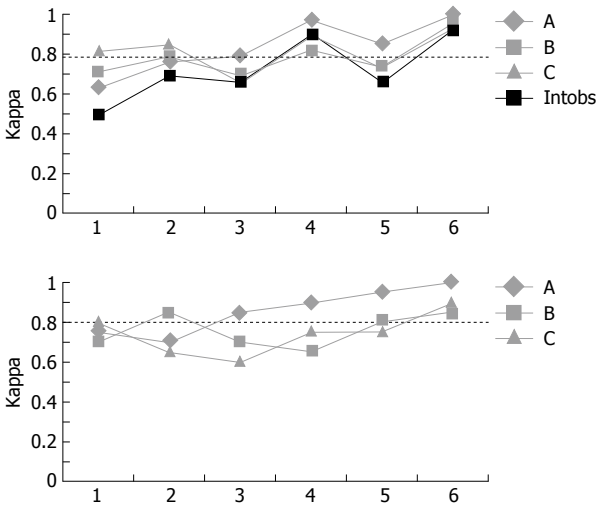

Figure 2 Variation of agreement with reference (Kappa) and inter-observer agreement (Black) (top graphic) and validity (bottom graphic) along sequential observations (1st to 6th) for the classification in subgroups [dashed line marks for 0.80 as the cutoff for almost perfect agreement (top graphic) and validity (bottom graphic)].

and bias adjusted kappa[35,36] and others advocate the use of either McNemar's test or the bias index (proportion of deviated ratings) to assess bias, following which Cohen's kappa could then be used. It seems reasonable to consider that both agreement measures (proportion of agreement and weighted Cohen's kappa) and validity measures. Thus, the proposed original classification could be considered either as the classification by a different observer and agreement with it by each observer would be evaluated for reliability or it may be considered as reference and common measures of validity would be used similarly to the paper of Tung and Tagashi.

In the present study, although 20 selected non-consecutive films were assessed, no observer bias was noticeable and the fact that all categories of classification groups were included for evaluation, may allow us, even though cautiously, to consider our classification of gastric mucosa both reliable and easily learnable.

The follow-up of patients with atrophic chronic gastritis and intestinal metaplasia[52,53] may lead to early diagnosis of gastric neoplastic lesions and improvement of patients' prognosis. Following the non-existence of distinctive symptoms[54-56], most authors based their studies (mostly) on morphologic evaluation through endoscopically performed multiple biopsies, because of the patchy characteristics of atrophic chronic gastritis and intestinalization of gastric mucosa[57-59]. However, with the exception of atrophic vascularization, most studies found that for conventional endoscopy, descriptions of 'gastritis' showed suboptimal validity[60-63] and unsatisfactory reliability[7,8,63].

New endoscopic methods are expected to optimise both the identification, in a (more) reproducible and valid measure for such lesions-'optic biopsy'. An increasing number of expert opinion texts, reviews and studies report the use of magnification chromoendoscopy through the gastrointestinal tract.

As far as colorectal lesions are concerned, in 1996 Kudo *et al*[15] defined a 7 patterns classification (type Ⅰ, Ⅱ, Ⅲs, ⅢL, Ⅳ, Vn, Vi) that showed consistently good sensitivity but highly heterogeneous results in its specificity[15,22]. Eight years have past and recently reproducibility was demonstrated[16], in an altered simplified three patterns classification with management consequences or prognosis implications: I and Ⅱ as non-neoplastic; ⅢL and IV as neoplastic; and Ⅲs and V as neoplastic possibly invasive.

However, in upper gastrointestinal tract, both for Barrett's mucosa and stomach mucosa, diverse classifications have been published and the need for their standardization stressed[23].

Endo *et al*[10] and Yagi *et al*[21] using methylene blue, Guelrud *et al*[12,14] and Toyoda *et al*[64] using acetic acid, and Sharma *et al*[13] using indigo carmine, described features of intestinal metaplasia and Sharma also reported endoscopic dysplasia. Good validity results were published by all authors, but Meining *et al*[11] showed a low inter-observer agreement, both for Endo and for Guelrud classifications (Cohen's kappa of 0.017 and 0.162).

In the stomach, our own group described the use of magnification chromoendoscopy with methylene blue for the diagnosis of intestinal metaplasia and gastric epithelial dysplasia in 2003. We subsequently found that a substantial agreement was observed on the classification of endoscopic images into groups (Ⅰ, Ⅱ, or Ⅲ), both for intra-observer (Pa = 0.91, Kc = 0.86) and for inter-observer agreement (Pa = 0.84, Kc = 0.74)[17]. Hereby, the stomach size and the presence of inflammation were considered limitations for chromoendoscopy and particularly for magnification[65].

However, concurrent results by others working in the field of gastric mucosa were consistent with ours. Recently, Yagi *et al*[66] described aspects for normal antral mucosa and for gastritis with H pylori similar to our group Ⅰ. Also Yang types A through D[67] and Kim types 1 through 3[18] may be compared with our group classification as Group Ⅰ. Furthermore, Kim's type 4 and Yang types D and E are very similar to Subgroups ⅡE and ⅡF. Tajiri *et al*[19,20] stressed that this procedure may have marked impact in the diagnosis of minute neoplastic flat 'gastritis-like' lesions and they described very similar features to our own research group's Group Ⅲ or pattern-less.

This means that, as with Kudo's classification in the colon, the existence of a unique and standardized classification for magnification chromoendoscopy (both in Barrett's and in the stomach) may have contributed to the dissemination of this technique and further use in even newer technologies.

In conclusion, a modified version of our classification for gastric mucosa diffuse changes and minute dysplastic lesions seems to be reliable and easily learnable. The web-based system hereby developed can be used for new diagnostic technology teaching and dissemination and for assessing the similarity between our own and other classifications, with the aim of the achievement of consensus.

ACKNOWLEDGMENTS

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COMMENTS

Background

Dissemination and teaching of image-based medical technologies depend on adequate training. Mostly, medical doctors perform specific training by visiting experts. New information technologies, namely those based on the internet, may circumvent such difficulties at least at early phases of training.

Research frontiers

Concerning digestive endoscopy, several studies addressed and derived classifications for endoscopic descriptions of precancerous and neoplastic lesions in the gastrointestinal tract. Web-based systems could also be used in this setting for dissemination and training.

Innovations and breakthroughs

As part of a multicenter trial, the training of endoscopists and teaching of this classification were planned using a web-based system. This manuscript reports the feasibility of such a system for the learning and dissemination of endoscopic classifications.

Applications

Similar systems could be used in other areas of medical technologies based on image. Furthermore, similar methodologies could even go further by gathering clinical data, other technologies could be used in medical decision analysis.

Peer review

This manuscript specifically addresses a new evaluation of the reliability of a classification for magnification chromoendoscopy. It describes the feasibility of a web-based system to be used as part of endoscopists' training in learning new technologies.

REFERENCES

1 **Levi F**, La Vecchia C, Lucchini F, Negri E. Cancer mortality in Europe, 1990-1992. *Eur J Cancer Prev* 1995; **4**: 389-417
2 **Schlemper RJ**, Riddell RH, Kato Y, Borchard F, Cooper HS, Dawsey SM, Dixon MF, Fenoglio-Preiser CM, Flejou JF, Geboes K, Hattori T, Hirota T, Itabashi M, Iwafuchi M, Iwashita A, Kim YI, Kirchner T, Klimpfinger M, Koike M, Lauwers GY, Lewin KJ, Oberhuber G, Offner F, Price AB, Rubio CA, Shimizu M, Shimoda T, Sipponen P, Solcia E, Stolte M, Watanabe H, Yamabe H. The Vienna classification of gastrointestinal epithelial neoplasia. *Gut* 2000; **47**: 251-255
3 **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992; **52**: 6735-6740
4 **Carneiro F**, Machado JC, David L, Reis C, Nogueira AM, Sobrinho-Simoes M. Current thoughts on the histopathogenesis of gastric cancer. *Eur J Cancer Prev* 2001; **10**: 101-102
5 **Kapadia CR**. Gastric atrophy, metaplasia, and dysplasia: a clinical perspective. *J Clin Gastroenterol* 2003; **36**: S29-S36; discussion S61-S62
6 **Dinis-Ribeiro M**, Lopes C, da Costa-Pereira A, Guilherme M, Barbosa J, Lomba-Viana H, Silva R, Moreira-Dias L. A follow up model for patients with atrophic chronic gastritis and intestinal metaplasia. *J Clin Pathol* 2004; **57**: 177-182
7 **Laine L**, Cohen H, Sloane R, Marin-Sorensen M, Weinstein WM. Interobserver agreement and predictive value of endoscopic findings for H. pylori and gastritis in normal volunteers. *Gastrointest Endosc* 1995; **42**: 420-423
8 **Belair PA**, Metz DC, Faigel DO, Furth EE. Receiver operator characteristic analysis of endoscopy as a test for gastritis. *Dig Dis Sci* 1997; **42**: 2227-2233
9 **Bruno MJ**. Magnification endoscopy, high resolution endoscopy, and chromoscopy; towards a better optical diagnosis. *Gut* 2003; **52** Suppl 4: iv7-i11
10 **Endo T**, Awakawa T, Takahashi H, Arimura Y, Itoh F, Yamashita K, Sasaki S, Yamamoto H, Tang X, Imai K. Classification of Barrett's epithelium by magnifying endoscopy. *Gastrointest Endosc* 2002; **55**: 641-647
11 **Meining A**, Rosch T, Kiesslich R, Muders M, Sax F, Heldwein W. Inter- and intra-observer variability of magnification chromoendoscopy for detecting specialized intestinal metaplasia at the gastroesophageal junction. *Endoscopy* 2004; **36**: 160-164
12 **Guelrud M**, Herrera I, Essenfeld H, Castro J. Enhanced magnification endoscopy: a new technique to identify specialized intestinal metaplasia in Barrett's esophagus. *Gastrointest Endosc* 2001; **53**: 559-565
13 **Sharma P**, Weston AP, Topalovski M, Cherian R, Bhattacharyya A, Sampliner RE. Magnification chromoendoscopy for the detection of intestinal metaplasia and dysplasia in Barrett's oesophagus. *Gut* 2003; **52**: 24-27
14 **Guelrud M**, Herrera I, Essenfeld H, Castro J, Antonioli DA. Intestinal metaplasia of the gastric cardia: A prospective study with enhanced magnification endoscopy. *Am J Gastroenterol* 2002; **97**: 584-589
15 **Kudo S**, Tamura S, Nakajima T, Yamano H, Kusaka H, Watanabe H. Diagnosis of colorectal tumorous lesions by magnifying endoscopy. *Gastrointest Endosc* 1996; **44**: 8-14
16 **Huang Q**, Fukami N, Kashida H, Takeuchi T, Kogure E, Kurahashi T, Stahl E, Kudo Y, Kimata H, Kudo SE. Interobserver and intra-observer consistency in the endoscopic assessment of colonic pit patterns. *Gastrointest Endosc* 2004; **60**: 520-526
17 **Dinis-Ribeiro M**, da Costa-Pereira A, Lopes C, Lara-Santos L, Guilherme M, Moreira-Dias L, Lomba-Viana H, Ribeiro A, Santos C, Soares J, Mesquita N, Silva R, Lomba-Viana R. Magnification chromoendoscopy for the diagnosis of gastric intestinal metaplasia and dysplasia. *Gastrointest Endosc* 2003; **57**: 498-504
18 **Kim S**, Harum K, Ito M, Tanaka S, Yoshihara M, Chayama K. Magnifying gastroendoscopy for diagnosis of histologic gastritis in the gastric antrum. *Dig Liver Dis* 2004; **36**: 286-291
19 **Tajiri H**, Doi T, Endo H, Nishina T, Terao T, Hyodo I, Matsuda K, Yagi K. Routine endoscopy using a magnifying endoscope for gastric cancer diagnosis. *Endoscopy* 2002; **34**: 772-777
20 **Tajiri H**, Ohtsu A, Boku N, Muto M, Chin K, Matsumoto S, Yoshida S. Routine endoscopy using electronic endoscopes for gastric cancer diagnosis: retrospective study of inconsistencies between endoscopic and biopsy diagnoses. *Cancer Detect Prev* 2001; **25**: 166-173
21 **Yagi K**, Nakamura A, Sekine A. Accuracy of magnifying endoscopy with methylene blue in the diagnosis of specialized intestinal metaplasia and short-segment Barrett's esophagus in Japanese patients without Helicobacter pylori infection. *Gastrointest Endosc* 2003; **58**: 189-195
22 **Tung SY**, Wu CS, Su MY. Magnifying colonoscopy in differentiating neoplastic from nonneoplastic colorectal lesions. *Am J Gastroenterol* 2001; **96**: 2628-2632
23 **Sharma P**. Magnification endoscopy. *Gastrointest Endosc* 2005; **61**: 435-443
24 **Bacro T**, Gilbertson B, Coultas J. Web-delivery of anatomy video clips using a CD-ROM. *Anat Rec* 2000; **261**: 78-82
25 **Mattheos N**, Nattestad A, Attstrom R. Local CD-ROM in interaction with HTML documents over the Internet. *Eur J Dent Educ* 2000; **4**: 124-127
26 **Cruz-Correia R**, Dinis-Ribeiro M, Fernandes S, Oliveira-Palhares E, Martins C, Costa-Pereira A. ALGA a Web-based gastrointestinal endoscopy learning curve evaluation

system. *Technol Health Care* 2003; **11**: 371-372
27 **Costa Santos C**, Costa Pereira A, Bernardes J. Agreement studies in obstetrics and gynaecology: inappropriateness, controversies and consequences. *BJOG* 2005; **112**: 667-669
28 **Cohen J**. Weighted kappa: Nominal scale agreement with provision for scaled disagreement or partial credit. *Psychol Bull* 1968; **70**: 213-220
29 **Uebersax JS**. A generalized kappa coefficient. *Educ Psychol Meas* 1982; **42**: 181-183
30 **Haley SM**, Osberg JS. Kappa coefficient calculation using multiple ratings per subject: a special communication. *Phys Ther* 1989; **69**: 970-974
31 **Fleiss JL**, Cohen J. The equivalence of weighted kappa and the intraclass correlation coefficient as measures of reliability. *Educ Psychol Meas* 1973; **33**: 613-619
32 **Markus H**, Bland JM, Rose G, Sitzer M, Siebler M. How good is intercenter agreement in the identification of embolic signals in carotid artery disease? *Stroke* 1996; **27**: 1249-1252
33 **Clopper CJ**, Pearson ES. The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika* 1934; **26**: 404-413
34 **Landis JR**, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33**: 159-174
35 **Ludbrook J**. Statistical techniques for comparing measurers and methods of measurement: a critical review. *Clin Exp Pharmacol Physiol* 2002; **29**: 527-536
36 **Ludbrook J**. Detecting systematic bias between two raters. *Clin Exp Pharmacol Physiol* 2004; **31**: 113-115
37 **Dinis-Ribeiro M**, Cruz-Correia R, Santos C, Fernandes S, Tavares C, Palhares E, Silva RA, Amaro P, Areia M, Ponchon T, Costa-Pereira A, Moreira-Dias L. Reproducibility and learning curve for a classification of magnification chromoendoscopy for gastric mucosal lesions-A web based evaluation. *Gastrointest Endosc* 2005; **12**: 15
38 **Vozenilek J**, Huff JS, Reznek M, Gordon JA. See one, do one, teach one: advanced technology in medical education. *Acad Emerg Med* 2004; **11**: 1149-1154
39 **de Lange T**, Svensen AM, Larsen S, Aabakken L. The functionality and reliability of an Internet interface for assessments of endoscopic still images and video clips: distributed research in gastroenterology. *Gastrointest Endosc* 2006; **63**: 445-452
40 **Mahmood T**, Darzi A. The learning curve for a colonoscopy simulator in the absence of any feedback: no feedback, no learning. *Surg Endosc* 2004; **18**: 1224-1230
41 **Rosser JC Jr**, Gabriel N, Herman B, Murayama M. Telementoring and teleproctoring. *World J Surg* 2001; **25**: 1438-1448
42 **Schlachta CM**, Mamazza J, Seshadri PA, Cadeddu M, Gregoire R, Poulin EC. Defining a learning curve for laparoscopic colorectal resections. *Dis Colon Rectum* 2001; **44**: 217-222
43 **Tekkis PP**, Senagore AJ, Delaney CP, Fazio VW. Evaluation of the learning curve in laparoscopic colorectal surgery: comparison of right-sided and left-sided resections. *Ann Surg* 2005; **242**: 83-91
44 **Eversbusch A**, Grantcharov TP. Learning curves and impact of psychomotor training on performance in simulated colonoscopy: a randomized trial using a virtual reality endoscopy trainer. *Surg Endosc* 2004; **18**: 1514-1518
45 **Eloubeidi MA**, Tamhane A. EUS-guided FNA of solid pancreatic masses: a learning curve with 300 consecutive procedures. *Gastrointest Endosc* 2005; **61**: 700-708
46 **Ramsay CR**, Grant AM, Wallace SA, Garthwaite PH, Monk AF, Russell IT. Statistical assessment of the learning curves of health technologies. *Health Technol Assess* 2001; **5**: 1-79
47 **Bland JM**, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; **1**: 307-310
48 **Chmura Kraemer H**, Periyakoil VS, Noda A. Kappa coefficients in medical research. *Stat Med* 2002; **21**: 2109-2129
49 **Thompson WD**, Walter SD. A reappraisal of the kappa coefficient. *J Clin Epidemiol* 1988; **41**: 949-958
50 **Byrt T**, Bishop J, Carlin JB. Bias, prevalence and kappa. *J Clin Epidemiol* 1993; **46**: 423-429
51 **Khan KS**, Chien PF. Evaluation of a clinical test. I: assessment of reliability. *BJOG* 2001; **108**: 562-567
52 **Genta RM**, Rugge M. Gastric precancerous lesions: heading for an international consensus. *Gut* 1999; **45** Suppl 1: I5-I8
53 **Genta RM**. Review article: Gastric atrophy and atrophic gastritis--nebulous concepts in search of a definition. *Aliment Pharmacol Ther* 1998; **12** Suppl 1: 17-23
54 **Westbrook JI**, McIntosh JH, Duggan JM. Accuracy of provisional diagnoses of dyspepsia in patients undergoing first endoscopy. *Gastrointest Endosc* 2001; **53**: 283-288
55 **Wallace MB**, Durkalski VL, Vaughan J, Palesch YY, Libby ED, Jowell PS, Nickl NJ, Schutz SM, Leung JW, Cotton PB. Age and alarm symptoms do not predict endoscopic findings among patients with dyspepsia: a multicentre database study. *Gut* 2001; **49**: 29-34
56 **Dinis-Ribeiro M**, Lomba-Viana H, Silva R, Fernandes N, Abreu N, Brandao C, Moreira-Dias L, da Costa-Pereira A. Should we exclude individuals from endoscopy based exclusively on the absence of alarm symptoms? *Scand J Gastroenterol* 2004; **39**: 910-911
57 **Rugge M**, Cassaro M, Di Mario F, Leo G, Leandro G, Russo VM, Pennelli G, Farinati F. The long term outcome of gastric non-invasive neoplasia. *Gut* 2003; **52**: 1111-1116
58 **Guarner J**, Herrera-Goepfert R, Mohar A, Sanchez L, Halperin D, Ley C, Parsonnet J. Interobserver variability in application of the revised Sydney classification for gastritis. *Hum Pathol* 1999; **30**: 1431-1434
59 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181
60 **Atkins L**, Benedict EB. Correlation of gross gastroscopic findings with gastroscopic biopsy in gastritis. *N Engl J Med* 1956; **254**: 641-644
61 **Heinkel K**. Correlation of gastroscopy, gastric photography and biopsy in diagnosis. *Gastrointest Endosc* 1969; **16**: 81-85
62 **Myren J**, Serck-Hanssen A. The gastroscopic diagnosis of gastritis with particular reference to mucosal reddening and mucus covering. *Scand J Gastroenterol* 1974; **9**: 457-462
63 **Sauerbruch T**, Schreiber MA, Schussler P, Permanetter W. Endoscopy in the diagnosis of gastritis. Diagnostic value of endoscopic criteria in relation to histological diagnosis. *Endoscopy* 1984; **16**: 101-104
64 **Toyoda H**, Rubio C, Befrits R, Hamamoto N, Adachi Y, Jaramillo E. Detection of intestinal metaplasia in distal esophagus and esophagogastric junction by enhanced-magnification endoscopy. *Gastrointest Endosc* 2004; **59**: 15-21
65 **Kiesslich R**, Fritsch J, Holtmann M, Koehler HH, Stolte M, Kanzler S, Nafe B, Jung M, Galle PR, Neurath MF. Methylene blue-aided chromoendoscopy for the detection of intraepithelial neoplasia and colon cancer in ulcerative colitis. *Gastroenterology* 2003; **124**: 880-888
66 **Yagi K**, Nakamura A, Sekine A. Characteristic endoscopic and magnified endoscopic findings in the normal stomach without Helicobacter pylori infection. *J Gastroenterol Hepatol* 2002; **17**: 39-45
67 **Yang JM**, Chen L, Fan YL, Li XH, Yu X, Fang DC. Endoscopic patterns of gastric mucosa and its clinicopathological significance. *World J Gastroenterol* 2003; **9**: 2552-2556

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BASIC RESEARCH

Extent of liver resection modulates the activation of transcription factors and the production of cytokines involved in liver regeneration

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Abstract

AIM: To investigate the molecular events involved in liver regeneration following subtotal hepatectomy (SH) as previous studies have largely focused on partial hepatectomy (PH).

METHODS: Male Wistar rats were subjected to 70% PH or 90% SH, respectively, and sacrificed at different times after surgery. Untreated and sham-operated animals served as controls. Serum and liver samples were obtained to investigate liver function, apoptosis (TUNEL assay) and transcription factors (NF-κB, Stat3; ELISA) or cytokines (HGF, TNF-α, IL-6, TGF-α, TGF-β; quantitative RT-PCR) involved in liver regeneration.

RESULTS: Serum levels of ALT and AST in animals with 70% PH differed significantly from sham-operated and control animals. We found that the peak concentration 12 h after surgery returned to control levels 7 d after surgery. LDH was increased only at 12 h after 70% PH compared to sham. Bilirubin showed no differences between the sham and 70% resection. After PH, early NF-κB activation was detected 12 h after surgery (313.21 ± 17.22 ng/mL), while there was no activation after SH (125.22 ± 44.36 ng/mL) compared to controls (111.43 ± 32.68 ng/mL) at this time point. In SH, however, NF-κB activation was delayed until 24 h (475.56 ± 144.29 ng/mL). Stat3 activation was similar in both groups. These findings correlated with suppressed and delayed induction of regenerative genes after SH (i.e. TNF-α 24 h postoperatively: 2375 ± 1220 in 70% and 88 ± 31 in 90%; IL-6 12 h postoperatively: 2547 ± 441 in 70% and 173 ± 82 in 90%). TUNEL staining revealed elevated apoptosis rates in SH (0.44% at 24 h; 0.63% at 7 d) compared to PH (0.27% at 24 h; 0.15% at 7 d).

CONCLUSION: The molecular events involved in liver regeneration are significantly influenced by the extent of resection as SH leads to suppression and delay of liver regeneration compared to PH, which is associated with delayed activation of NF-κB and suppression of proregenerative cytokines.

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Key words: Apoptosis; Cytokines; Liver regeneration; Major hepatectomy

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INTRODUCTION

Major hepatectomy is primarily used to treat malignant

liver disease and in living donor liver transplantation (LDLT). After resection the remaining liver can regenerate to a fully functional organ[1-3]. Tissue damage due to the surgical process further decreases the amount of healthy remnant liver tissue leading to an additional reduction in functional liver capacity in the postoperative period[4,5]. This situation can lead to liver insufficiency and, ultimately, to liver failure and death[6-8]. Therefore, patients have to fulfil certain criteria, such as the absence of end-stage liver disease, in order to be eligible for extended hepatectomy[1].

The same is true for donors in LDLT. While LDLT is a life saving procedure for the recipient, it is a potentially lethal operation for the donor. Therefore, very stringent criteria have to be fulfilled prior to hepatectomy to ensure donor safety[9-11]. For the donor, fast liver regeneration is imperative to reduce the probability of liver insufficiency[12,13]. Thus, an improvement in regenerative capacity would enhance donor safety and increase the possibility of including individuals who are not eligible for donation due to insufficient liver size[8,14].

As already stated the major concern in major hepatectomy or in the LDLT setting, is efficient regeneration of the remnant or transplanted partial liver, respectively. It has been demonstrated that graft size is of high importance in regenerative processes in the recipient[12]. Liver function parameters and regeneration are significantly better in patients undergoing a small resection than in patients undergoing a liver resection of more than 60%[15]. This effect exceeds a linear size correlation, which led to the conclusion that graft or remnant liver size influences regeneration. The underlying molecular mechanisms, however, are not well understood[16-19]. In particular, the role of proregenerative cytokines (e.g. IL-6 and TNF-α) and the role of transcription factors such as NF-κB are ambiguous[5,20-22].

While previous studies have largely focused on the molecular events after partial hepatectomy, the aim of this work was to investigate liver regeneration after subtotal hepatectomy. We analyzed whether the extent of liver resection has an impact on the activation of transcription factors and the expression of pro- and anti-regenerative cytokines using a rat resection model and compared 70% (partial hepatectomy, PH) and 90% resection (subtotal hepatectomy, SH), respectively.

MATERIALS AND METHODS

Resection

Six to eight-week-old male Wistar rats were anaesthetized with isoflurane. Seventy percent PH and 90% SH were performed under isoflurane anesthesia as described by Higgins *et al*[23] and Emond *et al*[24]. The rats were divided into 4 groups: control group (untreated), sham operation, 70% PH and 90% SH. Serum and liver tissue samples were obtained during surgery and 2 h, 12 h, 24 h, 48 h, 72 h and 7 d after resection (n = 4 at each time point, per group).

ALT, AST, LDH and bilirubin

Serum concentrations of liver related enzymes (ALT, AST, LDH and bilirubin) were assessed using commercially available enzyme activity tests [ALAT (GPT) FS (IFCC mod.); ASAT (GOT) FS (IFCC mod.); Bilirubin Auto Direct FS; LDH FS IFCC; DiaSys Diagnostic Systems; Holzheim, Germany].

Liver to body weight ratio

After the observation period, the remnant, regenerated liver was resected and weighed (A) and total body weight (B) was measured. The acquired data were expressed as a percentage of the ratio between the remnant liver weight divided by the total body weight multiplied by 100. Liver body weight ratio (LBW-r, %) = A/B × 100.

RNA isolation from liver tissue

Samples of liver tissue (approximately 2-5 g) were placed in 5 mL Trizol and homogenized with an Ultra Turrax (Janke & Kunkel, Staufen, Germany). One mL aliquots of the homogenized samples were used for RNA isolation using chloroform. RNA precipitation was performed using 100% isopropanol. The dry RNA pellet was resuspended in 100 μL Rnase-free water and purified with Rneasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration in the samples was measured by OD 260, purity was determined by OD 260/280.

Protein isolation

Samples of liver tissue (approximately 2-5 g) were placed in 3 mL lysis buffer (10 mmol/L HEPES, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.4% NP-40) and homogenized with an Ultra Turrax. One mL aliquots of the homogenized tissue were centrifuged at 4℃, 800 *g* for 5 min. Supernatants were centrifuged again at 4℃, 20 000 *g* for 30 min and the protein concentration was measured using a Bradford assay (BioRad, Munich, Germany). This fraction was referred to as the cytosolic fraction. The pellet obtained by centrifugation was resolved in 350 μL extraction buffer (20 mmol/L HEPES, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.2% NP-40) and repeatedly vortexed over a 30 min period. After centrifugation at 4℃, 20 000 *g* for 20 min, the supernatant was collected and the protein concentration measured with the Bradford assay. This fraction was referred to as the nucleic fraction. Proteinase inhibitors (Complete Mini EDTA free, Roche) were added to the extraction and lysis buffers 30 min prior to use.

Quantitative real-time PCR

Changes in mRNA expression were analyzed by quantitative real-time RT-PCR using the iCycler system (Bio-Rad, Munich, Germany). RT-PCR was performed with the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) to determine the cytokines involved in liver regeneration (TNF-α, IL-6, HGF, TGF-α, and TGF-β; Quantitect Primer Assays, Qiagen, Hilden,

Germany). Each PCR was performed using a total 30 μL volume of a mixture containing 2 μL of total RNA (20 ng to 200 ng). Beta-actin expression was chosen for normalization. The quantification was performed using the Pfaffl method[25] by calculating copy numbers from the ct-value for each gene per sample. Beta-actin mRNA levels were calculated in the same manner and the relation of target gene copies/100 000 copies of β actin are given. The data are shown as the mean of four separate experiments.

ELISA

NF-kappaB (NF-κB p65 ELISA KIT, BioSource™ CA, USA), and STAT3 [STAT3 (pY705) ELISA KIT, BioSource™, CA, USA] ELISAs were conducted according to the manufacturer's instructions. Negative and positive controls were included and a standard curve was determined for each assay. Sample size of the nucleic protein extract was 10 μL. Normalization was carried out by calculating relative protein concentrations.

TUNEL staining

Sections of paraffin-embedded tissue were dewaxed by heating to 60℃ for 30 min and subsequent washing in xylene. The slices were rehydrated through a grade series of ethanol (100%, 90%, 70%), permeabilized with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) and washed twice in PBS. Positive controls were incubated with DNase I for 10 min at room temperature prior to labeling. TUNEL reaction mixture was prepared according to protocol (*In Situ* Cell Death Detection Kit, Fluorescein; Roche, Germany). Labeling was conducted as described in the assay manual, samples were subsequently embedded in ProLong® Gold antifade reagent with DAPI (Invitrogen, CA, USA). Labeled cells per 10 fields of vision were counted on a fluorescence microscope and absolute numbers were compared.

Statistical analysis

Data are shown as mean ± SE. Differences between any two groups were determined by the Wilcoxon test for ELISA, qrt-PCR, TUNEL-assay and bilirubin. $P < 0.05$ was considered to be statistically significant. For differences between any two groups regarding the serum parameters ALT, AST and LDH the variance test was used and $F < 0.05$ was considered statistically significant.

RESULTS

Liver regeneration

The overall mean LBW-r was 4.06% ± 0.35% in control and sham-operated animals. After 70% resection, animals showed a continuous increase in LBW-r over 7 d starting from 0.74% ± 0.06% at the time of surgery, and reaching 2.70% ± 0.15% 7 d postoperatively. The earliest significant increase in LBW-r occurred between 2 h (0.88% ± 0.15%) and 12 h (1.39% ± 0.07%) with $P = 0.006$ (Figure 1).

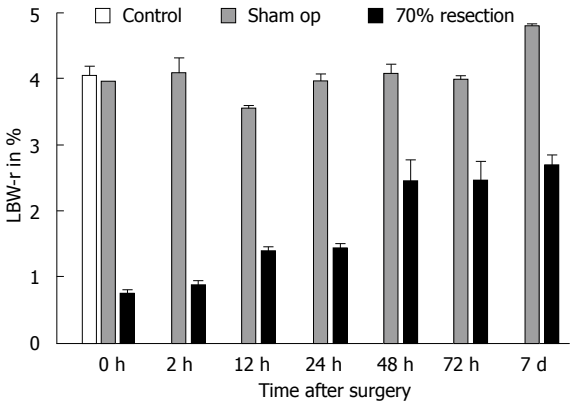

Figure 1 Liver-body-weight-ratio of control animals, sham-operated and 70% resected animals given in g per 100 g body weight. Controls had a LBW-r of 4.04% ± 0.15%.

Serum levels of liver enzymes

AST and ALT were significantly raised in the 70% resected animals compared to sham-operated rats (Figure 2A and B). Peak levels were found for both enzymes at 12 h postoperatively (AST, 12 h: 1055 ± 55 for 70% and 2204 ± 739 for 90%, $F = 0.011$; ALT, 12 h: 753 ± 110 for 70% and 1706 ± 725 for 90%, $F = 0.011$). Serum levels of both enzymes diminished over time, reaching control levels 7 d after surgery. LDH after 70% resection did not differ significantly from sham animals except at 7 d postoperatively (Figure 2C). LDH 7 d after 70% resection was 2060 U/I while the level in sham-operated animals was 890 U/I ($F = 0.033$). Seventy percent resection did not lead to a significant increase in bilirubin serum levels when compared to sham-operated animals (Figure 2D).

NF-κB activation

As described in the literature, NF-κB activation was observed after PH during the early phase of regeneration (0 h: 273.33 ± 24.45 pg, $P = 0.024$; 2 h: 285.34 ± 36.49 pg, $P = 0.009$) and 12 h postoperatively (313.21 ± 17.22 pg, $P = 0.001$). NF-κB remained activated until 7 d after surgery in this group. After SH, however, NF-κB activation was delayed until 24 h after the operation. NF-κB was significantly activated in the SH group 24h after surgery (475.66 ± 144.29, $P = 0.048$) with a peak at 48 h (747.18 ± 146.36 pg, $P = 0.02$). NF-κB activation was comparable in both groups at day 7 (Figure 3A).

STAT3 activation

Because we utilized a STAT3(pY705) ELISA, only phosphorylated STAT3 was measured in the assay.

Activation of STAT3 occurred during surgery in both the 70% (16-fold) and 90% (3-fold) resections. Two hours after surgery, STAT3 activation increased significantly in the PH (138-fold) and in the SH group (197-fold), decreasing thereafter and reaching preoperative levels 24 h after surgery. The differences between the two groups did not reach statistical significance (Figure 3B).

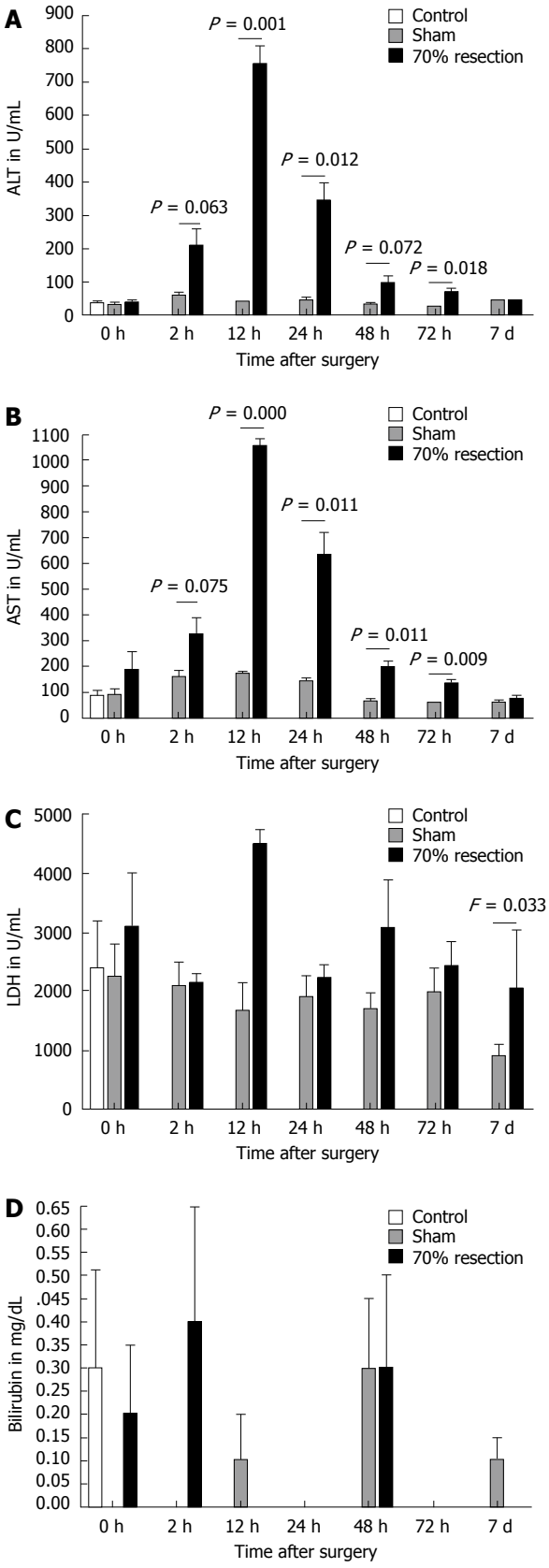

Figure 2 Serum levels of liver related enzymes, each given in U/L and mg/dL for bilirubin, respectively. A: ALT, base level in serum was 36.2 ± 5.2 U/mL; B: AST in controls was 84.0 ± 31.1 U/mL in serum; C: LDH, basal release of LDH into serum was 2416 ± 1088 U/mL; D: Bilirubin, with a baseline serum level of 0.3 ± 0.3 mg/dL.

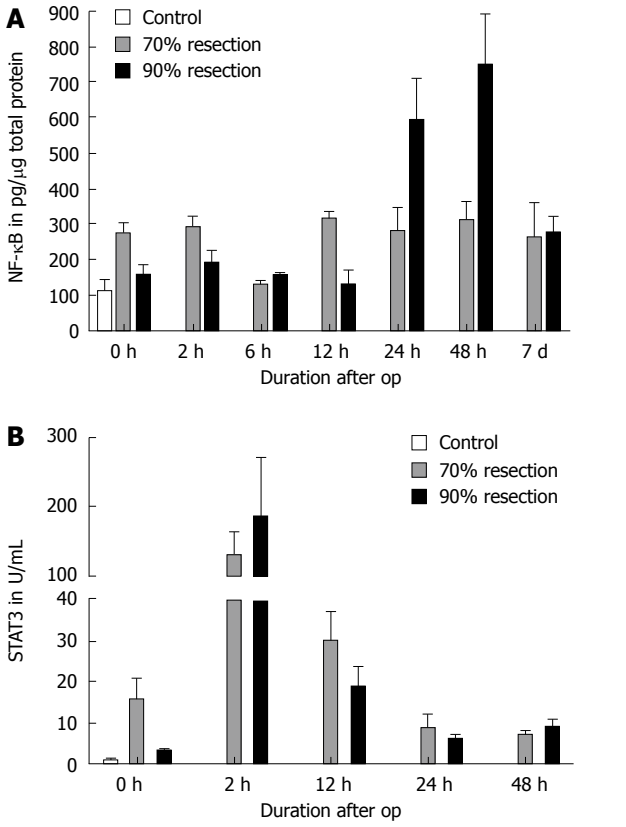

Figure 3 ELISA results of nucleic protein extracts. A: Active NF-κB. Baseline activation was 111 pg/μg total protein; B: Phosphorylated STAT3 (tyrosine 705) in total cellular protein. Activation in control animals was 4.5 U/mL. Protein was isolated from regenerating rat liver after 70% or 90% resection, respectively, at different time points after surgery. All data are normalized to an internal standard and are shown as mean values of four separate experiments. Significance is given *versus* control group.

Expression of pro- and anti-regenerative cytokines

In the group with 70% PH, 6 h after resection a rise in TNF-α expression was detected compared to controls, reaching a maximum after 24 h and decreasing thereafter to preoperative levels. In contrast, a significant rise in TNF-α expression was not detected after SH (Figure 4A). For IL-6, a biphasic expression pattern occurred in PH with high levels of expression at 2 h and 12 h postoperatively, while after SH a significant upregulation was only detected at 2 h after surgery (Figure 4B). Postoperatively, HGF expression increased steadily reaching a maximum at 12 h after surgery and returning to preoperative levels after 24 h in both groups (Figure 4C). A significant increase in early postoperative TGF-α expression was only detected after PH (12 h). At later time points, TGF-α expression was downregulated in this group while it increased up to 7 d after resection in SH (Figure 4D). We detected a slight upregulation in TGF-β expression in both resection groups at early time points (2 h, 6 h) with a strong peak at 12 h postoperatively which was detectable only in the PH group (8.25-fold compared to controls). Thereafter, TGF-β expression returned to control levels (Figure 4E).

Apoptosis

Control animals had a TUNEL index (percentage of TUNEL-positive cells) of approximately 0.12%. After PH, the rate of apoptosis reached a peak directly after surgery (0.44%), followed by a decrease to 0.27% at 24 h

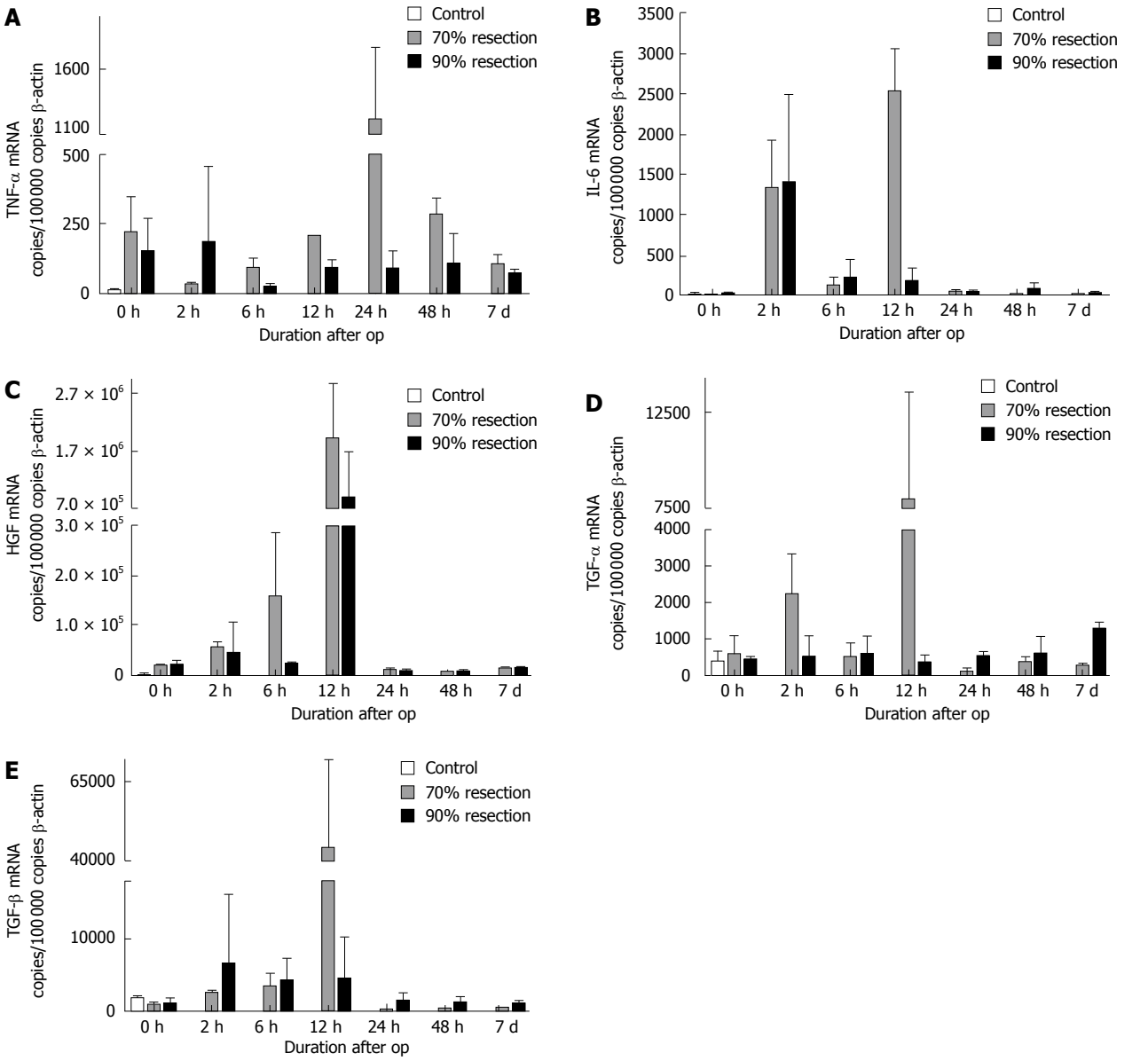

Figure 4 mRNA was isolated from regenerating liver tissue of rats at different time points after 70% or 90% resection, respectively. A: TNF-α (< 50 copies/100 000 copies β-actin); B: IL-6 (< 15 copies/100 000 copies β-actin); C: HGF (50 000 copies/100 000 copies β-actin); D: TGF-β (< 2500 copies/100 000 copies β-actin); E: TGF-β (< 5500 copies/100 000 copies β-actin). Measurement of cytokine and growth-factor expression was performed by quantitative real-time (rt) PCR. Copy numbers of each gene were calculated from ct values. Data shown are the mean of four separate experiments with standard error of mean. All statistical significances were calculated against control animals. Baseline expression of each gene is given in parentheses.

and to 0.20% at 48 h and returned to control levels at 7 d (0.15%). After SH, however, the apoptotic peak was delayed until 24 h after surgery, declining to 0.18% at 48 h. In contrast to PH, a second apoptotic peak (0.63%) was detected at 7 d in this group (Figures 5 and 6).

DISCUSSION

In this study, we analyzed the molecular events in liver tissue after subtotal hepatectomy (expression of proregenerative cytokines, activation of transcription factors and apoptosis) which are, in contrast to partial hepatectomy, not well understood. Our data indicate that activation of proregenerative genes like TGF-α and IL-6 is stronger after PH compared to SH. TNF-α which is also involved in liver regeneration, is induced by PH but not by SH. In addition, HGF expression was higher in PH than in SH. This was associated with stronger activation of NF-κB in PH during the early phase of regeneration. Finally, the apoptotic peak was delayed until 24 h after surgery and had a biphasic course in SH. In PH, apoptosis had a monophasic course and peaked directly after surgery.

These data are in accordance with other experiments from our group, in which we observed higher regenerative capacities and better liver function tests (ALT, AST, bilirubin) after 70% PH compared to 90% SH (Benkö *et al*, submitted). The time course of liver regeneration differed significantly between 70% PH and 90% SH. Animals with 70% resection showed a significant increase in LBW-r as early as 12 h after surgery. In the PH group, liver weight reached

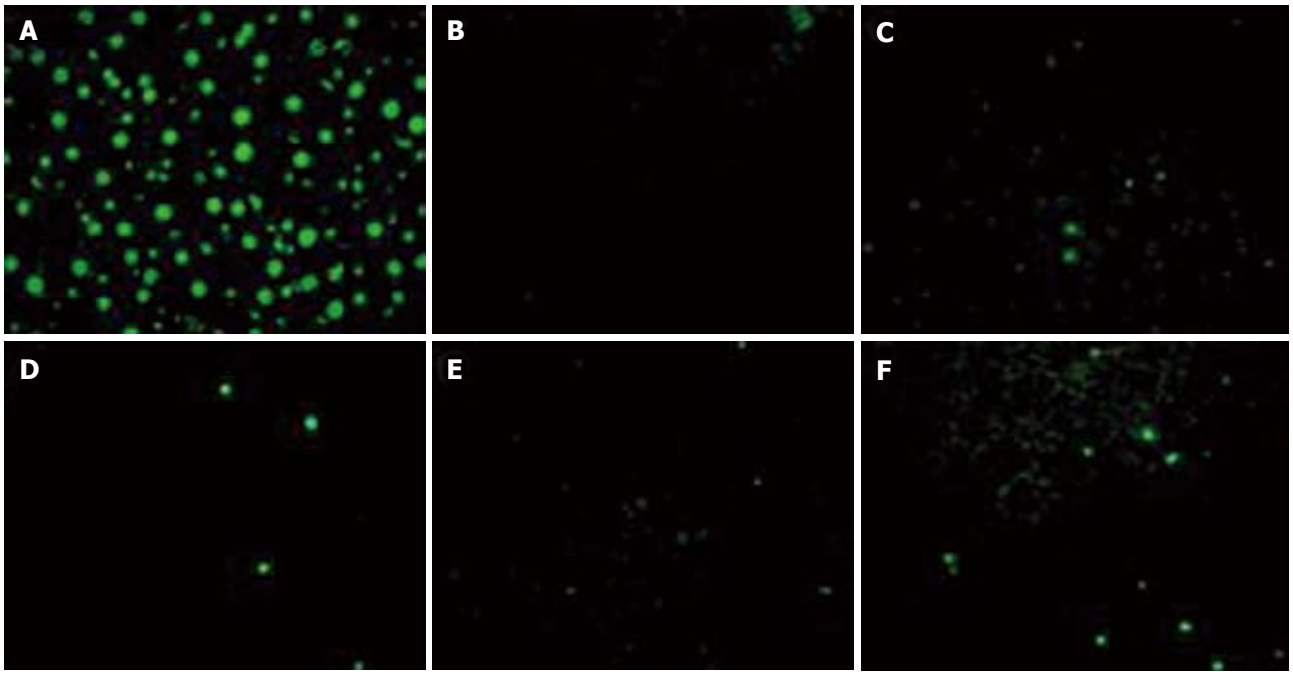

Figure 5 Representative images of TUNEL staining in liver tissue from the following groups. A: Positive control; B: Negative control; C: 70% resection, 24 h after surgery; D: 90% resection, 24 h after surgery; E: 70% resection 7 d after surgery; F: 90% resection, 7 d after surgery.

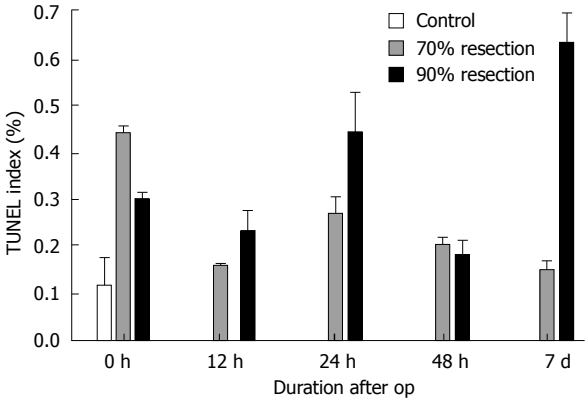

Figure 6 Results of TUNEL staining from paraffin embedded tissue sections. Paraffin embedded tissue slides from regenerating rat liver were dewaxed, washed and stained using the TUNEL method. The slides were covered in anti-fade medium with DAPI and analysed with a fluorescence microscope. False positive results were ruled out by comparison of the images with DAPI counterstaining (not shown). The TUNEL index is the number of TUNEL-positive cells divided by the total cell number. Cells were counted in ten fields of vision per section. Numbers shown are mean of four separate experiments in each group. TUNEL index for control animals (no resection) was approximately 0.12%.

65% of sham-operated controls 7 d after surgery. In contrast, after 90% resection, LBW-r started to increase 24 h after surgery and reached only 30% of the liver weight compared to controls at day 7. The time course in both groups for AST and ALT was similar although the release of both enzymes into serum was significantly higher after 90% SH, 12, 24 and 48 h postoperatively. LDH levels were similar in both groups until 12 h after surgery where the increase was more prominent in the 90% SH group than in the 70% PH group. At all later time points, LDH decreased in animals with 90% resection compared to controls and the 70% group. Serum bilirubin in the 70% resection group did not differ from the sham group. In animals with 90% resection we found a significant increase in bilirubin from 12 h to 72 h postoperatively. These findings show that damage to liver cells is increased after 90% hepatectomy compared to 70% resection.

Genes related to the initial phase of regeneration, such as TNF-α and IL-6, which prime hepatocytes into a state in which they are susceptible to growth factors[16,17], were expressed at later time points in our model. Nonetheless, both cytokines were expressed at lower levels or were delayed after SH compared to PH. As described previously[18,26-28], growth factors relevant to liver regeneration (HGF and TGF-β) were upregulated in PH, whereas no or only a reduced induction was observed after SH. This suggests that the expression of the factors relevant to the regeneration of liver tissue is influenced by the extent of resection.

For TGF-β we detected a distinct peak at 12 h after PH but not after SH. Since TGF-β has antiregenerative properties[18], high expression levels would be expected to counteract regeneration in this group. On the other hand, regeneration is a very tightly regulated process[19], which implies that high expression of TGF-β could lead to attenuation of ongoing regenerative processes in the PH group, whereas the regenerative stimuli which induce TGF-β expression are lacking in the SH group. This concept is supported by a very tight downregulation of TGF-β in the 70% hepatectomy group at later time points after surgery.

TUNEL indices were significantly raised directly after PH. Although we found a decrease in apoptosis over time, TUNEL indices remained elevated compared

to controls. Thus, the data indicate that apoptosis occurs earlier and in parallel with regeneration in animals with 70% resection. In contrast to this, animals with 90% hepatectomy showed slightly higher TUNEL indices at earlier time points and a strong increase in apoptosis 7 d after surgery. This could on the one hand, indicate that there was more tissue damage, which led to a higher number of apoptotic cells during the early postoperative phase. On the other hand, the significant rise in TUNEL indices 7 d after surgery leads to the conclusion that the main phase of remodelling occurs at later time points.

Our data suggest that liver regenerative processes after 90% SH are impaired by reduced or delayed activation of proregenerative factors compared to 70% PH. Activation of NF-κB as well as expression of important cytokines and growth factors depend on the amount of resected liver tissue. The underlying mechanisms are not yet clear, although they may be associated with the liver's ability to regenerate and to reduce tissue to fit current requirements[16,28].

It is still unclear, which role NF-κB plays during the regenerative process in the setting of extended hepatectomy. A continuous activation was detected at low levels after PH while a delayed and more pronounced activation could be seen after SH. While basal activation of NF-κB may be sufficient and important for regeneration and structural reformation after 70% resection[29], the strong NF-κB activation in animals with 90% hepatectomy may also favor inflammatory processes in the damaged tissue, which counteract the restoration of a functional liver[21,30,31].

In conclusion, our data suggest that the molecular events involved in liver regeneration are significantly influenced by the extent of resection, as subtotal hepatectomy leads to delayed activation of NF-κB and suppression of proregenerative cytokines compared to partial hepatectomy. Therefore, strategies to improve the activation of proregenerative transcription factors and the early production of proregenerative cytokines may improve clinical outcome after extended hepatectomy.

COMMENTS

Background

Liver resection is an important therapeutic measure to treat severe liver disease. Imperative for patient outcome is a timely regeneration of healthy liver tissue. Molecular events underlying liver regeneration are not completely understood but involve cytokines such as TNF-α and IL-6 to initiate and growth factors such as HGF and TGF-α to prolong regeneration.

Research frontiers

Although some hypotheses for the initiation of liver regeneration after tissue damage exist, it is not known which agents induce cell proliferation. Intrinsic LPS or contaminating bacteria have been discussed as initiating factors, activating TLR-systems in resident immune liver cells, known as Kupffer cells. One important factor in TLR-pathways is the transcription factor NF-κB, which regulates the expression of many genes including regenerative and anti-apoptotic effectors as well as proinflammatory cytokines. In this respect it is still unclear whether NF-κB activation promotes regeneration or contributes to further tissue damage by aggravating postoperative inflammation.

Innovations and breakthroughs

In our experiments we found a clear difference in regenerating liver after SH compared to PH regarding serum parameters and LBW-r. For the known cytokines and growth factors involved in liver regeneration, we detected reduced and/or delayed expression after SH. Furthermore, the SH group displayed a delayed activation of NF-κB. Until this work it was not known if the extent of liver resection had a direct influence on the regenerative capacity at a molecular and cellular level. Here, we demonstrated that the regenerative processes in the liver depend, at least partially, on the extent of resection.

Applications

A deeper insight into the molecular events underlying liver regeneration as well as into the relationship between the extent of resection and the regenerative processes may increase the possibility of enhancing regeneration pharmacologically. This would not only improve outcome for patients undergoing extensive hepatectomy but also make this therapeutic measure available for patients with severe liver tissue damage (i.e. cirrhosis), who otherwise would not be eligible for surgery.

Terminology

We used two models of liver resection. A 70% liver resection described as partial hepatectomy (PH) and a 90% liver resection model of extensive surgical intervention referred to as subtotal hepatectomy (SH).

Peer review

This manuscript focuses on the regulation of liver regeneration by the extent of resection. The authors of this study provide evidence that SH leads to delayed activation of NF-κB and suppression of proregenerative cytokines compared to PH. The manuscript is clearly written and findings are very interesting.

REFERENCES

1 **Kassahun WT**, Fangmann J, Harms J, Hauss J, Bartels M. Liver resection and transplantation in the management of hepatocellular carcinoma: a review. *Exp Clin Transplant* 2006; **4**: 549-558

2 **Kountouras J**, Boura P, Lygidakis NJ. Liver regeneration after hepatectomy. *Hepatogastroenterology* 2001; **48**: 556-562

3 **Chen MF**, Jeng LB. Partial hepatic resection for hepatocellular carcinoma. *J Gastroenterol Hepatol* 1997; **12**: S329-S334

4 **Tian Y**, Graf R, Jochum W, Clavien PA. Arterialized partial orthotopic liver transplantation in the mouse: a new model and evaluation of the critical liver mass. *Liver Transpl* 2003; **9**: 789-795

5 **Tian Y**, Jochum W, Georgiev P, Moritz W, Graf R, Clavien PA. Kupffer cell-dependent TNF-alpha signaling mediates injury in the arterialized small-for-size liver transplantation in the mouse. *Proc Natl Acad Sci USA* 2006; **103**: 4598-4603

6 **Dahm F**, Georgiev P, Clavien PA. Small-for-size syndrome after partial liver transplantation: definition, mechanisms of disease and clinical implications. *Am J Transplant* 2005; **5**: 2605-2610

7 **Zhong Z**, Schwabe RF, Kai Y, He L, Yang L, Bunzendahl H, Brenner DA, Lemasters JJ. Liver regeneration is suppressed in small-for-size liver grafts after transplantation: involvement of c-Jun N-terminal kinase, cyclin D1, and defective energy supply. *Transplantation* 2006; **82**: 241-250

8 **Florman S**, Miller CM. Live donor liver transplantation. *Liver Transpl* 2006; **12**: 499-510

9 **Pacheco-Moreira LF**, Enne M, Balbi E, Halpern M, Peixoto A, Cerqueira A, Moreira E, Araujo C, Pereira JL, Martinho JM. Selection of donors for living donor liver transplantation in a single center of a developing country: lessons learned from the first 100 cases. *Pediatr Transplant* 2006; **10**: 311-315

10 **Moreno Gonzalez E**, Meneu Diaz JC, Garcia Garcia I, Loinaz Segurola C, Jimenez C, Gomez R, Abradelo M, Moreno Elola A, Jimenez S, Ferrero E, Calvo J, Manrique A, Herrero ML. Live liver donation: a prospective analysis of exclusion criteria for healthy and potential donors. *Transplant Proc* 2003; **35**: 1787-1790

11 **Yokoi H**, Isaji S, Yamagiwa K, Tabata M, Nemoto A, Sakurai H, Usui M, Uemoto S. The role of living-donor liver transplantation in surgical treatment for hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2006; **13**: 123-130

12 **Bockhorn M**, Schollmann S, Opitz B, Sotiropoulos GC, Sheu

SY, Niehaus E, Trippler M, Frilling A, Broelsch CE, Schlaak JF. Vascular endothelial growth factor does not improve liver regeneration and survival after 90% subtotal liver resection. *Hepatol Res* 2007; **37**: 353-359

13 **Bockhorn M**, Goralski M, Prokofiev D, Dammann P, Grunewald P, Trippler M, Biglarnia A, Kamler M, Niehues EM, Frilling A, Broelsch CE, Schlaak JF. VEGF is important for early liver regeneration after partial hepatectomy. *J Surg Res* 2007; **138**: 291-299

14 **Lechler RI**, Sykes M, Thomson AW, Turka LA. Organ transplantation--how much of the promise has been realized? *Nat Med* 2005; **11**: 605-613

15 **Chijiiwa K**, Saiki S, Tanaka M. Serum interleukin-6 and hepatocyte growth factor levels in patients after hepatectomy. *Hepatogastroenterology* 2002; **49**: 467-471

16 **Pahlavan PS**, Feldmann RE Jr, Zavos C, Kountouras J. Prometheus' challenge: molecular, cellular and systemic aspects of liver regeneration. *J Surg Res* 2006; **134**: 238-251

17 **Qin SW**, Zhao LF, Chen XG, Xu CS. Expression pattern and action analysis of genes associated with the responses to chemical stimuli during rat liver regeneration. *World J Gastroenterol* 2006; **12**: 7285-7291

18 **Fausto N**. Growth factors in liver development, regeneration and carcinogenesis. *Prog Growth Factor Res* 1991; **3**: 219-234

19 **Fausto N**, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology* 2006; **43**: S45-S53

20 **Yuceturk H**, Yagmurdur MC, Gur G, Demirbilek M, Bilezikci B, Turan M, Karakayali H, Haberal M. Role of heparin on TNF-alpha and IL-6 levels in liver regeneration after partial hepatic resection. *Eur Surg Res* 2007; **39**: 216-221

21 **Luedde T**, Assmus U, Wustefeld T, Meyer zu Vilsendorf A, Roskams T, Schmidt-Supprian M, Rajewsky K, Brenner DA, Manns MP, Pasparakis M, Trautwein C. Deletion of IKK2 in hepatocytes does not sensitize these cells to TNF-induced apoptosis but protects from ischemia/reperfusion injury. *J Clin Invest* 2005; **115**: 849-859

22 **Coelho MC**, Tannuri U, Tannuri AC, Mello ES, dos Santos NA. Expression of interleukin 6 and apoptosis-related genes in suckling and weaning rat models of hepatectomy and liver regeneration. *J Pediatr Surg* 2007; **42**: 613-619

23 **Higgins GM**, Anderson RM. Experimental pathology of the liver 1: Restoration of the liver of the white rat following partial surgical removal. *Arch Pathol* 1931; **12**: 186-202

24 **Emond J**, Capron-Laudereau M, Meriggi F, Bernuau J, Reynes M, Houssin D. Extent of hepatectomy in the rat. Evaluation of basal conditions and effect of therapy. *Eur Surg Res* 1989; **21**: 251-259

25 **Pfaffl MW**. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29**: e45

26 **Fausto N**, Laird AD, Webber EM. Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. *FASEB J* 1995; **9**: 1527-1536

27 **Lowes KN**, Croager EJ, Olynyk JK, Abraham LJ, Yeoh GC. Oval cell-mediated liver regeneration: Role of cytokines and growth factors. *J Gastroenterol Hepatol* 2003; **18**: 4-12

28 **Mangnall D**, Bird NC, Majeed AW. The molecular physiology of liver regeneration following partial hepatectomy. *Liver Int* 2003; **23**: 124-138

29 **Yang WJ**, Zhang QY, Yu ZP, Song QT, Liang HP, Xu X, Zhu GB, Jiang FZ, Shi HQ. Effects of nuclear factor-kappaB on rat hepatocyte regeneration and apoptosis after 70% portal branch ligation. *World J Gastroenterol* 2005; **11**: 6775-6779

30 **Campbell JS**, Riehle KJ, Brooling JT, Bauer RL, Mitchell C, Fausto N. Proinflammatory cytokine production in liver regeneration is Myd88-dependent, but independent of Cd14, Tlr2, and Tlr4. *J Immunol* 2006; **176**: 2522-2528

31 **McAllister-Lucas LM**, Ruland J, Siu K, Jin X, Gu S, Kim DS, Kuffa P, Kohrt D, Mak TW, Nunez G, Lucas PC. CARMA3/Bcl10/MALT1-dependent NF-kappaB activation mediates angiotensin II-responsive inflammatory signaling in nonimmune cells. *Proc Natl Acad Sci USA* 2007; **104**: 139-144

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RAPID COMMUNICATION

Resveratrol attenuates oxidative stress and histological alterations induced by liver ischemia/reperfusion in rats

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Abstract

AIM: To investigate the effects of resveratrol on liver ischemia/reperfusion (I/R) injury in rats.

METHODS: A total of 40 male Sprague-Dawley rats weighing 240-290 g were randomized into four groups of ten: (1) controls: data from unmanipulated animals; (2) sham group: rats subjected to the surgical procedure, except for liver I/R, and given saline; (3) I/R group: rats underwent liver ischemia for 45 min followed by reperfusion for 45 min; (4) I-R/Resveratrol group: rats pretreated with resveratrol (10 μmol/L, iv). Liver tissues were obtained to determine antioxidant enzyme levels and for biochemical and histological evaluation.

RESULTS: Plasma aminotransferase activities were higher in the I/R group than in the I-R/Resveratrol group. Malondialdehyde levels and the hepatic injury score decreased, while superoxide dismutase, catalase, and glutathione peroxidase levels increased in group 4 compared to group 3. In group 4, histopathological changes were significantly attenuated in resveratrol-treated livers.

CONCLUSION: These results suggest that resveratrol has protective effects against hepatic I/R injury, and is a potential therapeutic drug for ischemia reperfusion-related liver injury.

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Key words: Injury; Ischemia/reperfusion; Liver; Resveratrol

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INTRODUCTION

Hepatic injury caused by ischemia/reperfusion (I/R) has been proposed as a key clinical problem associated with liver transplantation and major liver surgery[1]. Additionally, hepatic I/R injury also occurs in diverse situations, including heart failure, liver trauma, and blood occlusion to the liver[2,3]. The production of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radical, has been demonstrated in reperfusion injury[4]. I/R activates Kupffer cells (KC), the resident macrophages in the liver, to generate ROS, proinflammatory cytokines, and chemokines and to upregulate inducible nitric oxide synthase[5].

Resveratrol (3,4,5 tri-hydroxystilbene) is a naturally occurring phytoalexin present in high concentrations in the skin and seeds of grapes[6]. It occurs naturally in a trans-or cis-isoform[7-9]. Resveratrol has been reported to have several biologic effects such as a potent antioxidative effect *via* prevention of lipid peroxidation[10,11], anti-platelet activity[12], an estrogenic activity[13], and anti-inflammatory activity attributed to cyclooxgenase inhibition[6,14]. Previously, it was suggested that resveratrol stimulated nitric oxide production in endothelial cells, has a vasodilatory effect on blood vessels[15], and improves the energy metabolism system after spinal cord trauma[16].

The aim of this study was to evaluate the possible protective effect of resveratrol against I/R-induced hepatic injury, using biochemical and histological parameters.

MATERIALS AND METHODS

Forty male Sprague-Dawley rats weighing 240-290 g

were used in the study. All of the experimental protocols were performed according to the guidelines for the ethical treatment of experimental animals.

Animals and experimental protocol

The rooms where the animals were housed were windowless and were under controlled temperature (22 ± 2℃) and lighting conditions. The rats were housed individually in cages, and allowed free access to standard rat chow and water before and after the experiments. The animals were fasted overnight before the experiments, but were given free access to water. They were anesthetized using 100 mg/kg ketamine and 20 mg/kg xylazine body weight (ip), The right femoral vein was cannulated to administer drugs and saline.

The animals were randomized into four groups (n = 10 in each group): (1) controls: Unmanipulated animals, rats not subjected to any surgical procedure or liver manipulation; (2) sham group: Rats subjected to the surgical procedures described below, except for liver I/R, and administered saline vehicle and maintained under anesthesia for an equivalent duration (i.e. 45 min plus 45 min); (3) I/R group: Rats subjected to the surgical procedures described below and underwent liver ischemia for 45 min followed by reperfusion for 45 min (n = 10); (4) I-R/Resveratrol group: Rats received resveratrol 10 mg/kg dissolved in 2 mL 50% alcohol (Sigma Chemicals, St. Louis, MO, USA) by infusion pump 15 min before liver reperfusion.

Liver ischemia/reperfusion

As described previously[17], the ligament attachments connecting the liver, diaphragm, abdominal wall, and neighboring organs were divided. After the organ was carefully isolated, the liver hilus was exposed to find the common hepatic artery and portal vein. A vascular microclamp was used to interrupt the blood supply to three-quarters of the liver for 45 min, and this was followed by 45 min of reperfusion. Other rats were subjected to a sham operation (sham-operated), which was identical to the surgical procedure used for the I/R group rats without clamping; the rats were kept under anesthesia for the same length of time. At the end of the experiments, the rats were killed with an overdose of sodium pentobarbital.

Measuring serum liver enzymes

The abdominal aorta was punctured and 5 mL of blood was taken and placed in heparinized tubes. Plasma was separated by centrifugation (3000 r/min for 10 min at room temperature) for biochemical studies. The activities of alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury), and aspartate aminotransferase (AST, a nonspecific marker for hepatic injury) in plasma were determined in units per liter using standard auto-analyzer methods on an Abbott Aeroset (Abbott Laboratories, Abbott Park, IL, USA). Just before the rats were sacrificed, the livers were removed for histopathological evaluation.

Histopathological study

The livers were divided into two pieces. One was immediately placed in 10% formalin solution, left overnight, and then embedded in paraffin blocks. The blocks were cut into 4-μm sections and stained with hematoxylin-eosin, using standard protocols. The severity of hepatic injury in the sections was evaluated using a point-counting method on an ordinal scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration[18].

Biochemical analyses

The other piece of liver was washed in ice-cold 0.9% saline solution, weighed, and stored at -70℃. Tissue homogenates were prepared as 0.1 g/mL in 250 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DL-dithiothreitol, and 15 mmol/L Tris HCl (pH 7.4), using an all-glass Potter Elvehjem homogenizer (Selecta, Barcelona, Spain). Each homogenate was centrifuged for 20 min at 800 r/min. The resulting supernatant fraction was used to determine enzyme activities. The protein concentrations in the supernatant were determined using the Bradford method[19].

Malondialdehyde determination

Liver malondialdehyde (MDA) levels were determined using the method of Wasowicz *et al*[20] based on the reaction of MDA with thiobarbituric acid at 95 to 100℃. Fluorescence intensity was measured in the upper n-butanol phase using fluorescence spectrophotometry (F-4010; Hitachi, Tokyo, Japan) adjusted for excitation at 525 nm and emission at 547 nm. The arbitrary values obtained were compared with a series of standard solutions (1,1,3,3-tetramethoxypropane). The results are given in nanomoles per gram of wet tissue.

Superoxide dismutase, catalase, and glutathione peroxidase determination

Superoxide dismutase (SOD) activity was measured using the xanthine-oxidase-cytochrome c method, as described by McCord and Fridovich[21]. The final concentrations in the cuvettes were 50 mmol/L potassium phosphate (pH 7.8), 0.1 mmol/L EDTA, 10 mmol/L cytochrome c, 50 mmol/L xanthine, 50 or 2 mmol/L cyanide, 1 U catalase (CAT), and 0.05-0.1 mg of tissue. The reaction was initiated by adding 1 U xanthine-oxidase. The inhibition of xanthine-oxidase was followed spectrophotometrically at 550 nm. One unit of SOD activity was defined as the amount of enzyme that produced 50% inhibition of the control rate of cytochrome c reduction.

CAT activity was assayed according to the method of Beers and Sizer[22]. The final concentrations in the cuvettes were 500 mmol/L potassium phosphate (pH 7), 100 mmol/L H_2O_2, and 0.05-0.1 mg of tissue. The decrease in the absorbance at 240 nm after adding the substrate was followed spectrophotometrically.

GSH activity was assayed using a coupled enzyme system in which oxidized glutathione (GSSG) reduction

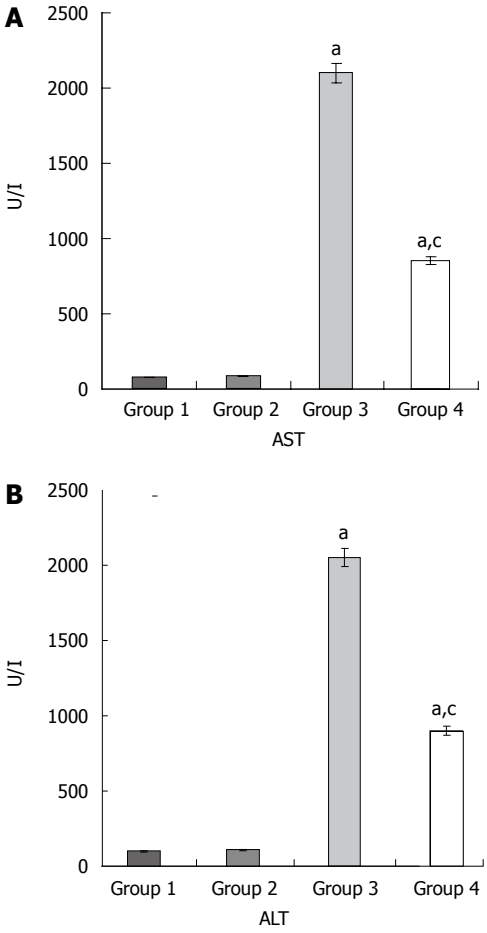

Figure 1 Effects of liver I/R and resvera-trol on liver function. A: The AST values; B: The ALT values $^{a}P < 0.05$ compared with Group 1 and 2; $^{c}P < 0.05$ compared with Group 3. The values are the mean ± SE. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

was coupled to NADPH oxidation by glutathione reductase[23]. The assay mixture contained 50 mmol/L potassium phosphate (pH 7.5), 1 mmol/L EDTA, 1 mmol/L NaN_3, 1 mmol/L reduced glutathione (GSH), 0.2 mmol/L NADPH, 1 U glutathione reductase, and tissue (0.05-0.2 mg). After a 5-min preincubation period (20-25℃), the reaction was initiated by adding 0.25 mmol/L H_2O_2. The decrease in absorbance at 340 nm was followed spectrophotometrically.

Protein assays

The protein content of the homogenates was determined using the procedure of Lowry *et al*[24].

Statistical analysis

Data were entered and analyzed on an IBM-compatible personal computer using SPSS version 9.0. All values were expressed as the mean ± SE. The significance of the data obtained was evaluated using analysis of variance (ANOVA). Differences between means were analyzed using the post-ANOVA test (Tukey's *b*). $P < 0.05$ was considered significant.

RESULTS

The ALT and AST levels were significantly increased in groups 3 and 4 in comparison with groups 1 and 2 ($P < 0.05$ in all cases). Moreover, the ALT and AST levels were significantly decreased in group 4 compared to group 3 ($P < 0.05$) (Figure 1).

The MDA, SOD, CAT, and GSH values for the different groups are shown in Figure 2. In group 3, MDA was significantly increased compared with groups 1, 2, and 4 ($P < 0.05$ in all cases). In addition, SOD, CAT, and GSH significantly decreased in group 3 compared with groups 1, 2, and 4 ($P <0.05$ in all cases).

The liver histopathological scores were 0.1 ± 0.2, 0.1 ± 0.2, 2.8 ± 0.1, and 1.1 ± 0.2 in groups 1 to 4, respectively. The histopathological scores were higher in groups 3 and 4 than in groups 1 and 2 ($P < 0.05$ in all cases). Moreover, the histopathological score was lower in group 4 than in group 3 ($P < 0.05$). Normal findings were obtained on histological examination of rats in groups 1 or 2 (Figure 3A and B). In group 3, focal necrosis with sinusoidal congestion and neutrophil accumulation were observed at the midzonal region (Figure 3C). In contrast, these changes were markedly attenuated in resveratrol-treated livers (Figure 3D).

DISCUSSION

Hepatic ischemia and reperfusion injury is observed following major liver surgery, transplantation, trauma and sepsis and may cause metabolic and structural hepatic damage[25,26]. This remains a significant problem in surgical procedures, and is a limitation of liver transplantation[27]. Several mechanisms have been considered to explain I/R injury of the liver. Activation of KC with enhanced formation of ROS and secretion of inflammatory cytokines and proteolytic enzymes are considered to play an important role in liver reperfusion injury[28,29]. Additionally, migration of activated polymorphonuclear leukocytes at the injury site may prolong the injury as a result of the production of inflammatory cytokines, adhesion molecules, and ROS[30,31]. The formation of ROS may initiate oxidative stress which can lead to lipid peroxidation[32]. Removal of oxidative stress is the primary intervention to decrease tissue injury. Superoxide dismutase, catalase, glutathione, α-tocopherol, and carotene are known endogenous antioxidants, but none of these endogenous antioxidants are sufficient to compensate oxidative stress. Several antioxidants have been studied and reported to decrease oxidative stress in hepatic and renal tissue in experimental obstructive jaundice, septic shock, and I/R models.

Resveratrol is a polyphenol phytoalexin (trans-3,5,4-trihydroxystilbene) that possesses diverse biochemical and physiological actions, including estrogenic, antiplatelet, and anti-inflammatory properties[33,34]. Recently, resveratrol was found to be a highly potent antioxidant which could inhibit free radical generation in brain, spinal cord, kidney, liver and red cell membrane[35-38]. It has been shown that resveratrol inhibits lipid peroxidation[39], and prevents apoptotic cell death induced by oxidative stress[40]. The end production of lipid peroxidation includes aldehydes, hydrocarbon gases, and MDA. MDA is a sensitive index of lipid peroxidation[18,41]. Bertelli *et al*[33] suggested that

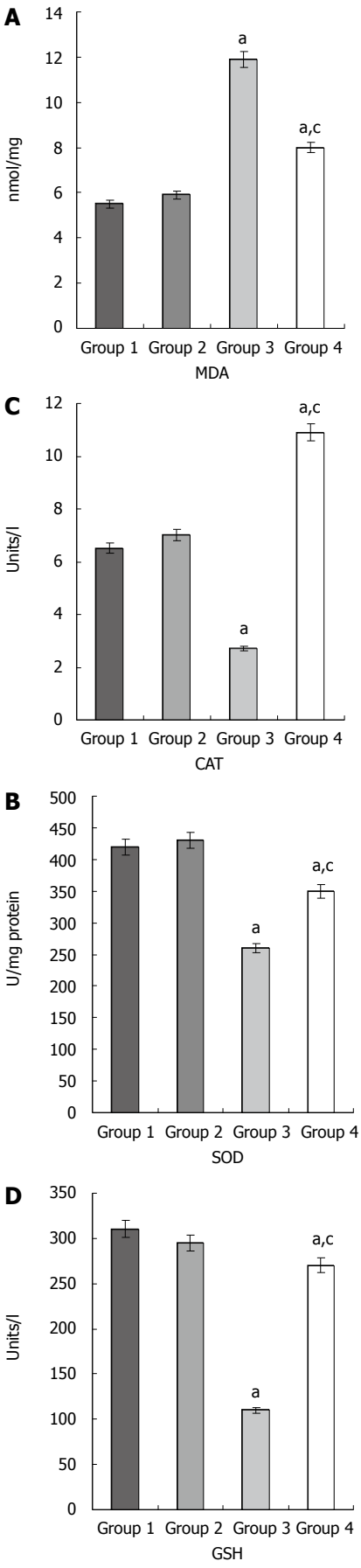

Figure 2 Effects of ischemia/reperfusion and resveratrol on the MDA (A), SOD (B), CAT (C), and GSH (D) levels in liver tissue. $^{a}P < 0.05$ compared with groups 1 and 2. $^{c}P < 0.05$ compared with group 3. The values are the mean ± SE. MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase; GSH: Glutathione peroxidase.

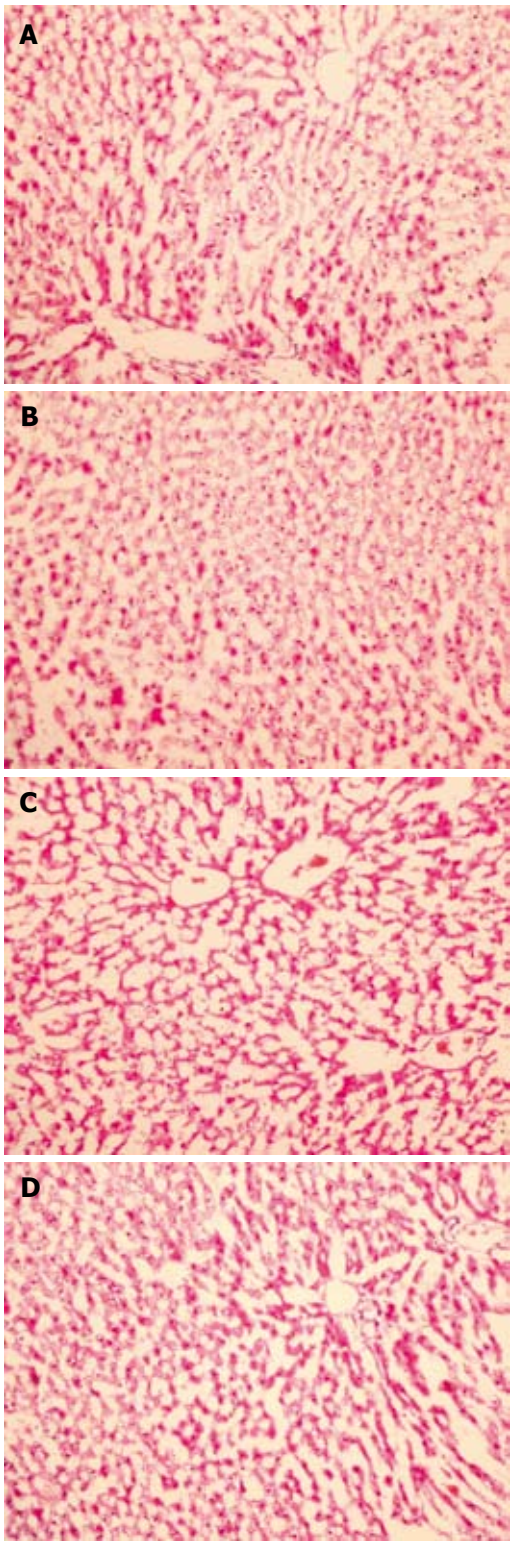

Figure 3 Effect of resveratrol on hepatic tissue injury after I/R evaluated by histological examination. In groups 1 and 2, normal findings were obtained on histological examination (A and B, respectively) (HE, x 200); C: In group 3, swollen hepatocytes with marked vacuolization and congestion were observed (HE, x 200); D: In group 4, the hepatocytes and sinusoids showed normal morphology, reflecting a well preserved liver parenchyma (HE, x 200).

MDA formation was reduced in the ischemic-reperfused myocardium of resveratrol-treated rats. In an experimental study by Kirimlioglu *et al*[42], MDA levels in liver tissue and plasma were higher in group B subjected to 70% partial hepatectomy than those of group A treated with

resveratrol before and after 70% partial hepatectomy. Although the exact mechanism is not known, it is thought that this was probably due to the antioxidant and inhibitory effects of resveratrol on ROS, which might be a biochemical mechanism related to the anti-inflammatory and anticarcinogenic properties of resveratrol[43]. To control the detrimental effects of ROS, organisms have developed a variety of antioxidant defense systems, especially the endogenous antioxidant enzymes system including SOD, CAT and GSH. The activities of these enzymes are higher in the liver than in other tissues. CAT is an oxidoreductase enzyme, which transforms H_2O_2 into H_2O and O_2. It can protect cells from damage induced by ischemia reperfusion by scavenging ROS[44]. GSH is considered to be the principal mitochondrial antioxidant and its depletion markedly enhances the sensitivity of mitochondrial structures to ROS-mediated injury[45]. A decrease in GSH level after reperfusion could reflect ROS-mediated consumption[46]. SOD catalyses dismutation of the superoxide anion (O_2^-) into H_2O_2; H_2O_2 can be transformed into H_2O and O_2 by CAT; GSH is a selenoprotein, which reduces lipidic or nonlipidic hydroperoxides as well as H_2O_2 while oxidizing GSH[47]. Plin *et al*[48] showed that resveratrol protects mitochondria and cells against cold preservation-warm reperfusion (CPWR)-induced injury. Resveratrol improved both mitochondrial coupling and ATP synthesis measured after CPWR as demonstrated by the increase in respiratory control ratio and ADP/oxygen values, respectively. These protective effects could be related to the antioxidant properties of the molecule which has been shown to be able to scavenge ROS[49].

In our study, liver tissue MDA levels in the I/R group increased compared with normal and sham groups. With resveratrol administration, liver tissue MDA levels decreased. Additionally, our results suggest that increased SOD, GSH and CAT activities may be attributed to reactive oxygen products such as superoxide anions and H_2O_2 in the resveratrol-treated group. These data indicate that resveratrol may provide protection to the liver during I/R injury, in part by improving activities of the endogenous antioxidant enzymes, which scavenge ROS and reduce their effects. Histopathologically, the resveratrol-treated group showed well preserved liver parenchyma with hepatocytes arranged radially around the central vein. In a study by Hassan-Khabbar *et al*[50], the effect of trans-resveratrol on liver injury induced by I/R was investigated. While Hassan-Khabbar performed measurements after 3 h of reperfusion, in the present study, measurements were performed after less than 1 h. Thus, the present study suggests that the protective effect of resveratrol may occur more rapidly than previously thought.

In conclusion, this study is the first to evaluate the hepatoprotective effects of resveratrol in hepatic I/R. The protective effects of resveratrol may be associated with its antioxidant activity and free radical scavenging activity which are released during the reperfusion period.

COMMENTS

Background

Hepatic injury caused by ischemia/reperfusion (I/R) has been proposed as a key clinical problem associated with liver transplantation and major liver surgery. Furthermore, hepatic I/R injury also occurs in diverse situations, including heart failure, liver trauma, and blood occlusion to the liver. The production of reactive oxygen species has been demonstrated in reperfusion injury. Resveratrol has been reported to have several biologic effects such as a potent antioxidative effect *via* prevention of lipid peroxidation. Therefore, the possible protective effects of resveratrol against I/R-induced hepatic injury were investigated.

Research frontiers

This study is the first to evaluate the hepatoprotective effects of resveratrol in hepatic I/R. The protective effects of resveratrol may be associated with its antioxidant activity and free radical scavenging activity which are released during the reperfusion period.

Innovations and breakthroughs

This study explained one of the possible mechanisms of the protective effects of resveratrol.This may be associated with its antioxidant activity and free radical scavenging activity released during the reperfusion period.

Applications

In this study, the implication of ROS in the pathology of hepatic I/R injury was explored. Protective effects of resveratrol were determined. This may represent a novel and attractive approach to determining the protective effects of resveratrol therapy on hepatic injury caused by ischemia/reperfusion

Peer review

In the present study, the authors tested the effect of Resveratrol on ischemia/reperfusion-induced liver injury in rats, and found a protective effect.

REFERENCES

1 **Ito K**, Ozasa H, Noda Y, Koike Y, Arii S, Horikawa S. Effect of non-essential amino acid glycine administration on the liver regeneration of partially hepatectomized rats with hepatic ischemia/reperfusion injury. *Clin Nutr* 2008; **27**: 773-780

2 **Jaeschke H**, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by neutrophils and Kupffer cells during in vivo reperfusion after hepatic ischemia in rats. *J Leukoc Biol* 1992; **52**: 377-382

3 **Tejima K**, Arai M, Ikeda H, Tomiya T, Yanase M, Inoue Y, Nagashima K, Nishikawa T, Watanabe N, Omata M, Fujiwara K. Ischemic preconditioning protects hepatocytes via reactive oxygen species derived from Kupffer cells in rats. *Gastroenterology* 2004; **127**: 1488-1496

4 **Lemasters JJ**. The mitochondrial permeability transition and the calcium, oxygen and pH paradoxes: one paradox after another. *Cardiovasc Res* 1999; **44**: 470-473

5 **Taniai H**, Hines IN, Bharwani S, Maloney RE, Nimura Y, Gao B, Flores SC, McCord JM, Grisham MB, Aw TY. Susceptibility of murine periportal hepatocytes to hypoxia-reoxygenation: role for NO and Kupffer cell-derived oxidants. *Hepatology* 2004; **39**: 1544-1552

6 **Soleas GJ**, Diamandis EP, Goldberg DM. Wine as a biological fluid: history, production, and role in disease prevention. *J Clin Lab Anal* 1997; **11**: 287-313

7 **Daniel O**, Meier MS, Schlatter J, Frischknecht P. Selected phenolic compounds in cultivated plants: ecologic functions, health implications, and modulation by pesticides. *Environ Health Perspect* 1999; **107** Suppl 1: 109-114

8 **Sobolev VS**, Cole RJ. trans-resveratrol content in commercial peanuts and peanut products. *J Agric Food Chem* 1999; **47**: 1435-1439

9 **Fremont L**. Biological effects of resveratrol. *Life Sci* 2000; **66**: 663-673

10 **Inoue H**, Umesono K, Nishimori T, Hirata Y, Tanabe T. Glucocorticoid-mediated suppression of the promoter activity of the cyclooxygenase-2 gene is modulated by expression of its receptor in vascular endothelial cells. *Biochem Biophys Res Commun* 1999; **254**: 292-298

11 **Sinha K**, Chaudhary G, Gupta YK. Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. *Life Sci* 2002; **71**: 655-665

12 **Inoue H**, Tanabe T, Umesono K. Feedback control of cyclooxygenase-2 expression through PPARgamma. *J Biol Chem* 2000; **275**: 28028-28032

13 **Huang SS**, Tsai MC, Chih CL, Hung LM, Tsai SK. Resveratrol reduction of infarct size in Long-Evans rats subjected to focal cerebral ischemia. *Life Sci* 2001; **69**: 1057-1065

14 **Bloomfield Rubins H**, Davenport J, Babikian V, Brass LM, Collins D, Wexler L, Wagner S, Papademetriou V, Rutan G, Robins SJ. Reduction in stroke with gemfibrozil in men with coronary heart disease and low HDL cholesterol: The Veterans Affairs HDL Intervention Trial (VA-HIT). *Circulation* 2001; **103**: 2828-2833

15 **Kimura Y**, Okuda H, Arichi S. Effects of stilbenes on arachidonate metabolism in leukocytes. *Biochim Biophys Acta* 1985; **834**: 275-278

16 **Yang YB**, Piao YJ. Effects of resveratrol on secondary damages after acute spinal cord injury in rats. *Acta Pharmacol Sin* 2003; **24**: 703-710

17 **Sepodes B**, Maio R, Pinto R, Marques C, Mendes-do-Vale J, McDonald MC, Thiemermann C, Mota-Filipe H. Tempol, an intracelullar free radical scavenger, reduces liver injury in hepatic ischemia-reperfusion in the rat. *Transplant Proc* 2004; **36**: 849-853

18 **Ozturk H**, Gezici A, Ozturk H. The effect of celecoxib, a selective COX-2 inhibitor, on liver ischemia/reperfusion-induced oxidative stress in rats. *Hepatol Res* 2006; **34**: 76-83

19 **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254

20 **Wasowicz W**, Neve J, Peretz A. Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. *Clin Chem* 1993; **39**: 2522-2526

21 **McCord JM**, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 1969; **244**: 6049-6055

22 **Beers RF Jr**, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* 1952; **195**: 133-140

23 **Lawrence RA**, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* 1976; **71**: 952-958

24 **Lowry OH**, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-275

25 **Shin T**, Kuboki S, Huber N, Eismann T, Galloway E, Schuster R, Blanchard J, Pritts TA, Lentsch AB. Activation of peroxisome proliferator-activated receptor-gamma during hepatic ischemia is age-dependent. *J Surg Res* 2008; **147**: 200-205

26 **van Gulik TM**, de Graaf W, Dinant S, Busch OR, Gouma DJ. Vascular occlusion techniques during liver resection. *Dig Surg* 2007; **24**: 274-281

27 **He XS**, Ma Y, Wu LW, Wu JL, Hu RD, Chen GH, Huang JF. Dynamical changing patterns of glycogen and enzyme histochemical activities in rat liver graft undergoing warm ischemia injury. *World J Gastroenterol* 2005; **11**: 2662-2665

28 **Shibuya H**, Ohkohchi N, Tsukamoto S, Satomi S. Tumor necrosis factor-induced, superoxide-mediated neutrophil accumulation in cold ischemic/reperfused rat liver. *Hepatology* 1997; **26**: 113-120

29 **Suzuki S**, Toledo-Pereyra LH. Interleukin 1 and tumor necrosis factor production as the initial stimulants of liver ischemia and reperfusion injury. *J Surg Res* 1994; **57**: 253-258

30 **Hughes H**, Farhood A, Jaeschke H. Role of leukotriene B4 in the pathogenesis of hepatic ischemia-reperfusion injury in the rat. *Prostaglandins Leukot Essent Fatty Acids* 1992; **45**: 113-119

31 **Jaeschke H**. Cellular adhesion molecules: regulation and functional significance in the pathogenesis of liver diseases. *Am J Physiol* 1997; **273**: G602-G611

32 **Aldemir M**, Bosnak M, Al B, Buyukbayram H, Tacyildiz I. Effects of molsidomine and lexipafant in hepatic ischaemia--reperfusion injury. *Injury* 2004; **35**: 232-237

33 **Bertelli AA**, Giovannini L, Bernini W, Migliori M, Fregoni M, Bavaresco L, Bertelli A. Antiplatelet activity of cis-resveratrol. *Drugs Exp Clin Res* 1996; **22**: 61-63

34 **Ferrero ME**, Bertelli AE, Fulgenzi A, Pellegatta F, Corsi MM, Bonfrate M, Ferrara F, De Caterina R, Giovannini L, Bertelli A. Activity in vitro of resveratrol on granulocyte and monocyte adhesion to endothelium. *Am J Clin Nutr* 1998; **68**: 1208-1214

35 **Yang YB**, Piao YJ. Effects of resveratrol on secondary damages after acute spinal cord injury in rats. *Acta Pharmacol Sin* 2003; **24**: 703-710

36 **Ray PS**, Maulik G, Cordis GA, Bertelli AA, Bertelli A, Das DK. The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury. *Free Radic Biol Med* 1999; **27**: 160-169

37 **Olas B**, Wachowicz B. Resveratrol and vitamin C as antioxidants in blood platelets. *Thromb Res* 2002; **106**: 143-148

38 **Chander V**, Tirkey N, Chopra K. Resveratrol, a polyphenolic phytoalexin protects against cyclosporine-induced nephrotoxicity through nitric oxide dependent mechanism. *Toxicology* 2005; **210**: 55-64

39 **Tadolini B**, Juliano C, Piu L, Franconi F, Cabrini L. Resveratrol inhibition of lipid peroxidation. *Free Radic Res* 2000; **33**: 105-114

40 **Chanvitayapongs S**, Draczynska-Lusiak B, Sun AY. Amelioration of oxidative stress by antioxidants and resveratrol in PC12 cells. *Neuroreport* 1997; **8**: 1499-1502

41 **Giakoustidis D**, Papageorgiou G, Iliadis S, Kontos N, Kostopoulou E, Papachrestou A, Tsantilas D, Spyridis C, Takoudas D, Botsoglou N, Dimitriadou A, Giakoustidis E. Intramuscular administration of very high dose of alpha-tocopherol protects liver from severe ischemia/reperfusion injury. *World J Surg* 2002; **26**: 872-877

42 **Kirimlioglu V**, Karakayali H, Turkoglu S, Haberal M. Effect of resveratrol on oxidative stress enzymes in rats subjected to 70% partial hepatectomy. *Transplant Proc* 2008; **40**: 293-296

43 **Jang DS**, Kang BS, Ryu SY, Chang IM, Min KR, Kim Y. Inhibitory effects of resveratrol analogs on unopsonized zymosan-induced oxygen radical production. *Biochem Pharmacol* 1999; **57**: 705-712

44 **Shen SQ**, Zhang Y, Xiang JJ, Xiong CL. Protective effect of curcumin against liver warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock protein and antioxidant enzymes. *World J Gastroenterol* 2007; **13**: 1953-1961

45 **Fernandez-Checa JC**, Garcia-Ruiz C, Colell A, Morales A, Mari M, Miranda M, Ardite E. Oxidative stress: role of mitochondria and protection by glutathione. *Biofactors* 1998; **8**: 7-11

46 **Jaeschke H**. Glutathione disulfide as index of oxidant stress in rat liver during hypoxia. *Am J Physiol* 1990; **258**: G499-G505

47 **Michiels C**, Raes M, Toussaint O, Remacle J. Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radic Biol Med* 1994; **17**: 235-248

48 **Plin C**, Tillement JP, Berdeaux A, Morin D. Resveratrol protects against cold ischemia-warm reoxygenation-induced damages to mitochondria and cells in rat liver. *Eur J Pharmacol* 2005; **528**: 162-168

49 **Leonard SS**, Xia C, Jiang BH, Stinefelt B, Klandorf H, Harris GK, Shi X. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. *Biochem Biophys Res Commun* 2003; **309**: 1017-1026

50 **Hassan-Khabbar S**, Cottart CH, Wendum D, Vibert F, Clot JP, Savouret JF, Conti M, Nivet-Antoine V. Postischemic treatment by trans-resveratrol in rat liver ischemia-reperfusion: a possible strategy in liver surgery. *Liver Transpl* 2008; **14**: 451-459

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RAPID COMMUNICATION

Identification of human papillomavirus in esophageal squamous papillomas

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Abstract

AIM: To investigate the presence of human papillomavirus (HPV) in esophageal squamous papilloma (ESP) and determine p16, p53 and Ki67 expression in a Mexican cohort.

METHODS: Nineteen cases diagnosed as ESP, corresponding to 18 patients were reviewed; nineteen cases of normal esophageal mucosa were used as negative controls. HPV detection was performed by amplified chromogenic in situ hybridization (ACISH) using a wide spectrum-cocktail probe and PCR.

RESULTS: The average age at presentation was 46.3 years (range 28-72 years). Patients included four (22.22%) males and 14 (77.77%) females. The most frequent location was upper third (11 cases), followed by middle third (3 cases) and unknown site (5 cases). Immunohistochemistry (IHC) revealed basal and focal p53 expression in 17 cases (89%); p16 was expressed in eight cases (42.10%) and the Ki67 index ranged from 10% to 30%. HPV was detected in 14 out of 16 cases (87.5%) by ACISH: Twelve showed diffuse nuclear patterns and two showed granular patterns. HPV DNA was identified by PCR in 12 out of 14 cases (85.7%). Low-risk HPV types were detected in the most of the cases.

CONCLUSION: This study provides identification of HPV infection in almost 80% of ESP using either ACISH or PCR; overall, all of these lesions show low expression of cell-cycle markers. We suggest ACISH as an alternative diagnostic tool for HPV detection in ESP.

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INTRODUCTION

Esophageal squamous papillomas (ESPs) are uncommon benign lesions, usually asymptomatic and often discovered as incidental findings. ESPs were first described by Adler in 1959[1-5]. The etiology and pathogenesis of ESP appear to be related to an inflammatory-reparatory type, such as chronic gastro-esophageal reflux, esophagitis, trauma, chemical irritants, and viruses[6,7]. Their malignant potential is still unclear, and it has been proposed that Human Papillomavirus (HPV) plays an etiopathogenic role; however, HPV is not consistently identified[8,9]. The detection of HPV in ESPs may have a great value, in terms of follow-up of lesions associated to genotypes implicated in the genesis of premalignant and malignant lesions in squamous epithelial-lined tissues, including nasal cavity, pharynx and the anogenital tract[7].

The Mexican population possesses a high prevalence of HPV-related cervical lesions. Previous studies have demonstrated high expression rates of $p16^{INK4a}$ in cervical lesions[10]; however, little is known on $p16^{INK4a}$ and the etiologic role of HPV in the development of ESPs. The purpose of this study is to investigate the presence of HPV through molecular techniques in a cohort of 19 ESPs cases and determine the relationship between HPV and the expression of cell cycle proteins.

MATERIALS AND METHODS

Nineteen specimens from 18 patients were retrieved from the Department of Pathology-UPAEP University

Table 1 Esophageal squamous papillomas: Clinical and pathological features

Age	Gender	Endoscopic indication	Location	ACISH	PCR	p16	p53	Ki67
33	F	Abdominal pain	Upper third	Diffuse	6/11	Positive	Positive	Positive
62	F[1]	Abdominal pain and reflux	Upper third	Diffuse	6/11	Positive	Positive	Positive
28	F	Abdominal pain	Upper third	Diffuse	6/11	Negative	Positive	Positive
72	M	Abdominal pain	Upper third	Diffuse	6/11	Negative	Positive	Positive
40	F	Abdominal pain	Upper third	Granular	16	Negative	Positive	Positive
57	F	Reflux	Upper third	Granular	16	Negative	Positive	Positive
46	M	Abdominal pain	Upper third	-	-	Negative	Positive	Positive
39	F	Abdominal pain	Upper third	Diffuse	6/11	Negative	Positive	Positive
32	F	Abdominal pain and reflux	Upper third	Diffuse	-	Negative	Positive	Positive
45	M	Reflux	Upper third	Diffuse	6/11	Negative	Positive	Positive
36	F	Abdominal pain	Upper third	Diffuse	6/11	Negative	Positive	Positive
66	F	Abdominal pain	Unknown	-	-	NA	NA	NA
42	F	Abdominal pain	Unknown	-	-	NA	NA	NA
61	F	Abdominal pain	Unknown	Diffuse	Negative	Positive	Positive	Positive
40	F	Reflux	Unknown	Diffuse	Negative	Positive	Positive	Positive
40	M	Reflux	Unknown	Diffuse	-	Positive	Positive	Positive
62	F[1]	Abdominal pain and reflux	Mid third	Diffuse	6/11	Positive	Positive	Positive
28	F	Abdominal pain	Mid third	Diffuse	6/11	Negative	Positive	Positive
28	F	Abdominal pain	Mid third	Diffuse	6/11	Negative	Positive	Positive

NA: Not available; ACISH: Amplified chromogenic in situ hybridization; PCR: Polymerase chain reaction; [1]Same patient.

Hospital files during a 12-year period. Endoscopic reports and clinical charts were reviewed to obtain information about age, gender, symptoms, endoscopic location and past medical history. Clinical and endoscopic follow-up were available for sixteen patients.

Multiple 4-μm thick sections from formalin-fixed paraffin-embedded tissue (FFPET) blocks were obtained. In addition to hematoxylin and eosin (HE) staining, the following immunostains were performed, according to manufacture's instructions: $p16^{INK4a}$ (Clone EGH4; 1:25; DakoCytomation, Carpinteria, California, USA), p53 (Clone DO-7; 1:100 DakoCytomation, Carpinteria, California, USA) and Ki67 (Clone MIB-1; 1:150; DakoCytomation, Carpinteria, California, USA). Negative and positive controls were reviewed. Slides were independently evaluated by two pathologists (OB, SSS). Discrepancies were resolved by consensus examination.

Molecular techniques were applied depending of availability of tissue. In 16 out of 19 cases, detection of HPV was performed by amplified chromogenic in situ hybridization (ACISH) (GenPoint/DakoCytomation, Carpinteria, California, USA), using a HPV biotinylated DNA probe. Nineteen samples of normal esophageal mucosa were used as negative controls.

Fourteen FFPET out of 19 ESPs were examined for HPV by PCR-RFLP, as described previously[11,12]. DNA was extracted from FFPET blocks (cases and negative control samples). After DNA extraction, amplifications were performed using consensus primers MY09 and MY11. To determine HPV types 6/11, 16, 18 and 31, the PCR products were digested using restriction endonucleases and analyzed by agarose gel electrophoresis.

RESULTS

The clinical and pathological features of the patients and their tumors are shown in Table 1. The average age at presentation was 46.3 years (range 28-72 years). Patients included four (22.22%) males and 14 (77.77%) females. The most frequent indication for endoscopy was abdominal pain (15 patients), followed by reflux (seven patients). In all cases, ESPs were incidental findings, discovered during the endoscopic procedure. In none of the cases was Barrett's esophagus or respiratory-tract papillomatosis reported. Clinical data showed no history of HPV-related diseases, immunosuppression or radiotherapy. At endoscopy, some patients also presented with Helicobacter pylori infection, chronic gastritis, peptic ulcer disease, hiatal hernia, esophagitis and reflux esophagitis.

Endoscopically, the most frequent location of ESP was the upper third (11 cases), followed by the middle third (3 cases) and unknown site (5 cases). All except one case were present as a single lesion. Patients underwent total endoscopic excision. Clinical and endoscopic follow-up was done in sixteen of the patients, and no recurrences have been documented to the date.

Grossly, ESPs present as polypoid, soft, smooth, whitish-pink tumors, ranging from 3 to 7 mm at their largest dimension. Histological examination revealed exophytic lesions, composed of branched fibrovascular core, covered by acanthotic squamous epithelium. No other growing pattern was observed. Basal cell hyperplasia and koilocytosis were present in all cases (100%). Vacuolated cells in the upper layers were detected in all cases. Parakeratosis, papillomatosis and intraepithelial lymphocytes and some eosinophils were also noted. Ten cases (52.63%) showed a prominent epithelial granular layer and intraepithelial capillary loops were present in nine cases (47.36%). Dyskeratotic cells and multinucleated giant cells were absent and no dysplastic changes were found in any of the patients.

HPV was detected in 14 of 16 cases (87.5%) by ACISH: Twelve showing a diffuse and complete nuclear

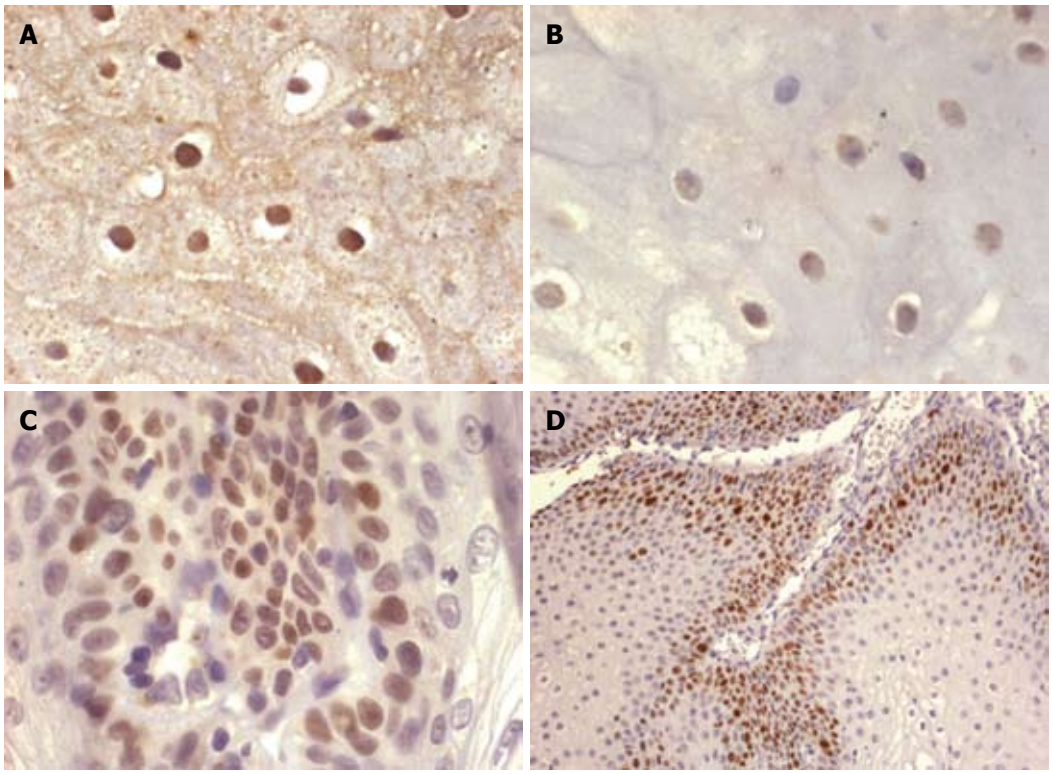

Figure 1 Esophageal squamous papillomas. A: Amplified chromogenic in situ hybridization, episomal infection with diffuse nuclear staining pattern; B: Amplified chromogenic in situ hybridization, nuclear granular staining pattern; C: p53 expression; D: Ki67 expression.

staining pattern (indicating the episomal state of the HPV viral genome, Figure 1A) and two a nuclear granular pattern (indicating the integrated form of the HPV viral genome, Figure 1B). HPV DNA was identified by PCR in 12 of 14 cases (85.7%). Infection by a low risk type HPV (6/11) was detected in 10 of 14 cases, whereas two of the 14 cases were infected by a high risk HPV type (16). Both high risk cases showed a granular staining pattern on ACISH, suggesting viral genomic infection. In two cases, ACISH demonstrated a diffuse pattern and HPV was not documented by PCR.

p53 was expressed in 17 cases (89%). The percent of p53-positive cells ranged from 10 to 15%, showing a basal and focal expression (Figure 1C). There was no difference between the p53 expression of non-infected and HPV-detected ESPs. In two cases, IHC for p53 was not performed (no tissue was present in the block). Eight cases (42.10%) expressed $p16^{INK4a}$ (both nuclear and cytoplasmic). Four of these cases were infected by 6/11 serotypes. In those cases, $p16^{INK4a}$ revealed a low rate of expression (< 20%), mostly at a basal layer location and focally extending into the medium third. Ki67 proliferative index in 17 cases ranged from 10 to 30% on basal cells (Figure 1D), and there was no difference on expression between non-HPV-related lesions and HPV high and low risk-related lesions.

DISCUSSION

This report of cases represents the first study performed in Mexico to determine the presence of HPV in an unusual esophageal lesion, whose relation with esophageal carcinoma is not completely understood. The etiology is still unclear, and the role of HPV in squamous cell lesions of the esophagus is difficult to establish. Since 1982, when Syrjanen *et al*[13] demonstrated the presence of HPV antigens in ESP, a certain percentage of ESPs have been reported as associated with HPV[2,7,14-16]; however, this relationship has not been consistently proven. Some authors hypothesize that mucosal injury, followed by regeneration, is the key role in the pathogenesis, supported by the coexistence of ESPs and gastro-esophageal reflux, esophagitis and hiatal hernia; also by the fact that most of the ESPs are located in lower level of the esophagus[7,17-18]. In a study, Talamini *et al*[9] found that in the Italian population, ESPs are located mostly in the middle third and secondarily, in the lower third; this study also reported a very low prevalence of cases associated with HPV and implied that alcohol intake and cigarette smoking were risks factors.

In contrast to previous reports, our study shows a female predominance, and a predominantly upper third location[8,9]. These findings raise the possibility that certain factors, including gastro-esophageal reflux, are unlikely to be the main etiological factors or are responsible for only a small proportion of cases. For that reason, some others conditions or perhaps, behavioral reasons, might be related in this specific population.

In our series of cases, we found that ten out of 11, were located in the upper third and were related to HPV-infection. In two of them, high grade serotypes (HPV 16) were detected. How does HPV get access to that location? This is still unknown. Jalal *et al*[19] have shown HPV DNA in the mouths of individuals with clinically normal oral mucosa. HPV is an epitheliotropic virus that requires the environment of a differentiating squamous epithelium for their life cycle. Peripheral blood mononuclear cells may serve as HPV carriers, spreading the virus *via* the blood[20]. The Mexican

population possesses a high prevalence of HPV-related cervical lesions, and some mechanism of infection probably exists, including viral transmission by direct contact (e.g. sexual) that might involve the infection of the upper third of the esophagus, but we could not confirm this hypothesis due to the non-availability of clinical information of the HPV-infection status in the patients' sexual partners.

According to Winkler *et al*[21], 31% of ESPs having histological findings of HPV infection, show positive reactions to HPV antigens by IHC; Odze *et al*[7] reported 50% positivity of HPV in 38 cases using PCR, mostly type 16, and proposed a multifactorial etiology including mucosal irritation and HPV association; Woo *et al*[18] also suggest a multifactorial cause and Poljak *et al*[8] believe that HPV DNA in ESPs is not the result of viral infection but the result of incidental HPV colonization which have been formed as result of other etiological factors; genetic association has also been mentioned[2]. In our study, we believe that presence of HPV-associated ESPs, reflects the high prevalence of the virus in developing countries, including Mexico, in contrast to western countries, and is in agreement with previous reports[22].

The malignant potential of ESPs has been described in some studies and is controversial[18]. According to Talamini *et al*[9], ESPs appear not to have a risk of development of malignancy or to be a marker of such risk. We found a predominance of HPV infection by low-risk serotypes and in two cases, high-risk serotype infection; however, no dysplastic changes, malignant transformation or recurrence was identified. Furthermore, we suggest that clinical and endoscopic follow-up should be recommended and performed on regular basis on patients with HPV high-risk serotypes lesions. These serotypes have been demonstrated by numerous epidemiologic and molecular studies to be the etiologic agents for an overwhelming majority of premalignant and malignant cervical cases[23] and in consequence, HPV might be related as a risk factor for esophageal carcinoma[7].

Previous reports have shown a correlation between $p16^{INK4a}$ expression in cases of cervical dysplasia HPV-related[10]. Although we did not find a relationship between HPV infection and protein expression of cell cycle markers, we believe that their utility will be mainly in the grading of dysplasia, and also in the evaluation of esophageal lesions when dysplasia or malignancy is suspected.

In summary, our study provides strong evidence that most ESPs are associated with HPV infection; however we believe that other pathogenetic mechanisms are related to the development of these lesions. PCR is currently considered to be the most sensitive method for the detection of HPV infection available[8] but is susceptible to false positives due to contamination. Although PCR is the gold-standard for HPV evaluation, PCR is not available in the pathological routine evaluation and ACISH might serve as a useful and alternative diagnostic tool for HPV detection.

COMMENTS

Background

Squamous papillomas are uncommon benign lesions of esophagus, of unknown etiology, and controversial malignant potential. Esophageal squamous papillomas (ESP) have been found to be associated with different conditions, including gastro-esophageal reflux, inflammation and trauma. Human papillomavirus (HPV) has been implicated in the genesis of malignant neoplasms in squamous epithelial-lined tissues, including the nasal cavity, pharynx and anogenital tract; however, previous studies have not consistently identified HPV in ESP.

Research frontiers

This study was designed to identify the presence of HPV in a series of 19 ESPs, from a population with high cervical HPV prevalence. Identification of HPV was performed by molecular techniques, and cell-cycle markers expression was assessed by immunohistochemistry.

Innovations and breakthroughs

Previous studies have not consistently identified HPV in ESP. To the best of our knowledge, this is the first study performed in Latin America to determine HPV infection in esophageal papillomas. In this Mexican cohort, we found that almost 80% of ESPs were associated with HPV infection.

Applications

Due to the well-known association of HPV with premalignant and malignant conditions, follow-up of patients with esophageal lesions with high-risk type of HPV and viral genomic infection should be implemented and guaranteed. Amplified chromogenic in situ hybridization (ACISH) can be used as an alternative diagnostic tool for routine HPV identification.

Terminology

HPV are a group of more than 100 viruses. Both high and low-risk types of HPV can cause the growth of abnormal cells, but only the high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73) lead to malignant transformation.

Peer review

This manuscript shows that the ACISH method is as useful as the standard PCR method in detecting HPV in esophageal squamous papillomas. The manuscript would be improved and more useful to the clinician if the author's would include photographs of gross esophageal squamous papillomas samples as well as photomicrographs for the ACISH results. The study is well conducted and the data support the conclusions of the author's that the use of ACISH represents an acceptable alternative to detect HPV in esophageal squamous papillomas.

REFERENCES

1 **Adler RH**, Carberry DM, Ross CA. of the esophagus: association with hiatal hernia. *J Thorac Surg* 1959; **37**: 625-635

2 **Carr NJ**, Bratthauer GL, Lichy JH, Taubenberger JK, Monihan JM, Sobin LH. Squamous cell papillomas of the esophagus: a study of 23 lesions for human papillomavirus by in situ hybridization and the polymerase chain reaction. *Hum Pathol* 1994; **25**: 536-540

3 **Szanto I**, Szentirmay Z, Banai J, Nagy P, Gonda G, Voros A, Kiss J, Bajtai A. [Squamous papilloma of the esophagus. Clinical and pathological observations based on 172 papillomas in 155 patients] *Orv Hetil* 2005; **146**: 547-552

4 **Colina F**, Solis JA, Munoz MT. Squamous papilloma of the esophagus. A report of three cases and review of the literature. *Am J Gastroenterol* 1980; **74**: 410-414

5 **Parnell SA**, Peppercorn MA, Antonioli DA, Cohen MA, Joffe N. Squamous cell papilloma of the esophagus. Report of a case after peptic esophagitis and repeated bougienage with review of the literature. *Gastroenterology* 1978; **74**: 910-913

6 **Orlowska J**, Jarosz D, Gugulski A, Pachlewski J, Butruk E. Squamous cell papillomas of the esophagus: report of 20 cases and literature review. *Am J Gastroenterol* 1994; **89**: 434-437

7 **Odze R**, Antonioli D, Shocket D, Noble-Topham S, Goldman H, Upton M. Esophageal squamous papillomas. A clinicopathologic study of 38 lesions and analysis for human papillomavirus by the polymerase chain reaction. *Am J Surg*

Pathol 1993; **17**: 803-812
8 **Poljak M**, Orlowska J, Cerar A. Human papillomavirus infection in esophageal squamous cell papillomas: a study of 29 lesions. *Anticancer Res* 1995; **15**: 965-969
9 **Talamini G**, Capelli P, Zamboni G, Mastromauro M, Pasetto M, Castagnini A, Angelini G, Bassi C, Scarpa A. Alcohol, smoking and papillomavirus infection as risk factors for esophageal squamous-cell papilloma and esophageal squamous-cell carcinoma in Italy. *Int J Cancer* 2000; **86**: 874-878
10 **Tringler B**, Gup CJ, Singh M, Groshong S, Shroyer AL, Heinz DE, Shroyer KR. Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia. *Hum Pathol* 2004; **35**: 689-696
11 **Shibata DK**, Arnheim N, Martin WJ. Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. *J Exp Med* 1988; **167**: 225-230
12 **Bauer HM**, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J, Reingold A, Manos MM. Genital human papillomavirus infection in female university students as determined by a PCR-based method. *JAMA* 1991; **265**: 472-477
13 **Syrjanen K**, Pyrhonen S, Aukee S, Koskela E. Squamous cell papilloma of the esophagus: a tumour probably caused by human papilloma virus (HPV). *Diagn Histopathol* 1982; **5**: 291-296
14 **Politoske EJ**. Squamous papilloma of the esophagus associated with the human papillomavirus. *Gastroenterology* 1992; **102**: 668-673
15 **Hording M**, Hording U, Daugaard S, Norrild B, Faber V. Human papilloma virus type 11 in a fatal case of esophageal and bronchial papillomatosis. *Scand J Infect Dis* 1989; **21**: 229-231
16 **Janson JA**, Baillie J, Pollock M. Endoscopic removal of esophageal condylomata acuminatum containing human papilloma virus. *Gastrointest Endosc* 1991; **37**: 367-370
17 **Quitadamo M**, Benson J. Squamous papilloma of the esophagus: a case report and review of the literature. *Am J Gastroenterol* 1988; **83**: 194-201
18 **Woo YJ**, Yoon HK. In situ hybridization study on human papillomavirus DNA expression in benign and malignant squamous lesions of the esophagus. *J Korean Med Sci* 1996; **11**: 467-473
19 **Jalal H**, Sanders CM, Prime SS, Scully C, Maitland NJ. Detection of human papilloma virus type 16 DNA in oral squames from normal young adults. *J Oral Pathol Med* 1992; **21**: 465-470
20 **Bodaghi S**, Wood LV, Roby G, Ryder C, Steinberg SM, Zheng ZM. Could human papillomaviruses be spread through blood? *J Clin Microbiol* 2005; **43**: 5428-5434
21 **Winkler B**, Capo V, Reumann W, Ma A, La Porta R, Reilly S, Green PM, Richart RM, Crum CP. Human papillomavirus infection of the esophagus. A clinicopathologic study with demonstration of papillomavirus antigen by the immunoperoxidase technique. *Cancer* 1985; **55**: 149-155
22 **Poljak M**, Cerar A, Seme K. Human papillomavirus infection in esophageal carcinomas: a study of 121 lesions using multiple broad-spectrum polymerase chain reactions and literature review. *Hum Pathol* 1998; **29**: 266-271
23 **Munoz N**, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003; **348**: 518-527

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RAPID COMMUNICATION

Immunolocalization of nestin in pancreatic tissue of mice at different ages

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Abstract

AIM: To localize nestin positive cells (NPC) in pancreatic tissue of mice of different ages.

METHODS: Paraffin sections of 6-8 μm of fixed pancreatic samples were mounted on poly-L-lysine coated slides and used for Immunolocalization using appropriate primary antibodies (Nestin, Insulin, Glucagon), followed by addition of a fluorescently labeled secondary antibody. The antigen-antibody localization was captured using a confocal microscope (Leica SP 5 series).

RESULTS: In 3-6 d pups, the NPC were localized towards the periphery of the endocrine portion, as evident from immunolocalization of insulin and glucagon, while NPC were absent in the acinar portion. At 2 wk, NPC were localized in both the exocrine and endocrine portions. Interestingly, in 4-wk-old mice NPC were seen only in the endocrine portion, towards the periphery, and were colocalized with the glucagon positive cells. In the pancreas of 8- wk-old mice, the NPC were predominantly localized in the central region of the islet clusters, where immunostaining for insulin was at a maximum.

CONCLUSION: We report for the first time the immunolocalization of NPC in the pancreas of mice of different ages (3 d to 8 wk) with reference to insulin and glucagon positive cells. The heterogeneous localization of the NPC observed may be of functional and developmental significance and suggest(s) that mice pancreatic tissue can be a potential source of progenitor cells. NPC from the pancreas can be isolated, proliferated and programmed to differentiate into insulin secreting cells under the appropriate microenvironment.

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Key words: Nestin; Insulin; Glucagon; Immunolocalization; Mice

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INTRODUCTION

Diabetes results from an inadequate mass of functional pancreatic β-cells and such inadequacy can result from a lack of selective autoimmune destruction of pancreatic β-cells (type 1)[1,2] or a lack of compensation to overcome Insulin resistance (type 2)[3,4]. In addition, an intrinsic β-cell defect, fragility, or shortened life span may be other possible impairments underlying the pathophysiology of diabetes[5]. Recently, adult pancreatic tissue (exocrine/endocrine) has emerged as a promising source of pancreatic stem cells, which are capable of differentiating into functional insulin secreting cells (ISC) given an appropriate microenvironment[6,7]. The introduction of new methods for *in vitro* generation of

β-cells from pancreatic tissue will help to provide greater numbers of viable ISC.

The promise of using adult tissue stem cells are attributed to their wide distribution in almost all tissues, modulation of their plasticity, ability to be modified genetically or reprogrammed and they are safer for transplantation[8]. They can also be immortalized or multiplied in culture for a number of passages and the ethical constraints on their use are minimal as compared to the use of embryonic stem cells[9]. Nestin has been unequivocally identified as a marker of neural stem cells or progenitors[10,11]. It is abundantly expressed in neuroepithelial cells during embryogenesis but it is nearly absent from all mature central nervous system cells[12,13]. In addition, nestin has been demonstrated in rat bone marrow[14], human embryonic stem cells[15,16], human islet explants[17], human fetal pancreas[18], and adult rat pancreas[19]. The varied expression and characterization of nestin positive cells (NPC) in different cell types is thought to have considerable functional significance during the developmental process. Despite several studies documenting the potential of NPC as stem cells/progenitors to differentiate into ISC, some studies have reported that NPC may not function as progenitors for ISC[20,21]. Nestin is an intermediate filament protein and might play a key role in imparting cytoskeletal functions to the cell[22]. In support of these reports, ductal epithelial cells (DEC), which comprise 10% of the pancreatic cells expressing the intermediate filament proteins cytokeratin 7 and 19, have been demonstrated to function as pancreatic progenitors due to their ability to generate ISC[7]. NPC present in the pancreas, in addition to imparting the structural functions, also participate in developmental regulation as progenitors, similarly to DEC[23].

Therefore, exploring *in vitro* system(s) for the precise characterization and propagation of NPC would be a feasible approach to harness the potential of NPC as progenitors capable of differentiation into ISC. The present investigation has been undertaken to characterize NPC in the pancreas of mice at different ages (3 d to 8 wk), which has not been previously reported. Hence, understanding the localization of NPC at different ages would help in identifying the source of the progenitors and can further facilitate their differentiation into ISC under an appropriate microenvironment.

MATERIALS AND METHODS

Experimental design

All animal experimental procedures were approved by the Institutional ethical committee on animal research. Male Swiss albino mice aged 3 d, 1, 2, 4 and 8 wk were obtained from the National Center for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad, India.

Tissue preparation

The animals were ethically sacrificed using CO_2 asphyxiation and their pancreas were removed and washed with PBS. After removal of the adhered fat, the tissue was fixed overnight in Bouins solution at room temperature[24,25]. The fixed tissue was washed twice in 70% Ethanol and paraffin sections of 6-8 μm were cut using a microtome before being mounted on to poly-L-lysine coated slides. They were dried overnight at 37℃ before proceeding to immunolocalization.

Sample preparation and immunolocalization for nestin/insulin/glucagon

The pancreatic sections from all the age groups were processed under identical conditions. The samples were de-paraffinized by heating at 60℃ for 30 min. They were then dehydrated by passing through a series of decreasing concentration of ethanol (100%, 90%, 70%, 50%, and 30%). This was followed by washing for 2 min each with double distilled water and PBS. They were then blocked with 4% Horse serum at room temperature for 1 h. Subsequently they were incubated with primary antibody overnight at 4℃ (Mouse anti Rat Nestin 1:200 BD Biosciences, USA, anti mouse glucagon 1:200 Santa Cruz and anti mouse insulin 1:500 Sigma, USA). After repeated washing with PBS containing Ca^{2+} and Mg^{2+}, the slides were treated with a secondary antibody tagged with an appropriate fluorescent dye (Goat anti guinea pig alexa 488, Goat anti rabbit alexa 546, Goat anti mouse alexa 633, Molecular Probes, USA). The fluorescence images were captured using a confocal microscope (Leica SP5 series) and fluorescent intensities units (FIU) were corrected using appropriate controls (primary antibody controls). The FIU have been quantitated as relative fluorescent units (RFU) and the experiments have been carried out independently in three sets of mice.

Statistical test

Results are expressed as mean ± SE using three independent experiments. One-way analysis of variance (ANOVA) was used, followed by a post-hoc LSD test with SPSS software to determine the significance.

RESULTS

Immunolocalization of nestin in mice pancreases at different ages is shown in Figure 1. In the early phase of postnatal period (3-6 d pups), the NPC were localized more towards the periphery of the endocrine pancreas, as evident from the colocalization of insulin and glucagon with NPC and by the absence of NPC in the acinar fraction (Figure 1A and B). In the 2-wk-old mice, the NPC were seen both in the exocrine as well as the endocrine pancreas (Figure 1C). Interestingly, in the 4-wk-old mice, the NPC were confined to the endocrine pancreas and were located more towards the periphery along with the glucagon positive cells (Figure 1D). In the 8-wk-old pancreatic tissue, the NPC localized predominantly in the central region of the islets clusters, where immunostaining for insulin was also predominant (Figure 1E). The RFU for the localization of nestin is shown in (Figure 2) where the NPC showed predominance in the insulin enriched fraction by eight week.

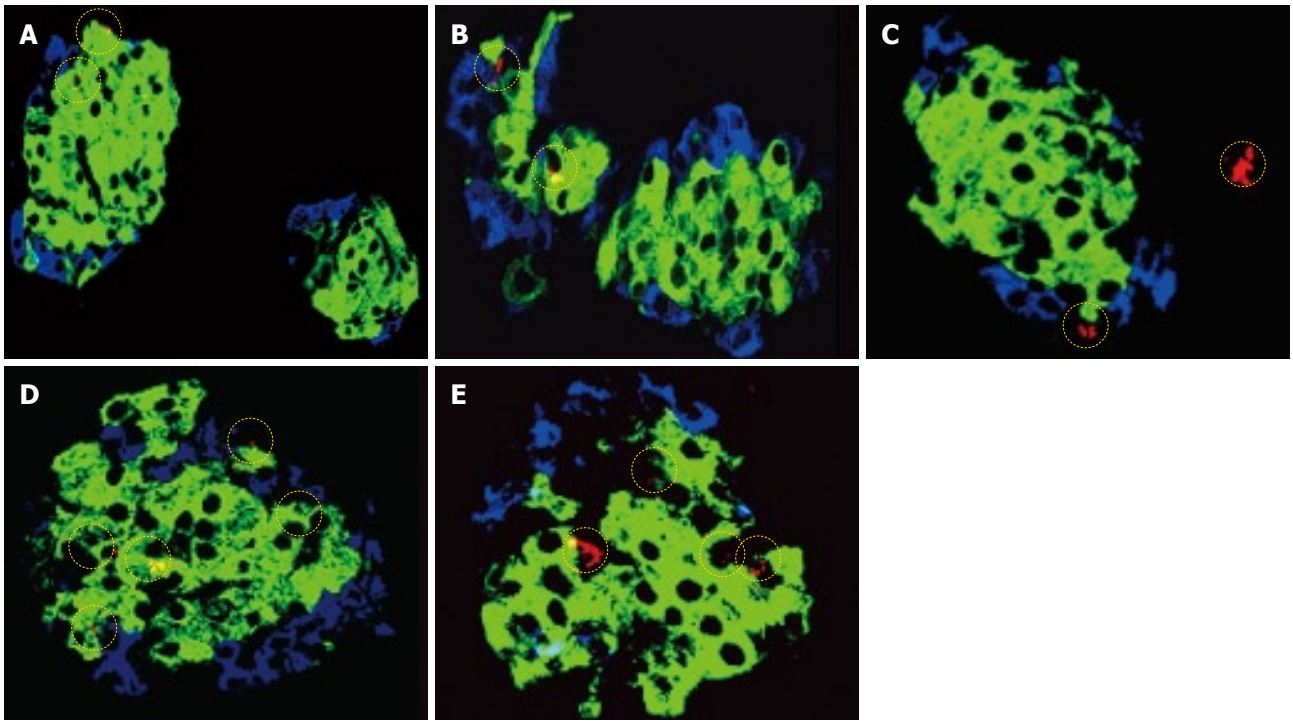

Figure 1 Immunolocalization of nestin in mice pancreases at different ages. A: Immunohistochemical localization of Nestin (red), Insulin (green), glucagon (blue) in three-day-old mouse pancreatic section, the presence of nestin is more towards the periphery of the endocrine fraction, magnification (x 400); B: One-week-old mouse pancreatic section, the presence of nestin is more towards the periphery of the endocrine fraction, magnification (x 400); C: Two-week-old mouse pancreatic section showing Nestin staining in both the exocrine as well as endocrine fraction, magnification (x 400); D: Four-week-old mouse pancreatic section showing Nestin staining only in the endocrine fraction confining more towards the periphery of the insulin stained cells, magnification (x 400); E: Eight-week-old mouse pancreatic section showing the Nestin localization predominant in the central region of the islets clusters where immunostaining for insulin was also significant, magnification (x 400).

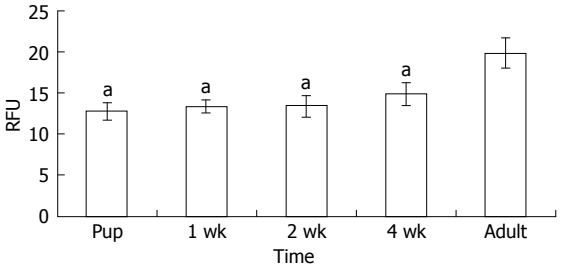

Figure 2 Quantitative relative fluorescence units of Nestin for different age groups performed in three different sections showing a higher level in the 8-wk-old pancreatic tissue. $^{a}P < 0.05$ in the adult as as compared to other four ages.

DISCUSSION

The present observations unequivocally suggest heterogeneity in the distribution of NPC within pancreatic tissue at different ages of mice (3 d to 8 wk) and this could be of functional and developmental significance. During embryogenesis, an increased expression of nestin has been reported in the neuroepithelial stem cells[26]. In line with these studies, the role of nestin has been demonstrated in the process of cellular rearrangements in the undifferentiated cells[27,28]. Studies in human islet explants, rat pancreatic tissue and embryonic stem cells have explored nestin as a marker of pancreatic progenitor stem cells, which have the renewal property and can be regulated to differentiate into insulin and glucagon positive cells[29,30].

The immunohistochemical localization of NPC (predominantly in the insulin-enriched fraction) has been depicted as RFU in Figure 2. These observations show that the endocrine portion of the pancreas serves as the primary enriched source of progenitors such as NPC, which, like DEC, can be expanded and differentiated into ISC with the appropriate growth factors in their microenvironment. In the present study, the application of reliable and specific immunocytochemical technique using specific antibodies and confocal microscopy has enabled the identification of NPC associated/colocalized with insulin and glucagon cells.

We report for the first time the significant localization of NPC (progenitors) in the endocrine pancreas at different ages of the mice. With increasing age of mice, the colocalization of NPC is more predominant in the insulin positive cells in the central region, unlike that seen at other ages. Identification of the sources and understanding the conditions and factors within the microenvironment of the pancreatic stem cells will be of therapeutic importance for the generation of ISC, which may be useful in the management of diabetes.

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COMMENTS

Background

The present study has been undertaken to investigate the precise localization and characterization of nestin positive cells (NPC) as a source of pancreatic progenitors that are capable of differentiating into insulin secreting cells (ISC). Nestin is an intermediate filament protein playing key role in imparting cytoskeletal functions to the cell and has been demonstrated as a progenitor in the neuronal tissue, bone marrow, embryonic stem cells, islet explants, fetal and adult pancreatic tissue.

Innovations and breakthroughs

This paper demonstrates the immunolocalization of NPC along with insulin and glucagon at different ages of mice for the first time.

Applications

Their data demonstrates the predominance of NPC in the insulin enriched fraction of 8-wk-old mice and these observations are significant due to the fact that NPC are one of the progenitors of adult pancreatic tissue. The NPC can be expanded and differentiated into ISC with the appropriate growth factors in its microenvironment.

Terminology

NPC standed for a pancreatic progenitor; ISC means the beta cells of islet that secrete insulin.

Peer review

The study investigates immunolocalization of nestin in the mouse pancreas. Nestin is a known marker of neural stem cells, which is also transiently expressed by many types of cells during development. Upon differentiation, nestin becomes downregulated. One best known instance of nestin expression in adult organisms, are the neuronal precursor cells of the subventricular zone. The study by Dorisetty *et al* suggests that nestin is localized to the endocrine pancreas. Based on this, the authors hypothesize that pancreatic endocrine tissue is a potent source of progenitor cells, which could be reprogrammed into insulin-producing cells and could be used for the treatment of diabetes. The idea of the study is interesting, and is supported by the literature data.

REFERENCES

1 **Lohr M**, Kloppel G. Residual insulin positivity and pancreatic atrophy in relation to duration of chronic type 1 (insulin-dependent) diabetes mellitus and microangiopathy. *Diabetologia* 1987; **30**: 757-762

2 **Pipeleers D**, Ling Z. Pancreatic beta cells in insulin-dependent diabetes. *Diabetes Metab Rev* 1992; **8**: 209-227

3 **DeFronzo RA**, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 1991; **14**: 173-194

4 **Kruszynska YT**, Olefsky JM. Cellular and molecular mechanisms of non-insulin dependent diabetes mellitus. *J Investig Med* 1996; **44**: 413-428

5 **Brownlee M**, Cerami A. The biochemistry of the complications of diabetes mellitus. *Annu Rev Biochem* 1981; **50**: 385-432

6 **Okuno M**, Minami K, Okumachi A, Miyawaki K, Yokoi N, Toyokuni S, Seino S. Generation of insulin-secreting cells from pancreatic acinar cells of animal models of type 1 diabetes. *Am J Physiol Endocrinol Metab* 2007; **292**: E158-E165

7 **Bonner-Weir S**, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, O'Neil JJ. In vitro cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci USA* 2000; **97**: 7999-8004

8 **Choi Y**, Ta M, Atouf F, Lumelsky N. Adult pancreas generates multipotent stem cells and pancreatic and nonpancreatic progeny. *Stem Cells* 2004; **22**: 1070-1084

9 Agarwal SS. Regulating stem cell research & therapy. *Indian J Med Res* 2006; **124**: 225-228

10 **Walcott JC**, Provis JM. Muller cells express the neuronal progenitor cell marker nestin in both differentiated and undifferentiated human foetal retina. *Clin Experiment Ophthalmol* 2003; **31**: 246-249

11 **Messam CA**, Hou J, Berman JW, Major EO. Analysis of the temporal expression of nestin in human fetal brain derived neuronal and glial progenitor cells. *Brain Res Dev Brain Res* 2002; **134**: 87-92

12 **Lendahl U**, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. *Cell* 1990; **60**: 585-595

13 **Dahlstrand J**, Zimmerman LB, McKay RD, Lendahl U. Characterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments. *J Cell Sci* 1992; **103** (Pt 2): 589-597

14 **Wislet-Gendebien S**, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. *Stem Cells* 2005; **23**: 392-402

15 **Abraham EJ**, Leech CA, Lin JC, Zulewski H, Habener JF. Insulinotropic hormone glucagon-like peptide-1 differentiation of human pancreatic islet-derived progenitor cells into insulin-producing cells. *Endocrinology* 2002; **143**: 3152-3161

16 **Huang H**, Tang X. Phenotypic determination and characterization of nestin-positive precursors derived from human fetal pancreas. *Lab Invest* 2003; **83**: 539-547

17 **Hunziker E**, Stein M. Nestin-expressing cells in the pancreatic islets of Langerhans. *Biochem Biophys Res Commun* 2000; **271**: 116-119

18 **Humphrey RK**, Bucay N, Beattie GM, Lopez A, Messam CA, Cirulli V, Hayek A. Characterization and isolation of promoter-defined nestin-positive cells from the human fetal pancreas. *Diabetes* 2003; **52**: 2519-2525

19 **Zulewski H**, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF. Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 2001; **50**: 521-533

20 **Piper K**, Ball SG, Turnpenny LW, Brickwood S, Wilson DI, Hanley NA. Beta-cell differentiation during human development does not rely on nestin-positive precursors: implications for stem cell-derived replacement therapy. *Diabetologia* 2002; **45**: 1045-1047

21 **Selander L**, Edlund H. Nestin is expressed in mesenchymal and not epithelial cells of the developing mouse pancreas. *Mech Dev* 2002; **113**: 189-192

22 **Street CN**, Lakey JR, Seeberger K, Helms L, Rajotte RV, Shapiro AM, Korbutt GS. Heterogenous expression of nestin in human pancreatic tissue precludes its use as an islet precursor marker. *J Endocrinol* 2004; **180**: 213-225

23 **Lumelsky N**, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 2001; **292**: 1389-1394

24 **Fukayama M**, Ogawa M, Hayashi Y, Koike M. Development of human pancreas. Immunohistochemical study of fetal pancreatic secretory proteins. *Differentiation* 1986; **31**: 127-133

25 **Erlandsen SL**, Hegre OD, Parsons JA, McEvoy RC, Elde RP. Pancreatic islet cell hormones distribution of cell types in the islet and evidence for the presence of somatostatin and gastrin within the D cell. *J Histochem Cytochem* 1976; **24**: 883-897

26 **Dahlstrand J**, Collins VP, Lendahl U. Expression of the class VI intermediate filament nestin in human central nervous

system tumors. *Cancer Res* 1992; **52**: 5334-5341
27 **Palm K**, Salin-Nordstrom T, Levesque MF, Neuman T. Fetal and adult human CNS stem cells have similar molecular characteristics and developmental potential. *Brain Res Mol Brain Re*s 2000; **78**: 192-195
28 **Rietze RL**, Valcanis H, Brooker GF, Thomas T, Voss AK, Bartlett PF. Purification of a pluripotent neural stem cell from the adult mouse brain. *Nature* 2001; **412**: 736-739
29 **Wang R**, Li J, Yashpal N, Gao N. Nestin expression and clonal analysis of islet-derived epithelial monolayers: insight into nestin-expressing cell heterogeneity and differentiation potential. *J Endocrinol* 2005; **184**: 329-339
30 **Lumelsky N**, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 2001; **292**: 1389-1394

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RAPID COMMUNICATION

Noninvasive assessment of liver fibrosis with combined serum aminotransferase/platelet ratio index and hyaluronic acid in patients with chronic hepatitis B

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Abstract

AIM: To construct a noninvasive assessment model consisting of routine laboratory data to predict significant fibrosis and cirrhosis in patients with chronic hepatitis B (CHB).

METHODS: A total of 137 consecutive patients with CHB who underwent percutaneous liver biopsy were retrospectively analyzed. These patients were divided into two groups according to their aminotransferase (ALT) level. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), the likelihood ratio (LR) of aminotransferase/platelet ratio index (APRI) $\geqslant$ 1.5 or < 1.5 in combination with different hyaluronic acid (HA) cut-off points were calculated for the presence of moderate to severe fibrosis/cirrhosis (fibrosis stages 2 and 4) and no to mild fibrosis/cirrhosis (fibrosis stages 0 and 1).

RESULTS: The APRI correlated with fibrosis stage in CHB patients. The APRI $\geqslant$ 1.5 in combination with a cut-off HA cut-off point > 300 ng/mL could detect moderate to severe fibrosis (stages 2-4) in CHB patients. The PPV was 93.7%, the specificity was 98.9%. The APRI < 1.5 in combination with different HA cut-off points could not detect no to mild fibrosis in CHB patients.

CONCLUSION: The APRI $\geqslant$ 1.5 in combination with a HA cut-off point > 300 ng/mL can detect moderate to severe fibrosis (stages 2-4) in CHB patients.

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Key words: Noninvasive assessment; Liver fibrosis; Chronic hepatitis B; Aminotransferase/platelet ratio index; Hyaluronic acid

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INTRODUCTION

About 350 million individuals are chronically infected with hepatitis B virus (HBV) worldwide[1]. There are 30 million patients with chronic hepatitis B (CHB) in China, which will progress to cirrhosis or hepatocellular carcinoma (HCC) in 10%-30% of CHB patients. Although antiviral treatment with interferon or nucleoside analogues has been widely adopted, it has significant side effects. Liver biopsy can help decide the treatment modality for patients infected with HBV, especially for those whose alanine aminotransferase (ALT) is under 2 of the upper limit of normal (ULN) or normal, but its value is questioned because of its potential risk and the concern of sampling errors[2]. Therefore, there is a growing tendency to use noninvasive measures instead of histopathological analysis of liver tissue for the evaluation of disease progression in patients with chronic liver diseases. Up to date, several laboratory tests, scores, and indices have been proposed for noninvasive prediction of hepatic fibrosis in CHB patients[3,4]. However, the results of such tests are different in

different study populations[5]. Aminotransferase/platelet ratio index (APRI) is easy to calculate, but it can only predict the severe hepatic fibrosis (F3, F4). Kuroiwa *et al*[6] reported that HA can predict all fibrosis stages, but its sensitivity and specificity are not very high. We hypothesized that APRI in combination with different hyaluronic acid (HA) cut-off points would be a better predictor of fibrosis than individual parameters.

MATERIALS AND METHODS

Patients

A total of 137 consecutive patients with CHB who underwent percutaneous liver biopsy at Shanghai Public Health Clinical Center (China) from 2005 to April 2008 were included in this study. Real-time PCR showed that all patients were positive for HBV DNA and had no chronic liver disease confirmed by standard clinical, serological, biochemical, and radiological criteria. Additional exclusion criteria were antiviral treatment before liver biopsy, alcohol consumption in excess of 40 g/d. Liver biopsies were obtained by either blind or ultrasound-guided techniques using a 16-gauge Klatskin needle. The length of biopsy samples was longer than 1.5 cm. All biopsies were read by a pathologist who had no clinical information on the CHB patients. Formalin-fixed and formalin-embedded liver tissues were cut into 4-μm thick sections with a microtome. One section was stained with hematoxylin and eosin for assessment of hepatic inflammatory activity and the other sections were stained with Gomori stain for evaluation of hepatic fibrosis. Biopsy specimens with at least 4 portal fields were considered representative and scored by a pathologist unaware of the laboratory results. Fibrosis was staged as no (0), mild (1), moderate (2), severe (3), and cirrhosis (4), using the METAVIR score[7]. Hepatic inflammatory activity was also scored.

Serum aspartate aminotransferase (AST), ALT, HA and platelet count in all patients within 2 wk after liver biopsy were routinely determined. The ULN for ALT was 50 U/L, and transformed into ULN for further analysis. The reference range for platelet count was 100 $\times 10^9$-300 $\times 10^9$/L. HA was measured using the RIA and the reference range was 9-119 ng/mL. According to the ULN of ALT, we divided the patients into two groups with their ALT $\geqslant$ 2ULN and < 2ULN, respectively. APRI was calculated as previously described[8,9].

Statistical analysis

Baseline demographic data were evaluated for comparability of the two groups using Fisher's exact test for categorical variables and Student's *t* test for continuous variables. Student's *t* test or analysis of variance was used to compare the means of different stage groups when appropriate. Correlation was evaluated by the Spearman correlation coefficient. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and likelihood ratio (LR) of APRI $\geqslant$ 1.5 and APRI < 1.5 in combination with different HA cut-off points were detected for the presence of moderate to severe fibrosis/cirrhosis (fibrosis stages 2 and 4) and no to mild fibrosis/cirrhosis (fibrosis stages 0 and 1). $P < 0.05$ was considered statistically significant.

Table 1 Characteristics of patients included in this study

	ALT $\geqslant$ 2ULN	ALT < 2ULN
Number of patients	78	59
Age (SD)	35.2 (7)	38.7 (7.4)
Gender		
Male	70.5%	69.5%
Female	29.5%	30.5%
Stage of fibrosis (%)		
F0 + F1	33 (42.3)	24 (40.7)
F2	26 (33.3)	20 (33.9)
F3	13 (16.7)	9 (15.2)
F4	6 (7.7)	6 (10.1)
Significant fibrosis ($\geqslant$ F2)	45 (57.3)	35 (59.3)
APRI[1](SD)		
F0 + F1	0.55 (0.82)	0.48 (0.33)
F2	1.44 (1.79)	1.21 (1.57)
F3	1.98 (2.34)	1.69 (1.62)
F4	2.11 (1.81)	1.97 (1.73)
Significant fibrosis ($\geqslant$ F2)	1.84 (1.38)	1.62 (1.45)
HA (SD)		
F0 + F1	131.3 (82.7)	129.9 (79.8)
F2	199.3 (158.2)	190.5 (149.9)
F3	285.3 (188.6)	285.7 (187.3)
F4	324.9 (212.6)	333.3 (224.1)
Significant fibrosis ($\geqslant$ F2)	269.8 (214.1)	268.9 (187.1)

[1]APRI: (AST/ULN) × 100/PLT.

RESULTS

Characteristics of patients

A total of 137 patients with CHB who underwent percutaneous liver biopsy were included in this study. In order to adequately estimate the predictive model, the patients were divided into 2 groups with their ALT $\geqslant$ 2ULN and ALT < 2ULN, respectively. There was no significant correlation between age, gender, HA, APRI and disease stage. The characteristics of patients in the two groups are shown in Table 1.

Stages of fibrosis

The distribution of fibrosis stages in the two groups is shown in Table 1. Of the 137 patients, 57 (41.6%) had no or only mild fibrosis (stages 0 and 1), 46 (33.6%) had moderate fibrosis (stage 2), and 34 (24.8%) had severe fibrosis or cirrhosis (stages 3 and 4). As expected, the APRI and HA cut-off points increased with the stage of fibrosis, but there was no significant difference between the two groups. The mean APRI was $\geqslant$ 1.5 in patients with moderate to severe fibrosis or cirrhosis (stages 2-4) and < 1.5 in patients with no or mild fibrosis (stages 0 and 1).

Among the patients with their APRI $\geqslant$ 1.5, the Spearman correlation coefficient was $r = 0.312$ and 0.344 between APRI and fibrosis stage ($P < 0.05$, Figure 1), and $r = -0.717$ and -0.812 between HA and fibrosis stage

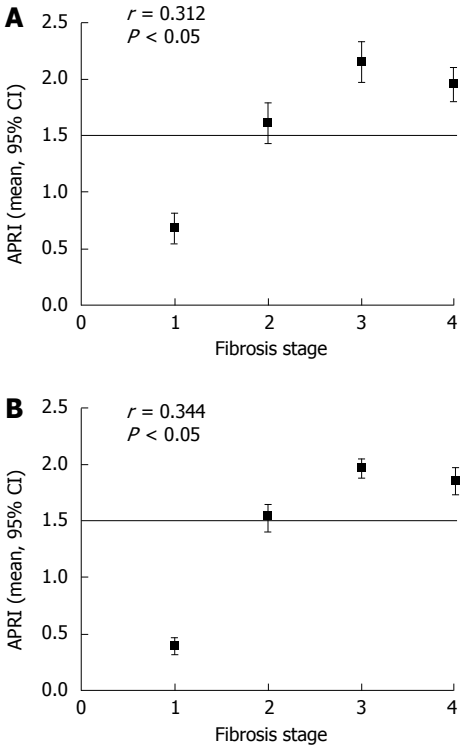

Figure 1 APRI and fibrosis stage in CHB patients with their ALT ⩾ 2ULN (A) or < 2ULN (B).

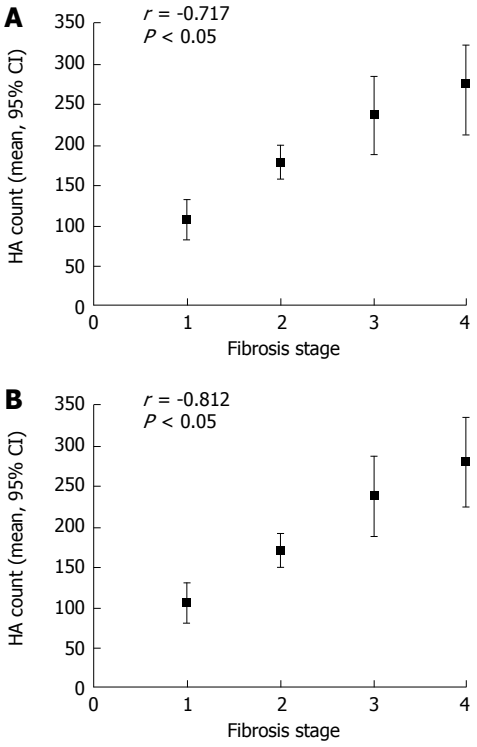

Figure 2 HA and fibrosis stage in CHB patients with their ALT ⩾ 2ULN (A) or < 2ULN (B).

Table 2 Sensitivity, specificity, PPV, and NPV of APRI > 1.5 in combination with different HA cut-off points for the detection of liver fibrosis (stages 2-4) in CHB patients

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	-LR
APRI ⩾ 1.5	44.7	84.3	41.3	84.7	2.80	0.66
+HA ⩾ 150	46.6	95.6	88.6	89.5	10.6	0.56
+HA ⩾ 200	46.8	97.8	90.2	89.9	21.3	0.54
+HA ⩾ 250	47.3	98.7	93.2	90.2	36.4	0.53
+HA ⩾ 300	45.3	98.9	93.7	91.3	41.2	0.55

Table 3 Sensitivity, specificity, PPV, and NPV of APRI ⩽ 1.5 in combination with different HA cut-off points for the detection of liver fibrosis (stages 0-1) in CHB patients

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	-LR
APRI < 1. 5	35.3	81.6	41.3	82.2	1.9	0.79
+HA < 300	45.7	86.2	44.6	83.1	3.3	0.63
+HA < 250	42.8	83.2	42.8	81.3	2.5	0.69
+HA < 200	38.5	80.8	40.8	80.3	2.0	0.76
+HA < 150	31.7	78.8	37.1	77.7	1.5	0.87

($P < 0.05$, Figure 2). However, there was no significant difference in patients with their APRI < 1.5 between APRI or HA count and fibrosis stage.

APRI ⩾ 1.5 in combination with different HA cut-off points could detect moderate to severe fibrosis (stages 2-4) in CHB patients (Table 2). The sensitivity, specificity, PPV, NPV, +LR and -LR were determined. APRI alone had a PPV of 41.3% and a specificity of 84.7%. When HA was added, the PPV and specificity increased significantly, indicating that APRI ⩾ 1.5 in combination with a HA cut-off point > 300 ng/mL can detect moderate to severe fibrosis or cirrhosis (stages 2-4) in CHB patients. In the present study, the PPV was 93.7% and the specificity was 98.9%. On the other hand, not all patients with moderate to severe fibrosis or cirrhosis could be correctly identified. The identification methods for patients with no or mild fibrosis (stages 0 and 1) using APRI < 1.5 in combination with different HA cut-off points are shown in Table 3. Since the sensitivity, specificity, PPV and NPV were low, mild fibrosis in CHB patients could not be detected using these laboratory parameters.

DISCUSSION

It is well known that the exact staging of liver fibrosis is crucial for the therapeutic decision and assessing the prognosis of CHB patients. The gold standard for fibrosis staging is liver biopsy. However, a simple noninvasive method for detection of fibrosis would be beneficial. Some studies have shown that APRI is only sensitive to F3-F4 stages of fibrosis[10,11].

HA, mainly metabolized in liver, is one of the important components of extracellular matrix, and can reflect the level of hepatic fibrosis in some degree. Guéchot *et al*[12] showed that the sensitivity and specificity of serum HA at a cut-off point of 110 μg/L for the diagnosis of hepatic fibrosis are 79% and 89%, respectively. Patel *et al*[13] reported that as a noninvasive valuable marker, serum HA concentration is correlated with hepatic fibrosis. Kuroiwa *et al*[6] showed that the

AUC value of HA for any fibrosis and cirrhosis is higher than 0.5. Montazeri *et al*[14] demonstrated that serum HA is a preferred marker of severe fibrogenesis and inflammation in CHB patients. HA at a cutoff point of 126.4 μg/L can detect severe fibrosis with a sensitivity of 90.9% and a specificity of 98.1%.

In this study, the APRI was correlated with fibrosis stage in CHB patients. The sensitivity, specificity, PPV, NPV, +LR and -LR of APRI in detecting were 35.3%, 81.6%, 41.3%, 82.2%, 1.9 and 0.79, respectively, for the detection of mild fibrosis (stage 1) in CHB patients. However, its sensitivity, specificity, PPV, NPV, +LR and -LR of APRI were 44.7%, 84.3%, 41.3%. 84.7%, 2.8 and 0.66, respectively, for the detection of moderate to severe fibrosis (stages 2-4) in CHB patients. The sensitivity, specificity, PPV, and NPV of HA were very low for the detection of fibrosis stages in CHB patients. APRI does not involve a complicated formula, thus allowing it to be quickly calculated. In addition, it uses 2 laboratory tests and is not associated with the added expense of a reference laboratory, and does not contain subjective parameters such as ethanol intake.

We established a noninvasive assessment model of liver fibrosis consisting of APRI and HA. APRI $\geqslant$ 1.5 in combination with different HA cut-off points was used to predict moderate to severe fibrosis (stages 2-4) in CHB patients. APRI alone had a PPV of 41.3% and a specificity of 84.7%. When different HA cut-off points were added, the PPV and specificity increased significantly, especially when a HA cut-off point was greater than 300 ng/mL, indicating that APRI $\geqslant$ 1.5 in combination with a HA cut-off point > 300 ng/mL can predict moderate to severe fibrosis (stages 2-4) in CHB patients. The PPV, specificity and LR of this model were 93.7%, 98.9% and 41.2, respectively. On the other hand, not all patients with moderate to severe fibrosis could be correctly identified. We want to know if serum ALT level is correlated with HA and APRI values. However, the APRI and HA increased with the stage of fibrosis, but there was no significant difference in patients with their ALT $\geqslant$ 2ULN or < 2ULN, respectively, which may be due to the small number of patients.

Since the rate of APRI < 1.5 in combination with different HA cut-off points for the detection of mild fibrosis (stage 1) in CHB patients was low in this study, liver biopsy was needed for the detection of mild fibrosis in CHB patients.

Finally, the model was established based on liver biopsy as the gold standard[15]. Since sampling error and inter-observer variability are known limitations of a liver biopsy, we should interpret the results of noninvasive tests for hepatic fibrosis with caution within a broader clinical context.

In conclusion, a predictive model for assessing the probability of significant hepatic fibrosis can be established in CHB patients. APRI $\geqslant$ 1.5 in combination with a HA cut-off point > 300 ng/mL can detect moderate to severe fibrosis (stages 2-4) in CHB patients.

COMMENTS

Background

Liver biopsy is the gold standard for hepatic fibrosis in chronic hepatitis B (CHB) patients, especially in those whose alanine aminotransferase (ALT) is under 2ULN or normal, but its value is questioned because of its potential risk and sampling error. Noninvasive markers of liver fibrosis have been recently proposed as substitutes for liver biopsy, but their reported accuracy is around 80%. They have been mostly validated in hepatitis C but not in hepatitis B. They applied their method in a cohort of patients with chronic hepatitis B.

Research frontiers

Since sampling error and inter-observer variability are known limitations of liver biopsy, some noninvasive methods for liver fibrosis have been proposed, but international guidelines still do not recommend a routine use of the markers due to lack of reproducibility and a misdiagnosis rate of 20%. Thus, a trusted method that avoids a number of liver biopsies by maintaining an excellent accuracy is urgently needed.

Innovations and breakthroughs

In this study, they established a noninvasive assessment model consisting of APRI and HA for the detection of liver fibrosis and cirrhosis in CHB patients. APRI $\geqslant$ 1.5 in combination with a HA cut-off point > 300 ng/mL could detect moderate to severe fibrosis (stages 2-4) in CHB patients. The PPV and specificity of this model were 93.7% and 98.9%, respectively, showing that it can be used as a non-invasive marker for the detection of liver fibrosis in CHB patients.

Applications

The predictive model can be used as a first line assessment of significant hepatic fibrosis in CHB patients, limiting liver biopsy to those who are unclassified or show a low predictive value. In the future, priority should be given to large scale validation studies and the most promising non-invasive markers in patients with all major etiologies of chronic liver disease and most frequent cofactors affecting the diagnostic performance of fibrosis markers.

Terminology

APRI: a simple test combining aspartate aminotransferase (AST) and platelet count for non-invasive prediction of significant fibrosis and cirrhosis in hepatitis C patients. It is a very simple and economic tool, but it is somehow less accurate than fibrotest.

Peer review

This is an interesting paper addressing a clinical problem in the management of chronic hepatitis B. The usefulness of this combination of non-invasive markers of fibrosis and its place in clinical practice needs to be further studied.

REFERENCES

1 **Lavanchy D**. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004; **11**: 97-107

2 **Bedossa P**, Dargere D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology* 2003; **38**: 1449-1457

3 **Rosenberg WM**, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, Hubscher S, Roskams T, Pinzani M, Arthur MJ. Serum markers detect the presence of liver fibrosis: a cohort study. *Gastroenterology* 2004; **127**: 1704-1713

4 **Forns X**, Ampurdanes S, Llovet JM, Aponte J, Quinto L, Martinez-Bauer E, Bruguera M, Sanchez-Tapias JM, Rodes J. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. *Hepatology* 2002; **36**: 986-992

5 **Giannini E**, Testa R. Noninvasive diagnosis of fibrosis: the truth is rarely pure and never simple. *Hepatology* 2003; **38**: 1312-1313; author reply 1313

6 **Kuroiwa Y**, Suzuki N, Yamamoto M, Hatakeyama N, Hori T, Mizue N. [Prognostic value of serum markers for liver fibrosis in transient abnormal myelopoiesis (TAM)] *Rinsho Ketsueki* 2005; **46**: 1179-1186

7 **Bedossa P**, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289-293

8 **Pohl A**, Behling C, Oliver D, Kilani M, Monson P, Hassanein

T. Serum aminotransferase levels and platelet counts as predictors of degree of fibrosis in chronic hepatitis C virus infection. *Am J Gastroenterol* 2001; **96**: 3142-3146

9 **Wai CT**, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 518-526

10 **Williams AL**, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. *Gastroenterology* 1988; **95**: 734-739

11 **Sebastiani G**, Vario A, Guido M, Alberti A. Sequential algorithms combining non-invasive markers and biopsy for the assessment of liver fibrosis in chronic hepatitis B. *World J Gastroenterol* 2007; **13**: 525-531

12 **Guéchot J**, Laudat A, Loria A, Serfaty L, Poupon R, Giboudeau J. Diagnostic accuracy of hyaluronan and type III procollagen amino-terminal peptide serum assays as markers of liver fibrosis in chronic viral hepatitis C evaluated by ROC curve analysis. *Clin Chem* 1996; **42**: 558-563

13 **Patel K**, Lajoie A, Heaton S, Pianko S, Behling CA, Bylund D, Pockros PJ, Blatt LM, Conrad A, McHutchison JG. Clinical use of hyaluronic acid as a predictor of fibrosis change in hepatitis C. *J Gastroenterol Hepatol* 2003; **18**: 253-257

14 **Montazeri G**, Estakhri A, Mohamadnejad M, Nouri N, Montazeri F, Mohammadkani A, Derakhshan MH, Zamani F, Samiee S, Malekzadeh R. Serum hyaluronate as a non-invasive marker of hepatic fibrosis and inflammation in HBeAg-negative chronic hepatitis B. *BMC Gastroenterol* 2005; **5**: 32

15 **Fung J**, Lai CL, Fong DY, Yuen JC, Wong DK, Yuen MF. Correlation of liver biochemistry with liver stiffness in chronic hepatitis B and development of a predictive model for liver fibrosis. *Liver Int* 2008; **28**: 1408-1416

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RAPID COMMUNICATION

Recurrent achalasia treated with Heller myotomy: A review of the literature

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Abstract

AIM: To evaluate the efficacy and safety of Heller myotomy (HM) for recurrent achalasia, performed after different methods of first-line treatment.

METHODS: We searched for studies published in PubMed from 1966 to March 2008 on treatment of recurrent achalasia with HM after failure with different methods of first-line treatment. The efficacy of HM was assessed by a pooled estimate of response rate with individual studies weighted proportionally to sample size.

RESULTS: Sixteen studies were eligible and included in the review. The results showed that HM has a better remission rate for recurrent achalasia after failure of HM [weighted mean (SD)] of 86.9% (21.8%) compared with 81.6% (23.8%) for pneumatic dilatation (PD). One study evaluated the efficacy of HM after failure of PD combined with botulinum toxin injection (83%). The most common complications were perforation and gastroesophageal reflux.

CONCLUSION: HM has the best efficacy in patients with recurrent achalasia who were treated with HM as first-line treatment. Future studies should focus on how to increase the success rate and decrease the complications of HM.

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Key words: Recurrent achalasia; Heller myotomy; Pneumatic dilatation

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INTRODUCTION

Achalasia is a severe neuromuscular disorder of the esophagus, characterized by the loss of peristalsis and an inability of the lower esophageal sphincter (LES) to reach optimal relaxation[1]. Although the etiology of achalasia remains elusive, the mainstay of therapy is directed towards reduction of LES pressure to improve esophageal emptying by gravity[2].

Treatment for achalasia includes drug therapy[3], botulinum toxin (BoTx) injection[4,5], pneumatic dilatation (PD)[6,7] and Heller myotomy (HM)[8-10]. Among these, PD and HM are the most common treatment methods for untreated achalasia[11]. However, none of the treatments reverses the underlying neuropathology of achalasia. There are still some patients who have persistent or recurrent dysphagia[12].

How to select an appropriate treatment for the patients of recurrent achalasia is a hot topic of debate. BoTx, PD and HM are the most popular options for recurrent achalasia. However, BoTx and PD do not achieve a good remission rate for recurrent achalasia in the long term. Moreover, perforation is a severe complication for patients treated with PD as second-line treatment. Therefore, HM is becoming increasingly popular for treatment of recurrent achalasia[13-16]. However, the efficacy of HM is very much dependent upon first-line treatment, for reasons of symptom relapse or failure, and age and co-morbidity of patients.

Therefore, our purpose was to compare the efficacy and safety of HM for patients with recurrent achalasia treated with different methods of first-line treatment, and to evaluate the influence of first-line treatment, including BoTx, PD and HM, on the efficacy of HM performed as second-line treatment.

Table 1 Clinical characteristics of studies included in the review

	No. of patient	Age (yr) (median)	Sex (M/F)	First therapy method	Second therapy method	Operation duration time (min)	Median hospital stay (d)	Symptom remission rate (*n*/N, %)
Ali *et al*[24]	19	33 (14-74)	12/7	19 PD	LM	104 (50-198)	3.2	14/19 (77.8)
Wills *et al*[25]	44	47 (12-87)	20/24	44 PD	LM	NA	NA	32/44 (72.7)
Rosati *et al*[26]	15	36 (12-54)	9/8	15 PD	LM + Dor	114 (60-200)	6 (3-14)	14/15 (93)
Pechlivanides *et al*[27]	29	47 (12-74)	12/17	29 PD	LM + Dor	82 (45-105)	2.6 (1-8)	26/29 (90)
Omura *et al*[28]	18	42 (16-65)	10/8	18 PD	LM + Dor	150 (120-205)	8 (4-18)	16/18 (88.9)
Gockel *et al*[29]	67	44 (14-87)	40/27	67 PD	HM + Dor	104 (50-198)	3.2	52/67 (77)
Ponce *et al*[30]	32	32 (18-67)	14/18	32 PD	HM + Dor	120 (90-210)	6 (3-11)	26/32 (81.3)
Beckingham *et al*[31]	10	30 (18-46)	4/6	10 PD	LM	90 (58-180)	3 (2-4)	9/10 (90)
Patti *et al*[32]	66	46 (20-72)	36/30	66 PD-BoTx	LM + Dor	156	3.5	54/66 (83)
Duffy *et al*[33]	5	39 (15-67)	3/2	5 LM	LM + Toupet	96 (87-120)	2	5/5 (100)
Iqbal *et al*[34]	15	42 (20-65)	8/7	15 LM	LM	NA	NA	12/15 (80)
Grotenhuis *et al*[35]	19	NA	6/13	19 LM	HM + Dor	101 (90-128)	4	17/19 (89.5)
Glatz *et al*[36]	8	52 (18-62)	3/5	8 HM	HM	90 (78-124)	3.5	6/8 (74)
Robinson *et al*[37]	3	34 (28-45)	1/2	3 TM	LM	NA	NA	100
Rakita *et al*[38]	12	40 (20-52)	5/7	10 TM	LM	98 (90-112)	4.5	73
Gorecki *et al*[39]	8	38 (24-60)	4/4	8 LM	8 LM	NA	NA	87.50

LM: Laparoscopic HM; NA: No analysis; HM + Dor: HM and Dor fundoplication; LM + Dor: Laparoscopic HM and Dor fundoplication; LM + Toupet: Laparoscopic HM and Toupet fundoplication.

MATERIALS AND METHODS

Literature search

A systematic review of the literature was conducted. Search tools included Elsevier Science Direct, Blackwell Synergy, Medline (OVID), Pub-Med and Springerlink. Search terms were "achalasia" combined with "treatment, Heller myotomy, pneumatic dilatation, botulinum toxin injection, recurrent, relapse, efficacy, or safety". We first reviewed the abstracts of all articles that reported the efficacy of HM for recurrent achalasia. Then, we retrieved the full articles when relevant. Additionally, reference lists of articles were checked for further relevant articles. We included studies that assessed the influence of first-line treatment methods for HM, in order to evaluate the efficacy and safety of HM as a second-line treatment for recurrent achalasia.

Study selection and data analysis

A study was considered eligible for inclusion if patients had undergone clinical, manometric, radiographic and endoscopic evaluation to confirm achalasia. The severity of symptoms was evaluated by a modified symptom score[17], which consisted of the sum of the scores for dysphagia, regurgitation and chest pain. Recurrent achalasia is defined as recurrence of severe symptoms of dysphagia, regurgitation or chest pain (symptom scores of 2 or 3), and the need for intervention again, or repeat HM[18-22].

Data regarding the first author, year of publication, first-line treatment, operation duration, median hospital stay, and symptoms remission rate, were extracted. Since there was no controlled trial to analyze efficacy and safety of HM for recurrent achalasia patients performed with different methods as the first treatment. There was no uniformity in assessment of efficacy among trials; therefore, we extracted the number of individuals with a good-to-excellent response, which was sustained until the end of the observation period without any further therapy after HM, regardless of criteria. These were considered HM treatment failures, when patients required further treatment or were converted to esophagectomy.

Statistical analysis

The efficacy of studies included in the review was assessed by a pooled estimate of response rate with individual studies weighted proportionally to sample size[23]. In calculating the weighted mean response for each treatment modality ($\hat{p}$), included studies were characterized by the number of subjects (n) and the response rate for those subjects (p). Ellipses represent scant data.

$$SE(\hat{p}) = [p_1(1 - p_1)/n_1 + p_2(1 - p_2)/n_2 + p_x(1 - p_x)/n_x]^{1/2}$$

$$\hat{p} = (n_1p_1 + n_2p_2 + n_xp_x)/(n_1 + n_2 + n_3)$$

RESULTS

Our literature searches identified 162 studies. Of these, 16 were eligible and included in the analysis. The clinical characteristics and efficacy of studies included in the review are shown in Table 1.

Study characteristics

There were five studies[24-28] and three studies[29-31] that assessed the efficacy of HM for failed PD as the first-line treatment. The remission rate was 77.8%-93%. Operation duration was 50-210 min. Median hospital stay was 2.6-8 d. One study[32] has reported the efficacy of HM for achalasia patients who failed combined treatment with PD and BoTx. The remission rate was 83%, operation duration was 156 min, and the hospital stay was 3.5 d. There were five[33-37] studies and two[38,39] studies that assessed the efficacy of HM for failed HM as the first-line treatment. The remission rate was 73%-100%. Operation duration was 90-128 min. Median hospital stay was 2-4 d. The reasons for recurrence of

HM were incomplete myotomy, myotomy fibrosis and fundoplication disruption.

Efficacy of HM for recurrent achalasia

Our review showed that HM has a better remission rate for recurrent achalasia performed with HM [weighted mean (SD)] is 86.9% (21.8%) effective versus 81.6% (23.8%) for PD as the first line treatment. Only one study evaluated the efficacy of HM for failed PD combined with BoTx (83%). We did not evaluate the efficacy of HM for failed BoTx injection because of the lack of data.

It is still controversial that prior treatment measures influence the efficacy of HM for the recurrent achalasia. Some researchers[40] consider that HM is easy to perform in PD patients, and the efficacy is as high as that in patients without any previous therapy. Good clinical results with HM are associated with a reduction of LES pressure to < 10 mmHg. Whereas, some studies[41-43] have shown that the technical difficulties of performing HM increase in some patients with previous dilatation. Preoperative PD represents a risk factor for laparoscopic HM. There was a trend toward a higher incidence of intraoperative esophageal perforation and recurrent dysphagia in patients with prior PD treatment. Moreover, some studies[44-47] have demonstrated that reoperative laparoscopic HM can be undertaken safely (in experienced hands), and can result in good outcomes, with a similar level of success as that seen after primary myotomy.

Complications of HM for recurrent achalasia

We did not evaluate the co-morbidity in patients with recurrent achalasia treated with HM after different methods of first-line treatment, because of a lack of detailed data. Therefore, we used a descriptive method. Patients who were treated with HM experienced intraoperative and postoperative complications. The most common intraoperative complication was gastrointestinal perforation, including gastric and esophageal perforation in 1.5%-20% of patients. Besides, some patients experienced pneumothorax (1.9%-6.7%). Early postoperative complications included pulmonary complications (1.3%-4%). Some patients experienced persistent and severe chest pain, which prolonged the time to hospital discharge. Gastroesophageal reflux is a frequent complication after HM, presenting at a rate of 2.6%-20%. Most patients could be controlled with medical therapy. Some patients presented with endoscopic esophagitis without reflux symptoms.

DISCUSSION

We have performed a review to analyze efficacy and safety of HM for recurrent achalasia performed after different methods of first-line treatment. Our results showed that HM has the best clinical efficacy in recurrent achalasia when performed after HM as first-line treatment (86.9%), followed by 83% for PD combined with BoTx and 81.6% for PD. Although the number of patients in this review was relatively small, it represents one of the largest series in the literature.

First, the efficacy of HM for recurrent achalasia depends on the method of first-line treatment. Traditionally, endoscopic therapies have been selected for first-line treatment over surgery, because of the morbidity associated with open or laparoscopic HM. In patients who have had prior endoscopic therapy, there is a notable difference in the submucosal dissection plane, especially near the squamocolumnar junction. Often the plane is obliterated, and it is very difficult to confidently and easily dissect down onto the mucosa, as can be accomplished in those who have not had prior therapy[48]. However, some studies have shown that previous PD does not determine a high failure rate or a high rate of complications with HM[49]. Therefore, it is urgent to build up a standard technique to decrease the risk of HM for recurrent achalasia.

Second, the efficacy of HM for recurrent achalasia depends on the reasons for symptom relapse or failure. Reasons for failure were incomplete myotomy (33%), myotomy fibrosis (27%), fundoplication disruption (13%), too tight fundoplication (7%) and a combination of myotomy fibrosis and incomplete myotomy (20%).

Third, the efficacy of HM for recurrent achalasia depends on the age and co-morbidity of patients. There is a strong association between age at which diagnosis of achalasia is first established and the requirement for HM[50]. For each year of increasing age, the odds ratio for the eventual requirement for myotomy decreases by 0.943 (95% CI: 0.90-0.98). As age increases, the need for surgical therapy progressively diminishes. The perioperative and postoperative morbidities also influence the efficacy of HM for recurrent achalasia.

Finally, the efficacy of HM for recurrent achalasia depends on the operative technique and postoperative management. In recent years, laparoscopic HM, with or without fundoplication, has increasingly been performed because of the lower morbidity and shorter recovery period associated with laparoscopic surgery[51]. Some surgeons[52] prefer a posterior partial fundoplication instead of an anterior hemifundoplication. It prevents reflux more effectively and keeps the edges of the myotomy separate. Whereas, some studies have shown that laparoscopic HM without fundoplication has the same or an even better effect on achalasia[53]. Finley *et al*[54] have reported that laparoscopic HM without anterior fundoplication shows significantly better upright esophageal clearance, with a trend toward improved dysphagia and regurgitation frequency, when compared with anterior fundoplication.

Recently, there have been some new methods to treat recurrent achalasia. Ponciano *et al*[55] have reported that the Serra-Dória procedure for the treatment of megaesophagus in patients who had already undergone cardiomyotomy, and whose symptoms recurred, has low morbidity and no mortality. It offered a significant relief of symptoms, with a decrease in the caliber of the esophagus in several patients. The patients also improved with regards to reflux esophagitis. Oelschlager

et al[56] have suggested that an extended gastric myotomy (3 cm) more effectively disrupts the LES, thus improving the results of surgical therapy for achalasia, without increasing the rate of abnormal GER, provided that a Toupet fundoplication is added. Postoperatively, LES pressure was significantly lower after extended gastric myotomy. There were no reoperations in the extended gastric myotomy and Toupet fundoplication group.

Our review suggests that HM has the best clinical efficacy in patients with recurrent achalasia who are treated with HM as first-line treatment. Future studies should focus on how to increase the success rate of HM and decrease the complications and adverse effects. It is necessary to promote and popularize the technique of HM in general hospitals.

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COMMENTS

Background

None of the treatment measures reverses the underlying neuropathology of achalasia. Heller myotomy (HM) is the most used technique for recurrent achalasia. However, the efficacy of HM is largely dependent on the method of first-line treatment.

Research frontiers

Authors aimed to evaluate the efficacy and safety of HM for patients with recurrent achalasia treated with different methods of first-line treatment.

Innovations and breakthroughs

Previous studies support the hypothesis that the efficacy of HM is very much dependent upon first-line treatment. However, there have been no studies to compare the efficacy of HM in patients with recurrent achalasia treated with different methods of first-line treatment. In this study, they found that HM has the best efficacy in recurrent achalasia treated with HM as the first-line therapy.

Applications

The results may provide a systematic analysis of the efficacy of HM for recurrent achalasia and identify HM as having the best efficacy in patients with achalasia treated with HM as first-line treatment. They can also offer assistance with the best treatment choice for achalasia in the future.

Terminology

Achalasia is a primary motor disorder characterized by incomplete relaxation of the lower esophageal sphincter (LES) and aperistalsis of the esophageal body, secondary to loss of inhibitory ganglion cells in the myenteric plexus. The etiology of the disease is unknown, with genetic, autoimmune, infectious, and environmental factors being implicated.

Peer review

This study is a systematic review of HM for recurrent achalasia. The authors show that HM achieves the best efficacy in patients with recurrent achalasia who were treated with HM as first-line therapy. It is a very interesting study.

REFERENCES

1 **Massey BT**. Management of idiopathic achalasia: short-term and long-term outcomes. *Curr Gastroenterol Rep* 2000; **2**: 196-200
2 **O'Connor JB**, Singer ME, Imperiale TF, Vaezi MF, Richter JE. The cost-effectiveness of treatment strategies for achalasia. *Dig Dis Sci* 2002; **47**: 1516-1525
3 **Bassotti G**, Annese V. Review article: pharmacological options in achalasia. *Aliment Pharmacol Ther* 1999; **13**: 1391-1396
4 **Zaninotto G**, Vergadoro V, Annese V, Costantini M, Costantino M, Molena D, Rizzetto C, Epifani M, Ruol A, Nicoletti L, Ancona E. Botulinum toxin injection versus laparoscopic myotomy for the treatment of esophageal achalasia: economic analysis of a randomized trial. *Surg Endosc* 2004; **18**: 691-695
5 **Annese V**, Bassotti G, Coccia G, Dinelli M, D'Onofrio V, Gatto G, Leandro G, Repici A, Testoni PA, Andriulli A. A multicentre randomised study of intrasphincteric botulinum toxin in patients with oesophageal achalasia. GISMAD Achalasia Study Group. *Gut* 2000; **46**: 597-600
6 **Vaezi MF**, Richter JE, Wilcox CM, Schroeder PL, Birgisson S, Slaughter RL, Koehler RE, Baker ME. Botulinum toxin versus pneumatic dilatation in the treatment of achalasia: a randomised trial. *Gut* 1999; **44**: 231-239
7 **Kostic S**, Kjellin A, Ruth M, Lönroth H, Johnsson E, Andersson M, Lundell L. Pneumatic dilatation or laparoscopic cardiomyotomy in the management of newly diagnosed idiopathic achalasia. Results of a randomized controlled trial. *World J Surg* 2007; **31**: 470-478
8 **Mattioli G**, Esposito C, Pini Prato A, Doldo P, Castagnetti M, Barabino A, Gandullia P, Staiano AM, Settimi A, Cucchiara S, Montobbio G, Jasonni V. Results of the laparoscopic Heller-Dor procedure for pediatric esophageal achalasia. *Surg Endosc* 2003; **17**: 1650-1652
9 **Iqbal A**, Haider M, Desai K, Garg N, Kavan J, Mittal S, Filipi CJ. Technique and follow-up of minimally invasive Heller myotomy for achalasia. *Surg Endosc* 2006; **20**: 394-401
10 **Richardson WS**, Kennedy CI, Bolton JS. Midterm follow-up evaluation after a novel approach to anterior fundoplication for achalasia. *Surg Endosc* 2006; **20**: 1914-1918
11 **Birgisson S**, Richter JE. Achalasia: what's new in diagnosis and treatment? *Dig Dis* 1997; **15** Suppl 1: 1-27
12 **Jafri M**, Alonso M, Kaul A, Dierig J, Racadio J, Inge T, Brown R, Ryckman F, Tiao G. Intraoperative manometry during laparoscopic Heller myotomy improves outcome in pediatric achalasia. *J Pediatr Surg* 2008; **43**: 66-70; discussion 70
13 **Palanivelu C**, Rangarajan M, Jategaonkar PA, Maheshkumaar GS, Vijay Anand N. Laparoscopic transhiatal esophagectomy for 'sigmoid' megaesophagus following failed cardiomyotomy: experience of 11 patients. *Dig Dis Sci* 2008; **53**: 1513-1518
14 **Ferulano GP**, Dilillo S, D'Ambra M, Lionetti R, Brunaccino R, Fico D, Pelaggi D. Short and long term results of the laparoscopic Heller-Dor myotomy. The influence of age and previous conservative therapies. *Surg Endosc* 2007; **21**: 2017-2023
15 **Schuchert MJ**, Luketich JD, Landreneau RJ, Kilic A, Gooding WE, Alvelo-Rivera M, Christie NA, Gilbert S, Pennathur A. Minimally-invasive esophagomyotomy in 200 consecutive patients: factors influencing postoperative outcomes. *Ann Thorac Surg* 2008; **85**: 1729-1734
16 **Bonavina L**, Incarbone R, Reitano M, Antoniazzi L, Peracchia A. Does previous endoscopic treatment affect the outcome of laparoscopic Heller myotomy? *Ann Chir* 2000; **125**: 45-49
17 **Vela MF**, Richter JE, Khandwala F, Blackstone EH, Wachsberger D, Baker ME, Rice TW. The long-term efficacy of pneumatic dilatation and Heller myotomy for the treatment of achalasia. *Clin Gastroenterol Hepatol* 2006; **4**: 580-587
18 **Gockel I**, Junginger T, Eckardt VF. Effects of pneumatic dilation and myotomy on esophageal function and morphology in patients with achalasia. *Am Surg* 2005; **71**: 128-131
19 **Parshad R**, Hazrah P, Saraya A, Garg P, Makharia G. Symptomatic outcome of laparoscopic cardiomyotomy without an antireflux procedure: experience in initial 40 cases. *Surg Laparosc Endosc Percutan Tech* 2008; **18**: 139-143
20 **Jeansonne LO**, White BC, Pilger KE, Shane MD, Zagorski S, Davis SS, Hunter JG, Lin E, Smith CD. Ten-year follow-up of laparoscopic Heller myotomy for achalasia shows durability. *Surg Endosc* 2007; **21**: 1498-1502

21 **Fernández AF**, Martínez MA, Ruiz J, Torres R, Faife B, Torres JR, Escoto CM. Six years of experience in laparoscopic surgery of esophageal achalasia. *Surg Endosc* 2003; **17**: 153-156
22 **Lai IR**, Lee WJ, Huang MT. Laparoscopic Heller myotomy with fundoplication for achalasia. *J Formos Med Assoc* 2002; **101**: 332-336
23 **Spiess AE**, Kahrilas PJ. Treating achalasia: from whalebone to laparoscope. *JAMA* 1998; **280**: 638-642
24 **Ali A**, Pellegrini CA. Laparoscopic myotomy: technique and efficacy in treating achalasia. *Gastrointest Endosc Clin N Am* 2001; **11**: 347-358
25 **Wills VL**, Hunt DR. Functional outcome after Heller myotomy and fundoplication for achalasia. *J Gastrointest Surg* 2001; **5**: 408-413
26 **Rosati R**, Fumagalli U, Bona S, Bonavina L, Pagani M, Peracchia A. Evaluating results of laparoscopic surgery for esophageal achalasia. *Surg Endosc* 1998; **12**: 270-273
27 **Pechlivanides G**, Chrysos E, Athanasakis E, Tsiaoussis J, Vassilakis JS, Xynos E. Laparoscopic Heller cardiomyotomy and Dor fundoplication for esophageal achalasia: possible factors predicting outcome. *Arch Surg* 2001; **136**: 1240-1243
28 **Omura N**, Kashiwagi H, Ishibashi Y, Yano F, Tsuboi K, Kawasaki N, Suzuki Y, Yanaga K. Laparoscopic Heller myotomy and Dor fundoplication for the treatment of achalasia. Assessment in relation to morphologic type. *Surg Endosc* 2006; **20**: 210-213
29 **Gockel I**, Junginger T, Bernhard G, Eckardt VF. Heller myotomy for failed pneumatic dilation in achalasia: how effective is it? *Ann Surg* 2004; **239**: 371-377
30 **Ponce J**, Juan M, Garrigues V, Pascual S, Berenguer J. Efficacy and safety of cardiomyotomy in patients with achalasia after failure of pneumatic dilatation. *Dig Dis Sci* 1999; **44**: 2277-2282
31 **Beckingham IJ**, Callanan M, Louw JA, Bornman PC. Laparoscopic cardiomyotomy for achalasia after failed balloon dilatation. *Surg Endosc* 1999; **13**: 493-496
32 **Patti MG**, Feo CV, Diener U, Tamburini A, Arcerito M, Safadi B, Way LW. Laparoscopic Heller myotomy relieves dysphagia in achalasia when the esophagus is dilated. *Surg Endosc* 1999; **13**: 843-847
33 **Duffy PE**, Awad ZT, Filipi CJ. The laparoscopic reoperation of failed Heller myotomy. *Surg Endosc* 2003; **17**: 1046-1049
34 **Iqbal A**, Tierney B, Haider M, Salinas VK, Karu A, Turaga KK, Mittal SK, Filipi CJ. Laparoscopic re-operation for failed Heller myotomy. *Dis Esophagus* 2006; **19**: 193-199
35 **Grotenhuis BA**, Wijnhoven BP, Myers JC, Jamieson GG, Devitt PG, Watson DI. Reoperation for dysphagia after cardiomyotomy for achalasia. *Am J Surg* 2007; **194**: 678-682
36 **Glatz SM**, Richardson JD. Esophagectomy for end stage achalasia. *J Gastrointest Surg* 2007; **11**: 1134-1137
37 **Robinson TN**, Galvani CA, Dutta SK, Gorodner MV, Patti MG. Laparoscopic treatment of recurrent dysphagia following transthoracic myotomy for achalasia. *J Laparoendosc Adv Surg Tech A* 2003; **13**: 401-403
38 **Rakita S**, Villadolid D, Kalipersad C, Thometz D, Rosemurgy A. Outcomes promote reoperative Heller myotomy for symptoms of achalasia. *Surg Endosc* 2007; **21**: 1709-1714
39 **Gorecki PJ**, Hinder RA, Libbey JS, Bammer T, Floch N. Redo laparoscopic surgery for achalasia. *Surg Endosc* 2002; **16**: 772-776
40 **Deb S**, Deschamps C, Allen MS, Nichols FC 3rd, Cassivi SD, Crownhart BS, Pairolero PC. Laparoscopic esophageal myotomy for achalasia: factors affecting functional results. *Ann Thorac Surg* 2005; **80**: 1191-1194; discussion 1194-1195
41 **Smith CD**, Stival A, Howell DL, Swafford V. Endoscopic therapy for achalasia before Heller myotomy results in worse outcomes than heller myotomy alone. *Ann Surg* 2006; **243**: 579-584; discussion 584-586
42 **Raftopoulos Y**, Landreneau RJ, Hayetian F, Papasavas P, Naunheim KS, Hazelrigg SR, Santos R, Gagné D, Caushaj P, Keenan RJ. Factors affecting quality of life after minimally invasive Heller myotomy for achalasia. *J Gastrointest Surg* 2004; **8**: 233-239
43 **Portale G**, Costantini M, Rizzetto C, Guirroli E, Ceolin M, Salvador R, Ancona E, Zaninotto G. Long-term outcome of laparoscopic Heller-Dor surgery for esophageal achalasia: possible detrimental role of previous endoscopic treatment. *J Gastrointest Surg* 2005; **9**: 1332-1339
44 **Gholoum S**, Feldman LS, Andrew CG, Bergman S, Demyttenaere S, Mayrand S, Stanbridge DD, Fried GM. Relationship between subjective and objective outcome measures after Heller myotomy and Dor fundoplication for achalasia. *Surg Endosc* 2006; **20**: 214-219
45 **Gockel I**, Junginger T, Eckardt VF. Persistent and recurrent achalasia after Heller myotomy: analysis of different patterns and long-term results of reoperation. *Arch Surg* 2007; **142**: 1093-1097
46 **Patti MG**, Molena D, Fisichella PM, Whang K, Yamada H, Perretta S, Way LW. Laparoscopic Heller myotomy and Dor fundoplication for achalasia: analysis of successes and failures. *Arch Surg* 2001; **136**: 870-877
47 **Yoo C**, Levine MS, Redfern RO, Laufer I, Buyske J. Laparoscopic Heller myotomy and fundoplication: findings and predictive value of early postoperative radiographic studies. *Abdom Imaging* 2004; **29**: 643-647
48 **Chapman JR**, Joehl RJ, Murayama KM, Tatum RP, Shi G, Hirano I, Jones MP, Pandolfino JE, Kahrilas PJ. Achalasia treatment: improved outcome of laparoscopic myotomy with operative manometry. *Arch Surg* 2004; **139**: 508-513; discussion 513
49 **Hunter JG**, Trus TL, Branum GD, Waring JP. Laparoscopic Heller myotomy and fundoplication for achalasia. *Ann Surg* 1997; **225**: 655-664; discussion 664-665
50 **Sweet MP**, Nipomnick I, Gasper WJ, Bagatelos K, Ostroff JW, Fisichella PM, Way LW, Patti MG. The outcome of laparoscopic Heller myotomy for achalasia is not influenced by the degree of esophageal dilatation. *J Gastrointest Surg* 2008; **12**: 159-165
51 **Perrone JM**, Frisella MM, Desai KM, Soper NJ. Results of laparoscopic Heller-Toupet operation for achalasia. *Surg Endosc* 2004; **18**: 1565-1571
52 **Sharp KW**, Khaitan L, Scholz S, Holzman MD, Richards WO. 100 consecutive minimally invasive Heller myotomies: lessons learned. *Ann Surg* 2002; **235**: 631-638; discussion 638-639
53 **Gockel I**, Junginger T, Eckardt VF. Long-term results of conventional myotomy in patients with achalasia: a prospective 20-year analysis. *J Gastrointest Surg* 2006; **10**: 1400-1408
54 **Finley C**, Clifton J, Yee J, Finley RJ. Anterior fundoplication decreases esophageal clearance in patients undergoing Heller myotomy for achalasia. *Surg Endosc* 2007; **21**: 2178-2182
55 **Ponciano H**, Cecconello I, Alves L, Ferreira BD, Gama-Rodrigues J. Cardiaplasty and Roux-en-Y partial gastrectomy (Serra-Dória procedure) for reoperation of achalasia. *Arq Gastroenterol* 2004; **41**: 155-161
56 **Oelschlager BK**, Chang L, Pellegrini CA. Improved outcome after extended gastric myotomy for achalasia. *Arch Surg* 2003; **138**: 490-495; discussion 495-497

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RAPID COMMUNICATION

Bone marrow-derived dendritic cells pulsed with tumor lysates induce anti-tumor immunity against gastric cancer *ex vivo*

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Abstract

AIM: To investigate whether bone marrow-derived dendritic cells pulsed with tumor lysates induce immunity against gastric cancer *ex vivo*.

METHODS: c-kit$^+$ hematopoietic progenitor cells were magnetically isolated with a MiniMACS separator from BALB/c mice bone marrow cells. These cells were cultured with cytokines GM-CSF, IL-4, and TNFα to induce their maturation. They were analysed by morphological observation, phenotype analysis, and mixed lymphocyte reaction (MLR). Bone marrow-derived DCs (BM-DCs) were pulsed with tumor cell lysate obtained by rapid freezing and thawing at a 1:3 DC:tumor cell ratio. Finally, cytotoxic T lymphocyte (CTL) activity and interferon gamma (IFNγ) secretion was evaluated *ex vivo*.

RESULTS: c-kit$^+$ hematopoietic progenitor cells from mice bone marrow cells cultured with cytokines for 8 d showed the character of typical mature DCs. Morphologically, observed by light microscope, these cells were large with oval or irregularly shaped nuclei and with many small dendrites. Phenotypically, FACS analysis showed that they expressed high levels of Ia, DEC-205, CD11b, CD80 and CD86 antigen, moderate levels of CD40, and negative for F4/80. Functionally, these cells gained the capacity to stimulate allogeneic T cells in MLR assay. However, immature DCs cultured with cytokines for 5 d did not have typical DCs phenotypic markers and could not stimulate allogeneic T cells. *Ex vivo* primed T cells with SGC-7901 tumor cell lysate-pulsed (TP) DCs were able to induce effective CTL activity against SGC-7901 tumor cells (E:T = 100:1, 69.55% ± 6.05% specific lysis), but not B16 tumor cells, and produced higher levels of IFNγ when stimulated with SGC-7901 tumor cells but not when stimulated with B16 tumor cells (1575.31 ± 60.25 pg/mL in SGC-7901 group *vs* 164.11 ± 18.52 pg/mL in B16 group, $P < 0.01$).

CONCLUSION: BM-derived DCs pulsed with tumor lysates can induce anti-tumor immunity specific to gastric cancer *ex vivo*.

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Key words: Dendritic cells; Cytokine; Gastric cancer; Cytotoxic T lymphocyte; Immunotherapy

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INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that both initiate and modulate the immune response[1]. DCs are cells in the pathway of antigen capture and presentation to T cells, with the unique

ability to directly prime naïve $CD4^+$ and $CD8^+$ T cells. They posses the ability to efficiently uptake, process, and present antigens on major histocompatibility complex (MHC) class I and Ⅱ molecules, together with co-stimulatory molecules such as B7 and CD40[2]. Therefore, DCs are believed to be essential for stimulating tumor-specific cytotoxic T lymphocyte (CTL) and inducing the protective and therapeutic anti-tumor immunity against cancer cells.

Gastric cancer is one of the most common cancers[3]. Although gastric cancer therapy has made great progress, it is still difficult to treat advanced gastric cancer, as it has spread to the lymph glands and metastasized. Currently, radical surgery represents the standard method of therapy. Adjuvant therapy such as chemotherapy and radiation therapy have been widely applied, but gastric cancer control at the advanced stage remains difficult[4,5]. A new adjuvant therapeutic method is needed in order to improve the 5-year survival rate of patients with gastric cancer. Currently, tumor immunotherapy for gastric cancer has potential.

Ishigami *et al*[6] examined 169 patients with gastric cancer by immunohistochemical staining of CD57 and S-100-protein and found that DCs infiltrate in the tissue of gastric caner, but cannot play an important role due to lacking Th cells in the tumor microenvironment. In addition, a poorer differentiation of gastric cancer corresponds to a the lower amount of DC infiltration in the tumor tissue. Patients with a high level of DCs infiltration had a lower positivity of lymph node metastases and lymphatic invasion than patients with lower level of DCs infiltration[7]. The 5-year survival rates of patients with many DCs infiltrated were 78% better than that of patients with fewer DCs infiltrated[6]. According to the function of DCs described above, we can state that the DCs are related to clinical stage, invasion, metastasis and prognosis of gastric cancer. Galetto *et al*[8] reported that T-cell memory against gastric carcinoma antigens can be triggered by tumor-loaded autologous DCs. Therefore, it is feasible that DCs-based tumor vaccines will become a new effective immunoadjuvant therapy for gastric cancer, which can decrease the incidence and recurrence rate after operation for gastric cancer[9].

In this study, we demonstrated that vaccination with bone marrow-derived DCs pulsed with tumor cell lysate induced tumor-specific CTL activity and anti-tumor immunity against SGC-7901 gastric cancer cell lines, suggesting promising strategy for gastric cancer immunotherapy.

MATERIALS AND METHODS

Animals

BALB/c and C57BL/6 (B6) mice (8-10 wk old) were purchased from the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). All mice were kept under pathogen-free conditions in the animal center of the Soochow University (Suzhou, China).

Cell culture

Gastric cancer cell line, SGC-7901, and melanoma cell line, B16, were purchased from the Shanghai Cell Biology Institutes, Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in RPMI (Roswell Park Memorial Institute) medium 1640 (GIBCO, USA) containing 10% fetal calf serum (FCS), penicillin G (100 U/mL), and streptomycin (100 μg/mL) at 37℃ in a humidified incubator supplemented with 50 mL/L CO_2.

Major

Murine granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, tumor necrosis factor-α (TNFα), IL-2, and IL-7 were purchased from Becton Dickinson (New Jersey, USA). Phenotypic analysis, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies (Mabs), such as MHC I (Ia), DEC-205, CD11b, CD40, CD80, CD86, and F4/80, were provided by Pharmigen (CA, USA). Mitomycin C (MMC) was purchased from Jingmei Biothe (Shenzhen, China).

Generation of bone marrow-derived DCs

Primary bone marrow-derived DCs (BM-DCs) were obtained from mouse bone marrow precursors according to a previously established protocol[10]. BALB/c mice bone marrow obtained from tibia and femurs by flushing with media. Tissue was minced in a single-cell suspension through a nylon mesh. BALB/c mice bone marrow cells were stained sequentially with biotinylated anti-c-kit MAb and Streptavidm MicroBeads (Dynal, Norway). $c\text{-}kit^+$ hematopoietic progenitor cells were magnetically isolated with a MiniMACS separator (Milteyi Biotec, Auburn, CA) from the BALB/c mice bone marrow cells. The cells (6×10^6) were cultured for 5 d in fresh RPMI medium 1640 containing 10% FCS, GM-CSF (4 ng/mL), and IL-4 (10 ng/mL). Then immature DCs were further cultured in GM-CSF (4 ng/mL) and TNFα (5 ng/mL) for 3 d to induce their maturation. Bone marrow-derived mature DCs were observed by light microscope (Nikon, Japan).

Immunofluorescence staining

DCs immunofluorescence analysis was performed as previously described[11]. In brief, DCs cultured for 5 d or 8 d as described above (2×10^5-4×10^5 cells) were incubated with rat anti-DEC-205 MAb followed by FITC-labeled goat anti-rat IgG $(Fab')^2$ antibodies or directly with FITC-labeled MAbs against CD40, F4/80, CD11b, or CD80 and PE-labeled MAbs against Ia, or CD86 followed by FACS analysis. The instrument compensation was set in each experiment using two-color stained samples.

Mixed leukocyte reaction assay

The mixed leukocyte reaction (MLR) assay was performed in accordance with previously described methods[12]. Immature DCs or mature DCs derived from bone marrow $c\text{-}kit^+$ cells were incubated in RPMI

Medium 1640 containing 10% FCS and mitomycin C (MMC; 15 μg/mL) in six-well plates at 37℃ for 3 h to arrest their proliferation. After several washes with PBS, these stimulator cells were suspended in RPMI-1640 containing 10% FCS at concentrations ranging from 1×10^2 to 5×10^4 cells/mL. One hundred microliters of the above stimulator cells suspension were added to each well of 96-well plates that contained allogeneic $CD4^+$ T cells (3×10^5 cells/100 μL per well) that had been magnetically isolated from B6 mice using CD4 Microbeads. Five days later, T-cell proliferation was determined by using an MTT assay. Fifteen μL of MTT (5 μg/mL in PBS) was added to each well, and the plates were incubated at 37℃ for an additional 4 h. The resultant absorbance at 550 nm was read by a microplate immunoreader. PBS alone was used as the negative control. Results are expressed as the mean of three wells from three individual experiments.

Pulsing DCs with tumor cell lysates

BM-DCs were pulsed with freeze-thawed tumor lysate at a 1:3 DC:tumor cell ratio. SGC-7901 tumor cells (6×10^6) were lysed by rapid freezing (liquid nitrogen) and thawing in a 37℃ bath three times. BM-DCs (2×10^6 cells/mL) were incubated in six-well plates in the presence of SGC7901 tumor lysates (6×10^6 cell equivalents/mL) in RPMI Medium 1640 containing 10% FCS, GM-CSF (4 ng/mL), and IL-4 (10 ng/mL) for one day at 37℃ and 50 mL/L CO_2. These SGC-7901 tumor cell lysate-pulsed (TP) DCs were used for vaccination after one day. B16 TP DCs were used as control.

Assays for cytotoxic T lymphocyte activity ex vivo

To confirm that tumor-specific cytotoxic T lymphocytes (CTLs) can be generated *ex vivo*, splenic $CD3^+$ T cells (1×10^6 cells/mL) were magnetically isolated from naïve BALB/c mice using CD3 microBeads. These cells were cultured in RPMI medium 1640 containing 10% FCS, then primed *ex vivo* in the presence of cytokines including IL-2 and IL-7 (5 ng/mL) at day 0, 7, and 14 with SGC-7901 TP DCs or B16 TP DCs at a 1:20 stimulator to responder cell ratio. Unpulsed DCs and SGC-7901 tumor lysates were used as controls. Fresh medium containing IL-2 and IL-7 was exchanged every 4 d. The primed T cells were effector cells; SGC-7901 or B16 tumor cells were target cells. On day 21, target cell suspension was added into 96 well plates, and effector cells were titrated to dilutions of target cells by serial dilutions (E-T mix, E:T, 1:1, 5:1, 10:1, 20:1, 50:1, 100:1). Supernatant from each well was collected after 20 h and cytolytic activity against target SGC-7901 tumor cells and B16 tumor cells was measured with a Cytotoxicity Detection Kit (LDH; Boehringer Mannheim, Mannheim, Germany). IFNγ production was determined with the IFNγ ELISA kit (Endogen, Woburn, MA, USA.) at a stimulator to responder cell ratio of 1:20.

Statistical analysis

Differences were evaluated using Statistical Package for Social Science 11.0 (SPSS11.0). Statistical analysis was performed using Student's *t*-test. Statistical tests were two-sided. $P < 0.05$ were considered to be statistically significant.

RESULTS

Morphological character of DCs

c-kit^+ hematopoietic progenitor cells from the BALB/c mice bone marrow cells were cultured in presence of GM-CSF, IL-4, or TNFα for 8 d, then observed by light microscopy. Results show large cells with oval or irregularly shaped nuclei and many small dendrites (Figure 1).

Phenotypic markers of DCs analysis by FACS

The phenotypic profile of a representative population of bone marrow DCs was determined by FACS. BM-DCs cultured for 5 d expressed moderate levels of co-stimulatory molecule CD40, high levels of CD11b, and very low levels of Ia, DEC-205, CD80, CD86, and were negative for F4/80 (Figure 2). However, when these cells were cultured in the presence of GM-CSF, IL-4, or TNFα for 8 d, they differentiated into mature DCs that expressed high levels of Ia, DEC-205, CD11b, CD80 and CD86 antigen, moderate levels of the co-stimulatory molecule CD40, and were negative for F4/80 (Figure 2).

The capacity of BM-DCs to enhance allogeneic MLR

Allogeneic mixed-leukocyte reactions were performed using splenic T cells purified from B6 mice as responder cells. BM-DCs were treated with MMC to arrest cell proliferation and were used as stimulator cells. T cell proliferation was determined by using an MTT assay. Results show BM-derived mature DCs have the capacity to stimulate allogeneic T cells (Figure 3); however, BM-derived immature DCs and PBS did not simulate allogeneic T cells (Stimulator cells: 5×10^4 cells/mL, OD 550 nm: 1.74 ± 0.15 in mature DCs group *versus* 0.22 ± 0.05 in immature DCs group or 0.16 ± 0.04 in PBS group, $P < 0.05$, Figure 3).

Generation of tumor-specific cytotoxic T cells induced by BM-DCs

To study the potential of in anti-tumor immunity *in vitro*, BM-derived DCs were prepared by pulsing BM-DCs with tumor lysates after 5 d of culture in the presence of GM-CSF and IL-4. Naïve mouse splenic T cells were primed *in vitro* with SGC-7901 TP DCs or B16 TP DCs in the presence of IL-2 and IL-7 to elicit cytolytic reactivity against tumor cells. The results show that T cells *in vitro* primed with SGC-7901 TP DCs were able to lyse efficiently and specifically parental SGC-7901 tumor cells, but not B16 tumor cells, and T cells primed with B16 TP DCs, SGC-7901 tumor lysates or unpulsed DCs did not induce specific CTL against SGC-7901 tumor cells (E:T = 100:1, 69.55% ± 6.05% specific lysis in the SGC-7901 TP DCs primed T cell/SGC-7901 group *versus* 15.72% ± 2.9% specific lysis in the SGC-7901 TP DCs primed T cell/B16 group, 13.75% ± 3.14% specific lysis in the B16 TP DCs primed T cell/SGC-7901 group,

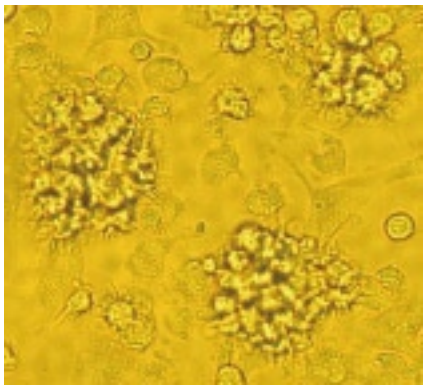

Figure 1 Morphological characteristic of BM-DCs. c-kit$^+$ hematopoietic progenitor cells from the BALB/c mice bone marrow cells were cultured in presence of GM-CSF, IL-4, or TNFα for 8 d, and then these cells were observed by light microscopy (× 200).

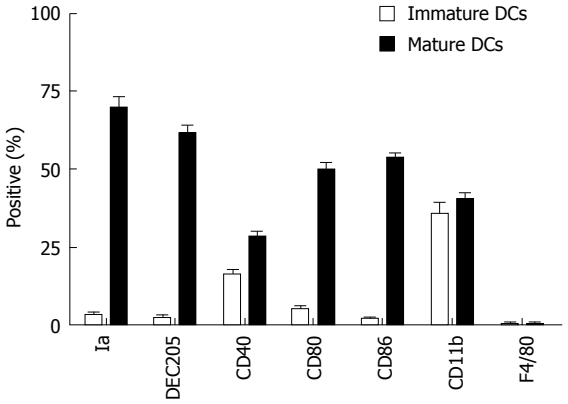

Figure 2 Immunophenotypic analysis of BM-DCs. BM-DCs (2×10^5-4×10^5 cells) cultured for 5 d or 8 d were incubated with FITC- or PE-labeled MAbs. The phenotypes of these cells were analyzed by immunofluorescence staining as described in the Materials and Methods. The results are representative of three independent experiments and the data are given as mean ± SE.

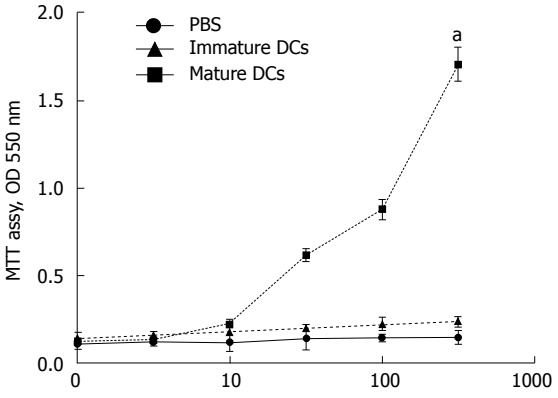

Figure 3 The capacity of BM-DCs to enhance allogeneic MLR. Allogeneic mixed-leukocyte reactions were performed using splenic T cells purified from B6 mice as responder cells. Immature or mature DCs were treated with MMC to arrest cell proliferation and were used as stimulator cells at the indicated cell numbers. PBS was used as a control. T-cell proliferation was determined by using an MTT assay after 5 d of culture. The results are representative of three independent experiments and the data are given as mean ± SE. $^aP < 0.05$, mature DCs group *versus* immature DCs group or PBS group.

10.6% ± 3.01% specific lysis in the SGC-7901 tumor lysates primed T cell/SGC-7901 group, or 8.75% ± 2.15% specific lysis in the unpulsed DCs primed T cell/SGC-7901 group, $P < 0.05$, Figure 4).

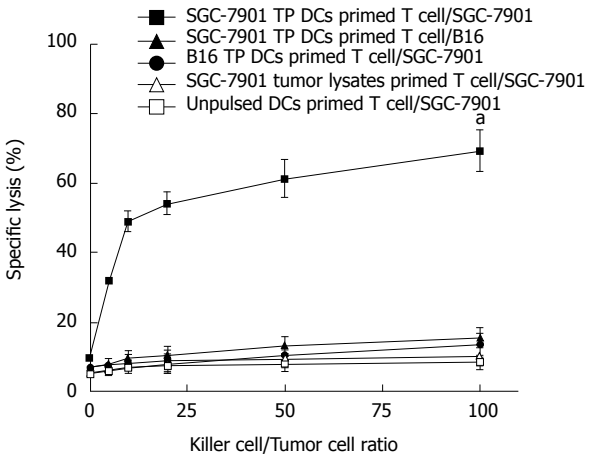

Figure 4 Generation of tumor-specific cytotoxic T cells *ex vivo*. Splenic CD3$^+$ T cells were isolated from naïve B6 mice with MACS. T cells were primed with SGC-7901 tumor cell lysate-pulsed BM-derived DCs as described in Materials and Methods. Unpulsed DCs and SGC-7901 tumor lysates were used as controls. The primed T cells (effector cells) were titrated by serial dilution (1:1, 5:1, 10:1, 25:1, 50:1, 100:1), and mixied with SGC-7901 or B16 target cells, and their lytic activity against SGC-7901 or B16 was assayed by a Cytotoxicity Detection Kit. Statistical analysis used the paired Student's *t* test. The results are representative of three independent experiments. Data are given as mean ± SE. $^aP < 0.05$, SGC-7901 TP DCs primed T cell/SGC-7901 group *versus* SGC-7901 TP DCs primed T cell/B16 group or other control groups.

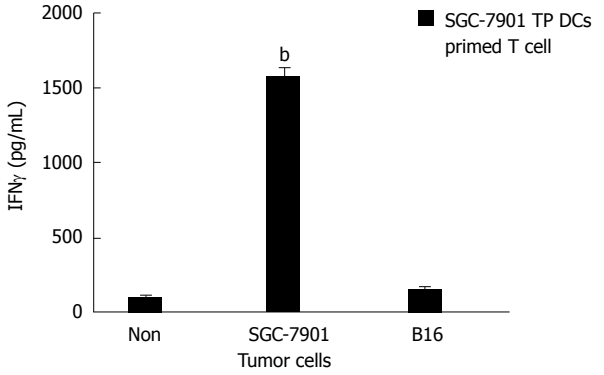

Figure 5 Assays for IFNγ secretion *ex vivo*. Splenic CD3$^+$ T cells were isolated from naïve BALB/c mice with MACS. T cells were primed with SGC-7901 tumor cell lysate-pulsed BM-derived DCs as described in Materials and Methods. The primed T cells (effector cells) were mixied and incubated with SGC-7901 or B16 target cells. Supernatant from each well was collected for measuring IFNγ production with the mouse IFNγ ELISA kit. Statistical analysis used the paired Student's *t* test. The results are representative of three independent experiments. Data are given as mean ± SE. $^bP < 0.01$, SGC-7901 *versus* B16 or Non.

Splenic CD3$^+$ T cells primed with SGC-7901 TP DCs produced higher levels of IFNγ *in vitro* when stimulated with SGC-7901 tumor cells; however, they did not produce higher levels of IFNγ when stimulated with B16 tumor cells (1575.31 ± 60.25 pg/mL in the SGC-7901 group *versus* 164.11 ± 18.52 pg/mL in the B16 group, $P < 0.01$, Figure 5).

DISCUSSION

In our experiments were obtained successfully from

BALB/c mouse bone marrow precursors. These BM-DCs were analysed by morphological observation, phenotype analysis, and mixed lymphocyte reaction (MLR). This study demonstrates the generation of an effective CTL response against gastric carcinoma cells by repeated *ex vivo* stimulation of T cells with tumor lysate-pulsed DCs.

DCs induce, sustain and regulate immune responses[13]. Four stages of their development have been delineated: (1) bone marrow progenitors; (2) precursor DCs, which patrol through the blood, lymphatics and lymphoid tissues; (3) tissue-resident immature DCs, which possess high endocytic and phagocytic capacity permitting antigen (Ag)-capture; and (4) mature DCs, present within secondary lymphoid organs, expressing high levels of co-stimulatory molecules permitting Ag-presentation[14].

Immature DCs are characterized by high capacity for antigen uptake but low T-cell stimulatory capacity. DCs mature because DC-mediated immune responses are more effective if DCs receive an activation signal. This signal can be microbial products such as lipopolysaccharide or unmethylated CpG motifs mimicking bacterial DNA[15,16], inflammatory mediators such as TNFα and IL-6[17,18], or T cell-derived signals such as CD40 ligand[19]. Matured DCs up-regulate co-stimulatory molecules, secrete the T-cell differentiation factor IL-12[20], and present antigens more effectively because of increased phenotypic stability and extended half-life of MHC class Ⅰ- and Ⅱ-molecules[21]. Furthermore, immature DCs bear the danger of inducing non-proliferating, IL-10-producing T cells, whereas mature DCs promote the development of Th1 cells[22].

In our experiment, c-kit$^+$ hematopoietic progenitor cells from mouse bone marrow cells cultured with GM-CSF, IL-4 or TNFα for 8 d showed the character of typical mature DCs. Morphologically, these large cells with oval or irregularly shaped nuclei and many small dendrites. Phenotypically, FACS analysis showed that they had typical mature DCs phenotypic markers, and expressed high levels of Ia, DEC-205, CD11b, CD80 and CD86 antigen, and moderate levels of CD40. Functionally, these cells gained the capacity to stimulate allogeneic T cells. However, immature DCs cultured with cytokines for 5 d did not possess typical DCs phenotypic markers and could not stimulate allogeneic T cells.

The use of a DC-based tumor vaccine as a cellular adjuvant to induce tumor-specific protective immunity holds great promise for cancer patients. This is important for advanced stage tumors with poor responsiveness to chemotherapy, such as gastric cancer. Currently, no defined tumor-specific antigen is available for many tumors, including gastric cancer. Now, DCs can be pulsed with synthetic peptides or proteins derived from known tumor associated antigens (TAA) such as MUC1, Her-2/neu, tyrosinase, CEA or Melan-A/MART[9].

In this study, tumor cell lysates were obtained by rapid freezing and thawing, and were regarded as tumor specific antigens. BM-derived DCs were pulsed with tumor lysates. Naïve mouse splenic T cells were primed *in vitro* with SGC-7901 TP DCs or B16 TP DCs to elicit cytolytic reactivity against tumor cells. The results showed that primed T cells *in vitro* with SGC-7901 TP DCs were able to induce specific CTL against SGC-7901 tumor cells, but not B16 tumor cells. Vaccination with DCs pulsed with tumor lysates has been shown to have efficient anti-tumor effects in many other tumor models and in clinical studies. Schnurr *et al*[23] report that T cells specific for pancreatic carcinoma cells can be generated *in vitro* by lysate-pulsed DCs and that the T-cell response can be enhanced by keyhole limpet hemocyanin (KLH). This *in vitro* model can be applied to compare different strategies in the development of DC-based tumor vaccines. Primary clinical studies were performed on melanoma patients using DCs pulsed with peptides or loaded with tumor cell lysates. Kono *et al*[24] report that tumor vaccination therapy with DCs pulsed with HER-2/neu-peptides may be a potential candidate for the novel treatment of gastric cancer patients. Nine gastric cancer patients with recurrent or unrescetable tumor were enrolled in the clinical trial. Vaccinations with DCs pulsed with HER-2 (p369) peptide were performed at 2-week intervals. In 3 of 9 patients, the tumor markers (CEA or CA19-9) were decreased after vaccination. Two patients had a tumor regression of more than 50%, and two presented a mixed response.

In summary, vaccination with bone marrow-derived dendritic cells pulsed with tumor cell lysates induced anti-tumor immunity specific to gastric cancer *ex vivo*. These results suggest that an evaluation of BM-DCs pulsed with tumor lysates against gastric cancer is an important next step *in vivo*. A trial evaluating this approach in mice is currently in preparation.

COMMENTS

Background

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that both initiate and modulate the immune response. DCs are cells in the pathway of antigen capture and presentation to T cells, with the unique ability to directly prime naïve CD4$^+$ and CD8$^+$ T cells. Gastric cancer is one of the most common cancers. Although gastric cancer therapy has made great progress, it is still difficult to treat advanced gastric cancer, as it has spread to the lymph glands and metastasized. Currently, tumor immunotherapy for gastric cancer has potential. DCs are believed to be essential for stimulating tumor-specific cytotoxic T lymphocyte (CTL) and inducing the protective and therapeutic anti-tumor immunity.

Research frontiers

Currently, no defined tumor specific antigen is available for many tumors, including gastric cancer. Now, DCs can be pulsed with synthetic peptides or proteins derived from known tumor associated antigens (TAA) such as MUC1, Her-2/neu, tyrosinase, CEA or Melan-A/MART. Some studies have shown that it is feasible that DCs-based tumor vaccines will become a new effective immunoadjuvant therapy for gastric cancer, which can decrease the incidence and recurrence rate after operation for gastric cancer. Primary clinical studies were performed on melanoma patients using DCs pulsed with peptides or loaded with tumor cell lysates. Kono *et al*[24] report that tumor vaccination therapy with DCs pulsed with HER-2/neu-peptides may be a potential candidate for the novel treatment of gastric cancer patients.

Innovations and breakthroughs

c-kit+ hematopoietic progenitor cells from mice bone marrow cells cultured with GM-CSF, IL-4 or TNFα for 8 d showed the character of typical mature

DCs. Gastric cancer cell lysates were obtained by rapid freezing and thawing, and were regarded as tumor specific antigens. Vaccination with bone marrow-derived dendritic cells pulsed with tumor cell lysates induced anti-tumor immunity specific to gastric cancer *ex vivo*.

Applications

Vaccination with bone marrow-derived dendritic cells pulsed with tumor cell lysates induced anti-tumor immunity specific to gastric cancer *ex vivo*. These results suggest that an evaluation of BM-DCs pulsed with tumor lysates against gastric cancer is an important next step *in vivo*. A trial evaluating this approach in mice is currently in preparation.

Terminology

DCs means dendritic cells, BM-DCs indicates bone marrow DCs, APCs stands for antigen presenting cells, MHC is major histocompatibility complex, CTL is cytotoxic T lymphocyte, IFNγ means interferon gamma.

Peer review

Using an *in vitro* system, the authors suggest a specific anti-tumor effect based on immunological mechanisms. The fatal prognosis of cancer requires the development of novel therapeutic concepts. Unfortunately, various promising strategies have failed in the clinical practice. Therefore, suitable animal models are strongly needed to investigate the possible effectiveness of the shown *ex vivo* principle for an anti-cancer treatment.

REFERENCES

1 **Gilboa E**. DC-based cancer vaccines. *J Clin Invest* 2007; **117**: 1195-1203
2 **Banchereau J**, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245-252
3 **Hohenberger P**, Gretschel S. Gastric cancer. *Lancet* 2003; **362**: 305-315
4 **Guida F**, Formisano G, Esposito D, Antonino A, Conte P, Bencivenga M, Persico M, Avallone U. [Gastric cancer: surgical treatment and prognostic score] *Minerva Chir* 2008; **63**: 93-99
5 **Liakakos T**, Fatourou E. Stage-specific guided adjuvant treatment for gastric cancer. *Ann Surg Oncol* 2008; **15**: 2622-2623
6 **Ishigami S**, Natsugoe S, Tokuda K, Nakajo A, Xiangming C, Iwashige H, Aridome K, Hokita S, Aikou T. Clinical impact of intratumoral natural killer cell and dendritic cell infiltration in gastric cancer. *Cancer Lett* 2000; **159**: 103-108
7 **Ishigami S**, Natsugoe S, Uenosono Y, Hata Y, Nakajo A, Miyazono F, Matsumoto M, Hokita S, Aikou T. Infiltration of antitumor immunocytes into the sentinel node in gastric cancer. *J Gastrointest Surg* 2003; **7**: 735-739
8 **Galetto A**, Contarini M, Sapino A, Cassoni P, Consalvo E, Forno S, Pezzi C, Barnaba V, Mussa A, Matera L. Ex vivo host response to gastrointestinal cancer cells presented by autologous dendritic cells. *J Surg Res* 2001; **100**: 32-38
9 **Wu Y**, Wang L, Zhang Y. Dendritic cells as vectors for immunotherapy of tumor and its application for gastric cancer therapy. *Cell Mol Immunol* 2004; **1**: 351-356
10 **Zhang Y**, Harada A, Wang JB, Zhang YY, Hashimoto S, Naito M, Matsushima K. Bifurcated dendritic cell differentiation in vitro from murine lineage phenotype-negative c-kit+ bone marrow hematopoietic progenitor cells. *Blood* 1998; **92**: 118-128
11 **Zhang Y**, Zhang YY, Ogata M, Chen P, Harada A, Hashimoto S, Matsushima K. Transforming growth factor-beta1 polarizes murine hematopoietic progenitor cells to generate Langerhans cell-like dendritic cells through a monocyte/macrophage differentiation pathway. *Blood* 1999; **93**: 1208-1220
12 **Zhang Y**, Yoneyama H, Wang Y, Ishikawa S, Hashimoto S, Gao JL, Murphy P, Matsushima K. Mobilization of dendritic cell precursors into the circulation by administration of MIP-1alpha in mice. *J Natl Cancer Inst* 2004; **96**: 201-209
13 **Ullrich E**, Ménard C, Flament C, Terme M, Mignot G, Bonmort M, Plumas J, Chaperot L, Chaput N, Zitvogel L. Dendritic cells and innate defense against tumor cells. *Cytokine Growth Factor Rev* 2008; **19**: 79-92
14 **Nouri-Shirazi M**, Banchereau J, Fay J, Palucka K. Dendritic cell based tumor vaccines. *Immunol Lett* 2000; **74**: 5-10
15 **Liu Q**, Shu X, Sun A, Sun Q, Zhang C, An H, Liu J, Cao X. Plant-derived small molecule albaconol suppresses LPS-triggered proinflammatory cytokine production and antigen presentation of dendritic cells by impairing NF-kappaB activation. *Int Immunopharmacol* 2008; **8**: 1103-1111
16 **Warren TL**, Bhatia SK, Acosta AM, Dahle CE, Ratliff TL, Krieg AM, Weiner GJ. APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. *J Immunol* 2000; **165**: 6244-6251
17 **Hartmann G**, Battiany J, Poeck H, Wagner M, Kerkmann M, Lubenow N, Rothenfusser S, Endres S. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. *Eur J Immunol* 2003; **33**: 1633-1641
18 **Su B**, Wang J, Wang X, Jin H, Zhao G, Ding Z, Kang Y, Wang B. The effects of IL-6 and TNF-alpha as molecular adjuvants on immune responses to FMDV and maturation of dendritic cells by DNA vaccination. *Vaccine* 2008; **26**: 5111-5122
19 **Gonzalez-Carmona MA**, Lukacs-Kornek V, Timmerman A, Shabani S, Kornek M, Vogt A, Yildiz Y, Sievers E, Schmidt-Wolf IG, Caselmann WH, Sauerbruch T, Schmitz V. CD40ligand-expressing dendritic cells induce regression of hepatocellular carcinoma by activating innate and acquired immunity in vivo. *Hepatology* 2008; **48**: 157-168
20 **He XZ**, Wang L, Zhang YY. An effective vaccine against colon cancer in mice: use of recombinant adenovirus interleukin-12 transduced dendritic cells. *World J Gastroenterol* 2008; **14**: 532-540
21 **Boes M**, Cerny J, Massol R, Op den Brouw M, Kirchhausen T, Chen J, Ploegh HL. T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature* 2002; **418**: 983-988
22 **Jalili A**. Dendritic cells and their role in cancer immunotherapy. *Iran J Immunol* 2007; **4**: 127-144
23 **Schnurr M**, Galambos P, Scholz C, Then F, Dauer M, Endres S, Eigler A. Tumor cell lysate-pulsed human dendritic cells induce a T-cell response against pancreatic carcinoma cells: an in vitro model for the assessment of tumor vaccines. *Cancer Res* 2001; **61**: 6445-6450
24 **Kono K**, Takahashi A, Sugai H, Fujii H, Choudhury AR, Kiessling R, Matsumoto Y. Dendritic cells pulsed with HER-2/neu-derived peptides can induce specific T-cell responses in patients with gastric cancer. *Clin Cancer Res* 2002; **8**: 3394-3400

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CASE REPORT

Acute hepatitis B or exacerbation of chronic hepatitis B-that is the question

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Abstract

Hepatitis B virus (HBV) infection constitutes a serious global health problem. In countries with intermediate or high endemicity for HBV, exacerbations of chronic hepatitis B may be the first presentation of HBV infection. Some of these patients may be diagnosed mistakenly as having acute hepatitis B. Accurate diagnosis in these cases is very important for deciding whether to start treatment or not, because acute hepatitis B does not require therapy, while exacerbation of chronic hepatitis may benefit from it. Clinical and routine laboratory findings cannot help distinguishing between these two conditions. Therefore, several assays have been proposed for this purpose during the last few years. The presence of high levels of anti-HBe antibodies, HBsAg and HBV DNA are typical of chronic disease, whereas high titers of IgM anti-HBc, together with their high avidity index, characterize acute HBV infection. Starting from the description of a patient with acute hepatitis B-who recently came to our observation-we critically review the currently available assays that may help distinguishing between the different conditions and lead to the optimal management of each patient.

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Key words: Hepatitis B; Anti-hepatitis B virus antibodies; Hepatitis B virus; Toxic hepatitis; autoimmune hepatitis

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INTRODUCTION

Many parts of the world are endemic for the hepatitis B virus (HBV) infection. In these countries, especially those with intermediate or high endemicity rates, patients may frequently present with acute or chronic HBV infection. In fact, exacerbations of chronic hepatitis B are common, and may even be the first presentation of infection, including in cases with compensated, previously asymptomatic cirrhosis. Sometimes these patients may be diagnosed mistakenly as suffering from acute hepatitis B. At first glance, it is difficult to distinguish between these two clinical conditions, due to the similar clinical features and serological profile[1,2]. It is estimated that, in endemic areas, acute exacerbations of chronic hepatitis constitute about 50% of cases diagnosed as primary infections[2-5]. Distinguishing between these two conditions is extremely important, because antiviral therapy is not recommended in cases of acute hepatitis (except for very severe ones), whereas it is indicated in cases of chronic hepatitis. Therefore, simple, effective and reliable assays are required for differentiating between these conditions. Some methods for differentiating between chronic and acute infections have been suggested in the last few years, but no review summarized or compared them until now. Staring from the description of a case with acute hepatitis-who recently came to our observation-we shortly review these methods. Our aim is to provide practicing physicians with the basic knowledge and the tools useful for distinguishing between acute and chronic hepatitis B, for the benefit of the patients.

CASE REPORT

A 38-year-old male presented to our emergency room

complaining of weakness, vomiting, sore throat and dark urines during the past few days. Several years before he had been diagnosed as having mixed connective tissue disease (MCTD), based on arthralgias and positive serology for ANA, anti-SSA/Ro and anti-SSB/La antibodies. He had been treated with plaquenil (hydroxychloroquine) for the previous 6 mo, but this drug had been discontinued two weeks before admission. The patient also had Wolff Parkinson White (WPW) syndrome for which he had never been treated. On admission, physical examination was unremarkable, except for the presence of a mild jaundice. Blood tests revealed extremely elevated liver enzymes, mostly hepatocellular, with ALT levels of 3069 U/L (normal: 6-53 U/L) and AST of 1215 U/L (normal: 2-60 U/L), while the indices of cholestasis were only mildly elevated, with alkaline phosphatase at 207 U/L (normal: 40-130 U/L) and GGT at 376 U/L (normal: 10-80 U/L). The total bilirubin was 35 mmol/L (normal: 0-17 mmol/L) and the LDH was 1200 U/L (normal: 300-620 U/L).

The patient denied traveling abroad, having unprotected sexual contact or recent viral infections. He had never received blood or blood-derived products, had never used drugs and he was not drinking alcohol. He had no fever and his electrocardiogram (ECG) was normal, with no signs of WPW syndrome.

What is the differential diagnosis in this patient?

The clinical presentation and the results of the laboratory tests call for a differential diagnosis of hepatitis. Since the patient had a diagnosis of MCTD the possibility of autoimmune hepatitis as an additional manifestation of his disease was raised[6]. In many of these cases, anti-smooth muscle antibodies (AMA) are positive, while in our patient they were negative. Another possible diagnosis would have been hepatic toxic injury due to plaquenil consumption, which is a reversible and dose-related cause of acute hepatitis[7]. Our patient had received plaquenil for 6 mo with no evidence of liver injury, and the treatment had been stopped 2 wk before admission. Furthermore, despite the discontinuation of the medication, liver tests were still worsening, making the diagnosis of plaquenil hepatotoxicity very unlikely. Thus, we had to look for infectious causes of hepatitis.

What further investigations are needed?

An abdominal ultrasound showed a liver of normal size and echogenicity, with an enlarged spleen (14.6 cm). The search for markers of autoimmunity revealed the presence of antinuclear (+2 of +4) and anti-SSA/Ro antibodies, but anti-SSB/La antibodies were negative and C3 levels were normal. Assays for ENA, anti-nRNP, anti-parietal, anti-mitochondria and anti-smooth muscle antibodies were negative. Blood tests for detecting infectious agents revealed the absence of serological markers of HAV, HCV and toxoplasmosis. However, there were markers suggesting a previous exposure to CMV and EBV (IgG antibodies). HBsAg and anti-HBc antibodies were positive. Further investigations revealed the presence of HBeAg, the absence of anti-HBe antibodies, and 2.65×10^9 IU/L of HBV DNA.

What does this serologic profile implicate?

Our patient had positive serology for HBV infection. The possibility of acute hepatitis B was raised, but the patient denied having unprotected sexual relations, using intravenous drugs, undergoing dental procedures or being exposed to blood products. Although in up to 30% of cases the exact mode of transmission cannot be identified, the lack of any risk factor for a recent infection does not support the diagnosis of acute infection. Therefore, we raised the possibility of an acute exacerbation of a chronic HBV infection. The differentiation between these two conditions is important because in cases of acute hepatitis, except for very severe ones, antiviral therapy is not recommended, whereas this is indicated in the case of an exacerbation of a chronic hepatitis. In both conditions, patients may suffer from flu-like prodromic symptoms, together with jaundice, abdominal discomfort and pruritus. Hepatosplenomegaly is also common in both situations. In addition, there are no significant differences as to the biochemical assays, such as peak bilirubin serum level, PT prolongation and serum albumin level. A tendency for higher levels of serum transaminases is seen in acute infection[8]. Some methods of differentiating between chronic and acute infections have been suggested over the past few years. A short review of some of them is described below, together with a discussion regarding their relevance to the present case.

Role of HBeAg in the differential diagnosis: HBeAg is a secretory protein that is processed from the precore protein and considered as a marker of HBV replication and infectivity[9]. The presence of HBeAg is usually associated with high levels of HBV DNA in serum and higher rates of transmission of infection from carrier mothers to their offspring and from patients to health care workers[10-13].

Seroconversion from HBeAg carrier to anti-HBe antibodies occurs early in patients with acute infection, prior to HBsAg to anti-HBs seroconversion[14]. However, HBeAg seroconversion may be delayed for years in patients with chronic HBV infection. In such patients, the presence of HBeAg is usually associated with the detection of HBV DNA in serum and active liver disease (except for HBeAg-positive patients with perinatally acquired HBV infection, who may have normal serum ALT concentrations and minimal inflammation in the liver)[15,16]. Seroconversion from HBeAg to anti-HBe is usually associated with a decrease in serum HBV DNA levels and liver disease remission[17].

HBeAg has been found more frequently in patients with acute infection compared with those with chronic infection, but the difference is not statistically significant. On the other hand, anti-HBe was found more frequently among patients suffering from an acute exacerbation of chronic infection than in those with acute infection. Levels of anti-HBe antibodies and HBeAg/anti-HBe

immune complexes are significantly higher in patients with exacerbation of chronic hepatitis[8,18]. However, the presence of anti-HBe as a diagnostic tool for chronic infection was found to have low sensitivity, specificity, NPV and PPV[8].

Our patient had positive HBeAg and negative anti-HBe antibodies, which supports the diagnosis of an acute infection.

Role of HBcAg in the differential diagnosis: The viral core antigen is expressed within the infected hepatic cells and cannot be detected in serum. Its corresponding antibody, on the other hand, can be detected in serum at different phases of infection. IgM anti-HBc is a single serum marker of HBV infection within the period between the disappearance of HBsAg and the appearance of anti-HB antibodies. Identification of IgM anti-HBc is considered diagnostic for the acute phase of infection, but it has been reported that it can remain present in serum for two years from the initial infection. Moreover, the titer of IgM anti-HBc can also rise and become detectable in exacerbations of chronic hepatitis[18].

Recently, it has been suggested that the titer of IgM anti-HBc can be useful for differentiating between acute and chronic HBV infection. Kumar *et al*[8] demonstrated that high titers of IgM anti-HBc are more common in patients with acute infection, and titers above 1:1000 can be seen in up to 80% of these patients. In about 70% of patients with chronic hepatitis, IgM anti-HBc titers were lower than 1:1000 or negative. Differentiation between these two conditions by measuring the titers did not prove to have high sensitivity, specificity, NPV or PPV. Therefore another assay was proposed that enables the standardization between different laboratories, i.e. the sample/cutoff ratio (S/CO)[19]. A ratio > 10 indicates acute infection whereas a ratio < 10 indicates chronic infection. It appears that in cases of acute infection the mean ratio is 25.96, as opposed to an average ratio of 2.95 in chronic infection, a difference that is statistically highly significant. A S/CO > 10 had a sensitivity and NPV of 100%, a specificity of 99% and PPV of 99.3% for diagnosing an acute infection.

In our patient there were high titers (> 1:1000) of IgM anti-HBc, supporting the diagnosis of acute infection, but the S/CO (3.2/1.2) was < 10, suggesting a chronic infection.

Role of HBsAg in the differential diagnosis: HBsAg is an important marker of HBV infection. It has been reported that changes in quantitative measurement of this marker depend on the phase of infection[20-22]. A difference is found between the high levels of HBsAg that are detected in patients who are hospitalized during chronic or acute infection and the low levels observed in subjects with inactive, chronic infection. Moreover, recent data support the fact that high level of HBsAg are related to viral replication and disease activity[8]. In acute hepatitis, the levels of HBsAg are generally above 1×10^7 IU/L and decrease sharply in the recovery phase. In chronic anti-HBe-positive cases, HBsAg levels are generally lower than 1×10^7 IU/L (mean 2655), whereas in 5 HBeAg positive chronic hepatitis patients the mean value was reported to be 7.8756×10^8 IU/L, with 90% of cases exceeding 1×10^7 IU/L.

Our patient had HBsAg levels of 2.22×10^9 IU/L, which does not support an acute infection, but positive HBeAg and negative anti-HBe antibodies, which in chronic cases correlate with high levels of HBsAg.

Role of HBV DNA in the differential diagnosis: A quantitative measurement of the viral DNA enables the evaluation of the level of viral replication. There are many assays for measuring HBV DNA, their sensitivity being dependent on the assay used. Currently, there are some attempts to standardize the different measurements and express results in IU/L. Low or undetectable levels have been seen in patients suffering from acute hepatitis[21], and a significant decline in HBV DNA levels has been reported even before the appearance of the disease. It has also been suggested that an acute infection may be diagnosed by finding undetectable HBV DNA in serum by the time medical aid is sought or in the presence of ALT levels lower than 400 IU/L. HBV DNA levels become detectable during reactivation of chronic hepatitis[22]. Recently, it has been shown that HBV DNA levels can help differentiate an acute infection progressing to recovery from an exacerbation of chronic infection, which requires therapy[8]. In this study, low levels of HBV DNA (< 0.5 pg/mL, equal to 141 500 copies/mL) were found in about 96% of patients with acute infection, as opposed to 13% in those with exacerbation of chronic hepatitis. Higher HBV DNA levels were found in 87% of patients with chronic infection compared to only 4% in those with acute infection. The sensitivity and specificity of low levels of HBV DNA for identifying an acute infection are 96% and 86.6%, respectively. The combination of high levels of HBV DNA with low titers of IgM anti-HBc yielded sensitivity, specificity, NPV and PPV of 100%, 97.9%, 96.3% and 100%, respectively, for diagnosing an exacerbation of a chronic hepatitis.

Our patient had high levels of HBV DNA (2 650 000 copies/mL), which supports the diagnosis of exacerbation of chronic infection.

What is the patient's diagnosis?

Our patient had positive HBeAg, negative anti-HBe antibodies, and high titers of IgM anti-HBc, which support the diagnosis of an acute infection (Table 1). However, the presence of IgM anti-HBc S/CO < 10, low levels of HBsAg and high levels of HBV DNA suggest an acute exacerbation of chronic infection. In order to make a progress in the diagnosis, we decided to investigate his family. Familial analysis revealed positive anti-HBc in the patient's mother. She also recalled having icteric disease about 40 years earlier. Therefore, the possibility of perinatal transmission was raised and a diagnosis of acute exacerbation of a chronic HBV infection was made. The patient was treated with

Table 1 Comparison of the serological markers in acute HBV and in exacerbation of chronic HBV infections

Serological markers	Acute infection	Chronic exacerbation	Comments
HBeAg	-	+	Not different, and also not helpful in infections with HBeAg-negative strains
Anti-HBe	-	+	Low sensitivity and specificity, and also not helpful in infections with HBeAg-negative strains
Anti-HBe level, and complexes of HBeAg/anti-HBe	↓	↑	Not helpful in infections with HBeAg-negative strains
IgM anti-HBc	> 1:1000	< 1:1000	Low sensitivity, specificity, NPV and PPV
Sample/cutoff ratio for IgM anti-HBc	> 10	< 10	High sensitivity, specificity, NPV and PPV
Anti-HBc avidity index	< 0.7	> 0.7	High sensitivity, specificity, NPV and PPV
HBsAg	↓	↑	Useful in HBeAg-negative cases
HBV-DNA	< 0.5 pg/mL	> 0.5 pg/mL	Sensitivity: 96%
	≤ 141 500 copies/mL	≥ 141 500 copies/mL	Specificity: 86%

lamivudine, 100 mg per day, for 4 mo. After 2 mo of treatment the liver enzymes returned to normal values. After 4 mo of treatment, an HBeAg seroconversion was seen and anti-HBs antibodies appeared. The treatment was discontinued and after one year liver enzymes were still normal. The HBsAg/anti-HBs ratio indicated a complete recovery from the HBV infection.

DISCUSSION

HBV infection remains a global public health problem, despite the availability of an effective vaccine. In most cases, HBV infection occurs in patients at high-risk, such as intravenous drug users, homosexual men and in certain groups where HBV is endemic. HBV infection can lead to an acute or chronic hepatitis, liver cirrhosis and hepatocellular carcinoma.

Acute HBV infection has a variable course, ranging from asymptomatic infection to fulminant hepatitis. The incubation period lasts from one to four months. A serum sickness-like syndrome may develop during the prodromal period, followed by systemic symptoms, anorexia, nausea, jaundice and right upper quadrant discomfort. The symptoms and jaundice generally disappear after one to three months. Some patients will develop chronic infection. The proportion of patients progressing to chronic infection is much higher in the newborn (up to 90%) compared to 1% to 5% among adults and intermediate values in young children. Most patients with chronic HBV infection are asymptomatic. However, in some patients, chronic HBV infection may be associated with extrahepatic manifestations such as panarteritis nodosa and glomerulonephritis. The natural history of chronic HBV infection is variable, depending upon age, mode of acquisition and ethnicity. Children in non-endemic countries frequently clear HBeAg and HBV DNA from serum during the first two decades of life. Children who seroconvert spontaneously tend to have higher ALT levels early in life[23]. By contrast, children from endemic countries in whom HBV was acquired perinatally usually remain HBeAg-positive and have high levels of viral replication, although histologic injury is typically mild[16,24]. These patients may experience acute exacerbations of hepatitis. Therefore, it may be difficult for their physicians to differentiate between those who have an acute hepatitis B from those with an exacerbation of chronic HBV infection. The guidelines provided here may help solving this problem since the therapeutic approach toward these conditions is different. Antiviral therapy is recommended in case of acute exacerbation of chronic hepatitis B, whereas, in most cases, supportive treatment is sufficient in acute hepatitis B.

Key learning points: (1) In endemic areas, acute exacerbations of chronic HBV infection constitute about 50% of cases diagnosed as having acute HBV infection; (2) the proportion of patients progressing to chronic HBV infection is much higher in the newborn (up to 90%) compared with children or adults; (3) the therapeutic approach differs between acute hepatitis B and acute exacerbation of chronic hepatitis B, since treatment is usually not recommended in case of acute infection; (4) available laboratory tests can be helpful in the differential diagnosis, in order to provide a better management of patients.

REFERENCES

1 **Chu CM**, Liaw YF, Pao CC, Huang MJ. The etiology of acute hepatitis superimposed upon previously unrecognized asymptomatic HBsAg carriers. *Hepatology* 1989; **9**: 452-456
2 **Tassopoulos NC**, Papaevangelou GJ, Sjogren MH, Roumeliotou-Karayannis A, Gerin JL, Purcell RH. Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. *Gastroenterology* 1987; **92**: 1844-1850
3 **Chu CM**, Sheen IS, Liaw YF. The aetiology of acute hepatitis in Taiwan: acute hepatitis superimposed on HBsAg carrier state as the main aetiology of acute hepatitis in areas with high HBsAg carrier rate. *Infection* 1988; **16**: 233-237
4 **Liaw YF**, Chu CM, Huang MJ, Chen TJ, Lin DY. The etiology of acute viral hepatitis in an endemic area of hepatitis A and B. *Am J Trop Med Hyg* 1983; **32**: 1401-1406
5 Davis GL, Hoofnagle JH. Reactivation of chronic type B hepatitis presenting as acute viral hepatitis. *Ann Intern Med* 1985; **102**: 762-765
6 **Aoki S**, Tada Y, Ohta A, Koarada S, Ushiyama O, Suzuki N, Nagasawa K. [Autoimmune hepatitis associated with mixed connective tissue disease: report of a case and a review of the literature] *Nihon Rinsho Meneki Gakkai Kaishi* 2001; **24**: 75-80
7 **Giner Galvan V**, Oltra MR, Rueda D, Esteban MJ, Redon J. Severe acute hepatitis related to hydroxychloroquine in a woman with mixed connective tissue disease. *Clin Rheumatol* 2007; **26**: 971-972
8 **Kumar M**, Jain S, Sharma BC, Sarin SK. Differentiating acute hepatitis B from the first episode of symptomatic exacerbation of chronic hepatitis B. *Dig Dis Sci* 2006; **51**:

594-599
9 **Miller DJ**. Seroepidemiology of viral hepatitis: correlation with clinical findings. *Postgrad Med* 1980; **68**: 137-141, 144-148
10 **Okada K**, Kamiyama I, Inomata M, Imai M, Miyakawa Y. e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. *N Engl J Med* 1976; **294**: 746-749
11 **Beasley RP**, Trepo C, Stevens CE, Szmuness W. The e antigen and vertical transmission of hepatitis B surface antigen. *Am J Epidemiol* 1977; **105**: 94-98
12 **Hwang LY**, Roggendorf M, Beasley RP, Deinhardt F. Perinatal transmission of hepatitis B virus: role of maternal HBeAg and anti-HBc IgM. *J Med Virol* 1985; **15**: 265-269
13 **Alter HJ**, Seeff LB, Kaplan PM, McAuliffe VJ, Wright EC, Gerin JL, Purcell RH, Holland PV, Zimmerman HJ. Type B hepatitis: the infectivity of blood positive for e antigen and DNA polymerase after accidental needlestick exposure. *N Engl J Med* 1976; **295**: 909-913
14 **Krugman S**, Overby LR, Mushahwar IK, Ling CM, Frosner GG, Deinhardt F. Viral hepatitis, type B. Studies on natural history and prevention re-examined. *N Engl J Med* 1979; **300**: 101-106
15 **Chang MH**, Hwang LY, Hsu HC, Lee CY, Beasley RP. Prospective study of asymptomatic HBsAg carrier children infected in the perinatal period: clinical and liver histologic studies. *Hepatology* 1988; **8**: 374-377
16 **Lok AS**, Lai CL. A longitudinal follow-up of asymptomatic hepatitis B surface antigen-positive Chinese children. *Hepatology* 1988; **8**: 1130-1133
17 **Hoofnagle JH**, Dusheiko GM, Seeff LB, Jones EA, Waggoner JG, Bales ZB. Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann Intern Med* 1981; **94**: 744-748
18 **Maruyama T**, Schodel F, Iino S, Koike K, Yasuda K, Peterson D, Milich DR. Distinguishing between acute and symptomatic chronic hepatitis B virus infection. *Gastroenterology* 1994; **106**: 1006-1015
19 **Rodella A**, Galli C, Terlenghi L, Perandin F, Bonfanti C, Manca N. Quantitative analysis of HBsAg, IgM anti-HBc and anti-HBc avidity in acute and chronic hepatitis B. *J Clin Virol* 2006; **37**: 206-212
20 **Frosner GG**, Schomerus H, Wiedmann KH, Zachoval R, Bayerl B, Backer U, Gathof GA, Sugg U. Diagnostic significance of quantitative determination of hepatitis B surface antigen in acute and chronic hepatitis B infection. *Eur J Clin Microbiol* 1982; **1**: 52-58
21 **Webster GJ**, Reignat S, Maini MK, Whalley SA, Ogg GS, King A, Brown D, Amlot PL, Williams R, Vergani D, Dusheiko GM, Bertoletti A. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. *Hepatology* 2000; **32**: 1117-1124
22 **Gayno S**, Marcellin P, Loriot MA, Martinot-Peignoux M, Levy P, Erlinger S, Benhamou JP. Detection of serum HBV-DNA by polymerase chain reaction (PCR) in patients before reactivation of chronic hepatitis B. *J Hepatol* 1992; **14**: 357-360
23 **Bortolotti F**, Cadrobbi P, Crivellaro C, Guido M, Rugge M, Noventa F, Calzia R, Realdi G. Long-term outcome of chronic type B hepatitis in patients who acquire hepatitis B virus infection in childhood. *Gastroenterology* 1990; **99**: 805-810
24 **Lok AS**, Lai CL, Wu PC, Lau JY, Leung EK, Wong LS. Treatment of chronic hepatitis B with interferon: experience in Asian patients. *Semin Liver Dis* 1989; **9**: 249-253

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CASE REPORT

Ascending colon adenocarcinoma with tonsillar metastasis: A case report and review of the literature

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Abstract

Metastatic palatine tonsil cancer is extremely rare, with nearly 100 such tumors reported in the English literature. The prognosis of metastatic palatine tonsil cancer is poor. A 53-year-old man presented with painless left palatine tonsillar swelling and a cervical mass following right hemicolectomy for an ascending colon adenocarcinoma. Physical examination showed an ulcerated mass located on the upper pole of the left palatine tonsil. A punch biopsy was taken for histological examination which showed a moderately-differentiated adenocarcinoma. The patient was treated with palliative radiotherapy and chemotherapy. He was still alive when we wrote this paper. Our case shows that immunohistochemical diagnosis of metastatic palatine tonsil cancer is essential.

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Key words: Tonsil neoplasm; Metastasis; Colon neoplasm; Adenocarcinoma; Immunohistochemistry

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INTRODUCTION

Metastatic palatine tonsil tumor is uncommon despite the palatine tonsil is rich in vasculature. It was reported that the incidence of metastatic palatine tonsil cancer is about 1%[1]. Metastatic palatine tonsil tumor generally occurs in lung[2], breast[3], stomach[4], and kidney[5,6]. Distant metastasis of palatine tonsil tumor in liver[7], brain[8], and lung[9] is usually found in colorectal cancer patients following a surgical resection. Metastatic palatine tonsil tumor is often accompanied with synchronous lesions in other organs, a sign needing aggressive palliative treatment[10].

It is often difficult to distinguish a metastatic tumor at an unusual site from a secondary cancer, especially after long-term complete remission. In this paper, we report a case of metastatic palatine tonsil tumor from an ascending colon carcinoma and reviewed the cases reported in the English literature.

CASE REPORT

A 53-year-old man with bellyache and changes in bowel habit was diagnosed having an adenocarcinoma of the ascending colon in January 2006. He underwent a right hemicolectomy, which showed a moderately-differentiated adenocarcinoma measuring 60 mm × 60 mm × 10 mm in size. The tumor invaded the subserosal layer (T_3) with three excised positive regional lymph nodes (N_1). The patient received 4 courses of adjuvant chemotherapy with fluorouracil and leucovorin and was regularly followed up, with physical examination, abdominal ultrasonography and detection of serum carcinoembryonic antigen (CEA) performed every 3 mo. One and a half years later, he was referred to our hospital due to painless left palatine tonsillar swelling and a cervical mass. He had no gastrointestinal and head symptoms. Physical examination showed an ulcerated mass located on the upper pole of the left palatine tonsil, about 2 cm in diameter. Several enlarged, fixed cervical lymph nodes were found in

Table 1 Reported cases of metastatic palatine tonsil tumor from colorectal cancer in the English literature

NO. (Ref)	Gender	Age	Primary site[1]	Differentiation[2]	Interval (mo)[3]	Location	Treatment	Prognosis(mo)
1 (13)	Female	55	R	Well	84	Right	Radiotherapy	nr
2 (14)	Male	53	R	Poorly	24	Right	Tonsillectomy	6 alive
3 (15)	Male	45	R	Poorly and signet-ring	0	Left	Radiotherapy and tonsillectomy	6
4 (16)	Male	36	R	Signet-ring	24	Right	Tonsillectomy	15 alive
5 (17)	Female	81	A	Moderately	0	Left	Tonsillectomy	12
6 (18)	Male	44	A	Signet-ring	0	Left	Radiotherapy	nr
7 (19)	Male	65	T	Poorly	0	Left	Radiotherapy	6
This case	Male	53	A	Moderately	19	Left	Radiotherapy	13 alive

[1]A = ascending colon, T = transverse colon, R = rectum, nr = not referred; [2]Well = well differentiated adenocarcinoma, moderately=moderately-differentiated adenocarcinoma, poorly = poorly-differentiated adenocarcinoma, Signet-ring = signet-ring cell carcinoma; [3]Interval = the time between the diagnosis of colorectal carcinoma and the development of metastatic palatine tonsil tumor.

the left area. The rest findings of physical examination were unremarkable. The CEA, CA125 and AFP levels were within the normal range. Magnetic resonance image (MRI) revealed an enlarged left palatine tonsil narrowing the oropharyngeal airway. At the same time, a lesion in the left temporal lobe of the brain was found, about 3 cm in diameter. Chest X-ray and abdominal ultrasonography also showed no metastasis in lung and liver. A punch biopsy was taken for histological examination which showed a moderately-differentiated adenocarcinoma. Immunohistochemistry showed that the tumor cells exhibited immunoreactive CDX2 and villin, an immunomarker. These features were consistent with those of metastatic colorectal adenocarcinoma. The patient was treated with palliative radiotherapy and chemotherapy.

DISCUSSION

The palatine tonsil is one of the most common sites of head and neck cancer and squamous cell carcinoma in adults[11] as well as lymphoma in children[12]. Metastatic palatine tonsil cancer is extremely rare, with only 100 such tumors reported in the English literature. We present a case of metastatic palatine tonsil cancer manifested as a left palatine tonsil mass from a primary ascending colon adenocarcinoma.

Seven cases of metastatic palatine tonsil tumor from colorectal carcinoma have been reported in the English literature[13-19] (Table 1). The age of these cases ranged 36-81 years with a median age of 54 years, and the male and female ratio was 5:2. Of the 7 cases, 4 had a primary rectal cancer[13-16], 2 had a primary ascending colon cancer[17,18] and 1 had a primary transverse colon cancer[19]. Our case was a 53-year old male patient with primary ascending colon cancer. Metastatic palatine tonsil cancer is often unilateral, with the left side more commonly involved than the right side[20]. Lymphatic routes have been proposed as the possible pathways of metastatic palatine tonsil cancer from colorectal cancer. Of the 7 reported cases, 5 had primary lesions with metastatic regional lymph nodes, 2 had enlarged cervical lymph nodes when the palatine tonsil mass was found. Similar findings were evident in our patient. Metastatic palatine tonsil tumor from colorectal cancer is generally considered a systematic disease with a poor prognosis. No matter it is treated with radiotherapy or tonsillectomy, the survival time of such patients is 6-15 mo. Radiotherapy remains the choice of treatment for palatine tonsil tumor[21]. Tsubochi *et al*[22] have reported a successfully-treated metastatic lingual tonsillar tumor from bronchial adenocarcinoma after external radiotherapy, and the patient was still alive 8 years after the treatment. However, the beneficial effects of radiotherapy on patients with metastatic palatine tonsil tumor remain unclear and should be further studied.

It is difficult to determine whether palatine tonsil tumor is primary or secondary, especially after long-term complete remission. In our case, metastatic palatine tonsil tumor had a carcinoma origin, because it was very similar to a colorectal adenocarcinoma. Also, to make sure that metastatic palatine tonsil tumor is originated from an ascending colon cancer, immunohistochemical staining for its tissue was performed. Immumarkers, such CDX2 CK20 and villin, are useful in determining the primary site of adenocarcinoma. Suh *et al*[23] reported that nearly all colorectal adenocarcinomas can express CDX2 and villin, which are of diagnostic values in distinguishing primary from secondary colorectal carcinoma.

In conclusion, metastatic palatine tonsil tumor from colorectal cancer rarely occurs, and immunohistochemical diagnosis of metastatic palatine tonsil tumor is essential.

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REFERENCES

1 **Crawford BE**, Callihan MD, Corio RL, Hyams VJ, Karnei RF. Oral pathology. *Otolaryngol Clin North Am* 1979; **12**: 29-43
2 **Hisa Y**, Yasuda N, Murakami M. Small cell carcinoma of the lung metastatic to the palatine tonsil. *Otolaryngol Head Neck Surg* 1997; **116**: 563-564
3 **Tueche SG**, Nguyen H, Larsimont D, Andry G. Late onset

of tonsillar metastasis from breast cancer. *Eur J Surg Oncol* 1999; **25**: 439-440
4 **Hurlstone DP**, Sanders DS, Smith A, Jones RB, Slater DN, Bardhan KD. Tonsillar metastasis: A rare presentation of gastric carcinoma. *Eur J Surg Oncol* 2001; **27**: 328-330
5 **Sood S**, Nair SB, Fenwick JD, Horgan K. Metastatic melanoma of the tonsil. *J Laryngol Otol* 1999; **113**: 1036-1038
6 **Stańczyk R,** Omulecka A, Pajor A. [A case of renal clear cell carcinoma metastasis to the oropharynx] *Otolaryngol Pol* 2006; **60**: 97-100
7 **Carnaghi C**, Tronconi MC, Rimassa L, Tondulli L, Zuradelli M, Rodari M, Doci R, Luttmann F, Torzilli G, Rubello D, Al-Nahhas A, Santoro A, Chiti A. Utility of 18F-FDG PET and contrast-enhanced CT scan in the assessment of residual liver metastasis from colorectal cancer following adjuvant chemotherapy. *Nucl Med Rev Cent East Eur* 2007; **10**: 12-15
8 **Sundermeyer ML**, Meropol NJ, Rogatko A, Wang H, Cohen SJ. Changing patterns of bone and brain metastases in patients with colorectal cancer. *Clin Colorectal Cancer* 2005; **5**: 108-113
9 **Muñoz Llarena A**, Carrera Revilla S, Gil-Negrete Laborda A, Pac Ferrer J, Barceló Galíndez R, López Vivanco G. [Prognostic factors associated with resectable pulmonary metastases from colorectal cancer] *Arch Bronconeumol* 2007; **43**: 309-316
10 **Bozza F**, Piantanida R, Pellini R, Spriano G. [Palatine tonsillar metastasis from small cell carcinoma of the lung] *Acta Otorhinolaryngol Ital* 2000; **20**: 281-283
11 **Golas SM**. Trends in palatine tonsillar cancer incidence and mortality rates in the United States. *Community Dent Oral Epidemiol* 2007; **35**: 98-108
12 **Berkowitz RG**, Mahadevan M. Unilateral tonsillar enlargement and tonsillar lymphoma in children. *Ann Otol Rhinol Laryngol* 1999; **108**: 876-879
13 **Sellars SL**. Metastatic tumours of the tonsil. *J Laryngol Otol* 1971; **85**: 289-292
14 **Goldenberg D**, Golz A, Arie YB, Joachims HZ. Adenocarcinoma of the rectum with metastasis to the palatine tonsil. *Otolaryngol Head Neck Surg* 1999; **121**: 653-654
15 **Vauléon E**, De Lajarte-Thirouard AS, Le Prisé E, Guihaire P, Raoul JL. Tonsillar metastasis revealing signet-ring cell carcinoma of the rectum. *Gastroenterol Clin Biol* 2005; **29**: 70-72
16 **Wang WS**, Chiou TJ, Pan CC, Chen WY, Chen PM. Signet-ring cell carcinoma of the rectum with tonsillar metastasis: a case report. *Zhonghua Yixue Zazhi* (Taipei) 1996; **58**: 209-212
17 **Vasilevsky CA**, Abou-Khalil S, Rochon L, Frenkiel S, Black MJ. Carcinoma of the colon presenting as tonsillar metastasis. *J Otolaryngol* 1997; **26**: 325-326
18 **Güvenç MG**, Ada M, Acioğlu E, Pamukçu M. Tonsillar metastasis of primary signet-ring cell carcinoma of the cecum. *Auris Nasus Larynx* 2006; **33**: 85-88
19 **Low WK**, Sng I, Balakrishnan A. Palatine tonsillar metastasis from carcinoma of the colon. *J Laryngol Otol* 1994; **108**: 449-451
20 **Brownson RJ**, Jaques WE, LaMonte SE, Zollinger WK. Hypernephroma metastatic to the palatine tonsils. *Ann Otol Rhinol Laryngol* 1979; **88**: 235-240
21 **Charbonneau N**, Gélinas M, del Vecchio P, Guertin L, Larochelle D, Tabet JC, Soulières D, Charpentier D, Nguyen-Tân PF. Primary radiotherapy for tonsillar carcinoma: a good alternative to a surgical approach. *J Otolaryngol* 2006; **35**: 227-234
22 **Tsubochi H**, Isogami K, Sato N, Imai T. Successfully treated lingual tonsillar metastasis from bronchial adenocarcinoma. *Jpn J Thorac Cardiovasc Surg* 2005; **53**: 455-457
23 **Suh N**, Yang XJ, Tretiakova MS, Humphrey PA, Wang HL. Value of CDX2, villin, and alpha-methylacyl coenzyme A racemase immunostains in the distinction between primary adenocarcinoma of the bladder and secondary colorectal adenocarcinoma. *Mod Pathol* 2005; **18**: 1217-1222

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Mesalamine hypersensitivity and Kounis syndrome in a pediatric ulcerative colitis patient

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Abstract

5-aminosalicylic acid (mesalamine) rarely induces hypersensitivity reactions. If chest pain associated with atypical electrocardiographic changes are seen during its administration, one should always bear in mind type I variant of Kounis syndrome. This variant includes patients, of any age, with normal coronary arteries, without predisposing factors for coronary artery disease, in whom the acute release of inflammatory mediators from mast cells can induce either sudden coronary artery narrowing, without increase of cardiac enzymes and troponins, or coronary artery spasm that progresses to acute myocardial infarction, with elevated cardiac enzymes and troponins.

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Key words: Drug hypersensitivity; Kounis syndrome; Mesalamine; Salicylates

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TO THE EDITOR

We have read with interest the article published recently in this journal[1] concerning a 12-year-old boy who developed severe chest pain after oral administration of 5-aminosalicylic acid (5-ASA, mesalamine). Electrocardiographic changes during the pain were not specific but inverted T-waves were present in the lateral chest leads. Additionally, cardiac enzymes, troponins, C-reactive protein and brain natriuretic peptide were normal. The authors concluded that this patient had suffered a rare hypersensitivity reaction to mesalamine.

It is known that mesalamine can, rarely, induce hypersensitivity reactions such as hypersensitivity myocarditis, hypersensitivity pneumonitis, angioedema, pericarditis, erythroderma, toxic epidermal necrolysis, palmar-plantar erythrodysesthesia, skin rashes and hypereosinophilia.

This case seems to be a characteristic case of type I variant of Kounis syndrome[2,3], the youngest reported so far. Another case of Kounis syndrome secondary to amoxicillin/clavulanic acid use has been recently reported in a 13-year-old child[4]. On the other hand, salicylate products such as aspirin are known to induce hypersensitivity reactions, and aspirin has been reported to induce Kounis syndrome[5].

Pathophysiologically, symptoms of salicylate hypersensitivity can be explained by over-production of leukotriene metabolites, since salicylate allergic patients who have come in contact with salicylates show a marked inhibition of cyclooxygenase (COX), which is continuously expressed in the human body. This leads to a diminished production of COX products such as prostacyclin and thromboxane, and can accelerate the metabolism of arachidonic acid towards lipoxygenase products such as leukotrienes. It is known that leukotrienes are powerful coronary arterial vasoconstrictors, and their biosynthesis is enhanced in the acute phase of unstable angina[2]. Atay *et al*[1] have correctly stated that arachidonic acid metabolites generated through both COX and lipoxygenase pathways are thought to be increased in patients with ulcerative colitis. Ideally, in this case, allergic screening with measurement of mast cell mediators such as histamine and arachidonic acid metabolites should have been carried out. However, this study shows that Kounis syndrome is not a very rare disease but a 'very rarely diagnosed' disease.

REFERENCES

1 **Atay O**, Radhakrishnan K, Arruda J, Wyllie R. Severe

chest pain in a pediatric ulcerative colitis patient after 5-aminosalicylic acid therapy. *World J Gastroenterol* 2008; **14**: 4400-4402

2 **Kounis NG**. Kounis syndrome (allergic angina and allergic myocardial infarction): a natural paradigm? *Int J Cardiol* 2006; **110**: 7-14

3 **Nikolaidis LA**, Kounis NG, Gradman AH. Allergic angina and allergic myocardial infarction: a new twist on an old syndrome. *Can J Cardiol* 2002; **18**: 508-511

4 **Biteker M**, Duran NE, Biteker FS, Erturk E, Aykan AC, Civan HA, Ozkan M. Kounis Syndrome secondary to amoxicillin/clavulanic acid use in a child. *Int J Cardiol* 2009; 136: e3-e5

5 **Kounis NG**, Kouni SN, Koutsojannis CM. Myocardial infarction after aspirin treatment, and Kounis syndrome. *J R Soc Med* 2005; **98**: 296

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Acellular extracellular matrix anal fistula plug: Results in high fistula-in-ano awaited

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Abstract

Song *et al* have reported a 100% success rate of acellular extracellular matrix (AEM) anal fistula plug in low fistula-in-ano. The results with this product in high fistula-in-ano are keenly awaited.

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Key words: Acellular extracellular matrix; Anorectal fistula; Rectal fistula; Recurrence

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To THE EDITOR

I read with great interest the work published by Song *et al*[1]. First of all, I congratulate the authors on achieving a 100% success rate with acellular extracellular matrix (AEM) anal fistula plug (AFP). This is an encouraging step to treat the notorious disease. However, there are few points that remain unanswered. First, the authors did not mention the source (origin) of the product and the company manufacturing the AEM. Second, how this product is different from the AFP (Surgisis, Cook Surgical Inc., Bloomington, Indiana, USA) is a matter of great interest. This assumes importance because various studies using Surgisis AFP have reported a success rate of 24%-87%[2,3]. Our study with Surgisis AFP in 21 patients with fistula-in-ano yielded a success rate of 71.4%. However, all of our patients had high fistulae[4]. Third, why did the authors specifically choose low fistulae in the study for which there are other effective treatment modalities available. Why high fistulae were not included in the study has not been explained in the paper. Fourth, the authors have pulled the plug from the secondary opening towards the primary opening. This is in contrast to the published studies with Surgisis AFP in which most of the authors pulled the plug from the primary opening to the secondary opening. Was there any specific reason for this variation or was it a random variation? Fifth, the authors did not explain as how did they use AEM material, like they rolled it and made a plug or they cut it into stripes and inserted those stripes into the fistula tract. The results reported are quite encouraging. However, the follow-up period of one month is too short to conclude anything convincingly. Further prospective studies with AEM plug in high fistulae would be required to substantiate these findings.

REFERENCES

1 **Song WL**, Wang ZJ, Zheng Y, Yang XQ, Peng YP. An anorectal fistula treatment with acellular extracellular matrix: a new technique. *World J Gastroenterol* 2008; **14**: 4791-4794
2 **Johnson EK**, Gaw JU, Armstrong DN. Efficacy of anal fistula plug vs. fibrin glue in closure of anorectal fistulas. *Dis Colon Rectum* 2006; **49**: 371-376
3 **Lawes DA**, Efron JE, Abbas M, Heppell J, Young-Fadok TM. Early experience with the bioabsorbable anal fistula plug. *World J Surg* 2008; **32**: 1157-1159
4 **Garg P**. To determine the efficacy of anal fistula plug in the treatment of high fistula-in-ano- an initial experience. *Colorectal Dis* 2009; **11:** 588-591

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
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June 11-13, Stockholm, Sweden
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June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
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June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
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July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
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September 13-16, New Delhi, India
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E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
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Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
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E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
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www.negf.org
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59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

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November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

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March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
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May 17-20, Denver, Colorado, USA
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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Books

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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

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[1]Passed away on October 20, 2007
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Contents

Contents

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EDITORIAL

Role of chemokines and their receptors in viral persistence and liver damage during chronic hepatitis C virus infection

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Abstract

Chemokines produced in the liver during hepatitis C virus (HCV) infection induce migration of activated T cells from the periphery to infected parenchyma. The milieu of chemokines secreted by infected hepatocytes is predominantly associated with the T-helper/T-cytotoxic type-1 cell (Th1/Tc1) response. These chemokines consist of CCL3 (macrophage inflammatory protein-1α; MIP-1α), CCL4 (MIP-1β), CCL5 (regulated on activation normal T cell expressed and secreted; RANTES), CXCL10 (interferon-γ–inducible protein-10; IP-10), CXCL11 (interferon-inducible T-cell α chemoattractant; I-TAC), and CXCL9 (monokine induced by interferon γ; Mig) and they recruit T cells expressing either CCR5 or CXCR3 chemokine receptors. Intrahepatic and peripheral blood levels of these chemokines are increased during chronic hepatitis C. The interaction between chemokines and their receptors is essential in recruiting HCV-specific T cells to control the infection. When the adaptive immune response fails in this task, non-specific T cells without the capacity to control the infection are also recruited to the liver, and these are ultimately responsible for the persistent hepatic damage. The modulation of chemokine receptor expression and chemokine secretion could be a viral escape mechanism to avoid specific T cell migration to the liver during the early phase of infection, and to maintain liver viability during the chronic phase, by impairing non-specific T cell migration. Some chemokines and their receptors correlate with liver damage, and CXCL10 (IP-10) and CXCR3 levels have shown a clinical utility as predictors of treatment response outcome. The regulation of chemokines and their receptors could be a future potential therapeutic target to decrease liver inflammation and to increase specific T cell migration to the infected liver.

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Key words: Chemokines; Chemokine receptors; Hepatitis C virus; Viral hepatitis pathogenesis; Persistent infection; Viral escape mechanism

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INTRODUCTION

The hepatitis C virus (HCV) is a hepatotropic non cytopathic virus very efficient in evading the host immune response. Hepatitis C infection is a major cause of chronic liver disease worldwide, affecting at least

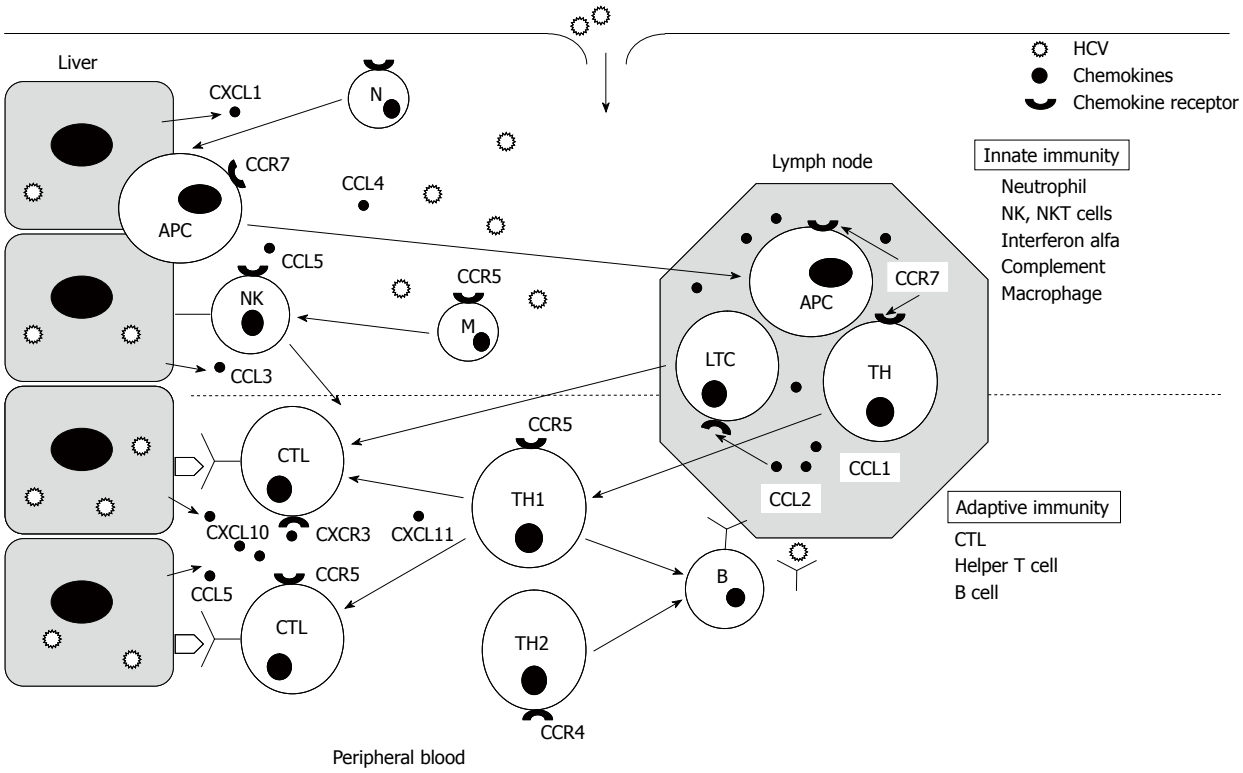

Figure 1 Innate and adaptive immune response. Importance of specific cytotoxic T cell response. In a non-cytopathic viral infection the development of a vigorous specific cytotoxic T lymphocyte (CTL) response is essential. Professional antigen presenting cells (APC) take up viral antigens and migrate from infected parenchyma to the lymph nodes to prime naive specific CTLs. These cells express the chemokine receptor CCR7 to reach the lymph nodes, attracted by CCL1 and CCL2 chemokines. After priming, specific CTLs lose CCR7 expression and up-regulate CCR5 and CXCR3 chemokine receptors, then migrate to the hepatic parenchyma to develop their effector function, attracted by the chemokines produced in the liver. During the early phase of infection, innate immunity is the first barrier for fighting against the virus. The cellular innate response is also recruited in the infected parenchyma by the interaction between chemokines and their receptors.

170 000 000 people[1]. Approximately three quarters of infected subjects develop a chronic infection, but only one third progress to cirrhosis, hepatocellular carcinoma and liver failure without treatment[2]. Nowadays around 50% of patients treated with pegylated-interferon plus ribavirin clear the virus[3,4]. To control HCV infection an adequate specific T cell response is necessary[5-7], with T cells that are able to migrate to the infected site to develop their effector functions (Figure 1). Nevertheless, specific T cells fail to remove the virus in the majority of patients[7,8], because often a non-specific T cell population is recruited to the infection site, and these cells are presumably responsible for the chronic damage[9] (Figure 2).

In both scenarios the attraction of leukocytes to the liver is controlled by chemokines, which are chemotactic cytokines secreted by infected cells and interact with their receptors expressed on the recruited leukocytes[10] (Figure 3). In an experimental model of influenza virus infection, the importance of appropriate chemotaxis to control viral infection was shown. In this model, cytotoxic specific CD8+ cells expressing inadequate chemokine receptors were not able to reach the infected site and clear the virus, while specific cytotoxic T cells expressing the correct chemokine receptors controlled the infection without tissue damage[11] (Figure 4). On the other hand, it has also been demonstrated that chemokines and their receptors can be involved in liver damage. In an animal model study it was reported that a massive hepatic infiltration by non-specific T cells, expressing chemokine receptors associated with the type-1 response, can cause acute liver failure[12] (Figure 5). Therefore, chemokines and their receptors are associated with viral control but are also associated with immune-mediated liver inflammation. Moreover, in a hepatotropic viral infection in humans, a huge intrahepatic non-specific mononuclear infiltrate during viral persistence was noticed, while this was not present in subjects with viral control[13] (Figure 2). In this last study, the intrahepatic chemoattraction of non-specific T cells perpetuated the liver damage. Consequently, also in humans, chemokines and their receptors develop an important role in viral clearance and in the development of chronic tissue inflammation. Obviously, the modulation of these pathways is important for generating an efficient immune response, and for participating in the inflammatory process during the chronic infection phase, but pathway modulation could also be a viral strategy used by HCV to escape from immune control[14]. This review introduces the advances obtained in the last decade on the role of chemokines and their receptors in chronic hepatitis C pathogenesis.

STRUCTURE AND FUNCTION OF CHEMOKINES AND THEIR RECEPTORS

Table 1 Chemokines and their receptors

	Chemokine receptor	Chemokine ligands	Target cells
Subfamily CC			
	CCR1	CCL3, CCL5, CCL7, CCL14	T cells, monocytes, basophils and eosinophils
	CCR2	CCL2, CCL8, CC7, CCL13, CCL16	Memory T cells, monocytes and dendritic cells
	CCR3	CCL11, CCL13, CCL7, CCL5, CCL8, CCL13	Eosinophils, basophils, mast cells, T helper 2 cells and platelets
	CCR4	CCL17, CCL22	T helper 2 cells, dendritic cells, basophils, macrophages and platelets
	CCR5	CCL3, CCL4, CCL5, CCL11, CCL14, CCL16	T cells, monocytes
	CCR6	CCL20	T cells, B cells and dendritic cells
	CCR7	CCL19, CCL21	T cells and dendritic cells
	CCR9	CCL25	T cells, plasma cells
	CCR10	CCL27, CCL28	T cells
Subfamily CXC			
	CXCR1	CXCL8, CXCL6	Neutrophils and monocytes
	CXCR2	CXCL8, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6	Neutrophils, monocytes and vascular endothelial cells
	CXCR3-A	CXCL9, CXCL10, CXCL11	T helper 1 cells, mast cells and mesangial cells
	CXCR3-B	CXCL4, CXCL9, CXCL10, CXCL11	Neoplastic cells and vascular endothelial cells
	CXCR4	CXCL12	Expressed in multiple cells
	CXCR5	CXCL13	B cells and T helper cells
	CXCR6	CXCL16	CD8+ cells, natural killer cells and memory CD4+ cells
Subfamily CX3C			
	CX3CR1	CX3CL1	Macrophages and smooth muscle cells
Subfamily XC			
	XCR1	XCL1, XCL2	T cells and natural killer cells

Chemokines are small heparin-binding proteins that direct the movement of mononuclear cells through the body to contribute to the development of an adaptive immune response and to the pathogenesis of inflammation. These molecules also play a role in angiogenesis, haematopoiesis, lymphoid organ development, wound healing and regulation of embryonic development. These proteins are 8-10 kDa in size with 20%-70% amino acid sequence homology and are secreted by resident cells at the inflammatory site[10]. Around 50 chemokines have been described and are subdivided into four families according to the position of the two N-terminal cysteine residues: CXC, CC, XC and CX3C[15,16,17] (Table 1). The CXC family has also been subdivided into two groups depending on the presence of the ELR motif (Glu-Leu-Arg). A systematic nomenclature has been adopted in the past few years for chemokines and their receptors[18,19]. Chemokines induce cell migration and activation by binding to specific G-protein-coupled cell-surface receptors on target cells, called chemokine receptors[10] (Table 1). Receptor triggering leads to a cascade of cellular activation, including the generation of inositol triphosphate, the release of intracellular calcium, and the activation of protein kinase C[20]. Chemokine receptor signalling also activates small guanosine triphosphate binding proteins of the Ras and Rho families[21]. Rho proteins are involved in cell motility through regulation of actin-dependent processes such as membrane ruffling, pseudopod formation, and assembly of focal adhesion complexes[15]. All these mechanisms propel cells in the appropriate direction. In humans different chemokine receptors subdivided into four families have been described: XC, CXC, CC and CX3C chemokine receptors. These receptors are expressed on different types of leukocytes, and some are constitutively expressed while others are induced, depending on the degree of leukocyte activation and differentiation[15]. Polarisation of chemokine receptor expression on T cells depending on the cytokine production profile has been demonstrated[22]. Chemokine receptors, such as CCR5 and CXCR3, are associated with the type-1 response, while CCR3, CCR4 and CCR8 are associated with the type-2 response[23,24,25,26]. Due to the preferential Th1/Tc1 response of liver infiltrating T cells during chronic hepatitis C[27,28], this review focuses on these two chemokine receptors, which bind chemokines from the non-ELR-CXC and CC subfamilies. The ligands for CXCR3 are interferon (IFN)-γ-inducible protein 10 (IP-10, CXCL10), monokine induced by IFN-γ (Mig, CXCL9), and IFN-inducible T-cell α chemoattractant (I-TAC, CXCL11). CXCR3 is expressed on activated T cells and natural killer cells[29]. The CCR5 ligands comprise regulated upon activation, normal T-cell expressed and secreted (RANTES, CCL5), macrophage inflammatory proteins 1α (MIP 1α, CCL3) and 1β (MIP 1β, CCL4). CCR5 is expressed predominantly on activated and memory T cells. Hereafter the systematic nomenclature for chemokines will be used.

CHEMOKINE SECRETION AND CHEMOKINE RECEPTOR EXPRESSION IN CHRONIC HEPATITIS C

The migration of lymphocytes to the liver is a complex process involving adhesion, rolling, triggering, and transendothelial migration. Chemokines and their

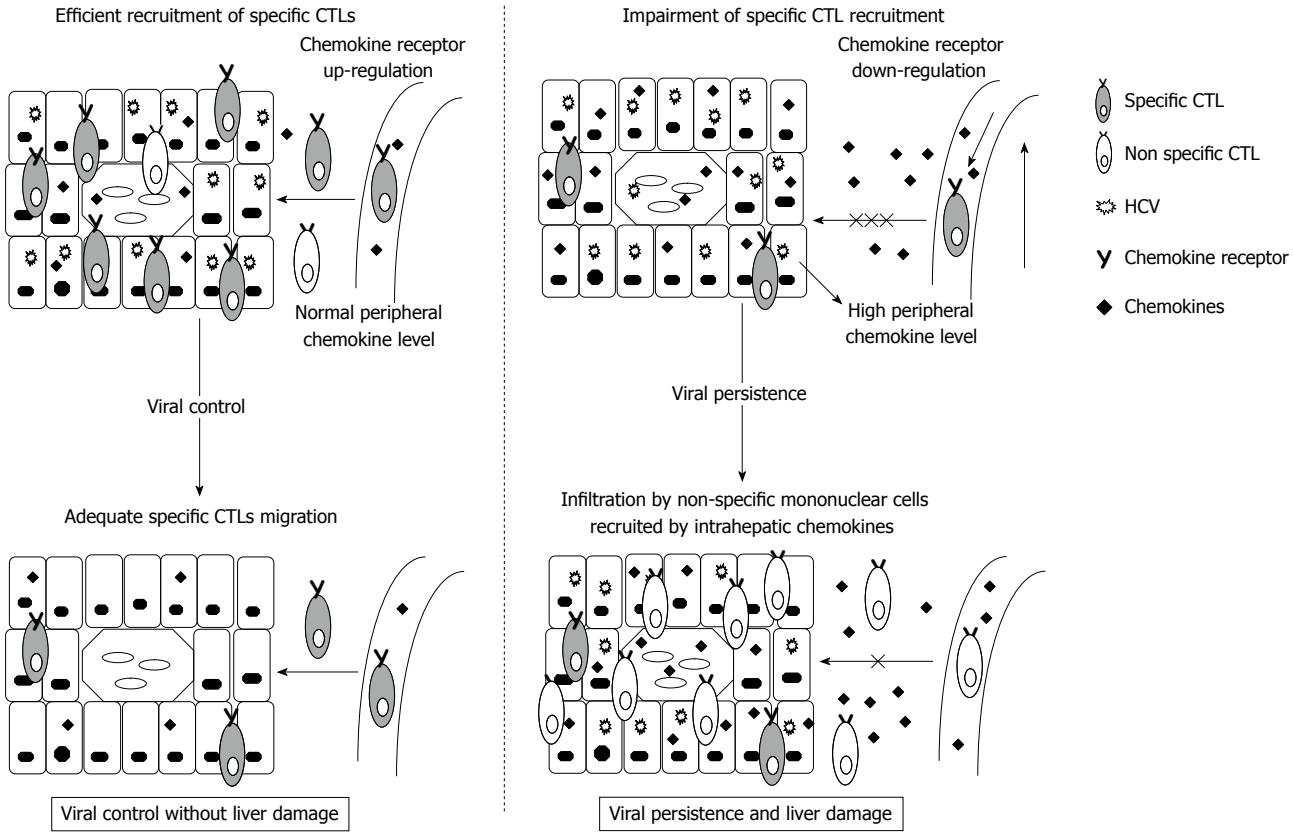

Figure 2 Different migration potential between resolved and persistent HCV infection. In a resolved infection HCV-specific cytotoxic T lymphocytes (CTL) migrate rapidly to the infected liver, attracted by the chemokines produced by hepatocytes, succeeding in controlling HCV infection without liver damage. In persistent infection the migration of T cells to the liver may be impaired. High chemokine levels during infection may induce chemokine receptor down-regulation on T cells by molecule internalization. This mechanism could impair the migration of specific T cells during primary infection, which could inhibit HCV clearance. During the chronic phase, the persistent presence of HCV in the liver could keep a long-standing chemokine production, which could attract non-specific mononuclear cells to the liver, and be responsible for chronic liver damage. The relative migration impairment due to chemokine receptor down-regulation on non-specific T cells could modulate inflammatory infiltration of the liver, inducing chronic low-grade liver damage, which could favour host and virus long-term survival.

receptors play an essential role in this multistep pathway[30,31]. In chronic hepatitis C, the expression of different chemokines in the liver has been described. CXCL10 is increased in the liver and peripheral blood during chronic hepatitis C[32,33,34,35]. This molecule is produced by hepatocytes and sinusoidal endothelial cells[33,34]. CXCL9 and CXCL11 are also increased in the serum and liver of subjects with chronic hepatitis C[33,36]. CXCL9 is detected primarily on sinusoidal endothelial cells, while CXCL11 is produced mainly by hepatocytes[33,37]. The intrahepatic expression of CCL5 is also elevated in chronic hepatitis C and it is produced by hepatocytes, sinusoidal endothelial cells and biliary epithelium[37]. Finally, several studies have reported an increased level of CCL3 and CCL4 either in the liver or in serum. These molecules are detected on endothelial cells, on some hepatocytes and on biliary epithelial cells[33,35,38]. The expression of all these chemokines in the liver can be induced directly by HCV. Previous reports have shown a high hepatocyte synthesis of CXCL10, CXCL9 and CCL5, induced by some HCV proteins such as NS5A and core[39], although a recent *in vitro* study suggests that HCV proteins could also decrease the expression of CCL5 and CXCL10 genes[40]. All these chemokines recruit T cells with a Th1/Tc1 phenotype expressing specific chemokine receptors such as CCR5 and CXCR3[24]. The non-ELR-CXC chemokine attracts CXCR3 expressing T cells while CC chemokine attracts CCR5 expressing T cells to the liver. Consequently, in chronic hepatitis C, an intrahepatic enrichment of CCR5 and CXCR3 expressing T cells, located in the hepatic lobule and portal tracts, has been shown while these populations are very infrequent in uninfected subjects[33,35,37] (Figure 6).

CORRELATION BETWEEN LIVER INFLAMMATION AND CHEMOKINE/ CHEMOKINE RECEPTOR

Persistent HCV infection is characterised by a non-specific inflammatory infiltrate in the liver, mainly composed of CD8+ cells[41,42], and responsible for liver damage[9]. These cells are attracted by the interaction between the intrahepatic secreted chemokines and the chemokine receptors expressed on T cells. Previous reports have shown a correlation between liver inflammation and liver infiltrating CXCR3/CCR5 expressing T cells[35,37] (Figure 7). The frequency of occurrence of these cells was positively correlated with portal and lobular inflammation but not with liver fibrosis. These data suggest that CCR5 and CXCR3

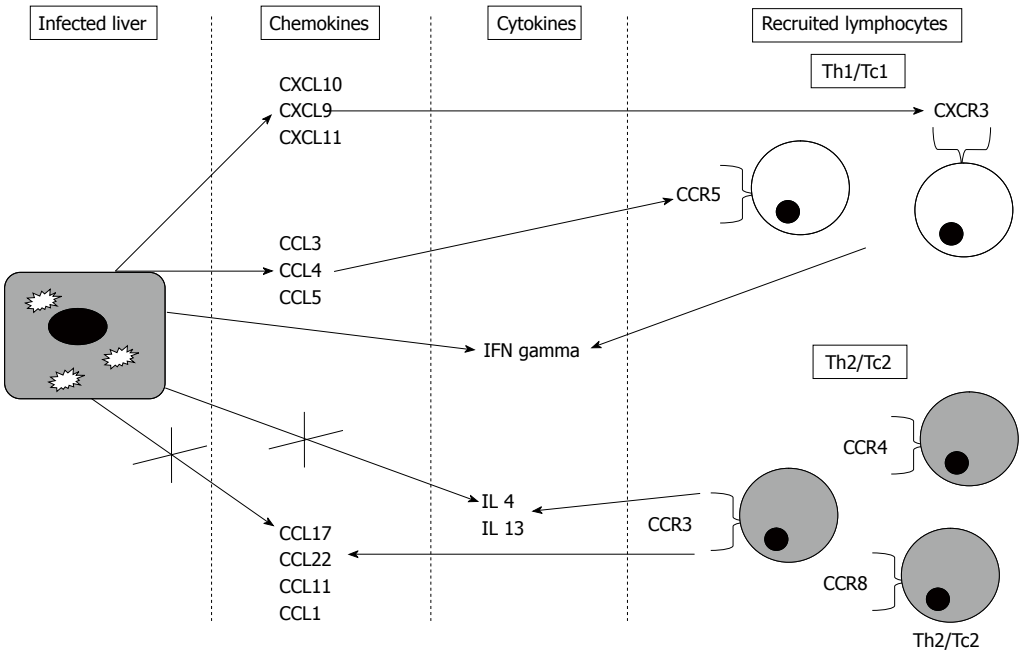

Figure 3 Chemokines and chemokine receptors related to chronic hepatitis C pathogenesis. In chronic hepatitis C, intrahepatic chemokines recruit Th1/Tc1 cells expressing CCR5/CXCR3 chemokine receptors. The X symbol indicates that chemokines and cytokines associated with a Th2/Tc2 response are not primarily produced in the HCV infected liver.

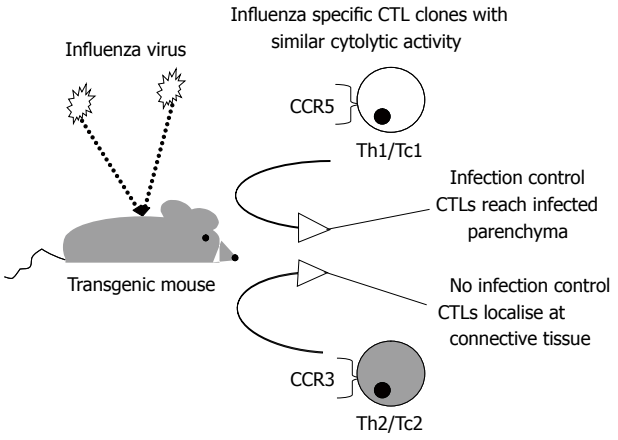

Figure 4 Adequate chemotaxis is necessary to control a viral infection. Specific cytotoxic T cells with active *in vitro* effector functionality are not able to control a viral infection if they do not express the appropriate chemokine receptor to reach the infected parenchyma. In a murine model of influenza virus infection, specific CCR5-expressing T cells were able to clear the virus while administration of specific CCR3-expressing T cells induced mouse death. CTLs: cytotoxic T lymphocytes.

could play an important role in chronic liver damage by means of recruitment of inflammatory T cells into the liver. Several previous studies have also shown a correlation between liver inflammation and chemokine levels. Intrahepatic CXCL10 mRNA levels are associated with intralobular inflammation[43]. Similarly, CXCL9 and CXCL11 correlate with the grade of liver inflammation[37,44]. Furthermore, CC chemokines are also correlated with intrahepatic inflammatory activity[45]. Clearly, intrahepatic CCL5-positive cells correlate with inflammatory activity but not with liver fibrosis[37]. The absence of correlation between liver fibrosis and liver infiltrating CXCR3/CCR5 expressing T cells and their ligands could be due to a statistical beta error, but also could suggest that it is necessary for something other than liver inflammation to occur in order to develop fibrosis, such as genetic and non-genetic factors[46]. Nevertheless, a recent study, examining a large sample of chronic hepatitis C patients, showed a positive correlation between CXCL9, CXCL10 and CXCL11 intrahepatic levels and the grade of fibrosis[47], indicating that persistent liver inflammation produced by the mononuclear cells attracted by these chemokines could finally induce the activation of a liver fibrosis cascade. Bearing in mind all the previous data, it is possible to speculate that chemokines are secreted in the infected liver to attract an adaptive immune response able to clear the virus. Unfortunately, when the specific response fails these chemokines also attract non-specific T cells, which are not able to remove the virus but produce liver inflammation (Figure 2). Therefore, as chemokines are nonspecific chemoattractants, the intrahepatic inflammatory infiltrate produced during chronic infection is mainly non-HCV-specific and consequently unable to eliminate HCV. It is, however, able to produce cytokines capable of initiating and perpetuating hepatic fibrogenesis[48]. The efficiency of this mechanism could play a role in determining why chronically infected individuals either do or do not progress to fibrosis. Certain polymorphisms in key chemokines known to be up-regulated in chronic HCV, such as CCL5, have been identified as correlates with the development of fibrosis[49].

MODULATION OF CHEMOKINE/CHEMOKINE RECEPTOR PATHWAY AS A VIRAL ESCAPE MECHANISM

HCV is usually able to evade the immune system efficiently. Several HCV escape mechanisms have been previously described, such as selection of escape mutations, induction of specific T cell anergy or resistance to the effects of α-interferon[5,50]. One

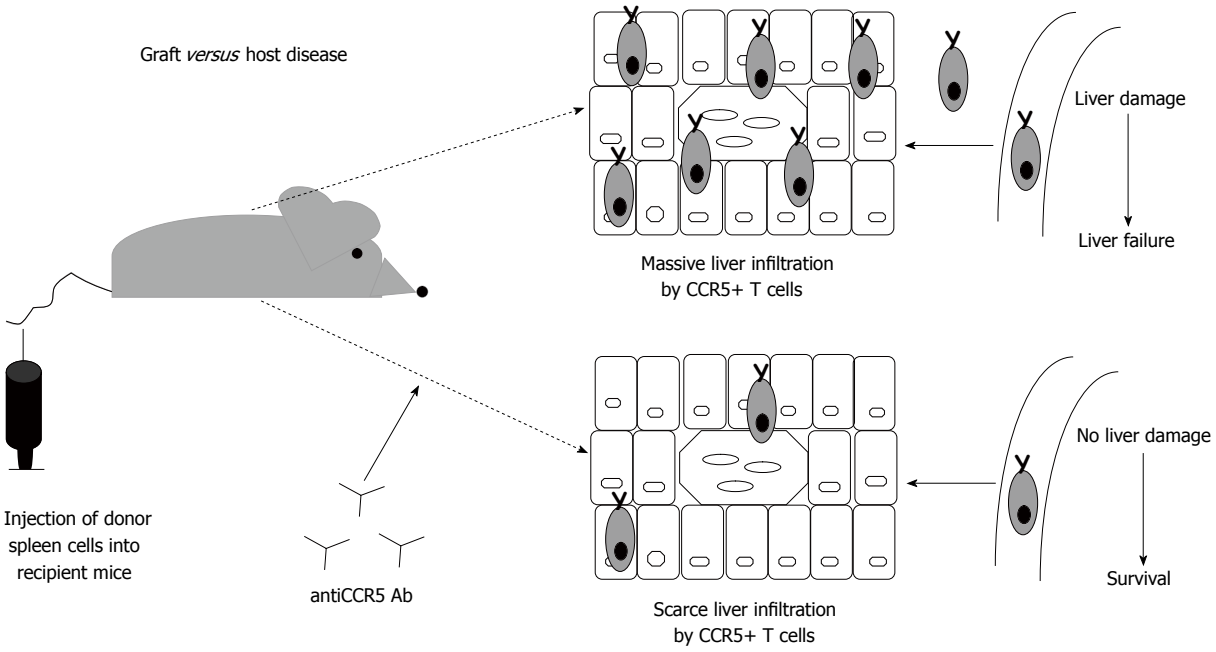

Figure 5 Intrahepatic chemoattraction of non-specific T cells causes liver damage. In a murine model of graft *versus* host disease the liver is infiltrated by CCR5-expressing T cells causing liver failure. This process can be blocked by using anti-CCR5 monoclonal antibodies.

hypothetical mechanism for HCV to survive could be to reduce hepatic chemotaxis of T lymphocytes to impair infection control during primary infection and also to decrease tissue damage during the chronic phase. The natural history of chronic hepatitis C means it can take up to three decades to develop liver cirrhosis[51]. This in turn means that the immunologically-mediated liver damage must be continuous but very light. For HCV it is essential to extend host survival as much as possible to assure its own viability. Considering these facts, it could be important for HCV to impair the expression of chemokine receptors associated with the type-1 response to improve its survival ability.

One mechanism to achieve this could be to reduce T cell migration into the liver through impairment of CCR5 and CXCR3 expression. To maximise the ability of the immune system to control the infection, a high frequency of HCV-specific CCR5+/CXCR3+ T cells should be expected during primary infection. Soon after HCV infection, prominent CD8+ cell responses are repeatedly observed and transient up-regulation of CCR5 and CXCR3 expression is seen[52,53]. During chronic hepatitis C, however, T cells show reduced surface expression of chemokine receptors associated with the Th1/Tc1 response while an intracellular increase of these molecules has been described[54]. This finding suggests the occurrence of HCV-mediated chemokine receptor internalization in chronically infected subjects. Moreover, a high serum concentration of CCL3 and CXCL10, associated with a normal or reduced peripheral frequency of CCR5- and CXCR3-positive T cells during chronic hepatitis C, has been also described[35,54]. During chronic HCV infection, the observed absence of an increase in the number of peripheral CCR5/CXCR3-positive T cells could be due to either an intrahepatic sequestration of CCR5/CXCR3 expressing T cells, caused by CCL3/CXCL10 attraction, or to a down-regulation of these chemokine receptors produced by the high serum concentration of their ligands.

It has been shown that GB virus C, a close relative of HCV[55], can reduce CCR5 expression on T cells by inducing CCL5 release[56,57]. This mechanism impairs the ability of HIV to infect CD4 T cells, thus extending host survival[57,58]. Another study into HCV infection described CCR5 down-regulation on CD8+ cells by receptor internalization[54]. It has been shown that the HCV-E2 protein, after binding to CD81[59], induces CCL5 secretion by CD8+ cells and the ensuing interaction between CCL5 and CCR5 is responsible for CCR5 down-regulation on these cells[60]. All these data suggest that HCV could modulate chemokine receptors associated with the Tc1 response to achieve a survival advantage. This mechanism could decrease HCV-specific T cell migration during acute infection, avoiding viral control, and could also impair non-specific T cell migration during persistent infection, modulating liver inflammation and fibrosis which could extend host and viral survival. If HCV is able to interfere with CCR5/CXCR3 expression, an increase in T cells expressing these chemokine receptors after viral load drop due to anti-viral treatment should be expected, together with a CXCL10/CCL3 decrease. To address this significant issue, a longitudinal analysis of CCR5/CXCR3 expressing CD8+ cells and CXCL10/CCL3 levels during treatment was performed[35]. In the majority of treated patients in this study, an increase in CCR5/CXCR3 expressing CD8+ cells was demonstrated. This finding was associated with a significant decrease in CXCL10 and CCL3 serum levels after 24 wk of treatment. A likely explanation for these data is that HCV control

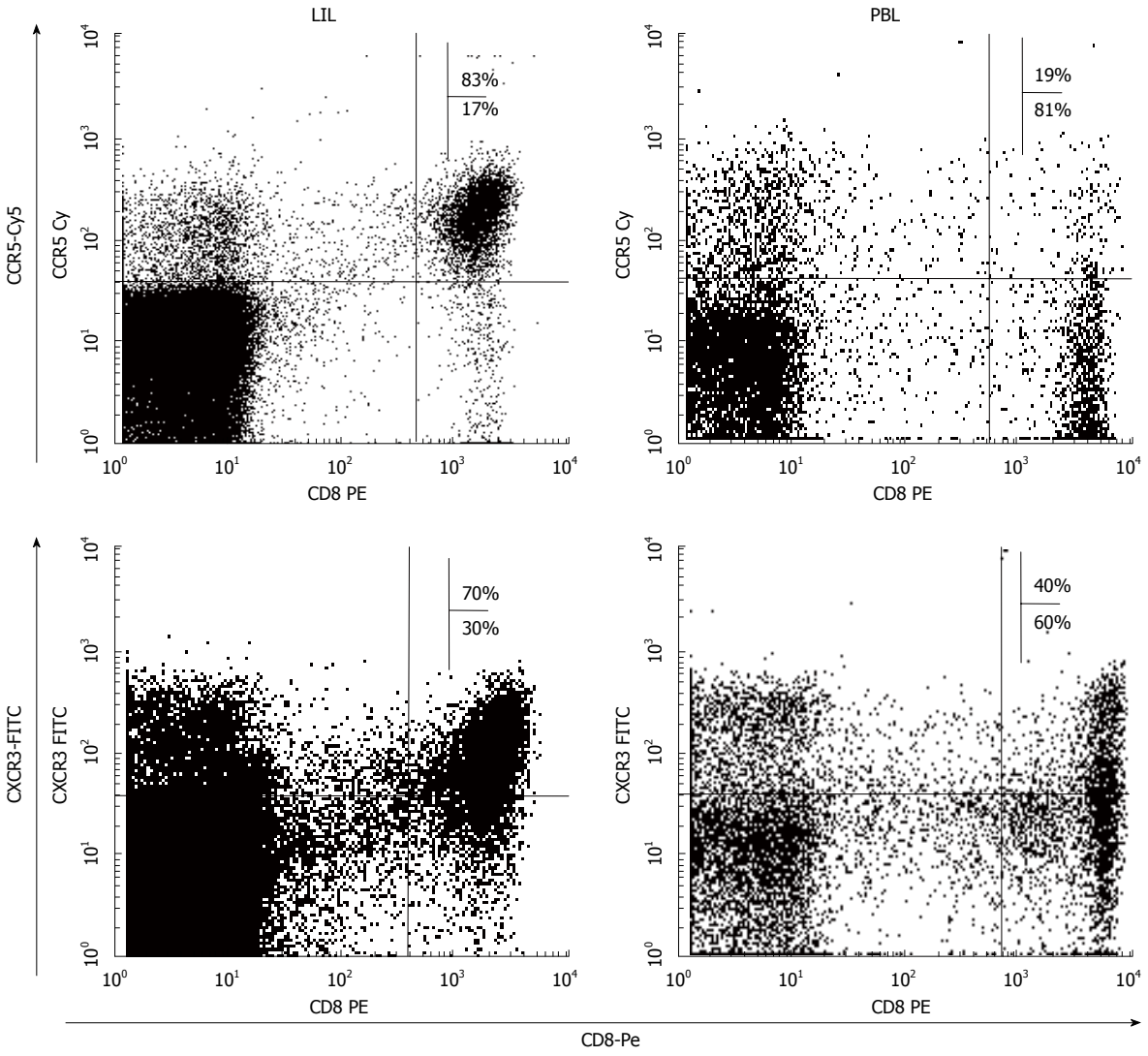

Figure 6 Intrahepatic enrichment of CCR5 and CXCR3 expressing T cells. FACScan® dot-plots of liver infiltrating (LIL), and peripheral blood (PBL) lymphocytes from a patient with chronic hepatitis C stained with labelled antibodies against CD8, CCR5 and CXCR3. The intrahepatic frequency of CCR5/CXCR3-expressing CD8+ cells is higher than in the peripheral blood.

during treatment could decrease CXCL10/CCL3 release, allowing CCR5/CXCR3 up-regulation on peripheral CD8+ cells. Actually, in this study a significant positive correlation between HCV viral load and CCL3 and an almost significant correlation with CXCL10 was shown. In summary, all these data taken together suggest that HCV could down-regulate CCR5 and CXCR3 expression on T cells by means of the secretion induction of their chemokine agonists. This strategy could favour HCV escape from immune control and could also decrease long-standing liver inflammation, allowing extended host and virus survival.

CHEMOKINE/CHEMOKINE RECEPTOR LEVELS AS TREATMENT RESPONSE OUTCOME PROGNOSTIC TOOL

As previously commented, Tc1/Th1 associated chemokines and their ligands can be modulated by HCV infection to impair the immune response. Therefore, the levels of these molecules may also influence treatment-mediated viral clearance. Previous studies have shown how baseline CXCL10 serum concentration is associated with the outcome of antiviral therapy in monoinfected[61-64] patients and in patients co-infected with HIV[65]. Elevated pre-treatment CXCL10 levels correlate with non-response to current therapy. Moreover, it was shown that the increase in CXCR3 expressing CD8+ cells during treatment is associated with SVR[35]. This suggests that for HCV, it is important to modulate the expression of this receptor not only to maintain liver viability but also to escape from immunological control. In addition, in this last study, a faster reduction in CXCL10 serum concentration was suggested in responders than in non-responders during the first 12 wk of treatment. Therefore, high CXCL10 may decrease the response of CXCR3 expressing T cells and have a negative influence on treatment outcome. Patients with either low base-line or rapid decrease of CXCL10 levels could promptly restore the response of CXCR3 expressing T cells which could assist in viral control. On the other hand, the absence of an increase in CXCR3

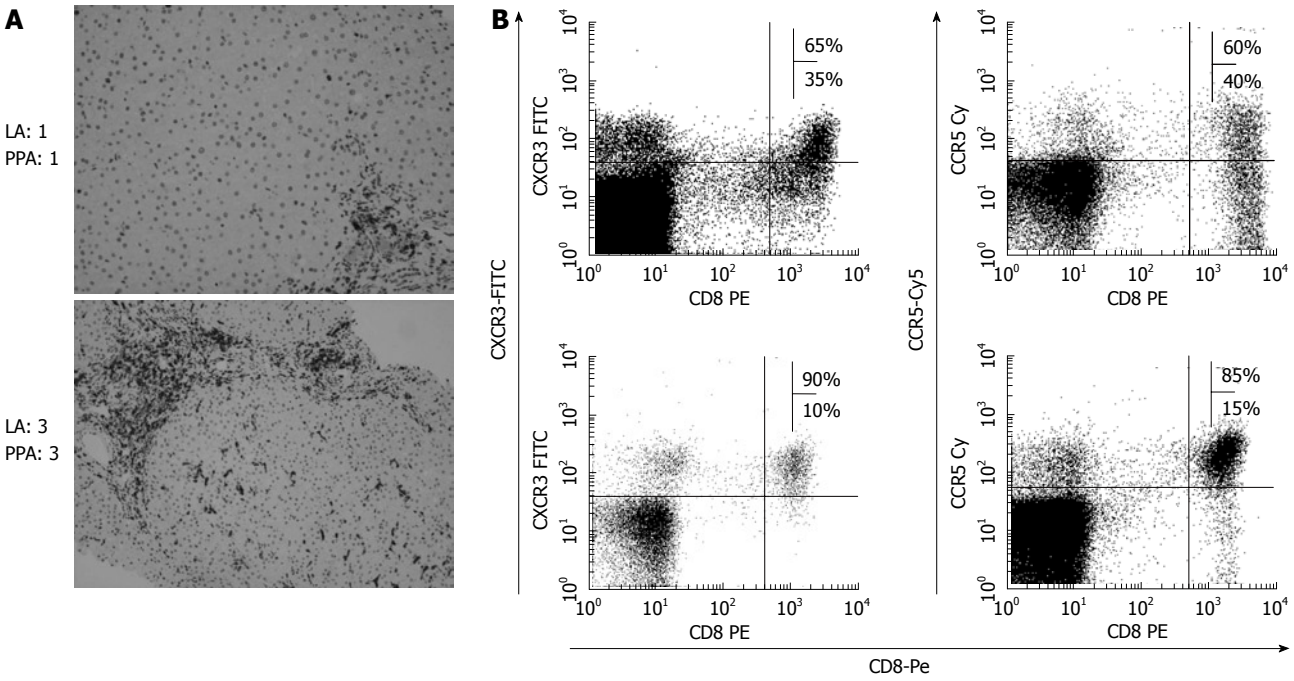

Figure 7 Correlation between liver inflammation and frequency of intrahepatic CCR5 and CXCR3 expressing T cells in chronic hepatitis C. A: Representative photomicrographs of liver immunostaining for CD8 from two chronic hepatitis C patients with different grades of inflammation. CD8 cells are stained in dark by the immunoperoxidase technique. Original magnification 400X. A patient with lobular activity (LA) 1 and porto-periportal activity (PPA) 1 showed little staining while a patient with LA 3 and PPA 3 presented intense CD8 staining; B: FACScan® dot-plots of intrahepatic lymphocytes from these two patients after staining with CD8-Pe and with either CCR5-Cy5 or CXCR3-FITC mAbs. In the upper right quadrant are represented the double positive cells. The frequency of CCR5/CXCR3 expressing CD8+ cells is higher in the patient with the higher histological liver inflammation.

expressing CD8+ cells after 24 wk of treatment is associated with a 100% negative predictive value of SVR[35]. This information may be clinically important in predicting non-response and allowing the termination of treatment in those patients with no increase in the frequency of CXCR3 expressing CD8+ cells after 24 wk of treatment. Nevertheless, with these data it is impossible to completely rule out the possibility that the observed change in chemokine patterns during treatment was an epiphenomenon due to a direct interferon effect[66]. This aspect could be clarified by studies based on patients treated with protease inhibitors. Finally, CCL20 and CXCL9 have also been suggested as tools to predict treatment outcome[61,67]. All these studies defined to predict treatment response provide preliminary information only since they are based on a very small number of patients. Therefore, these data should be re-confirmed by larger multivariate studies before applying these prognostic variables to daily clinical work.

ROLE OF CHEMOKINE/CHEMOKINE RECEPTOR POLYMORPHISMS

Several chemokine and chemokine receptor polymorphisms have been associated with different HCV infection outcomes. A CXCL11 polymorphism, defined by a 5-bp deletion in the CXCL11 promoter, has been associated with an increased risk of chronic HCV infection[68]. The distribution frequency of the allele was found to be significantly increased in a chronically HCV infected population compared to healthy controls. This deletion variant significantly reduced the transcriptional activity of the CXCL11 promoter in vitro in the presence of replicating HCV, which impaired T cell migration in vivo. Moreover, a CCR5 frameshift mutation, called CCR5-Δ32 that abrogates CCR5 expression also favoured HCV infection[69]. In this study an association between this homozygous mutation and an increased prevalence of HCV infection, as well as an increase in viral load, was documented. On the other hand, this mutation has also been associated with reduced liver inflammation[49,70,71]. These data suggest that the specific and non-specific T cell migration impairment due to the absence of CCR5 expression could favour HCV persistence and could also decrease liver damage. Similarly, a CCL5 deletion mutation which produces higher expression of CCL5 is associated with a lower grade of liver inflammation and fibrosis[70,71]. In this case the CCL5 over-expression may lead to CCR5 internalization and subsequent impairment of T cell migration. These mutations, which reduce chemokine receptor expression, are interesting proofs of the role of these molecules in the development of intrahepatic inflammation during chronic hepatitis C.

CHEMOKINE RECEPTORS AS A POTENTIAL THERAPEUTIC TARGET

Human monoclonal antibodies against CCR5, CXCR3 and their ligands have been used to treat different inflammatory and infectious diseases in humans and in animal models. CCR5 monoclonal antibodies have

been shown to be effective in avoiding T cell infection by CCR5-tropic HIV-1[72,73,74], and also in decreasing tissue inflammation in some animal models[75]. CXCL10 blocking is a successful treatment for experimental colitis[76,77]. Anti-CXCR3 monoclonal antibodies display an anti-inflammatory effect in an animal model of arthritis[78] and could also be a therapeutic target in inflammatory bowel disease[79]. These molecules have not been yet tested in chronic hepatitis C, due to the absence of an adequate animal model for proving their efficiency and safety. In the near future, an HCV permissive mouse model reconstituted with a human immune system will allow us to study mechanisms of chemokine/chemokine receptor immunopathogenesis[80]. Nevertheless, in a murine model of liver failure the administration of anti-CCR5 monoclonal antibodies has been shown to suppress intrahepatic liver inflammation, allowing mouse survival[12] (Figure 5). At least theoretically, all these previous data suggest that antibodies able to block the interaction between CCR5/CXCR3 and their ligands could decrease chronic liver inflammation in HCV infected subjects unable to clear the virus. Clearly, if the migration of non-specific T cells to the infected liver is impaired, liver damage will be reduced since HCV is not directly cytopathic. This strategy could be explored in patients without a sustained virologic response after current standard treatment. These future drugs could reduce liver fibrosis progression until new effective anti-HCV treatments are available. Obviously, the lessons learnt from HIV about the safety of these drugs should be the milestone for starting clinical trials in non-responder HCV patients[81].

In summary, the current knowledge about the role of chemokines and their receptors during chronic hepatitis C strongly suggests that they are implicated in persistent liver inflammation. Moreover, HCV seems able to modulate the expression of some chemokine receptors through the induction of their ligands. This strategy could be used by HCV as a survival mechanism. First of all, it could impair specific T cell migration to the infected liver during the primary infection, weakening HCV clearing. Later, the mechanism could interfere in non-specific T cell migration to the liver during the chronic phase of infection to extend host survival. From a practical point of view, some chemokines and their receptors have been shown to be prognostic tools in predicting anti-HCV treatment responses, and after new larger studies to re-confirm these data, these predictors could be added to daily clinical practice. Finally, the blocking of chemokines and chemokine receptor engagement is a therapeutic strategy that should be explored in the near future for non-responders to current anti-HCV therapy.

REFERENCES

1 **Lauer GM**, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41-52

2 **Afdhal NH**. The natural history of hepatitis C. *Semin Liver Dis* 2004; **24** Suppl 2: 3-8

3 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

4 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL Jr, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982

5 **Guidotti LG**, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006; **1**: 23-61

6 **Lechner F**, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klenerman P, Walker BD. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; **191**: 1499-1512

7 **Thimme R**, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; **194**: 1395-1406

8 **Thimme R**, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, Govindarajan S, Purcell RH, Chisari FV. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* 2002; **99**: 15661-15668

9 **Bertoletti A**, Maini MK. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? *Curr Opin Immunol* 2000; **12**: 403-408

10 **Charo IF**, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 2006; **354**: 610-621

11 **Cerwenka A**, Morgan TM, Harmsen AG, Dutton RW. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. *J Exp Med* 1999; **189**: 423-434

12 **Murai M**, Yoneyama H, Harada A, Yi Z, Vestergaard C, Guo B, Suzuki K, Asakura H, Matsushima K. Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. *J Clin Invest* 1999; **104**: 49-57

13 **Maini MK**, Boni C, Lee CK, Larrubia JR, Reignat S, Ogg GS, King AS, Herberg J, Gilson R, Alisa A, Williams R, Vergani D, Naoumov NV, Ferrari C, Bertoletti A. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. *J Exp Med* 2000; **191**: 1269-1280

14 **Price DA**, Klenerman P, Booth BL, Phillips RE, Sewell AK. Cytotoxic T lymphocytes, chemokines and antiviral immunity. *Immunol Today* 1999; **20**: 212-216

15 **Luster AD**. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; **338**: 436-445

16 **Rot A**, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol* 2004; **22**: 891-928

17 **Gerard C**, Rollins BJ. Chemokines and disease. *Nat Immunol* 2001; **2**: 108-115

18 **Bacon K**, Baggiolini M, Broxmeyer H, Horuk R, Lindley I, Mantovani A, Maysushima K, Murphy P, Nomiyama H, Oppenheim J, Rot A, Schall T, Tsang M, Thorpe R, Van Damme J, Wadhwa M, Yoshie O, Zlotnik A, Zoon K. Chemokine/chemokine receptor nomenclature. *J Interferon Cytokine Res* 2002; **22**: 1067-1068

19 **Murphy PM**, Baggiolini M, Charo IF, Hébert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 2000; **52**: 145-176

20 **Lodi PJ**, Garrett DS, Kuszewski J, Tsang ML, Weatherbee JA, Leonard WJ, Gronenborn AM, Clore GM. High-resolution solution structure of the beta chemokine hMIP-1 beta by multidimensional NMR. *Science* 1994; **263**: 1762-1767

21 **Laudanna C**, Campbell JJ, Butcher EC. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science* 1996; **271**: 981-983

22 **Bonecchi R**, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998; **187**: 129-134

23 **Qin S**, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* 1998; **101**: 746-754

24 **Mantovani A**. The chemokine system: redundancy for robust outputs. *Immunol Today* 1999; **20**: 254-257

25 **Nansen A**, Christensen JP, Andreasen SØ, Bartholdy C, Christensen JE, Thomsen AR. The role of CC chemokine receptor 5 in antiviral immunity. *Blood* 2002; **99**: 1237-1245

26 **Loetscher P**, Uguccioni M, Bordoli L, Baggiolini M, Moser B, Chizzolini C, Dayer JM. CCR5 is characteristic of Th1 lymphocytes. *Nature* 1998; **391**: 344-345

27 **Napoli J**, Bishop GA, McGuinness PH, Painter DM, McCaughan GW. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. *Hepatology* 1996; **24**: 759-765

28 **Bertoletti A**, D'Elios MM, Boni C, De Carli M, Zignego AL, Durazzo M, Missale G, Penna A, Fiaccadori F, Del Prete G, Ferrari C. Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. *Gastroenterology* 1997; **112**: 193-199

29 **Zeremski M**, Petrovic LM, Talal AH. The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection. *J Viral Hepat* 2007; **14**: 675-687

30 **Springer TA**. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994; **76**: 301-314

31 **Butcher EC**, Picker LJ. Lymphocyte homing and homeostasis. *Science* 1996; **272**: 60-66

32 **Patzwahl R**, Meier V, Ramadori G, Mihm S. Enhanced expression of interferon-regulated genes in the liver of patients with chronic hepatitis C virus infection: detection by suppression-subtractive hybridization. *J Virol* 2001; **75**: 1332-1338

33 **Shields PL**, Morland CM, Salmon M, Qin S, Hubscher SG, Adams DH. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *J Immunol* 1999; **163**: 6236-6243

34 **Narumi S**, Tominaga Y, Tamaru M, Shimai S, Okumura H, Nishioji K, Itoh Y, Okanoue T. Expression of IFN-inducible protein-10 in chronic hepatitis. *J Immunol* 1997; **158**: 5536-5544

35 **Larrubia JR**, Calvino M, Benito S, Sanz-de-Villalobos E, Perna C, Pérez-Hornedo J, González-Mateos F, García-Garzón S, Bienvenido A, Parra T. The role of CCR5/CXCR3 expressing CD8+ cells in liver damage and viral control during persistent hepatitis C virus infection. *J Hepatol* 2007; **47**: 632-641

36 **Bièche I**, Asselah T, Laurendeau I, Vidaud D, Degot C, Paradis V, Bedossa P, Valla DC, Marcellin P, Vidaud M. Molecular profiling of early stage liver fibrosis in patients with chronic hepatitis C virus infection. *Virology* 2005; **332**: 130-144

37 **Apolinario A**, Majano PL, Alvarez-Pérez E, Saez A, Lozano C, Vargas J, García-Monzón C. Increased expression of T cell chemokines and their receptors in chronic hepatitis C: relationship with the histological activity of liver disease. *Am J Gastroenterol* 2002; **97**: 2861-2870

38 **Apolinario A**, Diago M, Lo Iacono O, Lorente R, Pérez C, Majano PL, Clemente G, García-Monzón C. Increased circulating and intrahepatic T-cell-specific chemokines in chronic hepatitis C: relationship with the type of virological response to peginterferon plus ribavirin combination therapy. *Aliment Pharmacol Ther* 2004; **19**: 551-562

39 **Apolinario A**, Majano PL, Lorente R, Núñez O, Clemente G, García-Monzón C. Gene expression profile of T-cell-specific chemokines in human hepatocyte-derived cells: evidence for a synergistic inducer effect of cytokines and hepatitis C virus proteins. *J Viral Hepat* 2005; **12**: 27-37

40 **Sillanpää M,** Kaukinen P, Melén K, Julkunen I. Hepatitis C virus proteins interfere with the activation of chemokine gene promoters and downregulate chemokine gene expression. *J Gen Virol* 2008; **89**: 432-443

41 **Sprengers D**, van der Molen RG, Kusters JG, Kwekkeboom J, van der Laan LJ, Niesters HG, Kuipers EJ, De Man RA, Schalm SW, Janssen HL. Flow cytometry of fine-needle-aspiration biopsies: a new method to monitor the intrahepatic immunological environment in chronic viral hepatitis. *J Viral Hepat* 2005; **12**: 507-512

42 **Leroy V**, Vigan I, Mosnier JF, Dufeu-Duchesne T, Pernollet M, Zarski JP, Marche PN, Jouvin-Marche E. Phenotypic and functional characterization of intrahepatic T lymphocytes during chronic hepatitis C. *Hepatology* 2003; **38**: 829-841

43 **Harvey CE**, Post JJ, Palladinetti P, Freeman AJ, Ffrench RA, Kumar RK, Marinos G, Lloyd AR. Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. *J Leukoc Biol* 2003; **74**: 360-369

44 **Helbig KJ**, Ruszkiewicz A, Semendric L, Harley HA, McColl SR, Beard MR. Expression of the CXCR3 ligand I-TAC by hepatocytes in chronic hepatitis C and its correlation with hepatic inflammation. *Hepatology* 2004; **39**: 1220-1229

45 **Kusano F**, Tanaka Y, Marumo F, Sato C. Expression of C-C chemokines is associated with portal and periportal inflammation in the liver of patients with chronic hepatitis C. *Lab Invest* 2000; **80**: 415-422

46 **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218

47 **Zeremski M**, Petrovic LM, Chiriboga L, Brown QB, Yee HT, Kinkhabwala M, Jacobson IM, Dimova R, Markatou M, Talal AH. Intrahepatic levels of CXCR3-associated chemokines correlate with liver inflammation and fibrosis in chronic hepatitis C. *Hepatology* 2008; **48**: 1440-1450

48 **Friedman SL**. Liver fibrosis -- from bench to bedside. *J Hepatol* 2003; **38** Suppl 1: S38-S53

49 **Promrat K**, McDermott DH, Gonzalez CM, Kleiner DE, Koziol DE, Lessie M, Merrell M, Soza A, Heller T, Ghany M, Park Y, Alter HJ, Hoofnagle JH, Murphy PM, Liang TJ. Associations of chemokine system polymorphisms with clinical outcomes and treatment responses of chronic hepatitis C. *Gastroenterology* 2003; **124**: 352-360

50 **Lloyd AR**, Jagger E, Post JJ, Crooks LA, Rawlinson WD, Hahn YS, Ffrench RA. Host and viral factors in the immunopathogenesis of primary hepatitis C virus infection. *Immunol Cell Biol* 2007; **85**: 24-32

51 **Alter HJ**. HCV natural history: the retrospective and prospective in perspective. *J Hepatol* 2005; **43**: 550-552

52 **Gruener NH**, Lechner F, Jung MC, Diepolder H, Gerlach T, Lauer G, Walker B, Sullivan J, Phillips R, Pape GR, Klenerman P. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol* 2001; **75**: 5550-5558

53 **Wiegand J**, Cornberg M, Aslan N, Schlaphoff V, Sarrazin C, Kubitschke A, Buggisch P, Ciner A, Jaeckel E, Manns MP, Wedemeyer H. Fate and function of hepatitis-C-virus-specific T-cells during peginterferon-alpha2b therapy for acute hepatitis C. *Antivir Ther* 2007; **12**: 303-316

54 **Lichterfeld M**, Leifeld L, Nischalke HD, Rockstroh JK, Hess L, Sauerbruch T, Spengler U. Reduced CC chemokine receptor (CCR) 1 and CCR5 surface expression on peripheral blood T lymphocytes from patients with chronic hepatitis C infection. *J Infect Dis* 2002; **185**: 1803-1807

55 **Linnen J**, Wages J Jr, Zhang-Keck ZY, Fry KE, Krawczynski

KZ, Alter H, Koonin E, Gallagher M, Alter M, Hadziyannis S, Karayiannis P, Fung K, Nakatsuji Y, Shih JW, Young L, Piatak M Jr, Hoover C, Fernandez J, Chen S, Zou JC, Morris T, Hyams KC, Ismay S, Lifson JD, Hess G, Foung SK, Thomas H, Bradley D, Margolis H, Kim JP. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science* 1996; **271**: 505-508

56 **Nattermann J**, Nischalke HD, Kupfer B, Rockstroh J, Hess L, Sauerbruch T, Spengler U. Regulation of CC chemokine receptor 5 in hepatitis G virus infection. *AIDS* 2003; **17**: 1457-1462

57 **Xiang J**, George SL, Wünschmann S, Chang Q, Klinzman D, Stapleton JT. Inhibition of HIV-1 replication by GB virus C infection through increases in RANTES, MIP-1alpha, MIP-1beta, and SDF-1. *Lancet* 2004; **363**: 2040-2046

58 **Williams CF**, Klinzman D, Yamashita TE, Xiang J, Polgreen PM, Rinaldo C, Liu C, Phair J, Margolick JB, Zdunek D, Hess G, Stapleton JT. Persistent GB virus C infection and survival in HIV-infected men. *N Engl J Med* 2004; **350**: 981-990

59 **Pileri P**, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. *Science* 1998; **282**: 938-941

60 **Nattermann J**, Nischalke HD, Feldmann G, Ahlenstiel G, Sauerbruch T, Spengler U. Binding of HCV E2 to CD81 induces RANTES secretion and internalization of CC chemokine receptor 5. *J Viral Hepat* 2004; **11**: 519-526

61 **Butera D**, Marukian S, Iwamaye AE, Hembrador E, Chambers TJ, Di Bisceglie AM, Charles ED, Talal AH, Jacobson IM, Rice CM, Dustin LB. Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C. *Blood* 2005; **106**: 1175-1182

62 **Lagging M**, Romero AI, Westin J, Norkrans G, Dhillon AP, Pawlotsky JM, Zeuzem S, von Wagner M, Negro F, Schalm SW, Haagmans BL, Ferrari C, Missale G, Neumann AU, Verheij-Hart E, Hellstrand K. IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. *Hepatology* 2006; **44**: 1617-1625

63 **Diago M**, Castellano G, García-Samaniego J, Pérez C, Fernández I, Romero M, Iacono OL, García-Monzón C. Association of pretreatment serum interferon gamma inducible protein 10 levels with sustained virological response to peginterferon plus ribavirin therapy in genotype 1 infected patients with chronic hepatitis C. *Gut* 2006; **55**: 374-379

64 **Romero AI**, Lagging M, Westin J, Dhillon AP, Dustin LB, Pawlotsky JM, Neumann AU, Ferrari C, Missale G, Haagmans BL, Schalm SW, Zeuzem S, Negro F, Verheij-Hart E, Hellstrand K. Interferon (IFN)-gamma-inducible protein-10: association with histological results, viral kinetics, and outcome during treatment with pegylated IFN-alpha 2a and ribavirin for chronic hepatitis C virus infection. *J Infect Dis* 2006; **194**: 895-903

65 **Zeremski M**, Markatou M, Brown QB, Dorante G, Cunningham-Rundles S, Talal AH. Interferon gamma-inducible protein 10: a predictive marker of successful treatment response in hepatitis C virus/HIV-coinfected patients. *J Acquir Immune Defic Syndr* 2007; **45**: 262-268

66 **Yang YF**, Tomura M, Iwasaki M, Ono S, Zou JP, Uno K, Shearer GM, Fujiwara H, Hamaoka T. IFN-alpha acts on T-cell receptor-triggered human peripheral leukocytes to up-regulate CCR5 expression on CD4+ and CD8+ T cells. *J Clin Immunol* 2001; **21**: 402-409

67 **Yamauchi K**, Akbar SM, Horiike N, Michitaka K, Onji M. Increased serum levels of macrophage inflammatory protein-3alpha in chronic viral hepatitis: prognostic importance of macrophage inflammatory protein-3alpha during interferon therapy in chronic hepatitis C. *J Viral Hepat* 2002; **9**: 213-220

68 **Helbig KJ**, George J, Beard MR. A novel I-TAC promoter polymorphic variant is functional in the presence of replicating HCV in vitro. *J Clin Virol* 2005; **32**: 137-143

69 **Woitas RP**, Ahlenstiel G, Iwan A, Rockstroh JK, Brackmann HH, Kupfer B, Matz B, Offergeld R, Sauerbruch T, Spengler U. Frequency of the HIV-protective CC chemokine receptor 5-Delta32/Delta32 genotype is increased in hepatitis C. *Gastroenterology* 2002; **122**: 1721-1728

70 **Hellier S**, Frodsham AJ, Hennig BJ, Klenerman P, Knapp S, Ramaley P, Satsangi J, Wright M, Zhang L, Thomas HC, Thursz M, Hill AV. Association of genetic variants of the chemokine receptor CCR5 and its ligands, RANTES and MCP-2, with outcome of HCV infection. *Hepatology* 2003; **38**: 1468-1476

71 **Wald O**, Pappo O, Ari ZB, Azzaria E, Wiess ID, Gafnovitch I, Wald H, Spengler U, Galun E, Peled A. The CCR5Delta32 allele is associated with reduced liver inflammation in hepatitis C virus infection. *Eur J Immunogenet* 2004; **31**: 249-252

72 **Schols D**. HIV co-receptors as targets for antiviral therapy. *Curr Top Med Chem* 2004; **4**: 883-893

73 **Huber M**, Olson WC, Trkola A. Antibodies for HIV treatment and prevention: window of opportunity? *Curr Top Microbiol Immunol* 2008; **317**: 39-66

74 **Lederman MM**, Penn-Nicholson A, Cho M, Mosier D. Biology of CCR5 and its role in HIV infection and treatment. *JAMA* 2006; **296**: 815-826

75 **Gong X**, Feng H, Zhang S, Yu Y, Li J, Wang J, Guo B. Increased expression of CCR5 in experimental autoimmune myocarditis and reduced severity induced by anti-CCR5 monoclonal antibody. *J Mol Cell Cardiol* 2007; **42**: 781-791

76 **Suzuki K**, Kawauchi Y, Palaniyandi SS, Veeraveedu PT, Fujii M, Yamagiwa S, Yoneyama H, Han GD, Kawachi H, Okada Y, Ajioka Y, Watanabe K, Hosono M, Asakura H, Aoyagi Y, Narumi S. Blockade of interferon-gamma-inducible protein-10 attenuates chronic experimental colitis by blocking cellular trafficking and protecting intestinal epithelial cells. *Pathol Int* 2007; **57**: 413-420

77 **Singh UP**, Singh S, Taub DD, Lillard JW Jr. Inhibition of IFN-gamma-inducible protein-10 abrogates colitis in IL-10-/- mice. *J Immunol* 2003; **171**: 1401-1406

78 **Mohan K**, Issekutz TB. Blockade of chemokine receptor CXCR3 inhibits T cell recruitment to inflamed joints and decreases the severity of adjuvant arthritis. *J Immunol* 2007; **179**: 8463-8469

79 **Singh UP**, Venkataraman C, Singh R, Lillard JW Jr. CXCR3 axis: role in inflammatory bowel disease and its therapeutic implication. *Endocr Metab Immune Disord Drug Targets* 2007; **7**: 111-123

80 **Barth H**, Robinet E, Liang TJ, Baumert TF. Mouse models for the study of HCV infection and virus-host interactions. *J Hepatol* 2008; **49**: 134-142

81 **Lalezari J**, Yadavalli GK, Para M, Richmond G, Dejesus E, Brown SJ, Cai W, Chen C, Zhong J, Novello LA, Lederman MM, Subramanian GM. Safety, pharmacokinetics, and antiviral activity of HGS004, a novel fully human IgG4 monoclonal antibody against CCR5, in HIV-1-infected patients. *J Infect Dis* 2008; **197**: 721-727

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Crypt region localization of intestinal stem cells in adults

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Abstract
The intestinal epithelial lining plays a central role in the digestion and absorption of nutrients, but exists in a harsh luminal environment that necessitates continual renewal. This renewal process involves epithelial cell proliferation in the crypt base and later cell migration from the crypt base to the luminal surface. This process is dependent on multi-potent progenitor cells, or stem cells, located in each crypt. There are about 4 to 6 stem cells per crypt, and these stem cells are believed to generate distinct end-differentiated epithelial cell types, including absorptive cells, goblet cells, enteroendocrine cells and Paneth cells, while also maintaining their own progenitor cell state. Earlier studies suggested that intestinal stem cells were located either in the crypt base interspersed between the Paneth cells [i.e. crypt base columnar (CBC) cell model] or at an average position of 4 cells from the crypt base [i.e. label-retaining cells (LRC +4) model]. Recent studies have employed biomarkers in the *in vivo* mammalian state to more precisely evaluate the location of these progenitor cells in the intestinal crypt. Most notable of these novel markers are *Lgr5*, a gene that encodes a G-protein-coupled receptor with expression restricted to CBC cells, and *Bmi 1*, which encodes a chromatin remodeling protein expressed by LRC. These studies raise the possibility that there may be separate stem cell lines or different states of stem cell activation involved in the renewal of normal mammalian intestinal tract.

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Key words: Crypt base columnar cells; Intestinal epithelial cell renewal; *Lgr5* gene; Polycomb bmi1 protein; Progenitor cells; Stem cells; +4 stem cell model

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INTRODUCTION

An improved understanding of stem cell biology and its possible application in the treatment of diseases has emerged as an important fundamental area in clinical medicine. The intestinal epithelial lining is relatively unique in its ability to rapidly regenerate. This has led to an ever increased focus on the crypt cell as a potential model of adult stem cell biology. The mammalian intestinal epithelial lining plays a critical role in digestion and in the absorption of nutrients and requires constant renewal due to the very harsh luminal environment. This renewal process involves rapid and continuous proliferation of epithelial cells in the crypt base with subsequent migration of these cells to the luminal surface. This process of epithelial cell renewal within the intestine appears to be entirely dependent upon a limited number of long-lived multi-potent intestinal progenitor cells or stem cells.

An intestinal epithelial stem cell may be broadly defined as a cell having at least 2 important properties: firstly, an ability to generate distinct differentiated epithelial cell types found in the intestine; and secondly, an ability to maintain itself as a progenitor cell over prolonged periods. It appears that each intestinal crypt contains approximately 4 to 6 stem cells. Each of these cells is believed to be capable of essential regenerative activity which is required to produce all the distinctive end-differentiated epithelial cell types found in the intestine. However, recent studies employing specific biomarkers for identification of stem cells have raised new and intriguing issues which have significant implications for the regenerative processes in the digestive tract involved in both health and disease.

INTESTINAL STEM CELL IDENTIFICATION

Over 30 years ago, Cheng and Leblond identified small cycling epithelial cells interspersed between the Paneth cells, or the so-called crypt base columnar (CBC) cells, using morphological methods in mammalian intestine[1,2]. Later, Bjerkness and Cheng provided additional information on these specialized cells using elegant clonal marking techniques[3].

These investigators theorized that the CBC cells located within the stem cell zone of the crypt base might represent the actual intestinal epithelial stem cells[3,4]. All of the end-differentiated intestinal epithelial cells were hypothesized to develop from these CBC cells including intestinal columnar cells, intestinal goblet cells, enteroendocrine cells and Paneth cells.

An alternative hypothesis has also suggested that intestinal stem cells were actually located elsewhere at a position that averaged +4 from the bottom of the crypts with the lowest three positions generally relegated to the terminally differentiated Paneth cells. Evidence supporting this hypothesis of the +4 stem cell model was provided by Potten *et al*[5,6]. These investigators, using the DNA-labeling reagents, bromo-deoxyuridine or (^{3}H)-thymidine, on radiation-sensitive, label-retaining cells (LRC) showed that the LRC were located specifically at the +4 position in the intestinal crypt region, precisely at the origin of the migratory epithelial cell column.

INTESTINAL STEM CELL SIGNALING

Intestinal stem cells, although compartmentalized into the crypt region, do not function in isolation independent of specific regulatory controls. Clearly, these cells play critical roles in the proliferation and differentiation of normal and neoplastic epithelial cells, However, the complex regulation of these cells involves a wide array of critical signaling pathways, recently well reviewed elsewhere[7]. These include Wnt, BMP, PTEN-controlled PI3K/Akt and Notch pathways. While studies are needed to further elucidate all of the specific mechanisms involved in each signaling pathway, recent reports focused on the elusive position of these progenitor cells have served to add a new dimension to the intricate biology of these cell processes.

INTESTINAL STEM CELL LOCALIZATION

In 2007, Barker and Clevers reported a highly restricted molecular marker for intestinal stem cells[8]. These investigators identified *Lgr5*, a gene encoding a specific G-protein-coupled receptor, which was expressed specifically in CBC cells. Later, using engineered mice, a DNA marker on *Lgr5*-positive cells was created which permitted subsequent tracing of their lineage into long-lived epithelial clones with all of the cell types in normal ratios. Interestingly, CBC cells were not quiescent (as might have been expected) but completed a cell cycle in about one day suggesting that these CBC cells would have to undergo many hundreds or thousands of cell divisions during the lifetime of the animal without loss of genetic information or malignant transformation. The *Lgr5* gene was also found to be expressed in the stomach and throughout the intestinal tract as well as in colon cancer cells leading to speculation that *Lgr5* might be a cancer stem cell marker. Moreover, as the *Lgr5* gene encoded a receptor on the cell surface, it was suggested that this might permit recognition with a monoclonal antibody and a means of eventually eradicating *Lgr5*-positive cancer stem cells[9].

In a recent 2008 report, another new stem cell marker was reported by Sangiorgi *et al*[10]. In their studies, *Bmi 1*, a Polycomb group protein known to play an important role in the renewal of hematopoietic and neural stem cells, characterized the progeny derived from *Bmi 1*-positive cells using a similar lineage tracing approach employed for Lgr5-positive cells. The *Bmi 1* locus marked long-lived cell clones by all intestinal cell types. Ablation of *Bmi 1*-positive cells resulted in depletion from the epithelium in entire intestinal crypts. Of note, *Bmi 1* was expressed in cells in the +4 position region, not at the location of the CBC cells, and primarily in the proximal small intestine, suggesting that a different stem cell population may have been characterized. Moreover, crypt *Bmi 1*-positive cells appeared to have slow turnover with relatively slow kinetics, another difference from *Lgr5*-positive cells which rapidly turnover[11].

FUTURE STUDIES

The possible relationship of these two apparently distinctive intestinal stem cell populations requires further definition. In addition, studies for other new stem cell markers are likely to already be in progress. Are these studies likely to indicate that there are multiple stem cell populations in the intestine similar to other stem cell organs, such as the epidermis, with distinctive stem cell populations? Or, alternatively, is this simply the expression of a single stem cell population in different proliferative states? Further definition of this issue is essential. The regenerative processes in the intestinal epithelial lining in health and disease are both fascinating and complex. More importantly, the possible implications for clinical medicine may be enormous. As clinicians caring for patients, close attention to this rapidly progressing fundamental investigative endeavour is needed.

REFERENCES

1 **Cheng H**, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. *Am J Anat* 1974; **141**: 461-479
2 **Cheng H**, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat* 1974; **141**: 537-561
3 **Bjerknes M**, Cheng H. Clonal analysis of mouse intestinal epithelial progenitors. *Gastroenterology* 1999; **116**: 7-14
4 **Bjerknes M**, Cheng H. Gastrointestinal stem cells. II. Intestinal stem cells. *Am J Physiol Gastrointest Liver Physiol*

2005; **289**: G381-G387
5 **Potten CS**. Extreme sensitivity of some intestinal crypt cells to X and gamma irradiation. *Nature* 1977; **269**: 518-521
6 **Potten CS**, Kovacs L, Hamilton E. Continuous labelling studies on mouse skin and intestine. *Cell Tissue Kinet* 1974; **7**: 271-283
7 **Scoville DH**, Sato T, He XC, Li L. Current view: intestinal stem cells and signaling. *Gastroenterology* 2008; **134**: 849-864
8 **Barker N**, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; **449**: 1003-1007
9 **Barker N**, Clevers H. Tracking down the stem cells of the intestine: strategies to identify adult stem cells. *Gastroenterology* 2007; **133**: 1755-1760
10 **Sangiorgi E**, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* 2008; **40**: 915-920
11 **Batlle E**. A new identity for the elusive intestinal stem cell. *Nat Genet* 2008; **40**: 818-819

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REVIEW

Management of Hinchey Ⅱ diverticulitis

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Abstract

Colonic diverticulosis can either be asymptomatic or present with complications resulting in significant morbidity and mortality. A key presentation of complicated disease is abscess formation (Hinchey type Ⅱ). The natural course of this is unclear and therefore treatments range from conservative approach with antibiotics and percutaneous guided drainage (PCD) to surgery. There is no clear consensus on the exact management strategy. A Medline based literature search specifically looking at studies dealing with Hinchey type Ⅱ diverticulitis and its management was carried out. For comparison, five-year retrospective data of diverticular abscesses from our institution was collected and the outcome analysed. Various studies have looked into this aspect of the disease, elaborating on the significance of the size and location of the abscesses, the role of PCD, recurrence rates and the controversies regarding the need for elective surgery. Conservative treatment with antibiotics alone is effective in a majority of cases with a role for PCD in large safely accessible abscesses. Variable recurrence rates have been reported in literature and elective surgery should be planned for selected groups of patients.

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Key words: Diverticulosis; Diverticular abscess; Hinchey classification; Percutaneous drainage; Recurrent diverticulitis

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INTRODUCTION

The prevalence of colonic diverticulosis has gradually yet steadily increased in the western world and ranges from 30%-50% of population over the age of 50 and more than two thirds over the age of 80[1].The sigmoid colon is the most common site to be involved. These diverticulae are primarily pulsion type of pseudo-diverticulae characterised by outpouching of mucosa and peritoneum through the sites of penetration of blood vessels in the colonic wall[2]. Though the exact aetiology is not known, abnormal colonic structure and motility along with genetic, dietary and changes in intestinal flora are considered to play a role in its patho-physiology[3].

There is lack of clarity about the natural course of the disease. Its different manifestations ranging from being completely asymptomatic to an array of complicated diverticular disease makes it vital to acquire a good understanding of this condition. Complicated diverticulitis (CD), accounting for 20%-30% of total prevalence of diverticulosis, causes significant morbidity and mortality in patients in addition to considerable healthcare costs in the western world[4]. This includes the gamut of diverticulitis either with or without phlegmon, abscess, fistula, obstruction, bleeding and perforation with purulent/faecal peritonitis[5,6].

The clinical presentation usually comprises of a triad of primarily left lower quadrant pain, fever and leucocytosis, although other associated features like nausea, vomiting, change in bowel habits, rectal bleeding and dysuria may be present. The clinical classification scheme[7] by the European association for endoscopic surgeons is based on the clinical severity and is detailed in Table 1. Other classifications do additionally incorporate the clinical and pathological aspects with the computerized tomography (CT) findings and aid in the planning of treatment strategies for this condition. The universally accepted is the Hinchey classification[8],

which descriptively characterises the various stages of diverticulitis and its acute complications. Type I and Type Ⅱ refer to inflammatory phlegmon and paracolic abscesses while type Ⅲ and Ⅳ refer to purulent and faeculent peritonitis respectively. This classification has been further modified as given in Table 2.

The sequence of pathological changes complicating a colonic diverticulum starts from a microperforation in its structure[9]. However the progression beyond this can be unpredictable either leading to a phlegmon or to frank faeculent peritonitis. The management is dictated by the stage of the disease at the time of its presentation and by the observed response to the initiated treatment[10]. Conservative measures characterised by bowel rest, antibiotics and clinical monitoring are usually effective in the treatment of early stages of diverticulitis especially modified Hinchey stage 0 and stage I a. There is also a clear consensus on open or laparoscopic surgical management of type Ⅲ and Ⅳ as these stages are unlikely to respond to conservative measures. However the exact management strategy for type I b and Ⅱ (specifically diverticular abscesses i.e. paracolic or pelvic) needs more clarity.

The general treatment option for these stages (I a & Ⅱ) starts from conservative management to CT guided percutaneous drainage (PCD) and then on to surgery based on progression of the disease with reliance on clinical and laboratory parameters. It is important to understand that this severe condition has an overall mortality of 5%-10% and higher rates in emergency surgery (> 25%)[13].

The problems associated with management of these stages relate to the difficulty in gauging if the condition will respond to conservative treatment, the exact indications for PCD and identifying the cohort of patients who will need early emergency surgery. Additional hurdles to management include problems with PCD, primarily the difficulty in access, documented failure rate and its associated complications. Most of the studies in literature have dealt with the different stages of CD and the main impetus has been on the various surgical approaches to the management of this condition and its impact on morbidity and mortality. This article has specifically reviewed the management of Hinchey Ⅱ diverticulitis associated with pericolic and pelvic abscesses.

MEDLINE BASED LITERATURE SEARCH

A Medline based review of the literature was performed specifically looking at studies which dealt with diverticular abscesses (Hinchey type Ⅱ diverticulitis) and its outcome. A five-year retrospective analysis of data of patients admitted with diverticular abscess (Hinchey type Ⅱ) admitted to our institution was also collected and analysed for comparison.

OUTCOME ANALYSIS

The rationale behind the use of antibiotics and PCD in the management of diverticular abscesses is based on the treatment of intra-abdominal sepsis and the reduction of the abdomino-pelvic inflammatory milieu to facilitate single stage operations. CT has revolutionised the approach to diverticulitis due to it's high sensitivity and specificity in the diagnosis[14] planning of management and also a therapeutic role in guided drainage of abscesses. Before the advent of PCD, 10%-15% of operations for diverticulitis was for drainage of abscesses[15] and multistage surgical procedures were being performed for diverticular abscesses[16,17]. The diagnostic findings on CT include inflammation of pericolic fat, colonic wall thickening, mesocolic and pelvic abscesses, perforation with free intraperitoneal air and bowel obstruction (Figure 1).

CT in addition enables to exclude other causes of acute abdomen, however, the reliable differentiation of acute diverticulitis from colonic malignancy can be difficult[18]. It has been reported that the findings such as pericolic stranding, involvement of more than 10 cm of the colon or the absence of pericolic nodes associated with colonic thickening and pericolonic inflammatory changes are likely to point to diverticulitis rather than cancer[18]. The CT classification by Ambrosetti[19] as shown in Table 3 is valuable in assessing the severity of the disease and planning optimal management.

The preferred approach is usually transabdominal, either anterior or lateral taking precautions to avoid the inferior epigastric and the deep circumflex iliac vessels, respectively[20]. Alternative approaches include transgluteal[21,22], transperineal[23,24], transvaginal and

Table 1 Clinical classification of diverticulitis (adapted from Köhler *et al*[7])

Grade	Clinical features	Symptoms
I	Symptomatic uncomplicated disease	Fever, abd pain, CT evidence of diverticulitis
Ⅱ	Recurrent symptomatic disease	Recurrence of above symptoms
Ⅲ	Complicated disease	GI bleeding Phlegmon Abscess Peforation-purulent/ faecal peritonitis Stricture Fistula Obstruction

Table 2 Modified hinchey classification (adapted from Wasvery *et al* [11,12])

Modified Hinchey classification	
0	Mild clinical diverticulitis
I a	Confined pericolic inflammation-phlegmon
I b	Confined pericolic abscess
Ⅱ	Pelvic, intrabdominal or retrocolic abscess
Ⅲ	Generalized purulent peritonitis
Ⅳ	Faecal peritonitis
Fistula	Colo-vesical/-vaginal/-enteric/-cutaneous
Obstruction	Large/small bowel obstruction

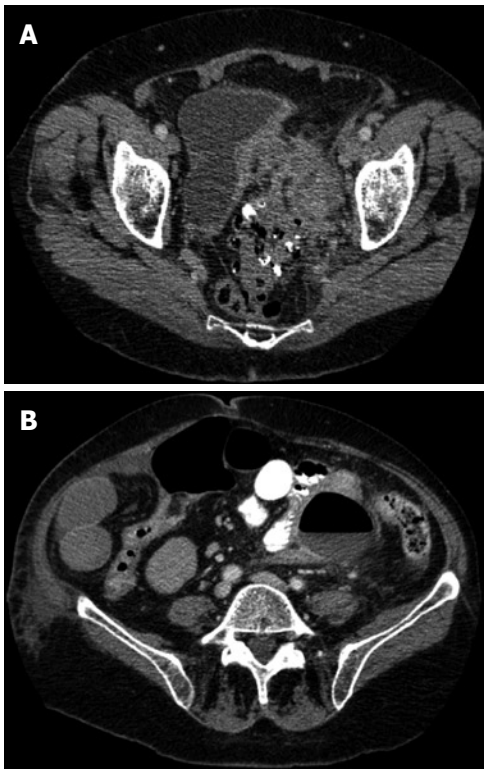

Figure 1 Abdomen and pelvis CT. A: Pelvic abscess secondary to complicated diverticulitis; B: Paracolic abscess secondary to complicated diverticulitis.

transrectal[25] for access. PCD primarily was used as a temporary measure and bridge to surgery though recent suggestions questioning the necessity of surgery after conservative treatment have generated further debates. Although it is presently acknowledged that CT plays a pivotal role in guided PCD, operator skill and the location of the abscess are paramount in determining success of the intervention. Guided PCD is the choice of intervention for simple uninoculated abscesses with success rate of more than 80%, but is known to have a high failure rate in cases of complex abscesses which are defined as abscesses with loculations, associated with fistulae and infected fluid collections whose drainage route traverses normal organs[20].

Despite innovative improvements in modalities, the response to conservative treatment of Hinchey Ⅱ is not consistently successful, as various unexplained factors seem to modify the clinical course. Based on deterioration in clinical parameters, patients undergo surgery preferably receiving a single stage colectomy or in some cases two stage procedures, a colectomy with diversion stoma. Attempts to identify the exact indications surrounding the modality of approach are currently ongoing and can be appreciated from the interesting literature on this issue. Tables 4 and 5 feature some of the important publications and their findings dealing specifically with diverticular abscesses.

Stabile *et al*[26] in his series of 19 patients with a follow up of 17.4 mo showed that PCD was successful in 14 patients who subsequently had elective single stage operations. The average size of the abscesses in this series

Table 3 CT staging of diverticulitis (adapted from Ambrosetti *et al*[19])

Ambrosetti's CT staging of diverticulitis	
Moderate diverticulitis	**Severe diverticulitis**
Localised sigmoid wall thickening (5 mm or more)	Abscess
Inflammation of pericolic fat	Extraluminal air/contrast

Table 4 Results of a retrospective audit from our institution over a five-year period

Series of diverticulitis case	*n*
CT confirmed diverticulitis (*n*)	69
Hinchey type Ⅱ (*n*)	28
Average size of abscesses (cm)	5
Average size of abscess for PCD (cm)	7
Rate of surgical intervention (%)	46

was quite large at 8.7 cm. Meuller *et al*[27] also reported successful results with PCD and on retrospective chart review of 87 patients with diverticulitis prior to the use of PCD found that more than 50% of those who underwent two stage surgical procedures would have benefited from PCD. Similar studies by Neff *et al*[28] and Saini *et al*[29] have reinforced the contribution of PCD in facilitating single stage surgical procedures.

Ambrosetti *et al*[30] from his study of 73 cases of diverticular abscesses with a follow up of 43 mo found that 59% of total cases eventually needed surgery either during the acute admission or as an elective procedure. The rest of the group did not need any form of surgical intervention after conservative treatment either with or without PCD. In his study, antibiotics alone without PCD was effective in 30 patients and 19 out of 73 patients underwent PCD. The study also compared the prognosis of mesocolic abscesses with pelvic ones and revealed that pelvic abscesses exhibited an aggressive behaviour and therefore needed to be rapidly drained percutaneously and were likely to require surgery. In addition, it was stated that the indication for secondary colectomy after successful treatment of mesocolic abscess with or without drainage was not peremptory.

In a 10-year study and a mean follow up of 46.5 mo by Kaiser *et al*[11] CT guided PCD was found to be successful in 93% of cases. In this series of 99 cases, 16 (16.2%) of them were amenable for PCD. Out of 99 patients, 23 patients failed conservative treatment with antibiotics ± PCD and needed emergency surgery during the same admission. Overall recurrence rate for the entire group was 18.2 % with especially high rates for early stages (40% for stage I b + PCD, and stage Ⅱ ± PCD). Interestingly, the recurrence in the stage I b group after CT guided PCD was high at 40% when compared to the overall stage I b recurrence of 12.7%. The exact reason for this was not clear although the size of the abscesses in the drained group was significantly larger thus making the possibility of more severe disease in the drained group likely. The key finding of a high

Table 5 Results showing the total number, numbers drained, age, site and size (average for PCD) of abscesses from studies in literature

Name	Total	Drained	Mean age yr (/range)	Site of abscess			Size for PCD (cm)
				Paracolic	Pelvic	Others	
Kumar *et al*[31]	30	12	39	15	5	10	6.5
Stabile *et al*[26]	19	19	63.8	8	9	2	8.7
Kaiser *et al*[11]	99	16	-	74	25	-	7.1
Ambrosetti *et al*[30]	73	19	66.9	45	28	-	6.7
Brandt *et al*[13]	66	34	71	-	-	-	6
Bahadursingh *et al*[32]	25	10	61	9	9	7	-
Siewert *et al*[33]	30	4	54.2	-	-	-	5.9
Neff *et al*[28]	16	16	42-86	2	13	1	> 5
Alvarez *et al*[34]	59	-	64	37	22	-	-

Table 6 Results showing the total number, numbers drained, response to conservative treatment and details of emergency/elective/semi-elective surgery from studies in literature

Name	Total	Drained	Antibiotics ± PCD	Surgery same admission	Elective/semi-elective
Kumar *et al*[31]	30	12	17	5	-
Stabile *et al*[26]	19	19	-	3	14
Mueller *et al*[27]	24	21	1	6	12
Kaiser *et al*[11]	99	16	56	23	20
Ambrosetti *et al*[30]	73	19	30	18	25
Brandt *et al*[13]	66	34	21	15	28
Siewert *et al*[33]	30	4	17	-	13
Cinat *et al*[37]	13	13	-	5	-
Saini *et al*[29]	17	8	-	1	7
Alvarez[34]	59	-	33	26	-

incidence of recurrence in these groups highlighted the assertion that successful PCD should be considered as just a temporary measure rather than definitive treatment. The authors recommended that considering the high rates of recurrence, CT confirmed abscess was a strong indication for consideration of elective/semi-elective surgery.

There are several studies in literature detailing the role of PCD in diverticular abscesses but very few comparing treatment with antibiotics alone or antibiotics with PCD. Brandt *et al*[13] retrospectively looked at this issue comparing two groups with CT confirmed abscesses, one who had antibiotics alone and the other antibiotics with PCD. Interestingly, the results show that the patients treated with antibiotics alone achieved an outcome similar to patients treated with PCD. However the average abscess size was larger in the PCD group (4 cm in the antibiotic only group compared to 6 cm in PCD group). Failure rate of PCD in this series was 33% which was comparable to other series[35,36]. The results from this study showed that antibiotics alone were effective in more than 80% of patients presenting with Hinchey type Ⅱ diverticulitis.

A similar study by Siewert *et al*[33] reported that antibiotics alone was effective in resolving acute symptoms for abscess size less than 3 cm. In this study, the abscess size was comparatively smaller than in other studies as most patients presented early in the course of the disease and CT was performed on the day of presentation in the emergency department. Kumar *et al*[31] in his series of 30 patients with abscesses found that patients with an average abscess diameter of 4 cm improved on antibiotics alone.

In the series of 69 cases of CT confirmed diverticulitis (Table 4) from our institution over a period of 5 years (unpublished), we found comparable rates of abscesses to that in literature (28/69 patients with diverticular abscess). The average size of abscesses in our patients was 5.0 cm. Only a small percentage of abscesses were accessible for PCD (3/28 cases) in our series and the average size of the abscesses in these cases was 7 cm. The rate of acute surgical intervention during the initial admission was 46% in our series and was comparable to other series in literature.

As seen in Tables 5 and 6, in a majority of studies, the average size of abscesses for PCD intervention was more than 6 cm with an overall increased prevalence of paracolic abscesses over pelvic and distant abscesses. The percentage of PCD intervention ranged from about 13% to 51%, though in a few studies it was not clear whether all abscesses were taken into consideration or the only ones amenable to drainage were reported. Interestingly, conservative treatment using antibiotics with or without PCD was successful as definitive treatment in about 30% to 56% in a majority of studies while it was effective in tiding over the acute situation in a range of 56% to 76%. The collective data also suggests that more than 50% of cases may eventually need semi-elective or elective surgery due to recurrent symptoms.

Issues in management

Despite the wealth of literature detailing the management of this condition, certain aspects of decision making and

management have remained controversial, especially the indications for PCD, single stage operations and planning of elective surgery.

Size and location of abscesses

Significant parameters like the size and location of the abscesses do influence the success of conservative or PCD intervention. Most of the evidence in literature shows that the size of the abscess is directly proportional to the need for intervention. Some of the large series reported in literature are given in Table 4. It is generally observed in various studies that abscesses with a size of up to 4 cm seem to respond better to antibiotics alone[13,31,33]. In the context of PCD, as an average, size equal to or more than 5 cm with safe access seems to be an indication[11,30].

Regarding the significance of the abscess location, there has been emphasis on better prognosis with mesocolic and paracolic abscesses[30,34] though this has been disputed by some studies[31]. It is feasible that mesocolic abscesses are the result of early disease resulting in local colonic inflammatory changes with pus collection contiguous with the mesentery and therefore respond better to conservative treatment. Single stage surgical procedures are also sufficient in these circumstances as these abscesses can be removed en bloc with the specimen[27]. Pelvic abscesses are probably the result of a slightly larger perforation with potential to complicate further by exceeding the confines of the mesentery onto the surrounding pelvis and peritoneum. This may benefit by PCD but could further deteriorate to necessitate surgery or on resolution of the acute episode need elective surgery[30]. Therefore, the pelvic location of the abscess could be a potential indicator for further elective surgery.

Failure of conservative treatment with or without PCD

The early Hinchey stages like stage 0 or I a can also fail conservative treatment and progress to emergency surgery. Kaiser *et al*[11] in his series found that 6.8% of these stages needed surgical intervention during the first admission. In his series, 22.2% of stage I b and Ⅱ failed conservative treatment and PCD. The failure rate of PCD ranges from 15%-30% in different series[11,38,39]. Although there are no clear early indicators to potential failure of conservative treatment, large sized abscesses with systemic features of inflammation could predict failure. In this context, Kumar *et al*[31] found in his series that an abscess size more than 6.75 cm associated with leucocytosis of more than 14.8×10^9/L and fever more than 102°1 F could predict a failure in conservative treatment.

Complications

Complications with PCD have been reported in the range of about 5% in most series[31] and they include bleeding, perforation of viscus, solid organ injury and fistulation. High rates of mortality as much as 75% were seen in earlier series due to complications of PCD[40]. Although faeculent drainage from the catheters were reported in some series[26], a majority did show response to conservative treatment and did not cause any secondary problems[27]. Recurrent abscesses after PCD have been reported which can interfere with elective operation[6].

Recurrence and elective surgery

It is known that recurrence is a problem after conservative management of diverticulitis. The manifestations of recurrence may range from episodic cramps without CT evidence to complicated disease and about 30% are likely to develop recurrent diverticulitis[2,41,42].Variable recurrence rates ranging from about 7% to 35% have been reported in literature[42]. The potential to recurrence is high in these early Hinchey stages as the later stages would have had emergency surgery as definitive treatment[11]. Recurrent symptoms of diverticulitis have also been linked to the initial size of the abscesses[33] though there is no clear pattern in its presentation. High relative recurrence rates of 53.8% were seen in non-operated cases in some series[11,32]. Considering the high rates of recurrence and the low complication rates for elective surgery, it has been suggested that elective surgery should be undertaken to achieve relief of symptoms[43].

Questions regarding the indications for elective colectomy after conservative treatment remain unanswered. Resection is commonly recommended after two attacks of uncomplicated diverticulitis[44] in order to reduce the morbidity and mortality of complicated disease. However, the pattern of the first presentation of complicated disease can be very variable. Although pericolic abscesses and inflammatory phlegmon were found to be significantly associated with at least one prior episode of diverticulitis[5], studies have shown that previous history of diverticulitis was present in less than 20% of acute diverticulitis admissions[34]. Interestingly, Chapman *et al*[5] in her series on CD found that 53.4% of patients presented with CD as their first episode and 89.5% of patients who died due to diverticular perforation had no previous history of diverticulitis. Therefore, prophylactic colectomy to reduce morbidity and mortality from CD may not serve its intended purpose.

Studies have also shown that about 40%-50% of patients admitted with abscesses responded well to conservative treatment and eventually did not need surgery[30]. Therefore, elective surgery should be considered for patients who develop persistent or recurrent symptoms. Although there is no consensus on this issue, it is acknowledged that some subgroups may be at high risk and should be offered surgery especially young patients and patients with co morbidities such as diabetes, collagenous disorders and immunosuppressed[45-47].

CONCLUSION

Interpreting the results of various studies, the essential consistent finding is that about 20%-30% of the CD

cases present with abscesses out of which about a similar percentage (20%-30%) are amenable to PCD. In addition, the failure rate in PCD is about 20%-30%. Considering the whole spectrum of the Hinchey type Ⅱ diverticulitis, primary broad-spectrum antibiotics alone will remain the choice of primary therapy and interventions like PCD should be planned based on the abscess size and feasibility of drainage and also depend on the local expertise available to undertake the procedure. The role of PCD, though beneficial, will still be nominal. However, considering the large and increasing numbers of hospital admissions with this pathology, the contribution of PCD will remain paramount in terms of ameliorating the clinical picture. Considering the high rates of recurrent diverticulitis and its morbidity, assessment for elective surgery should be done on a case-by-case basis.

REFERENCES

1 **Roberts P**, Abel M, Rosen L, Cirocco W, Fleshman J, Leff E, Levien D, Pritchard T, Wexner S, Hicks T. Practice parameters for sigmoid diverticulitis. The Standards Task Force American Society of Colon and Rectal Surgeons. *Dis Colon Rectum* 1995; **38**: 125-132

2 **Stollman N**, Raskin JB. Diverticular disease of the colon. *Lancet* 2004; **363**: 631-639

3 **Simpson J**, Scholefield JH, Spiller RC. Pathogenesis of colonic diverticula. *Br J Surg* 2002; **89**: 546-554

4 **Sandler RS**, Everhart JE, Donowitz M, Adams E, Cronin K, Goodman C, Gemmen E, Shah S, Avdic A, Rubin R. The burden of selected digestive diseases in the United States. *Gastroenterology* 2002; **122**: 1500-1511

5 **Chapman J**, Davies M, Wolff B, Dozois E, Tessier D, Harrington J, Larson D. Complicated diverticulitis: is it time to rethink the rules? *Ann Surg* 2005; **242**: 576-581; discussion 581-583

6 **Wedell J**, Banzhaf G, Chaoui R, Fischer R, Reichmann J. Surgical management of complicated colonic diverticulitis. *Br J Surg* 1997; **84**: 380-383

7 **Köhler L**, Sauerland S, Neugebauer E. Diagnosis and treatment of diverticular disease: results of a consensus development conference. The Scientific Committee of the European Association for Endoscopic Surgery. *Surg Endosc* 1999; **13**: 430-436

8 **Hinchey EJ**, Schaal PG, Richards GK. Treatment of perforated diverticular disease of the colon. *Adv Surg* 1978; **12**: 85-109

9 **Berman LG**, Burdick D, Heitzman ER, Prior JT. A critical reappraisal of sigmoid peridiverticulitis. *Surg Gynecol Obstet* 1968; **127**: 481-491

10 **Ferzoco LB**, Raptopoulos V, Silen W. Acute diverticulitis. *N Engl J Med* 1998; **338**: 1521-1526

11 **Kaiser AM**, Jiang JK, Lake JP, Ault G, Artinyan A, Gonzalez-Ruiz C, Essani R, Beart RW Jr. The management of complicated diverticulitis and the role of computed tomography. *Am J Gastroenterol* 2005; **100**: 910-917

12 **Wasvary H**, Turfah F, Kadro O, Beauregard W. Same hospitalization resection for acute diverticulitis. *Am Surg* 1999; **65**: 632-635; discussion 636

13 **Brandt D**, Gervaz P, Durmishi Y, Platon A, Morel P, Poletti PA. Percutaneous CT scan-guided drainage vs. antibiotherapy alone for Hinchey II diverticulitis: a case-control study. *Dis Colon Rectum* 2006; **49**: 1533-1538

14 **Ambrosetti P**, Grossholz M, Becker C, Terrier F, Morel P. Computed tomography in acute left colonic diverticulitis. *Br J Surg* 1997; **84**: 532-534

15 **Rodkey GV**, Welch CE. Changing patterns in the surgical treatment of diverticular disease. *Ann Surg* 1984; **200**: 466-478

16 **Greif JM**, Fried G, McSherry CK. Surgical treatment of perforated diverticulitis of the sigmoid colon. *Dis Colon Rectum* 1980; **23**: 483-487

17 **Hackford AW**, Schoetz DJ Jr, Coller JA, Veidenheimer MC. Surgical management of complicated diverticulitis. The Lahey Clinic experience, 1967 to 1982. *Dis Colon Rectum* 1985; **28**: 317-321

18 **Chintapalli KN**, Chopra S, Ghiatas AA, Esola CC, Fields SF, Dodd GD 3rd. Diverticulitis versus colon cancer: differentiation with helical CT findings. *Radiology* 1999; **210**: 429-435

19 **Ambrosetti P**, Jenny A, Becker C, Terrier TF, Morel P. Acute left colonic diverticulitis--compared performance of computed tomography and water-soluble contrast enema: prospective evaluation of 420 patients. *Dis Colon Rectum* 2000; **43**: 1363-1367

20 **Golfieri R**, Cappelli A. Computed tomography-guided percutaneous abscess drainage in coloproctology: review of the literature. *Tech Coloproctol* 2007; **11**: 197-208

21 **Harisinghani MG**, Gervais DA, Maher MM, Cho CH, Hahn PF, Varghese J, Mueller PR. Transgluteal approach for percutaneous drainage of deep pelvic abscesses: 154 cases. *Radiology* 2003; **228**: 701-705

22 **Ryan JM**, Murphy BL, Boland GW, Mueller PR. Use of the transgluteal route for percutaneous abscess drainage in acute diverticulitis to facilitate delayed surgical repair. *AJR Am J Roentgenol* 1998; **170**: 1189-1193

23 **Michalson AE**, Brown BP, Warnock NG, Simonson TM. Presacral abscesses: percutaneous transperineal drainage with use of bone landmarks and fluoroscopic guidance. *Radiology* 1994; **190**: 574-575

24 **Sperling DC**, Needleman L, Eschelman DJ, Hovsepian DM, Lev-Toaff AS. Deep pelvic abscesses: transperineal US-guided drainage. *Radiology* 1998; **208**: 111-115

25 **Gazelle GS**, Haaga JR, Stellato TA, Gauderer MW, Plecha DT. Pelvic abscesses: CT-guided transrectal drainage. *Radiology* 1991; **181**: 49-51

26 **Stabile BE**, Puccio E, vanSonnenberg E, Neff CC. Preoperative percutaneous drainage of diverticular abscesses. *Am J Surg* 1990; **159**: 99-104; discussion

27 **Mueller PR**, Saini S, Wittenburg J, Simeone J, Hahn PF, Steiner E, Dawson SL, Butch RJ, Stark DD, Ottinger LW. Sigmoid diverticular abscesses: percutaneous drainage as an adjunct to surgical resection in 24 cases. *Radiology* 1987; **164**: 321-325

28 **Neff CC**, vanSonnenberg E, Casola G, Wittich GR, Hoyt DB, Halasz NA, Martini DJ. Diverticular abscesses: percutaneous drainage. *Radiology* 1987; **163**: 15-18

29 **Saini S**, Mueller PR, Wittenberg J, Butch RJ, Rodkey GV, Welch CE. Percutaneous drainage of diverticular abscess. An adjunct to surgical therapy. *Arch Surg* 1986; **121**: 475-478

30 **Ambrosetti P**, Chautems R, Soravia C, Peiris-Waser N, Terrier F. Long-term outcome of mesocolic and pelvic diverticular abscesses of the left colon: a prospective study of 73 cases. *Dis Colon Rectum* 2005; **48**: 787-791

31 **Kumar RR**, Kim JT, Haukoos JS, Macias LH, Dixon MR, Stamos MJ, Konyalian VR. Factors affecting the successful management of intra-abdominal abscesses with antibiotics and the need for percutaneous drainage. *Dis Colon Rectum* 2006; **49**: 183-189

32 **Bahadursingh AM**, Virgo KS, Kaminski DL, Longo WE. Spectrum of disease and outcome of complicated diverticular disease. *Am J Surg* 2003; **186**: 696-701

33 **Siewert B**, Tye G, Kruskal J, Sosna J, Opelka F, Raptopoulos V, Goldberg SN. Impact of CT-guided drainage in the treatment of diverticular abscesses: size matters. *AJR Am J Roentgenol* 2006; **186**: 680-686

34 **Alvarez JA**, Baldonedo RF, Bear IG, Otero J, Pire G, Alvarez P, Jorge JI. Presentation, management and outcome of acute

sigmoid diverticulitis requiring hospitalization. *Dig Surg* 2007; **24**: 471-476
35 **Bernini A**, Spencer MP, Wong WD, Rothenberger DA, Madoff RD. Computed tomography-guided percutaneous abscess drainage in intestinal disease: factors associated with outcome. *Dis Colon Rectum* 1997; **40**: 1009-1013
36 **Schechter S**, Eisenstat TE, Oliver GC, Rubin RJ, Salvati EP. Computerized tomographic scan-guided drainage of intra-abdominal abscesses. Preoperative and postoperative modalities in colon and rectal surgery. *Dis Colon Rectum* 1994; **37**: 984-988
37 **Cinat ME**, Wilson SE, Din AM. Determinants for successful percutaneous image-guided drainage of intra-abdominal abscess. *Arch Surg* 2002; **137**: 845-849
38 **Aydin HN**, Remzi FH. Diverticulitis: when and how to operate? *Dig Liver Dis* 2004; **36**: 435-445
39 **vanSonnenberg E**, Wittich GR, Goodacre BW, Casola G, D'Agostino HB. Percutaneous abscess drainage: update. *World J Surg* 2001; **25**: 362-369; discussion 370-372
40 **Belmonte C**, Klas JV, Perez JJ, Wong WD, Rothenberger DA, Goldberg SM, Madoff RD. The Hartmann procedure. First choice or last resort in diverticular disease? *Arch Surg* 1996; **131**: 612-615; discussion 616-617
41 **Chautems RC**, Ambrosetti P, Ludwig A, Mermillod B, Morel P, Soravia C. Long-term follow-up after first acute episode of sigmoid diverticulitis: is surgery mandatory?: a prospective study of 118 patients. *Dis Colon Rectum* 2002; **45**: 962-966
42 **Parks TG**. Natural history of diverticular disease of the colon. *Clin Gastroenterol* 1975; **4**: 53-69
43 **Mueller MH**, Glatzle J, Kasparek MS, Becker HD, Jehle EC, Zittel TT, Kreis ME. Long-term outcome of conservative treatment in patients with diverticulitis of the sigmoid colon. *Eur J Gastroenterol Hepatol* 2005; **17**: 649-654
44 **Wong WD**, Wexner SD, Lowry A, Vernava A 3rd, Burnstein M, Denstman F, Fazio V, Kerner B, Moore R, Oliver G, Peters W, Ross T, Senatore P, Simmang C. Practice parameters for the treatment of sigmoid diverticulitis--supporting documentation. The Standards Task Force. The American Society of Colon and Rectal Surgeons. *Dis Colon Rectum* 2000; **43**: 290-297
45 **Chapman JR**, Dozois EJ, Wolff BG, Gullerud RE, Larson DR. Diverticulitis: a progressive disease? Do multiple recurrences predict less favorable outcomes? *Ann Surg* 2006; **243**: 876-830; discussion 880-883
46 **Mpofu S**, Mpofu CM, Hutchinson D, Maier AE, Dodd SR, Moots RJ. Steroids, non-steroidal anti-inflammatory drugs, and sigmoid diverticular abscess perforation in rheumatic conditions. *Ann Rheum Dis* 2004; **63**: 588-590
47 **Perkins JD**, Shield CF 3rd, Chang FC, Farha GJ. Acute diverticulitis. Comparison of treatment in immuno-compromised and nonimmunocompromised patients. *Am J Surg* 1984; **148**: 745-748

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TOPIC HIGHLIGHT

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Laser ablation of hepatocellular carcinoma-A review

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Abstract

A wide range of local thermal ablative therapies have been developed in the treatment of non resectable hepatocellular carcinoma (HCC) in the last decade. Laser ablation (LA) and radiofrequency ablation (RFA) are the two most widely used of these. This article provides an up to date overview of the role of laser ablation in the local treatment of HCC. General principles, technique, image guidance and patient selection are discussed. A review of published data on treatment efficacy, long term outcome and complication rates of laser ablation is included and comparison with RFA made. The role of laser ablation in combination with transcatheter arterial chemoembolization is also discussed.

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Key words: Liver; Carcinoma; Hepatocellular; Laser therapy

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INTRODUCTION

Surgical resection or transplantation have in the past been considered the gold standard for treatment of hepatocellular carcinoma (HCC). The overall resectability rate for such lesions is very low due to a combination of underlying chronic liver disease, lesion location and multifocal nature of HCC. Similarly, surgical resection carries a significant associated morbidity and mortality, as well as a disease recurrence rate of up to 75%[1,2]. Local thermal ablative techniques have gained popularity over the last decade proving to be an effective and safe alternative in many patients. These techniques are also cost effective in comparison to other treatments and are able to maximise the preservation of surrounding liver parenchyma whilst minimizing in-patient hospitalization[3]. Laser ablation (LA) represents one of a range of currently available loco-ablative techniques with the majority of reported data coming from Italy, Germany and the UK.

GENERAL PRINCIPLES & TECHNIQUES

The term laser ablation refers to the thermal tissue destruction of tissue by conversion of absorbed light (usually infrared) into heat and includes various technical variations on this theme including "laser coagulation therapy", "laser interstitial tumour therapy" and "laser interstitial photocoagulation"[4].

Infrared energy penetrates tissue directly for a distance of between 12-15 mm, although heat is conducted beyond this range creating a larger ablative zone[5]. Optical penetration has been shown to be increased in malignant tissue compared to normal parenchyma[6]. Temperatures above 60℃ cause rapid coagulative necrosis and instant cell death, but irreversible cell death can also be achieved at lower hyperthermic temperatures (> 42℃) although longer durations (30-60 min) are required[7]. Temperatures above 100℃ will cause vaporisation from evaporation of tissue water and above 300℃ tissue carbonisation occurs. Overheating is thus best avoided as carbonization decreases optical penetration and heat conduction and limits the size of lesion produced[6]. Local tissue properties, in particular perfusion, have a significant impact on the size of ablative zone. Highly perfused tissue and large vessels act

as a heat sink, as laser light is absorbed by erythrocytic heme and transported from the local area[8]. This phenomenon makes native liver parenchyma relatively more resilient to LA than tumor tissue and is the basis for the use of hepatic inflow occlusion techniques in conjunction with laser therapy such as local arterial embolization[9,10].

The most widely used device for LA techniques is the Nd-YAG (neodymium: yttrium-aluminium-garnet) laser with a wavelength of 1064 nm, because penetration of light is optimal at the near-infrared range of the spectrum[11]. More recently, more compact, less expensive diode lasers with shorted wavelengths (800-980 nm) have been used, although the lower tissue penetration produces a smaller volume of destruction.

Light is delivered *via* flexible quartz fibres of diameter from 300-600 μm. Conventional bare tip fibres provides a near spherical lesion of about 15 mm diameter at their ends, but have been largely replaced by interstitial fibres which are quartz fibres that have flat or cylindrical diffusing tips and are 10-40 mm long, providing a much larger ablative area of up to 50 mm[12,13]. The use of beam splitting devices allows the use of up to four fibres at once with corresponding increase in ablative volume, but requires multiple fibres to be placed and only works effectively at lower powers. Beam splitters are, therefore, rarely used with interstitial fibres. With increasing laser power, comes better light transmission and larger ablative zones. However, it also causes increased local temperature rise, risking overheating and carbonization of the adjacent tissue. The use of water cooled laser application sheaths allows a higher laser power output (up to 50 W compared with 5 W) while preventing carbonization[14]. Thus, the use of multiple water-cooled higher power fibres allows ablative zones of up 80 mm diameter. Water cooled sheaths do require wider bore cannulas, but are commonly placed *via* a coaxial dilation system from an 18 G puncture.

IMAGE GUIDANCE

A range of different imaging modalities have been used to guide percutaneous laser ablation techniques determined largely by local experience and resource availability. Ultrasound (US) guided needle placement has the advantage that it is quick, portable, and widely available, as well as being familiar to those used to performing US guided biopsies. The major disadvantage is that it offers little reliable indication of the temperature or extent of the ablative zone being created.

In contrast, magnetic resonance (MR) guided LA, often in conjunction with liver specific contrast agent, e.g. teslascan [mangafodipir trisodium (MnDPDP) Nycomed Imaging, Oslo, Norway], offers real time thermal mapping that allows the operator to visualise the size, location and temperature of the ablation zone[15,16]. This technique tends to be used in conjunction with the higher power water cooled laser systems as the increased energy (for example 40 000 J) can be delivered in a safe and controlled manner[17,18]. By contrast, US guided laser systems tend to use multiple lower power laser fibre with a lower total energy delivery[19]. However, MR guided LA is limited by machine availability and represents a longer procedure.

Thermal imaging can be performed on most MR systems, with thermal changes being easier to demonstrate as magnet field strength increases. There are several methods of measuring tissue temperature changes with MR. The simplest, and most widely used technique, is measuring alterations in T1 value of tissues which decreases in a linear relationship with increasing tissue temperature up to approximately 55℃[20,21]. Techniques that measure changes in the tissue diffusion coefficient are more accurate, up to ± 1℃, but require long acquisition times and, therefore, suffer from patient motion artifacts[22]. Another technique measures changes in the proton resonance frequency shift (phase shift). Again this measures temperature changes very accurately, but is not suitable for use in fatty tissue and is less suited to open MR units as it requires a homogenous magnetic field[23]. All of these techniques are best used in conjunction with subtraction techniques, i.e. pretreatment image subtraction from heating image, which allows very accurate assessment of lesion size, but is sensitive to motion and misregistration artifacts[24].

In addition to thermometry, the use of "open" MR magnets allows real time imaging of needle placement in a truly multiplanar manner [unlike computed tomography (CT)] and is not limited by the presence of bone or gas (unlike US) with the disadvantage that the inherent lower field and gradient strengths of open systems reduce image quality and increase scan time. Conventional closed magnets require fibre placement using CT or US with subsequent transfer into the MR scanner for thermal mapping. This has the disadvantages involved with patient transfer mid procedure, but provides faster imaging and thermal mapping than an open system. The use of MR guidance and thermometry is currently only feasible with LA systems, as it uses a completely metal free system and does not produce any radiofrequency (RF) interference. Most RFA systems are currently not suitable for MR usage, both because of steel within the electrodes and the degradation of image quality by extraneous RF noise produced by the RF generators. Although MR compatible systems have been developed in practice the RF noise remains problematic[16].

There is variation across the literature regarding patient analgesia during laser ablation with some groups providing conscious sedation and intravenous analgesia whilst others preferring general anaesthesia. General anaesthesia allows higher tolerable energy delivery and better control of respiration, but has significant resource implications, has its own associated morbidity rates and may affect patient inclusion/selection.

The use of local ablative techniques in combination with surgery has been explored using RFA and cryoablation[25]. While technically feasible, there is little recently published experience of LA usage at laparoscopy or laparotomy.

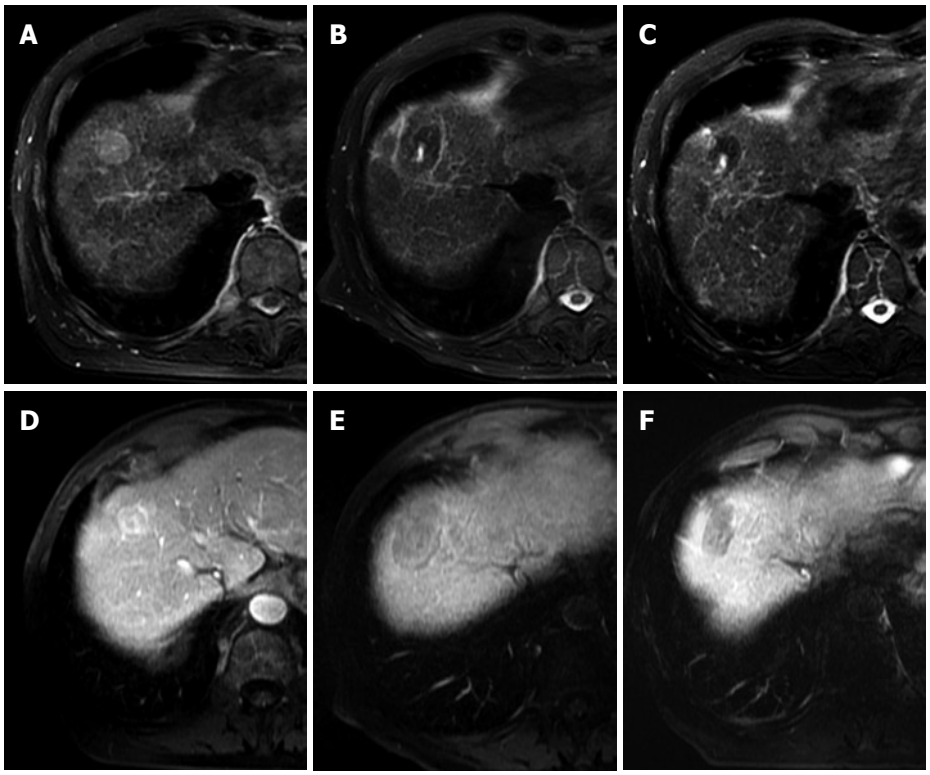

Figure 1 Typical MRI appearances pre and post laser ablation of a small HCC in a 73-year-old male patient with hepatitis C cirrhosis. T2-weighted axial images demonstrate a high signal 2.5 cm segment Ⅷ HCC (A). At one month post treatment (B) a typical low signal laser burn is demonstrated with high signal centre which has shrunk in size at 10 mo follow up (C). T1-weighted post contrast images of the same lesion demonstrate heterogeneous arterial enhancement pre-treatment (D), with non-enhancing scar at 1 mo (E) and persistent non-enhancement at 10 mo (F).

PATIENT SELECTION

Selection criteria vary from unit to unit dependant on the technique used and facilities available, but are broadly similar to those for other local ablative techniques and are based on size, number and site of HCC in patients who are deemed unsuitable for surgical resection or transplant. Laser ablation is also considered a valid interim treatment while awaiting transplant surgery. Patients are generally considered if there are less than 5 lesions of 5 cm or less. Lesions larger than this can be considered, particularly if using a high power system, though they may require more than one treatment. The ideal lesions are those less than 3 cm and deep within liver parenchyma. Lesions adjacent to major vessels, biliary ducts, bowel or diaphragm can be treated with caution. MR guided techniques allow confident ablation of anatomically more problematic lesions with the use of real time thermometry and multiplanar MR targeting. Severe liver disease (Childs C) and coagulopathy are relative contraindications to be considered in each individual patient and extra hepatic disease is generally considered an absolute contraindication.

EFFECTIVENESS

Several factors make interpretation of published outcome data difficult. While the majority of patients have HCC secondary to viral hepatitis, they represent a diverse cohort in terms of disease severity. Differing laser fiber systems with differing energy delivery levels make comparison of both efficacy and complication rates difficult to compare. Again, the range of imaging modalities used at follow-up, combined with a variety of definitions of treatment success, make comparison of data difficult.

Lastly, current guidelines suggest that formal tissue biopsy of HCC is not recommended in every case due to potential risks of tumour seeding. Many groups, therefore, assume the diagnosis of HCC based on history, imaging appearances and rising alpha fetoprotein (αFP) levels. The inevitable occasional inclusion of some lesions that are not in fact HCCs may, therefore, favorably skew outcome data.

When assessing the literature regarding efficacy of laser ablation, a clear distinction must be drawn between primary effectiveness rates and long term outcome rates. Primary effectiveness relates to complete lesion ablation as assessed by either CT or MR at a defined point post procedure and after a defined number of treatments. This does not necessarily correspond to improved long term outcomes. However, data regarding long term survival rates for LA is scarce and in part reflects the novelty of the treatment and the rapid advances in technology, particularly in relation to the laser fibres. Pacella *et al*[26] reported long term survival rates of 89%, 52% and 27% for 1, 3 and 5 years, respectively, in a series of 169 sub 40 mm lesions in 148 patients (144 biopsy proven HCC) treated with 239 sessions. They quote an overall 82% complete lesion ablation rate[26]. The same group reported an earlier series of 74 patients with 92 biopsy proven sub 4 cm HCCs with a complete ablation rate of 97% and survival rates of 99%, 68% and 15% for 1, 3 and 5 years, respectively[27] (Figure 1).

Results reported by those groups using water cooled higher power MR guided laser ablation have been promising. Eichler *et al*[28] reported mean survival rates of 4.4 years (95% CI: 3.6-5.2) in a series of 39 patients with 61 presumed HCCs with a complete ablation rate

of 97.5%. Although the same groups have provided compelling long term survival data for the ablation of hepatic metastases, no such data has to date been published for HCC ablation[29].

COMPARISON WITH OTHER ABLATIVE TECHNIQUES

A report by Ferrari *et al*[30] is the only randomized prospective study comparing LA with RF ablation. They treated 81 cirrhotic patients with 95 biopsy proven, sub 4 cm HCCs. Two matched groups were randomised to US guided RF or LA under general anaesthetic. LA was *via* multiple 5 W fibres delivering a maximum of 1800 J per fibre per treatment. Post treatment, lesions were evaluated with CT. They reported no significant difference overall in survival rates between the two techniques with cumulative rates of 91.8%, 59.0%, and 28.4% at 1, 3 and 5 years, respectively. They did, however, demonstrate a statistically significant higher survival rate for RFA over LA for Child A patients ($P = 0.9966$) and nodules $\leqslant$ 25 mm ($P = 0.0181$). They reported no significant complications[30]. This work added to the same groups' previous published improved survival rates in patients treated with LA, compared to those treated with either transcatheter arterial chemoembolization (TACE) or percutaneous ethanol injection (PEI)[31].

COMBINATION WITH TACE

The rationale of combining LA with TACE is attractive, particularly for large lesions. Pacella *et al*[19] described the use of LA followed by TACE in the treatment of 30 large (3.5-9.6 cm) HCCs. They achieved 90% ablation rate with survival rates of 92%, 68% and 40% at 1, 2, and 3 years, respectively. No significant complications were reported, leading the authors to conclude that combination therapy was a safe and effective palliative therapy in the treatment of large HCCs[19]. Ferrari *et al*[32] supported this suggestion demonstrating improved ablation rates and survival in patients with HCCs > 5 cm given combination treatment over LA alone.

COMPLICATIONS

Reported complication rates for laser ablation compare favourably to surgical rates.

Arienti *et al*[33] reported complication rates for 520 patients, with 647 presumed HCCs treated with 1004 laser sessions (local anaesthetic, US guided). They report 0.8% deaths and 1.5% major complication rate. An earlier report by Vogl *et al*[34] included 899 patients (of which 42 had HCC), with 2520 lesions treated with 2132 laser sessions (local anaesthetic, CT/MRI guided) and reported 0.1% mortality and 1.8% major complication rate. Included in the major complications were liver failure/segmental infarction, hepatic abscess/cholangitis, bile duct injury, and haemorrhage (intrahepatic/peritoneal/gastrointestinal). Both groups reported common side effects of asymptomatic pleural effusion (7.3% & 6.9%), post procedural fever (33.3% & 12.3%) and severe pain (7.5% & 11.5%). While tumor seeding after percutaneous biopsy and ablative therapies is well recognized, it has rarely been reported after laser ablation and neither of the above groups reported this complication.

CONCLUSION

LA of HCC remains a safe and effective local therapy for patients in which surgical resection is not possible or appropriate. Nevertheless, further data from randomized trials are required to establish long term survival rates, particularly for higher power water cooled systems before the treatment can be fully validated as a standard treatment. Further data is required to establish its role in combination therapy, either with other percutaneous treatments and/or surgery. At this stage, RFA remains the standard of care, but as the technology and experience progress the technique will be in a position to be randomised against RFA as an effective alternate primary treatment of HCC in an attempt to establish equivalent efficacy/long term survival.

REFERENCES

1 **Zhao WH**, Ma ZM, Zhou XR, Feng YZ, Fang BS. Prediction of recurrence and prognosis in patients with hepatocellular carcinoma after resection by use of CLIP score. *World J Gastroenterol* 2002; **8**: 237-242

2 **Llovet JM**, Fuster J, Bruix J. Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. *Hepatology* 1999; **30**: 1434-1440

3 **Llovet JM**, Mas X, Aponte JJ, Fuster J, Navasa M, Christensen E, Rodes J, Bruix J. Cost effectiveness of adjuvant therapy for hepatocellular carcinoma during the waiting list for liver transplantation. *Gut* 2002; **50**: 123-128

4 **Goldberg SN**, Grassi CJ, Cardella JF, Charboneau JW, Dodd GD 3rd, Dupuy DE, Gervais D, Gillams AR, Kane RA, Lee FT Jr, Livraghi T, McGahan J, Phillips DA, Rhim H, Silverman SG. Image-guided tumor ablation: standardization of terminology and reporting criteria. *Radiology* 2005; **235**: 728-739

5 **Jacques SL**. Laser-tissue interactions. Photochemical, photothermal, and photomechanical. *Surg Clin North Am* 1992; **72**: 531-558

6 **Germer CT**, Roggan A, Ritz JP, Isbert C, Albrecht D, Muller G, Buhr HJ. Optical properties of native and coagulated human liver tissue and liver metastases in the near infrared range. *Lasers Surg Med* 1998; **23**: 194-203

7 **Thomsen S**. Pathologic analysis of photothermal and photomechanical effects of laser-tissue interactions. *Photochem Photobiol* 1991; **53**: 825-835

8 **Whelan WM**, Wyman DR, Wilson BC. Investigations of large vessel cooling during interstitial laser heating. *Med Phys* 1995; **22**: 105-115

9 **Muralidharan V**, Malcontenti-Wilson C, Christophi C. Effect of blood flow occlusion on laser hyperthermia for liver metastases. *J Surg Res* 2002; **103**: 165-174

10 **Wacker FK**, Reither K, Ritz JP, Roggan A, Germer CT, Wolf KJ. MR-guided interstitial laser-induced thermotherapy of hepatic metastasis combined with arterial blood flow reduction: technique and first clinical results in an open MR system. *J Magn Reson Imaging* 2001; **13**: 31-36

11 **Wyman DR**, Whelan WM, Wilson BC. Interstitial laser photocoagulation: Nd:YAG 1064 nm optical fiber source compared to point heat source. *Lasers Surg Med* 1992; **12**: 659-664

12 **Huang GT**, Wang TH, Sheu JC, Daikuzono N, Sung JL, Wu MZ, Chen DS. Low-power laserthermia for the treatment of small hepatocellular carcinoma. *Eur J Cancer* 1991; **27**: 1622-1627

13 **Muralidharan V**, Malcontenti-Wilson C, Christophi C. Interstitial laser hyperthermia for colorectal liver metastases: the effect of thermal sensitization and the use of a cylindrical diffuser tip on tumor necrosis. *J Clin Laser Med Surg* 2002; **20**: 189-196

14 **Vogl TJ**, Straub R, Zangos S, Mack MG, Eichler K. MR-guided laser-induced thermotherapy (LITT) of liver tumours: experimental and clinical data. *Int J Hyperthermia* 2004; **20**: 713-724

15 **Joarder R**, de Jode M, Lamb GA, Gedroyc WM. The value of MnDPDP enhancement during MR guided laser interstitial thermoablation of liver tumors. *J Magn Reson Imaging* 2001; **13**: 37-41

16 **Gedroyc WM**. Magnetic resonance guidance of thermal ablation. *Top Magn Reson Imaging* 2005; **16**: 339-353

17 **Vogl TJ**, Eichler K, Straub R, Engelmann K, Zangos S, Woitaschek D, Bottger M, Mack MG. Laser-induced thermotherapy of malignant liver tumors: general principals, equipment(s), procedure(s)--side effects, complications and results. *Eur J Ultrasound* 2001; **13**: 117-127

18 **Dick EA**, Joarder R, de Jode M, Taylor-Robinson SD, Thomas HC, Foster GR, Gedroyc WM. MR-guided laser thermal ablation of primary and secondary liver tumours. *Clin Radiol* 2003; **58**: 112-120

19 **Pacella CM**, Bizzarri G, Cecconi P, Caspani B, Magnolfi F, Bianchini A, Anelli V, Pacella S, Rossi Z. Hepatocellular carcinoma: long-term results of combined treatment with laser thermal ablation and transcatheter arterial chemoembolization. *Radiology* 2001; **219**: 669-678

20 **Botnar R**. Temperature sensitive MR sequences. In: Debatin J, Adam G, eds. Interventional Magnetic Resonance Imaging. Heidelberg, Germany: Springer, 1998: 171-177

21 **Matsumoto R**, Mulkern RV, Hushek SG, Jolesz FA. Tissue temperature monitoring for thermal interventional therapy: comparison of T1-weighted MR sequences. *J Magn Reson Imaging* 1994; **4**: 65-70

22 **MacFall J**, Prescott DM, Fullar E, Samulski TV. Temperature dependence of canine brain tissue diffusion coefficient measured in vivo with magnetic resonance echo-planar imaging. *Int J Hyperthermia* 1995; **11**: 73-86

23 **Peters RD**, Hinks RS, Henkelman RM. Heat-source orientation and geometry dependence in proton-resonance frequency shift magnetic resonance thermometry. *Magn Reson Med* 1999; **41**: 909-918

24 **Dick EA**, Wragg P, Joarder R, de Jode M, Lamb G, Gould S, Gedroyc WM. Feasibility of abdomino-pelvic T1-weighted real-time thermal mapping of laser ablation. *J Magn Reson Imaging* 2003; **17**: 197-205

25 **Tait IS**, Yong SM, Cuschieri SA. Laparoscopic in situ ablation of liver cancer with cryotherapy and radiofrequency ablation. *Br J Surg* 2002; **89**: 1613-1619

26 **Pacella CM**, Bizzarri G, Francica G, Forlini G, Petrolati A, Valle D, Anelli V, Bianchini A, Nuntis SD, Pacella S, Rossi Z, Osborn J, Stasi R. Analysis of factors predicting survival in patients with hepatocellular carcinoma treated with percutaneous laser ablation. *J Hepatol* 2006; **44**: 902-909

27 **Pacella CM**, Bizzarri G, Magnolfi F, Cecconi P, Caspani B, Anelli V, Bianchini A, Valle D, Pacella S, Manenti G, Rossi Z. Laser thermal ablation in the treatment of small hepatocellular carcinoma: results in 74 patients. *Radiology* 2001; **221**: 712-720

28 **Eichler K**, Mack MG, Straub R, Engelmann K, Zangos S, Woitaschek D, Vogl TJ. [Oligonodular hepatocellular carcinoma (HCC): MR-controlled laser-induced thermotherapy] *Radiologe* 2001; **41**: 915-922

29 **Vogl TJ**, Straub R, Eichler K, Sollner O, Mack MG. Colorectal carcinoma metastases in liver: laser-induced interstitial thermotherapy--local tumor control rate and survival data. *Radiology* 2004; **230**: 450-458

30 **Ferrari FS**, Megliola A, Scorzelli A, Stella A, Vigni F, Drudi FM, Venezia D. Treatment of small HCC through radiofrequency ablation and laser ablation. Comparison of techniques and long-term results. *Radiol Med* (Torino) 2007; **112**: 377-393

31 **Ferrari FS**, Stella A, Pasquinucci P, Vigni F, Civeli L, Pieraccini M, Magnolfi F. Treatment of small hepatocellular carcinoma: a comparison of techniques and long-term results. *Eur J Gastroenterol Hepatol* 2006; **18**: 659-672

32 **Ferrari FS**, Stella A, Gambacorta D, Magnolfi F, Fantozzi F, Pasquinucci P, Civeli L, Pieraccini M. Treatment of large hepatocellular carcinoma: comparison between techniques and long term results. *Radiol Med* (Torino) 2004; **108**: 356-371

33 **Arienti V**, Pretolani S, Pacella CM, Magnolfi F, Caspani B, Francica G, Megna AS, Regine R, Sponza M, Antico E, Di Lascio FM. Complications of laser ablation for hepatocellular carcinoma: a multicenter study. *Radiology* 2008; **246**: 947-955

34 **Vogl TJ**, Straub R, Eichler K, Woitaschek D, Mack MG. Malignant liver tumors treated with MR imaging-guided laser-induced thermotherapy: experience with complications in 899 patients (2,520 lesions). *Radiology* 2002; **225**: 367-377

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Gamma-aminobutyric acid promotes human hepatocellular carcinoma growth through overexpressed gamma-aminobutyric acid A receptor α3 subunit

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Abstract

AIM: To investigate the expression pattern of gamma-aminobutyric acid A (GABAA) receptors in hepatocellular carcinoma (HCC) and indicate the relationship among gamma-aminobutyric acid (GABA), gamma-aminobutyric acid A receptor α3 subunit (GABRA3) and HCC.

METHODS: HCC cell line Chang, HepG2, normal liver cell line L-02 and 8 samples of HCC tissues and paired non-cancerous tissues were analyzed with semiquantitative polymerase chain reaction (PCR) for the expression of GABAA receptors. HepG2 cells were treated with gamma-aminobutyric acid (GABA) at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L), and their proliferating abilities were analyzed with the 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, cell doubling time test, colon formation assay, cell cycle analysis and tumor planted in nude mice. Small interfering RNA was used for knocking down the endogenous GABRA3 in HepG2. Proliferating abilities of these cells treated with or without GABA were analyzed.

RESULTS: We identified the overexpression of GABRA3 in HCC cells. Knockdown of endogenous GABRA3 expression in HepG2 attenuated HCC cell growth, suggesting its role in HCC cell viability. We determined the *in vitro* and *in vivo* effect of GABA in the proliferation of GABRA3-positive cell lines, and found that GABA increased HCC growth in a dose-dependent manner. Notably, the addition of GABA into the cell culture medium promoted the proliferation of GABRA3-expressing HepG2 cells, but not GABRA3-knockdown HepG2 cells. This means that GABA stimulates HepG2 cell growth through GABRA3.

CONCLUSION: GABA and GABRA3 play important roles in HCC development and progression and can be a promising molecular target for the development of new diagnostic and therapeutic strategies for HCC.

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Key words: Hepatocellular carcinoma; Proliferation; Gamma-aminobutyric acid; Gamma-aminobutyric acid A receptor α3 subunit; RNAi

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide[1]. Although liver resection and local ablation are regarded as potentially curative treatment[2], its prognosis is poor. Up to 40% of patients are diagnosed at an advanced stage, and only palliative treatment can be offered.

To change this situation, the development of new molecular therapies against good targets is an urgent issue. To this direction, we previously used a method by combining the *in silico* screen and experimental verification to identify genes that are differently expressed in cancers compared with their corresponding normal tissues. Among genes that are overexpressed in

HCC cells, we focused on the gene gamma-aminobutyric acid (GABA) A receptor α3 subunit (GABRA3). GABRA3 is a subunit of the GABAA receptors that may associate with other GABAA receptor subunits to form a functional chloride channel that mediates the inhibitory synaptic transmission in the mature central nervous system (CNS). GABA primarily functions as an inhibitory neurotransmitter in the mature CNS by activating the GABA receptor, but can also modulate the proliferation, migration and differentiation of neuronal cells during CNS development[3-5] and the proliferation of peripheral non-neuronal cells[6,7]. GABA and GABAA receptors are also present in peripheral tissues, including cancerous cells, but their precise functions are poorly defined.

This study demonstrates that GABRA3 overexpressed in HCC and GABA promoted the proliferation of cancer cells through GABRA3.

MATERIALS AND METHODS

Cell lines

HCC cell line Chang, HepG2 and normal liver cell line L-02 were maintained by our lab. All cell lines were cultured in DMEM supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Cells were maintained at 37℃ in an atmosphere of humidified air with 5% CO_2.

Collection of tissues

All samples of HCC tissues and paired non-cancerous tissues (5 cm away from tumor) were obtained during surgical resection from the First Affiliated Hospital of Xiangya School of Medicine. Written consent was obtained from the patients, who agreed with the collection of tissue samples. The resected tissue samples were immediately cut into small pieces, and snap-frozen in liquid nitrogen until use. All tumor tissue and paired non-cancerous tissue samples were pathologically confirmed.

Semiquantitative polymerase chain reaction (PCR)

Total RNA from HepG2, Chang L-02 cell lines and liquid-nitrogen-frozen tissue samples were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 2 μg of DNase I -treated total RNA using oligo (dT) primer with SuperscriptTM Ⅱ reverse transcriptase (Invitrogen) for 60 min at 42℃. We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring β-actin as a quantitative control. The sets of primer for GABAA receptor subunits are shown in Table 1, 5'-AGCAAGAGAGGCATCCTCA-3' and 5'-TCAGGCAGCTCGTAGCTCT-3' for β-actin, 554bp. One μL of each cDNA product was amplified in a mixture containing 5 pmol primers, 200 μmol/L dNTPs, and 1 unit Taq DNA polymerase with reaction buffer in a final volume of 20 μL. PCR was performed using the following parameters: initial denaturation at 95℃ for 1 min, 30 cycles of 30 s at 95℃, 30 s at 60℃, and 1 min 30 s at 68℃, followed by a final 10 min extension at 68℃. Reaction products were separated on 1.5% agarose gels containing ethidium bromide and the level of amplification was determined using a Phosphor Imager.

Table 1 Primers for GABAA receptor subunits

Subunit	Primer	Product size (bp)
α1		902
Sense	5'-TCGTCACCAGTTTCGGACC-3'	
Antisense	5'-GGTTGCTGTTGGAGCGTAA-3'	
α2		722
Sense	5'-TTCACAATGGGAAGAAATCAGTAG-3'	
Antisense	5'-TGCATAAGCGTTGTTCTGTATCA-3'	
α3		561
Sense	5'-TCGGTCTCTCCAAGTTTGTGC-3'	
Antisense	5'-TTCCGTTGTCCACCAATCTGA-3'	
α4		750
Sense	5'-TGAAATTCGGGAGTTATGCCTATC-3'	
Antisense	5'-GGCTGAATGGGTTTGGACTG-3'	
α5		826
Sense	5'-CACCATGCGCTTGACCATCTCT-3'	
Antisense	5'-GCCGAACAAGACTGGGAATA-3'	
α6		764
Sense	5'-TGAGGCTTACCATCAATGCTGA-3'	
Antisense	5'-GACAGGTGTTGATTGTAAGATGGG-3'	
β1		603
Sense	5'-GTTCTCTATGGACTCCGAATCACA-3'	
Antisense	5'-ATTGGCACTCTGGTCTTGTTTG-3'	
β2		640
Sense	5'-AGCTTAAGAGAAACATTGGCTACT-3'	
Antisense	5'-CGATCTATGGCATTCACATCA-3'	
β3		633
Sense	5'-AGTGCTGTATGGGCTCAGAATCAC-3'	
Antisense	5'-CCCGGTTGCTTTCGCTCTT-3'	
γ1		262
Sense	5'-GTGTTTTGCAGCCTTGATGG-3'	
Antisense	5'-TGGCAATGCGTATGTGTATCCT-3'	
γ2		605
Sense	5'-AAGTCCTCCGATTGAACAGCAACA-3'	
Antisense	5'-CGCTGTGACATAGGAGACCTT-3'	
γ3		767
Sense	5'-ACACTCCTGCCCGCTGATT-3'	
Antisense	5'-TGTCTATGTGAATACGCCCTTTCC-3'	
Δ		654
Sense	5'-TCACCATCACCAGCTACCACTTCA-3'	
Antisense	5'-GGGCGTAAATGTCAATGGTGTC-3'	
ε		632
Sense	5'-GCAGGCGGTTTGGCTATGT-3'	
Antisense	5'-CGAGTAGTTATCCAGGCGGTAG-3'	
θ		465
Sense	5'-TCGAGTTCTCCTCTGCTGTG -3'	
Antisense	5'-TATGCAGATCCAGGGACAA-3'	
π		250
Sense	5'-CGTCGAGGTCGGCAGAAGT-3'	
Antisense	5'-GCGGGCATCCAGAGTGAAG-3'	

RNA interference

To knock down GABRA3 expression, we used pGCsi-U6/Neo/GFP vector encoding a small hairpin RNA directed against the target gene in HepG2. The target sequences for GABRA3 were 5'-GCACTGACA ACATCACTAT-3' (Si-1), 5'-CTGAGACCAAGACCT

ACAA-3' (Si-2), 5'-GATCCTTCCACTGAACAAT-3' (Si-3). As a negative control, we used shRNA vector without hairpin oligonucleotides (Si-Mock). Human HCC cell line HepG2 was plated onto 6-well plates, and transfected with these small interfering RNA (siRNA) expression vectors using FuGENE6 (Roche) according to the instructions of the manufacturer, followed by 800 μg/mL of neomycin selection. The cells were harvested 10 d later to analyze the knockdown effect on GABRA3 by RT-PCR using the primers shown in Table 1 and by flow cytometry (FCM) using rabbit anti-human polyclone antibody against GABRA3 (Chemicon).

Cell proliferation test *in vitro*

Growth experiments of HepG2, HepG2/Si-1, and HepG2/Si-Mock were performed using the 3-(4,5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, cell doubling time test, soft agar cloning formation assay and were reconfirmed with cell cycle analysis, which was performed by flow cytometry.

In the MTT assay, cells were seeded with serum-free medium at a density of 10^3 cells/well in 96-well plates ($n = 6$), grown overnight, washed in PBS, and incubated with GABA (Sigma-Aldrich) at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L) in appropriate medium supplemented with 1% FBS. The samples were tested every 24 h for 6 d. MTT was added (50 μg/well) for 4 h. Formazan products were solubilized with DMSO, and the optical density was measured at 570 nm.

In the cell doubling time test, cells were seeded at a density of 10^4 cells/well in 24-well plates ($n = 3$), grown with serum-free medium overnight, and incubated with GABA at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L) in DMEM medium with 1% FBS. Cells were collected 6 d later, and cell densities were assessed by counting the cells in a hemocytometer. Cell doubling time (TD) was calculated with the following formula: TD=t × [lg2/(lgNt-lgNo)] [t: Period of culturing (hour); No: cell density when the cells were seeded; Nt: cell density when the cells were cultured "t" hours].

Soft agar colony formation assay was used to assess the anchorage-independent growth ability of cells. The cells were incubated with GABA at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L) in DMEM medium overnight. A total of 1000 single-cell suspension cells were resuspended in 1 mL growth media containing 0.3% low melting temperature agarose and GABA at different concentrations (0, 1, 10, 20, 40 and 60 μmol/L) respectively and were plated in triplicate on 6-well plates over a base layer of 1 mL growth media containing 0.6% low melting temperature agarose. Colonies > 50 μm were counted 14 d after plating.

For flow cytometry, cells were incubated with serum-free medium for 24 h, and then cultured in DMEM with 10% FBS in the presence or absence of 40 μmol/L of GABA for 48 h. Cells were harvested and resuspended in fixation fluid at a density of 10^6/mL, 1500 μL propidium iodide (PI) solution was added, and the cell cycle was detected by FACS Caliber (Becton Dickinson).

Tumor implanted in nude mice

Male 8-wk-old BALB/c nude (nu/nu) mice were purchased from Slac Laboratory Animal Co. Ltd (Shanghai, China). HepG2, HepG2/Mock and HepG2/Si-1 cells were treated with or without GABA (40 μmol/L) for 24 h first, and then the cells (3×10^6) were suspended in 0.2 mL of extracellular matrix gel and injected sc in the left back flank of these animals. The mice were divided into six groups: (a) the mice were injected with HepG2 and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2, $n = 5$); (b) the mice were injected with HepG2 and treated with GABA injections (40 μmol/L in 150 μL of 0.9% NaCl) into the tumor (HepG2 + GABA, $n = 5$); (c) the mice were injected with HepG2/Mock and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2/Si-Mock, $n = 5$); (d) the mice were injected with HepG2/Mock and treated with GABA injections (40 μmol/L in 150 μL of 0.9% NaCl) into the tumor (HepG2/Si-Mock + GABA, $n = 5$); (e) the mice were injected with HepG2/Si-1 and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2/Si-1, $n = 5$); (f) the mice were injected with HepG2/Si-1 and treated with GABA injections (40 μmol/L in 150 μL of 0.9% NaCl) into the tumor (HepG2/Si-1 + GABA, $n = 5$). The same operator did the injections every other day starting from "day 0" when the tumors were implanted. Tumor variables were measured every three days by an electronic caliper, and volume was determined as tumor volume (mm^3) = length (mm) × width^2 (mm^2) × 0.523. The measurement started from the first week, when the tumor mass was well established. A third operator, in a coded and blinded fashion, evaluated the morphometric variables.

Statistics

Results were expressed as mean ± SD. Student's t test was used, and $P < 0.05$ was considered statistically significant.

RESULTS

Expression of GABAA receptors

We documented GABAA mRNA expression in 8 pairs of HCC and adjacent non-tumor tissues as well as HepG2, Chang, L-02 cell lines. The results of semiquantitative RT-PCR on a scale of + (limited expression) to +++ (highly expressed) are shown in Table 2.

GABAA-α3, β3, δ, ε, and θ receptor subunits were detected in HepG2 and α3, δ, ε, and θ in Chang liver cells by RT-PCR. In L-02, only ε subunit was detected. In tissues adjacent to HCC, GABAA receptor expression was limited to GABAA-β3, ε and π. In HCC tissues, although GABAA-ε expression remained largely unchanged, GABAA-α3 mRNA expression was consistently (6/8) and significantly increased, and GABAA-δ and θ isoforms were also detected.

Effect of GABRA3-siRNA on the growth of HCC cells

To investigate the biological significance of GABRA3

Table 2 GABAA receptor subunits mRNA expression in HepG2, Chang, L-02 and 8 sets of human HCC and adjacent nontumor tissues

Set	GABAA receptor subunits															
	α1	α2	α3	α4	α5	α6	β1	β2	β3	γ1	γ2	γ3	Δ	ε	θ	π
Cell line																
HepG2			+++						+				+	++	++	
Chang			+++										+	++	+	
L-02														++		
Tumor																
1			+++						+				+	++		
2			++						+		+			+		+
3	+		+++										+	++		++
4									+				+	++		
5			++			+			++							+
6			++						+		+		+	+		
7													+	++		+
8			+						+				+	++		
Non-tumor																
1									+					++		+
2									++					++		+
3									+					++		
4									+					+		+
5									+					++		
6									++					++		++
7									+					+		
8									++					+		

Table 3 Cell cycle of HepG2/Si-1 and HepG2/Si-Mock (mean ± SD)

	Si-1	Si-Mock
G1/G0%	56.1 ± 3.36	50.9 ± 3.14
G2/M%	14.3 ± 1.72	14.2 ± 1.43
S%[1]	29.5 ± 2.26	34.5 ± 2.37

[1]$n = 3$, $P < 0.05$.

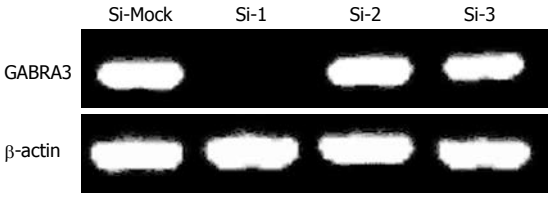

Figure 1 RT-PCR confirmed the knockdown effect on GABRA3 expression by Si-1, but not by Si-2, Si-3 and a negative control Si-Mock in HepG2 cells. β-actin was used to quantify RNAs.

overexpression in HCC cells, we constructed three siRNA expression vectors (Si-1, Si-2, and Si-3) specific to GABRA3 transcripts and transfected them into HepG2 cells that endogenously expressed high levels of GABRA3 as shown in Table 2. A knockdown effect was observed by RT-PCR when we transfected Si-1, but not Si-2, Si-3 or a negative control Si-Mock (Figure 1). MTT assays (Figure 2A) and colony formation (Figure 2B and C) revealed a drastic reduction in the number of cells transfected with Si-1 compared with Si-Mock for which no knockdown effect was observed. The cell doubling time of the cells transfected with Si-1 was 8.7 ± 0.183 d, while Si-Mock was 5.3 ± 0.125 d (n =3, $P < 0.05$). Cell proliferation detected by flow cytometry is shown in Figure 2D and Table 3. This result was consistent with the results above. The tumor size of the nude mice injected with HepG2/Si-1 (Group e) was significantly smaller than the mice injected with HepG2/Si-Mock (Group c) (Figure 2E).

Effect of GABA on the growth of HCC cells

To examine the effect of GABA on the growth of GABRA3-expressing HCC cells, we treated GABRA3-positive HCC cells HepG2 with GABA at several concentrations. As shown in Figure 3 and Table 4, the addition of GABA in the culture media enhanced the proliferation of HepG2 cells in a dose-dependent manner. The promoting effect on HCC cell proliferation was more evident with the GABA concentration from 1 μmol/L to 40 μmol/L. When the GABA concentration was up to 60 μmol/L, the promoting effect became insignificant. We also tried GABA at concentrations of 100 μmol/L, 200 μmol/L and 400 μmol/L, but there was no significant effect (data not shown). The effect of GABA persisted up to 5 d. The cell doubling times of HepG2 cells treated with 0, 1, 10, 20, 40 and 60 μmol/L were 5.1 ± 0.128, 2.73 ± 0.052, 2 ± 0.057, 1.51 ± 0.044, 1.3 ± 0.064 and 4.1 ± 0.085 d.

In the nude mice implanted with tumors (injected with HepG2 cells), a significant difference in tumor size was found in GABA-treated (at the concentration of 40 μmol/L) mice compared with mice injected with 0.9% NaCl only (Figure 3E).

GABA stimulated HCC cell proliferation through GABRA3

To examine the function of GABRA3 as a GABA receptor on the growth of GABRA3-expressing HCC

Table 4 Cell cycle of HepG2 treated with GABA at serial concentrations (mean ± SD)

	0 μmol/L	1 μmol/L	10 μmol/L	20 μmol/L	40 μmol/L	60 μmol/L
G1/G0%	59.7 ± 3.82	58.5 ± 3.63	55.6 ± 3.46	56.5 ± 3.69	45.2 ± 3.07	57.6 ± 3.83
G2/M%	13.5 ± 1.14	18.6 ± 1.27	12.5 ± 1.15	7.6 ± 0.79	17.3 ± 1.48	12.7 ± 1.53
S%[1]	27.2 ± 2.19	31.3 ± 2.24	32.4 ± 2.58	35.8 ± 2.47	37.6 ± 2.64	30.4 ± 2.32

[1]$n = 3$, $P < 0.05$.

Table 5 Cell cycle of HepG2/Si-Mock and HepG2/Si-1 treated with or without GABA at a concentration of 40 μmol/L (mean ± SD)

	HepG2/Si-Mock	HepG2/Si-Mock + GABA	HepG2/Si-1	HepG2/Si-1 + GABA
G1/G0%	51.7 ± 3.53	49.3 ± 3.23	54.5 ± 3.37	59.4 ± 3.85
G2/M%	15.7 ± 1.75	7.2 ± 0.92	17.6 ± 1.64	15.6 ± 1.69
S%	32.4 ± 2.12	43.6 ± 2.84[1]	27.6 ± 1.92	25.5 ± 2.06

[1]HepG2/Si-Mock *vs* HepG2/Si-Mock + GABA, $n = 3$, $P < 0.05$.

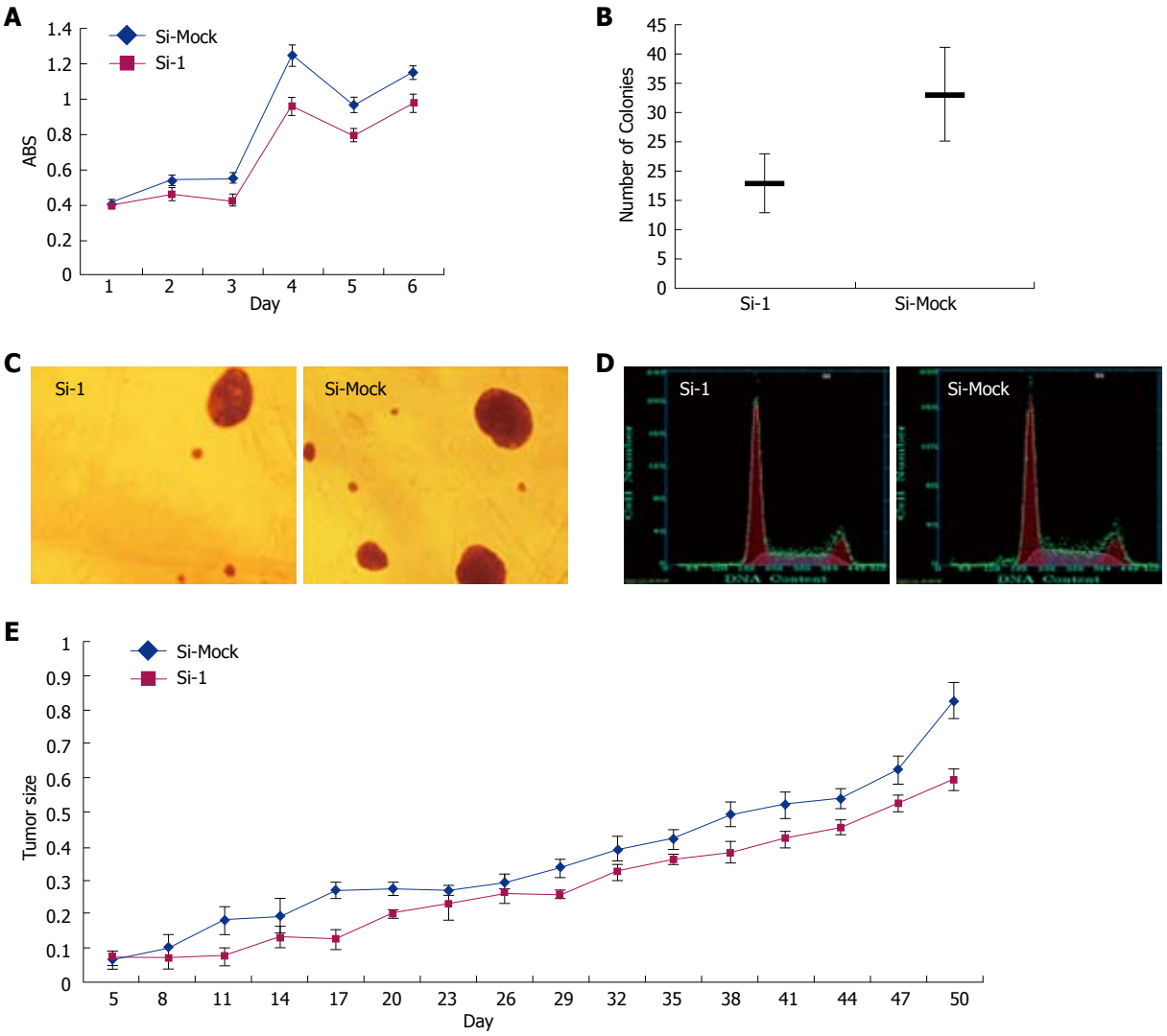

Figure 2 Effect of GABRA3-siRNA on the growth of HCC cells. A: MTT assay of HepG2 cells transfected with Si-1 vectors to GABRA3 and a negative control vector (Si-Mock). Y-axis: Average value of absorbance (ABS) at 570 nm, measured with a microplate reader ($n = 6$, $P < 0.05$); B and C: Soft agar colony formation assay of HepG2/Si-1 and HepG2/Si-Mock. Same cells were incubated in low-melting agarose as described in Materials and Methods. Two weeks later, colonies were photographed and numbers of colonies were counted. ($n = 3$, $P < 0.05$); D: Cell cycle of HepG2/Si-1 and HepG2/Si-Mock measured by flow cytometry; E: Tumor volume in nude mice with HepG2/Si-1 or HepG2/Si-Mock injected (mm^3).

cells, we treated HepG2/Si-1 and HepG2/Si-Mock cells with or without GABA (40 μmol/L). As shown in Figure 4

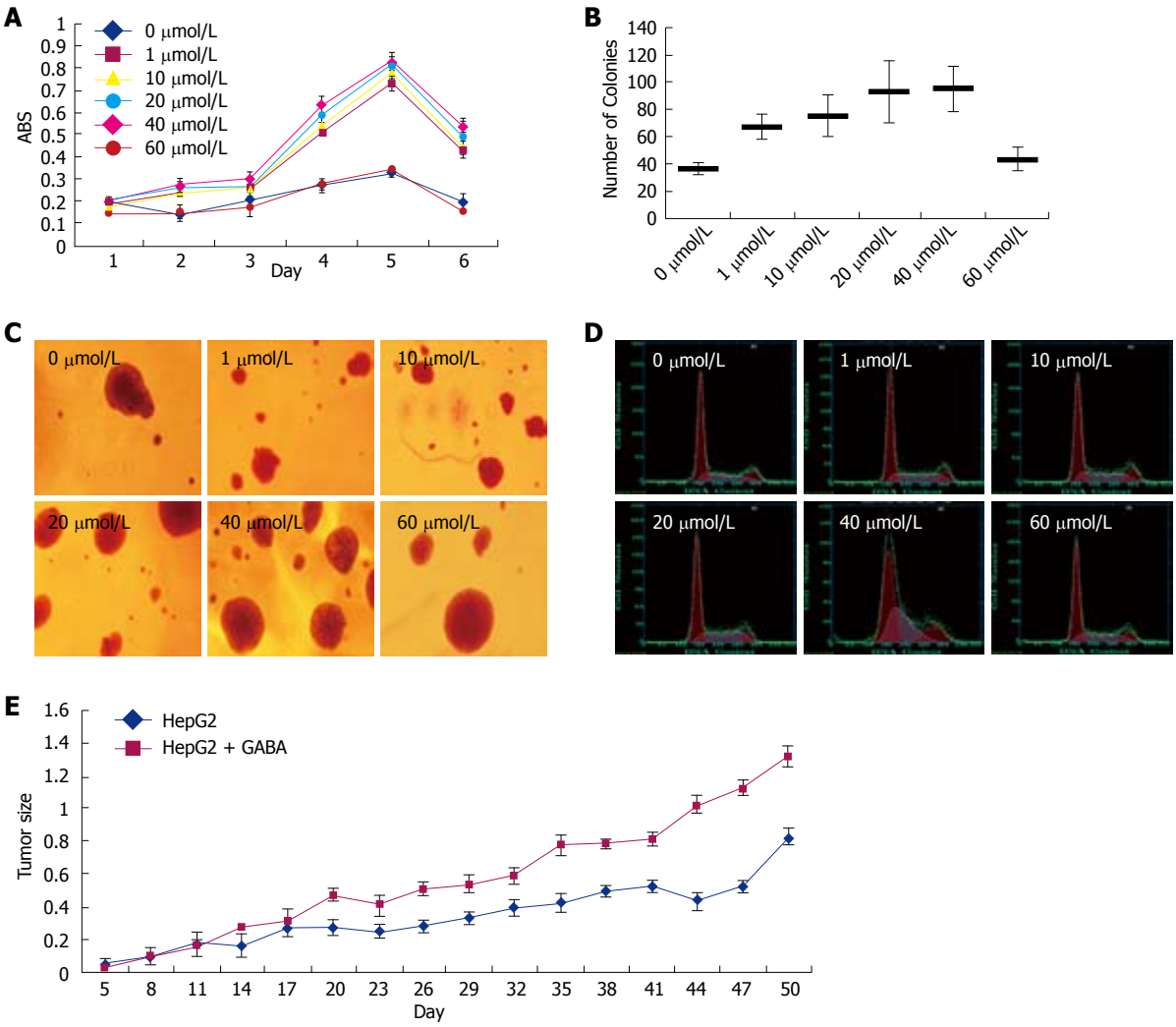

Figure 3 Effect of GABA on the growth of HCC cells. HepG2 cells were treated with GABA at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L). A: MTT assay. Y-axis: Average value of absorbance (ABS) at 570 nm, measured with a microplate reader (n = 6, P < 0.05); B and C: Soft agar colony formation assay. Same cells were incubated in low-melting agarose as described in Materials and Methods. Two weeks later, colonies were photographed and numbers of colonies were counted. (n = 3, P < 0.05); D: Cell cycle measured by flow cytometry; E: Tumor volume in nude mice with HepG2 and GABA (0 μmol/L and 20 μmol/L) injected (mm^3).

and Table 5, GABA enhanced the growth of HepG2/Si-Mock compared with the HepG2/Si-Mock without GABA. On the other hand, the proliferating ability of HepG2/Si-1, which did not express GABRA3, was not enhanced but lowered by GABA. In the nude mice injected with HepG2/Si-Mock, the tumor size of the mice treated with GABA was much larger than the mice treated without GABA, while the result was opposite in the mice injected with HepG2/Si-1.

DISCUSSION

Although GABA and GABA receptors function as an inhibitory neurotransmitter in the mature CNS, abnormal levels of gene and protein expression of some GABA receptor subunits have been detected in many malignant tumors, such as π subunit in pancreatic cancer[8]. This indicates that GABAergic system may play an important role in the pathogenesis and development of malignant tumors, and their expression levels are important for prognosis. In this study, we found the overexpression of GABRA3 in more than half of the HCC tissues compared with the adjacent non-tumor liver tissues, and GABRA3 was expressed in malignant liver cell lines HepG2 and Chang, but not in normal cell line L-02, implicating that GABRA3 may be a good molecular target for the diagnosis of HCC.

GABAA-β3 receptor expression is absent in malignant hepatic cell lines, and the proliferating ability of Chang cells, which were stably transfected with GABAA-β3 receptor, was significantly decreased[9]. On the other hand, GABAB receptors were increased and GABAB receptor agonist promoted proliferation of hepatocytes[10]. It means that even in HCC, different GABA receptor subunits may have different functions. Functional analysis using siRNA of GABRA3 strongly supported its involvement in the development and progression of HCC. In our study, the proliferation rate of HepG2 cells after GABRA3 knockdown was significantly reduced, whereas proliferation of HepG2/Si-Mock cells was not inhibited. This result indicated that GABRA3 may increase the proliferating ability of

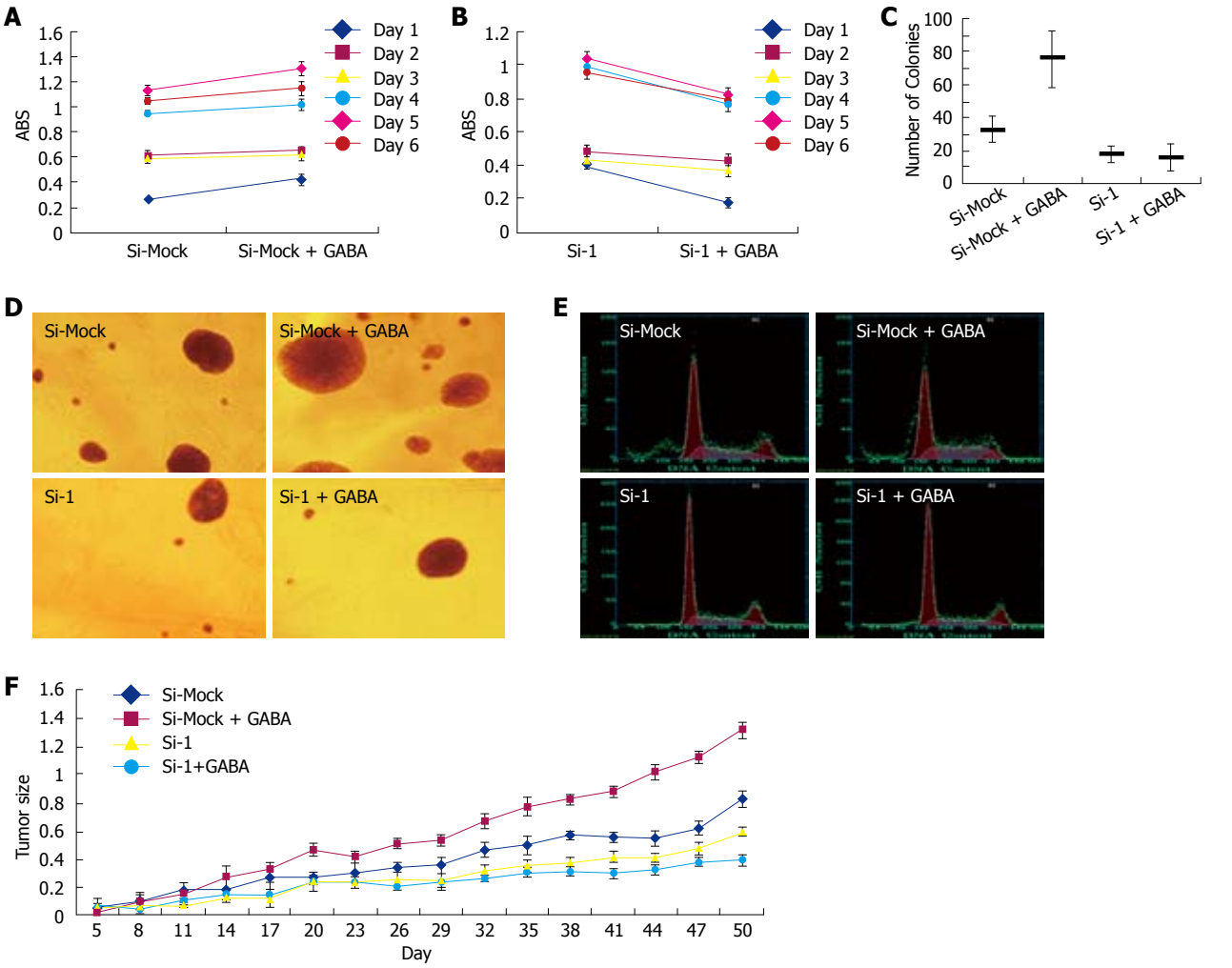

Figure 4 GABA stimulated HCC cell proliferation through GABRA3. HepG2/Si-1 and HepG2/Si-Mock cells were treated with or without GABA (20 μmol/L). A: MTT assay of HepG2/Si-Mock cells treated with or without GABA. (n = 6, P < 0.05); B: MTT assay of HepG2/Si-1 cells treated with or without GABA. (n = 6); C and D: Soft agar colony formation assay. (n = 6, P < 0.05 for HepG2/Si-Mock *vs* HepG2/Si-Mock + GABA); E: Cell cycle measured by flow cytometry; F: Tumor volume in nude mice (mm^3).

hepatocytes.

Biju *et al* [10] reported that, in N-nitrosodiethylamine-induced neoplasia in the rat liver, GABAB receptors were increased and the GABAB receptor agonist baclofen increased EGF-mediated DNA synthesis in hepatocytes. Thus, GABA-associated pathways also could act positively in the regulation of cancer cell behavior. Our findings in this study also support the theory that GABA and GABRA3 promoted HepG2 cell proliferation *in vivo* and *in vitro*. Although GABA usually induces hyperpolarization in adult neurons, GABA has been shown to exert depolarizing responses in the immature CNS structures and CNS tumors[11,12]. And Minuk *et al*[13] reported that human HCC tissues were depolarized compared with adjacent non-tumor tissues. In particular, GABA increased the proliferation of immature cerebellar granule cells through the activation of GABAA receptors and voltage-dependent calcium channels[14]. Takehara *et al*[14] reported that GABA stimulated pancreatic cancer growth through GABRP by increased intracellular Ca^{2+} levels and activating the mitogenactivated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) cascade[8]. From the results above, we deduce that GABA may promote the HepG2 cell proliferation through GABRA3 by voltage-dependent calcium channels. Interestingly, GABAA receptor antagonist bicuculline methiodide also could promote the proliferation of HepG2 cells (data not shown), indicating that it might activate some other signal transductions.

By comparing the proliferative activity of the GABRA3-knockdown HepG2 cells treated with GABA, we found that GABA stimulated HepG2 cell growth through GABRA3. The proliferating ability of the cells treated with GABA was not enhanced compared with the cells without GABA treatment. And interestingly, GABA inhibited the growth of the GABRA3-knockdown HepG2 cells. It indicates that GABA activates some other receptors to inhibit the proliferation without GABRA3, which is identical to some previous reports[15,16,17]. But GABA primarily activates GABRA3 if it exists.

Regarding the expression of other GABAA receptor subtypes/isoforms, the GABAA-δ expression detected

in the majority of HCC tissues has not been previously described, and the significance of this finding has yet to be elucidated. Although GABAA-α1 (1 case), α3 (1 case), β3, γ2 (2 cases), ε and π expression was also detectable, it is unlikely that expression of these isoforms results in the formation of functional GABA-gated receptors with GABRA3. The reason is that GABA could produce opposite effects on HepG2 cells with or without GABRA3 expression, which means that GABA could activate GABA receptors. Moreover, the detection of the subunits was not unexpected, because previous reports have documented that the spectrum of GABAA receptor isoform expression correlates with the state of cell differentiation[18,19].

In conclusion, relative to adjacent non-tumor tissues, HCC tissues have increased GABAA-α3 receptor expression. Knockdown of GABRA3 expression in receptor expressing malignant hepatocytes results in attenuated *in vivo* and *in vitro* tumor growth. Moreover, GABA promotes hepatocyte proliferation through GABRA3. These findings highlight the importance of elucidating the role of GABAergic activity in the pathogenesis of HCC. They also raise the potential for new therapeutic and diagnostic approaches to HCC in humans.

COMMENTS

Background

Gamma-aminobutyric acid A receptor α3 subunit (GABRA3) is a subunit of the gamma-aminobutyric acid A (GABAA) receptors that may associate with other GABAA receptor subunits to form a functional chloride channel that mediates the inhibitory synaptic transmission in the mature central nervous system (CNS). GABA functions as an inhibitory neurotransmitter for activating GABA receptors.

Research frontiers

Recently, abnormal levels of gene and protein expression of some GABA receptor subunits have been detected in many malignant tumors. This indicates that GABAergic system may play an important role in the pathogenesis and development of malignant tumors.

Innovations and breakthroughs

This study demonstrated the overexpression of GABRA3 in Hepatocellular carcinoma (HCC) tissues, which has not been previously described, and illustrated that GABA stimulated HCC cell proliferation through GABRA3.

Applications

The findings raise the potential for new therapeutic and diagnostic approaches to HCC in humans.

Terminology

GABA stands for gamma-aminobutyric acid, which is an inhibitory neurotransmitter. GABRA3 stands for gamma-aminobutyric acid A receptor α3 subunit.

Peer review

In this work, the authors have explored the expression pattern of GABAA receptors in hepatocellular carcinoma cells, identifying the overexpression of GABRA3 in HCC cells and tissues.They also demonstrate that GABA increases proliferation of GABRA3 positive cells, which is cancelled when GABRA-3 expression is knock-down. Results are new and show relevance to understand the molecular mechanisms that control hepatocarcinogenesis.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108

2 **Song TJ**, Ip EW, Fong Y. Hepatocellular carcinoma: current surgical management. *Gastroenterology* 2004; **127**: S248-S260

3 **Haydar TF**, Wang F, Schwartz ML, Rakic P. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. *J Neurosci* 2000; **20**: 5764-5774

4 **Behar TN**, Schaffner AE, Scott CA, Greene CL, Barker JL. GABA receptor antagonists modulate postmitotic cell migration in slice cultures of embryonic rat cortex. *Cereb Cortex* 2000; **10**: 899-909

5 **Meier J**, Akyeli J, Kirischuk S, Grantyn R. GABA(A) receptor activity and PKC control inhibitory synaptogenesis in CNS tissue slices. *Mol Cell Neurosci* 2003; **23**: 600-613

6 **Tamayama T**, Maemura K, Kanbara K, Hayasaki H, Yabumoto Y, Yuasa M, Watanabe M. Expression of GABA(A) and GABA(B) receptors in rat growth plate chondrocytes: activation of the GABA receptors promotes proliferation of mouse chondrogenic ATDC5 cells. *Mol Cell Biochem* 2005; **273**: 117-126

7 **Erlander MG**, Tobin AJ. The structural and functional heterogeneity of glutamic acid decarboxylase: a review. *Neurochem Res* 1991; **16**: 215-226

8 **Takehara A**, Hosokawa M, Eguchi H, Ohigashi H, Ishikawa O, Nakamura Y, Nakagawa H. Gamma-aminobutyric acid (GABA) stimulates pancreatic cancer growth through overexpressing GABAA receptor pi subunit. *Cancer Res* 2007; **67**: 9704-9712

9 **Sun D**, Gong Y, Kojima H, Wang G, Ravinsky E, Zhang M, Minuk GY. Increasing cell membrane potential and GABAergic activity inhibits malignant hepatocyte growth. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G12-G19

10 **Biju MP**, Pyroja S, Rajeshkumar NV, Paulose CS. Enhanced GABA(B) receptor in neoplastic rat liver: induction of DNA synthesis by baclofen in hepatocyte cultures. *J Biochem Mol Biol Biophys* 2002; **6**: 209-214

11 **Ganguly K**, Schinder AF, Wong ST, Poo M. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell* 2001; **105**: 521-532

12 **Labrakakis C**, Patt S, Hartmann J, Kettenmann H. Functional GABA(A) receptors on human glioma cells. *Eur J Neurosci* 1998; **10**: 231-238

13 **Minuk GY**, Zhang M, Gong Y, Minuk L, Dienes H, Pettigrew N, Kew M, Lipschitz J, Sun D. Decreased hepatocyte membrane potential differences and GABAA-beta3 expression in human hepatocellular carcinoma. *Hepatology* 2007; **45**: 735-745

14 **Fiszman ML**, Borodinsky LN, Neale JH. GABA induces proliferation of immature cerebellar granule cells grown in vitro. *Brain Res Dev Brain Res* 1999; **115**: 1-8

15 **Zhang M**, Gong Y, Assy N, Minuk GY. Increased GABAergic activity inhibits alpha-fetoprotein mRNA expression and the proliferative activity of the HepG2 human hepatocellular carcinoma cell line. *J Hepatol* 2000; **32**: 85-91

16 **Tatsuta M**, Iishi H, Baba M, Nakaizumi A, Ichii M, Taniguchi H. Inhibition by gamma-amino-n-butyric acid and baclofen of gastric carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats. *Cancer Res* 1990; **50**: 4931-4934

17 **Tatsuta M**, Iishi H, Baba M, Yano H, Uehara H, Nakaizumi A. Effect of selective and non-selective muscarinic blockade on baclofen inhibition of gastric carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats. *Carcinogenesis* 1996; **17**: 293-296

18 **Laurie DJ**, Wisden W, Seeburg PH. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* 1992; **12**: 4151-4172

19 **Neelands TR**, Zhang J, Macdonald RL. GABA(A) receptors expressed in undifferentiated human teratocarcinoma NT2 cells differ from those expressed by differentiated NT2-N cells. *J Neurosci* 1999; **19**: 7057-7065

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CLINICAL RESEARCH

Usefulness of contrast-enhanced ultrasonography in determining treatment efficacy and outcome after pancreatic cancer chemotherapy

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Abstract

AIM: To investigate if contrast-enhanced ultrasonography (CE-US) is useful for determining treatment efficacy and outcome in the early stages of pancreatic cancer chemotherapy by assessing changes in intratumor hemodynamics using CE-US with a contrast agent.

METHODS: The subjects were 34 patients with unresectable advanced pancreatic cancer treated by chemotherapy. CE-US was assessed after every treatment (course) completion under the same conditions, and patients were divided into two groups according to the intratumor enhancement pattern: Vascular rich (R) group and vascular poor (P) group.

RESULTS: After the second course of treatment, R group in intratumor hemodynamics had 18 patients, and P group had 16 patients. The reduction rates of serum CA19-9 level after chemotherapy which decreased to half or less of the baseline level were 2/15 (0.1%) in P group, but 11/16 (69%) in R group (P = 0.006). When the mean number of courses of chemotherapy and outcome were compared, P group had a mean number of courses of 4.9 (R group, 10.2) and mean survival time (MST) of 246 d (R group, 402 d), showing that outcome was significantly better in R group (P = 0.006).

CONCLUSION: CE-US revealed that the change in intratumor blood flow correlated with both serum CA19-9 level and outcome. Patients with serum CA19-9 that decreased to less than half the baseline level, and patients with an abundant intratumor blood flow, had a significantly better outcome. Thus, CE-US is potentially useful for evaluating treatment efficacy and outcome in the early stages of pancreatic cancer chemotherapy.

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Key words: Pancreatic cancer; Chemotherapy; Gemcitabine; Contrast-enhanced ultrasonography; Outcome

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INTRODUCTION

The yearly mortality due to pancreatic cancer exceeds 200 000[1] worldwide and is increasing. Even with recent advances in diagnostic image technology, most cases of pancreatic cancer are only discovered at an unresectable stage, when the prognosis is poor and the 5-year survival rate is no more than 1%[1]. To date, multimodality therapy consisting of chemotherapy with 5-fluorouracil (5-FU)[2-7] and radiotherapy[8-10] has been the standard treatment for unresectable pancreatic cancer. Since the results of this treatment have not been satisfactory, gemcitabine (GEM) (Eli Lilly Japan, Kobe, Japan) has recently become the standard chemotherapy medication

for unresectable pancreatic cancer, and GEM is expected to both increase antitumor efficacy[11-15] and improve clinical benefit response (CBR)[13].

The present study seeks to determine the antitumor effects of GEM in the early stages of treatment. To do so, we compared the enhancement patterns of pancreatic tumors visualized by contrast-enhanced ultrasonography (CE-US) using a microbubble contrast agent[16,17]. We investigated whether assessment of changes in intratumor hemodynamics by CE-US is useful for determining treatment efficacy and prognosis in the initial stages of chemotherapy for pancreatic cancer.

MATERIALS AND METHODS

Materials

The subjects were 34 patients (17 Stage Ⅲ and 17 Stage Ⅳ) with performance status 0-2 unresectable advanced pancreatic cancer that had visited this hospital and had undergone CE-US over the past 5 years. Staging of the disease was in accordance with The International Union Against Cancer (UICC) staging system (6th edition). The drugs and doses used were 1000 mg/m^2 for GEM and 10 mg/m^2 for cisplatin (CDDP). Seven patients showed adverse effects of GEM treatment, so the dose was reduced to 800 mg/m^2. One course of treatment consisted of the following: GEM alone given to 18 patients, GEM + CDDP given to 13 patients, and GEM + CDDP with radiotherapy (50-60 Gy) given to three patients once per week for three weeks followed by a one-week rest. In addition, a histological diagnosis by endoscopic ultrasonography-fine needle aspiration (EUS-FNA), which has been reported to be useful[18], was performed for all the subjects prior to the chemotherapy treatment. After the chemotherapy treatment of patients who had received four courses or more, re-biopsy was again performed for eight patients that gave informed consent to do so. All patients provided written informed consent before the study. Our Institutional Review Board reviewed and approved the entire study.

CE-US

The ultrasound equipment used was either a Sequoia 512 (Siemens-Acuson; Mountain View, CA, USA) or Aplio (Toshiba Medical Systems, Tokyo, Japan). Levovist R (2.5 g) (Shering; Tokyo, Japan) was prepared at a dose of 300 mg/mL, and a bolus of 7 mL was injected through the median cubital vein at a speed of 1 mL/s. After detailed observation of the lesion in B-mode, the vascular image was depicted for 60 s after injection of the contrast agent, at a speed of 5 frames/s (fps) with breath held, focusing on the blood flow dynamics in the lesion and surrounding pancreatic tissue. Thereafter, perfusion images were depicted for 180 s after injection with intermittent transmissions for periods of 2-10 s to observe the enhancement of the lesion. The color Doppler gain level was standardized as 50. We observed the blood vessels of the lesion in the vascular image, and the actual perfusion of the lesion through the capillaries in the perfusion image.

According to our previous report[16], both the vascular and perfusion images of the tumor blood flow signal were compared with the non-tumor regions of pancreas and classified depending on the signal intensities into the following three groups: hypervascularity (hyper), isovascularity (iso), and hypovascularity (hypo) (Figure 1).

Assessment of CE-US for chemotherapy

CE-US enhancement patterns after chemotherapy were classified into the following patterns according to our previous assessment prior to the present study. CE-US was assessed after every treatment (course) completion under the same conditions, and patients were divided into four types: an increased intratumor blood flow type (from hypo to iso, HI type); an abundant intratumor blood flow that remained the same before and after treatment type (from iso to iso; II type), a no increase in intratumor blood flow type (from hypo to hypo, HH type), and a decreased intratumor blood flow type (from hyper/iso to hypo, IH type). Furthermore, these four types were further divided into two groups; a vascular rich (R) group of the HI and II types with a high post-treatment intratumor blood flow, and vascular poor (P) group of the HH and IH types with a poor post-treatment intratumor blood flow. The assessment of intratumor blood flow luminance was performed at the same time as the analysis of luminance where the midline of the tumor, which was the region of interest (ROI), was compared over time with adjacent non-tumor regions using Adobe Photoshop (Adobe Systems; San Jose, CA). This was done to measure the amount of blood flow objectively. When the luminance score (LS) (luminance of tumor area divided by luminance of normal area) is 20% or less, there is the possibility of increased luminance due to artifacts. Therefore, a conclusion of increased blood flow was reached if the LS increased by 20% or more compared to the baseline value. The images for all the patients were recorded and luminance evaluated from the same images by three physicians (T.I., T.K., and F.M.) experienced in CE-US.

Endpoint

Treatment efficacy and outcome were evaluated from the viewpoint of serum CA19-9 level and CE-US enhancement pattern in the R and P groups. The definition of response to treatment was evaluated after the second course of chemotherapy.

Statistical analysis

Student's *t* test, Fisher's exact test, Wilcoxon's test, and the log-rank test were used for the statistical analysis. A P value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

All the tumors were visualized in B-mode by CE-US, and mean tumor diameter was 39.4 mm (15-80 mm). When the CE-US enhancement patterns of the tumors before

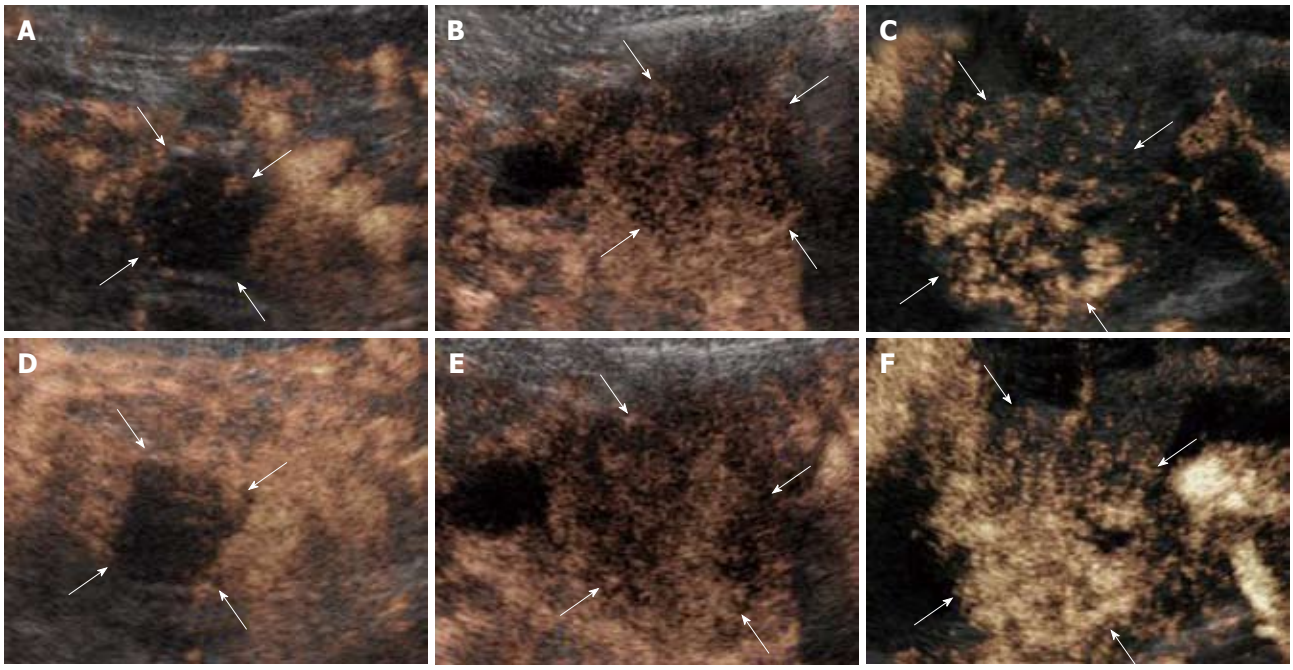

Figure 1 Enhancement pattern of CE-US in pancreatic cancer. A-C: Vascular image; D-F: Perfusion image; A, D: Hypovascularity; B, E: Isovascularity; C, F: Hypervascularity.

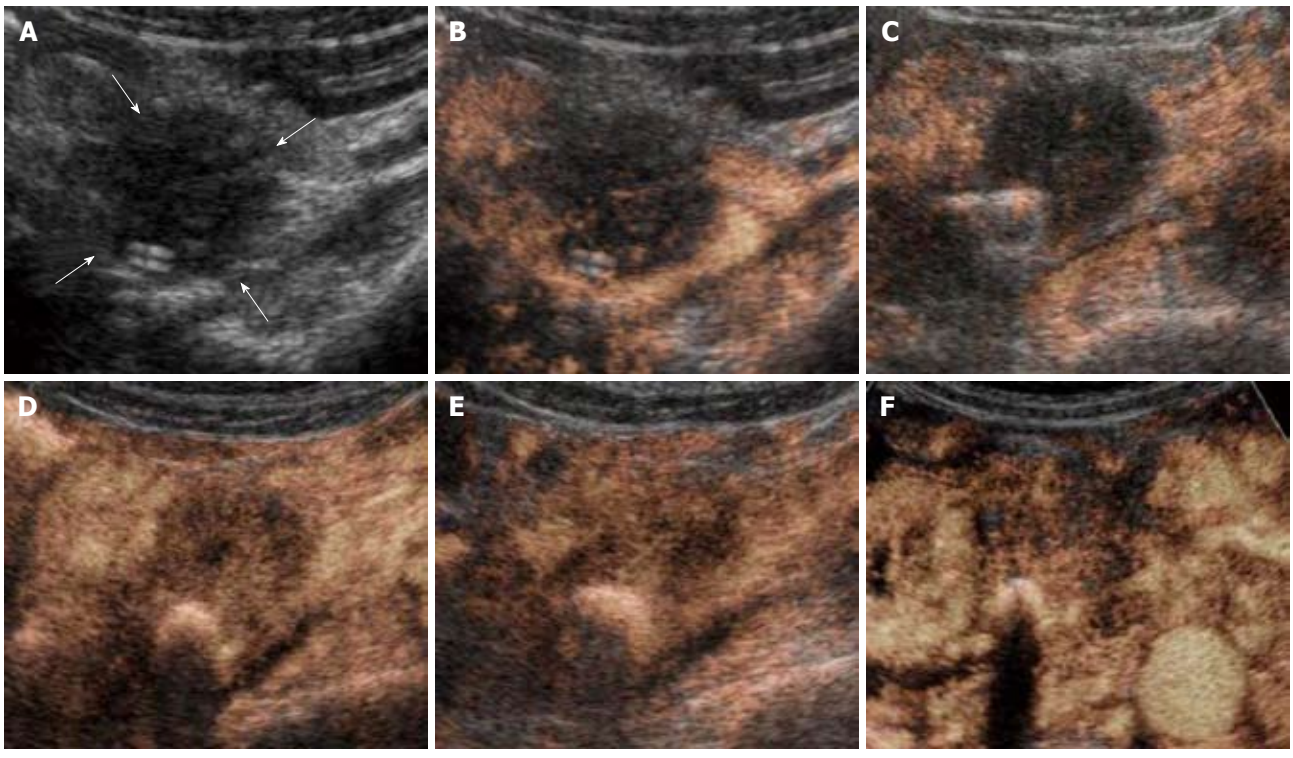

Figure 2 CE-US changes over time in the R (HI) group (luminance score). A: B-mode; B: Pre (0.30); C: Post 2 course (0.28); D: Post 4 course (0.78); E: Post 7 course (0.62); F: Post 16 course (0.46).

and after treatment were compared, intratumor blood flow before treatment was of the hypovascularity type in 28 (82%) patients and of the isovascularity type in 6 (18%) patients. After treatment, changes in intratumor hemodynamics after the second course were seen in 12 (35%) patients, all of whom had increased intratumor blood flow (HI type). There was no change in the hemodynamics in 22 (65%) patients (16 in the HH type and 6 in the II type). Blood flow did not decrease in any of the patients. Twelve patients in the HI type and 6 in the II type, making a total of 18 (53%) patients, were combined to form the R group. The CE-US changes over time in the R group are shown in Figures 2 and 4, and the CT changes in Figures 3 and 5. Similarly, for the P group (HH type), the CE-US and CT changes over time for 16 patients are shown in Figures 6 and 7. After

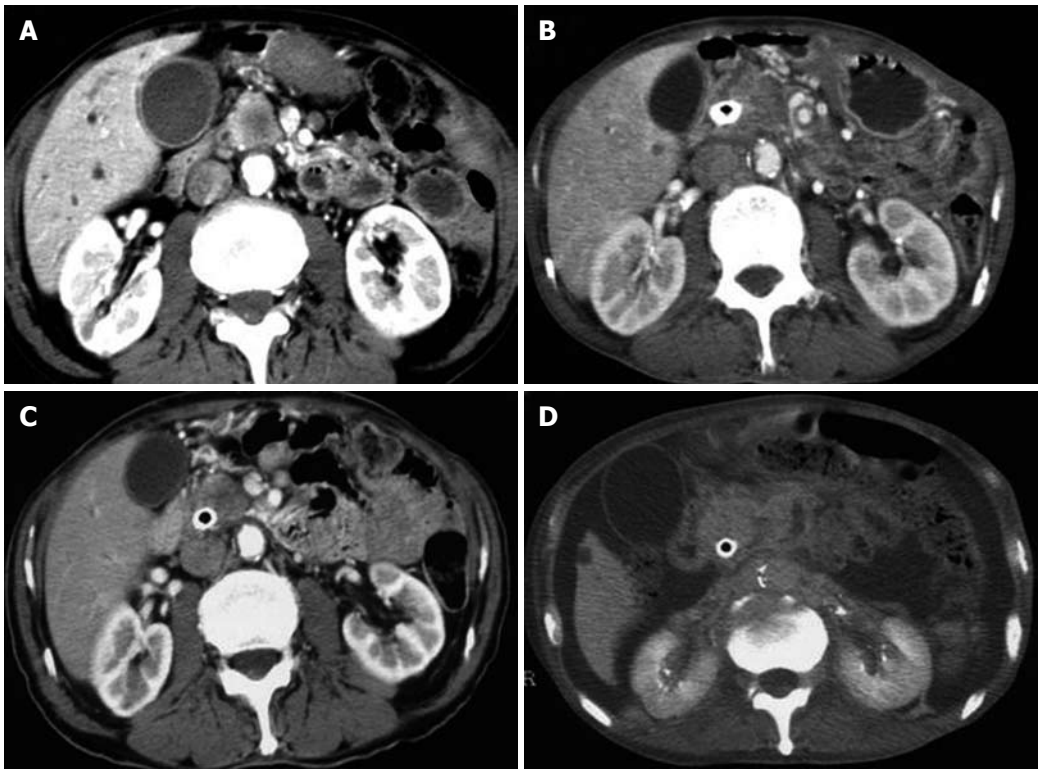

Figure 3 CT changes over time in the R (HH) group. A: Pre; B: Post 4 course; C: Post 7 course; D: Post 16 course.

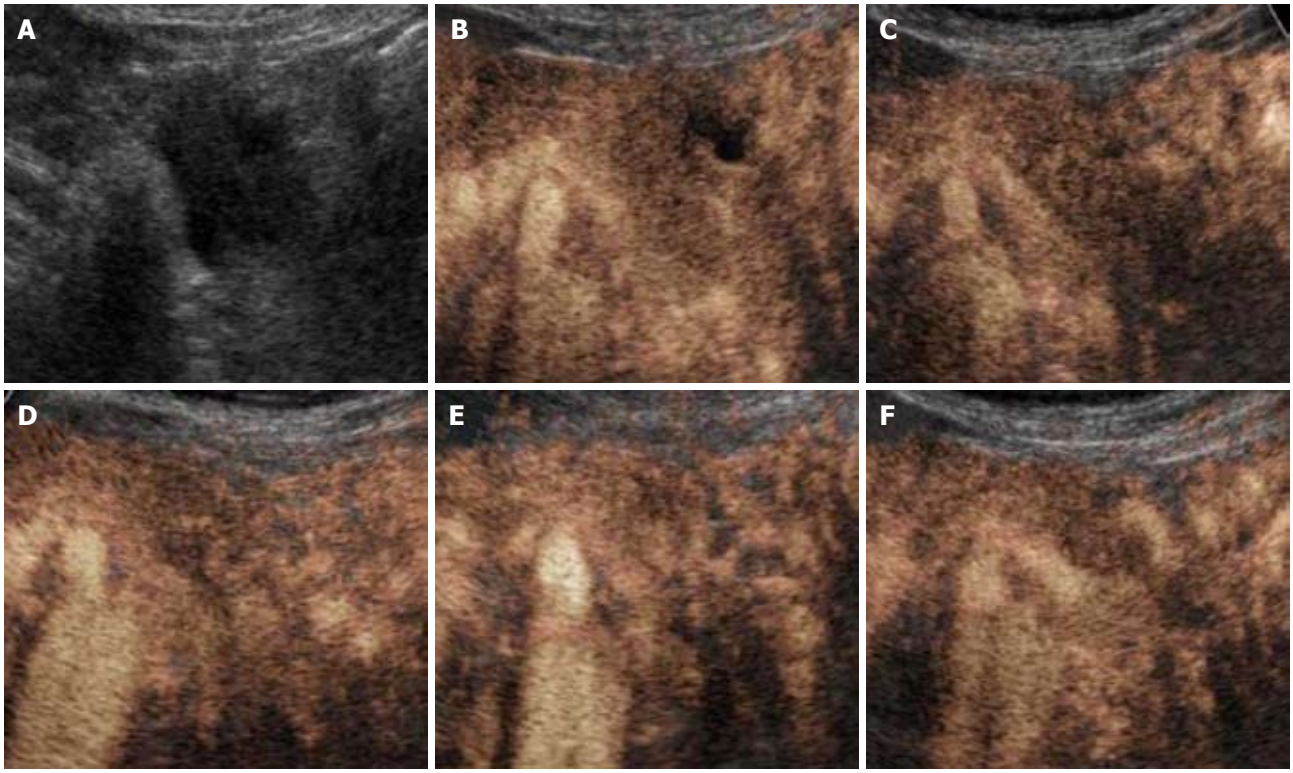

Figure 4 CE-US changes over time in the R (II) group (luminance score). A: B-mode; B: Pre (0.96); C: Post 2 course (0.99); D: Post 4 course (1.0); E: Post 7 course (0.93); F: Post 11 course (1.0).

treatment, there was increased imaging performance by both CE-US and CT in the HI type, in other words, the changes after treatment showed correlation in terms of images in 33% (4/12) patients. In addition, there was no significant difference in the tissue types.

There was no difference between the R and P groups in relation to performance status or chemotherapy regimen. When mean tumor diameter before and after treatment in the R and P groups were compared, the values before treatment were 37.2 mm *vs* 41.8 mm and after treatment were 31.9 mm *vs* 37.5 mm, with no significant difference between the groups ($P = 0.891$).

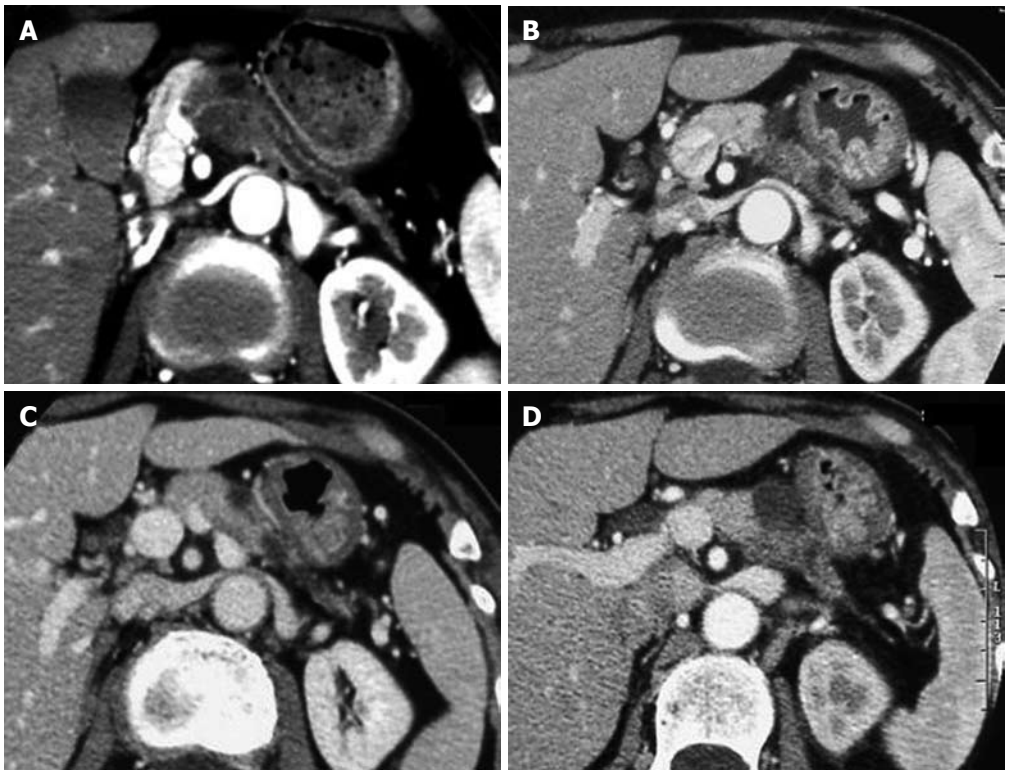

Figure 5 CT changes over time in the R (II) group. A: Pre; B: Post 2 course; C: Post 7 course; D: Post 11 course.

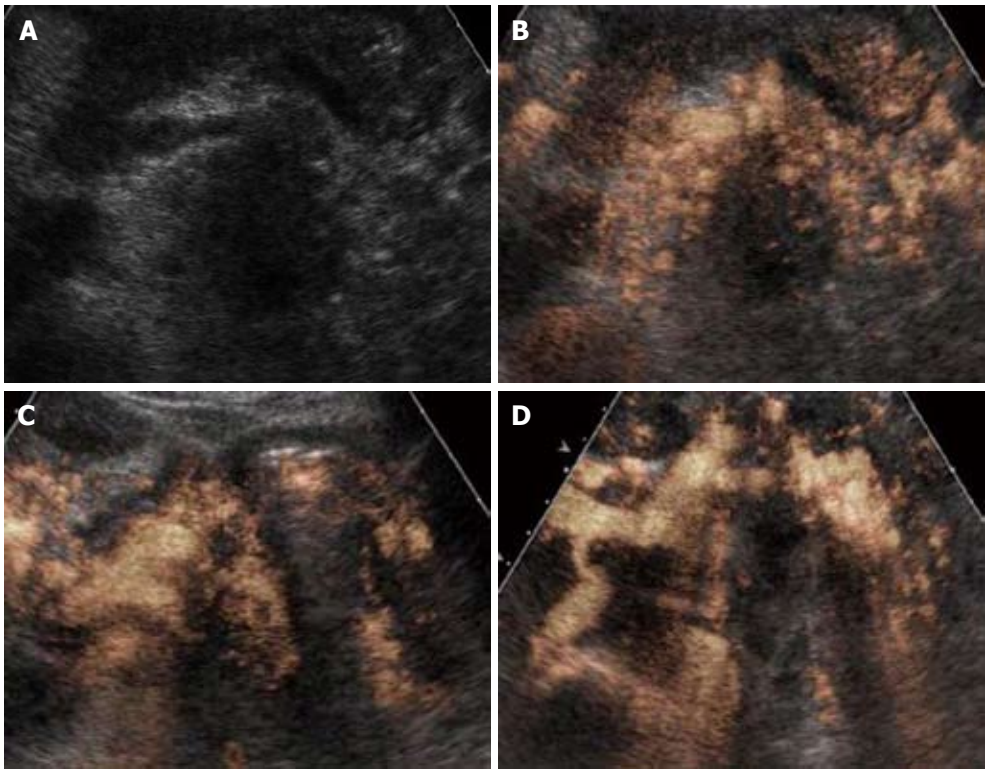

Figure 6 CE-US changes over time in the P (HH) group (luminance score). A: B-mode; B: Pre (0.29); C: Post 2 course (0.46); D: Post 3 course (0.29).

Tumor response based on Response Evaluation Criteria In Solid Tumors (RECIST) in the R group was rated as a complete response (CR) in one patient, partial response (PR) in 5 patients, stable disease (SD) in 10 patients and progressive disease (PD) in 2 patients. In the P group, results were PR in one patient, SD in 6 and PD in 9 patients. Although there was no significant difference between the groups, the response rate in the R group was high compared with P group (33% *vs* 6%, $P = 0.051$). These results are shown in Table 1. The grade of histological differentiation in the R group was well-moderately differentiated type in 12 patients, poorly differentiated medullary type in five patients and poorly differentiated scirrhous type in one patient. In the P group, it was well-moderately differentiated adenocarcinoma in 16 patients. There was no significant difference in grade of histological differentiation between the two groups ($P = 0.072$). In the 8 patients

Table 1 Results of hemodynamic change in CE-US, response, and outcome after chemotherapy

	Hemodynamic change in CE-US	No. of cases (n = 34)	CEM (n = 18)	CEM + CDDP (n = 13)	Chemo + radiation (n = 3)	Response				CA19-9 reduction of less than half (n = 31)	Average dosage (course)	Outcome (d)
						CR	PR	SD	PD			
R group	Total	18	9	8	1	1	5	10	2	11 (61%)[a]	10.2[a]	402.0[a]
	Increase (HI)	12	6	5	1	1	3	8	0	8 (67%)[a]	10.7 (6-18)[a]	411.6 (239-6.4)[a]
	No change (II) iso-iso	6	3	3	0	0	2	2	2	3 (50%)[a]	9.0 (1-27)[a]	383.5 (109-831)[a]
P group	Total	16	9	5	2	0	1	6	9	2 (0.1%)[a]	4.9[a]	246.0[a]
	No change (HH) hypo-hypo	16	9	5	2	0	1	6	9	2 (0.1%)	4.9 (1-12)	246.0 (119-351)
	Decrease (IH)	0	0	0	0	0	0	0	0	0	0	0

[a]$P < 0.05$.

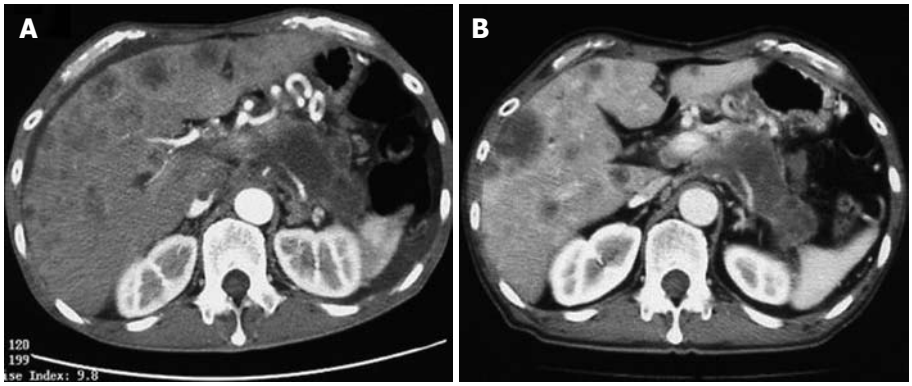

Figure 7 CT changes over time in the P (HH) group. A: pre; B: post 3 course.

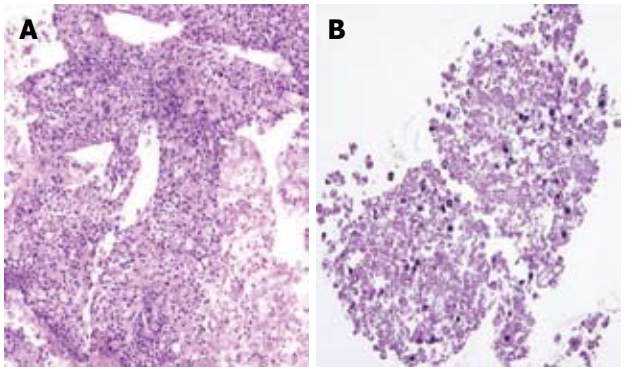

Figure 8 Histological change of post-treatment tissue biopsy pre-chemotherapy (A) and post-chemotherapy (B).

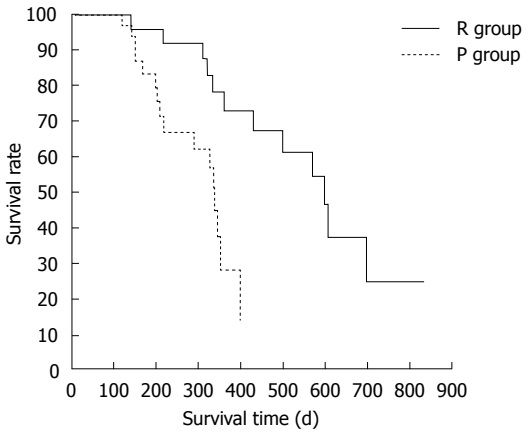

Figure 9 Kaplan-Meier survival curves by enhancement pattern of CE-US and outcome after chemotherapy.

(6 in group R and 2 in group P) that had post-treatment tissue biopsy, the histological findings revealed only necrotic tissue except in one R group case showing viable malignant tissue (Figure 8). The patients whose serum CA19-9 level decreased to half the baseline level or less were 2/15 (0.1%) in group P but 11/16 (69%) in group R, showing a significant decrease in group R [P = 0.006; 95% confidence interval (CI), 41-88]. When mean courses of chemotherapy and outcome were compared, group P had a mean course number of 4.9 (range, 1-12) and mean survival time (MST) of 246 d (range, 119-351 d), whereas group R had a mean course number of 10.2 (range, 1-27) and MST of 402 d (range, 109-831 d), showing that outcome tended to be significantly better in group R (P = 0.006, 95% CI, 48-265). These results are shown in Table 1. The correlation between CE-US imaging pattern and MST based on these results is presented as a Kaplan Meier survival curve (Figure 9).

DISCUSSION

In this investigation, we demonstrated that patients with abundant intratumor blood flow (the R group) had significantly better GEM treatment results than the P group with a poor intratumor blood flow (P group *vs* R group; mean number of treatment courses: 4.9 *vs* 10.2; MST: 246 d *vs* 402 d). Patients with increased intratumor blood flow were commonly seen after the second course of treatment. With regard to tumor response, the GEM

response rate in the R group was 33.3% while that in the P group was no more than 6.3%.

Halm[19] and Heinemann *et al*[20] reported good treatment efficacy in patients where serum CA19-9 level decreased by 20% or more of the baseline value. In the present study, serum CA19-9 level decreased to half the baseline value or less in 69% of patients in R group, where treatment efficacy was good. However, in P group, the decrease was only 0.1%. Serum CA19-9 is the sialylated Lewis blood group antigen that is expressed by the monoclonal antibody 1116 NS 19-9[21]. When this antigen is negative, an increase in serum CA19-9 level cannot be seen, making a clear interpretation difficult. These results suggest that changes in intratumor blood flow after treatment, as revealed by CE-US, are related to serum CA19-9 level and prognosis, and that prognosis is good in patients with serum CA19-9 levels that decrease to half the baseline level or less after treatment, and those with abundant intratumor blood flow.

GEM became commercially available in the late 1990s as a chemotherapy agent for unresectable pancreatic cancer, and is now positioned as a first-choice drug. We have used GEM as a primary chemotherapy agent but have sometimes experienced cases where the treatment has not been effective. Although the reason for that is not clear, recently there are reports that gemcitabine resistance has been associated with the ribonucleotide reductase subunit M2 (RRM2)[22]. If the status of treatment efficacy can be determined in the early stage of antitumor drug treatment, more effective chemotherapy can be selected. Physicians could then switch patients to other drugs, and select other treatment options such as concomitant therapy or combined treatment with radiotherapy. The burden that unnecessary anticancer drug treatments place on the body, as well as the time wasted, could thus be avoided.

Based on our previous investigations[16,17] of the CE-US imaging patterns of pancreatic tumor disease, it was assumed that the qualitative intratumor histological changes that were caused by treatment were reflected in the hemodynamics seen in CE-US. Therefore, CE-US could be applied to the evaluation of treatment efficacy after chemotherapy of pancreatic cancer.

The CE-US imaging patterns of pancreatic ductal cancer are basically classified into three types-hypo, iso, and hypervascular-with the most common pattern being hypovascularity[16]. Although the extent of the patterns in the tumors differed, a punctate or dendritical blood flow signal was seen in most patients. This indicates that the pancreatic tumor is an ischemic tumor, but demonstrates that much of the blood flow is at the capillary level. In the relationship between CE-US imaging pattern and histological differentiation, tissue type in the case of a hypovascularity pattern was moderately or poorly differentiated adenocarcinoma (scirrhous type). In the case of an isovascularity pattern, the tissue type was poorly differentiated adenocarcinoma (medullary type), whereas in the case of a hypervascularity pattern it was papillary adenocarcinoma[17]. These differences in imaging pattern are related to inflammatory changes, tumor development morphology and grade of histological differentiation, fibrosis and amount of interstitial connective tissue, vascular occlusion and degree of patency due to invasion of tumor vessels, and density.

Pancreatic cancers are generally anemic tumors and so respond poorly to anticancer treatment. It has become possible to visualize the microvascular flow in tumors by CE-US, and it is now evident that much of the intratumor blood flow is at the capillary level. With an abundant intratumor blood flow, more drugs will readily enter the tumor and thus increase treatment efficacy. The bubble diameter of the contrast agent is 2-3 μm, which is the same as the diameter of the capillaries that can be visualized. It is therefore preferable for the diameter of the particle system of anticancer drugs to be lower than that. According to the manufacturer (Eli Lilly Japan), the particle diameter of GEM is 0.2 μm or less. This is also important from the perspective of tissue transferability; GEM migrates up to the capillary level of tumors and so is assumed to show antitumor effect in tissues. The increase in intratumor blood flow in CE-US may be the reason for the intratumor histological changes and changes at the capillary level. The post-treatment results on EUS-FNA tissue showed that the tumor had changed into necrotic tissue and had decreased in volume. A reactive inflammatory change is triggered, causing disintegration of the tumor, which then becomes necrotic tissue. Furthermore, the volume of the tumor decreases and there is improvement in vascular occlusion and exclusion caused by tumor exclusion and vascular invasion. This is assumed to be the mechanism whereby intratumor blood flow improves. However, there was no significant difference between antitumor efficacy and grade of histological differentiation in this study, which suggests that antitumor efficacy not only correlates with the grade of histological differentiation, but also with other factors such as the amount of interstitial connective tissue and density.

In this study, the CT and CE-US imaging patterns before and after treatment were not always in concordance. The rate of concordance of the imaging patterns before treatment was 92% (31/34 patients), and after treatment it was 76% (26/34 patients). Moreover, in the R group, where treatment efficacy was good and CE-US revealed abundant blood flow, the CT imaging after treatment did not necessarily show change. In the HI type in particular, only 33% (4/12 patients) showed a correlation in imaging changes after treatment. The reasons for this include the fact that the contrast agents for CT and those for CE-US are different; the CT agents are predisposed to extravascular interstitial leakage, and there is a difference in the imaging time phase of the agents. There were some cases in which there was no change in tumor volume and enhancement pattern of CT after the treatment, but the outcome was comparatively good. The CE-US of such patients commonly revealed abundant intratumor blood flow, and also it showed that such patients were good outcome in our present study. Therefore, there was a difference of

assessment between CT and CE-US.

At present, although the UICC guidelines suggest that antitumor effects should be evaluated based on volume change as estimated by CT, such evaluation is sometimes questionable. The results of the present investigation demonstrated that, apart from volume change by CT, there is the possibility that the CE-US imaging pattern is also one important parameter in the evaluation of the post-treatment effect. It can at least be said that CT-based estimation of antitumor effect leaves much to be desired. Moreover, there will be a problem of radiation exposure related to CT in the future. However, the role of CE-US imaging in prognostic evaluation needs to be investigated further in a larger patient cohort.

In conclusion, CE-US revealed that the change in intratumor blood flow after GEM treatment was correlated with serum CA19-9 level and outcome. Patients with serum CA19-9 levels decreasing to less than half the baseline level, and patients with an abundant intratumor blood flow, tended to have a good outcome. Thus, CE-US is potentially useful for evaluating treatment efficacy and outcome in the early stages of pancreatic cancer chemotherapy.

ACKNOWLEDGMENTS

The authors are indebted to Professor J Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript.

COMMENTS

Background

The yearly mortality due to pancreatic cancer exceeds 200 000 worldwide and is increasing. Even with recent advances in diagnostic image technology, most cases of pancreatic cancer are only discovered at an unresectable stage, when the prognosis is poor and the 5-year survival rate is no more than 1%.

Research frontiers

To date, multimodality therapy consisting of chemotherapy with 5-fluorouracil (5-FU) and radiotherapy has been the standard treatment for unresectable pancreatic cancer. Since the results of this treatment have not been satisfactory, gemcitabine (GEM) has recently become the standard chemotherapy medication for unresectable pancreatic cancer, and GEM is expected to both increase antitumor efficacy and improve clinical benefit response (CBR).

Innovations and breakthroughs

The present study seeks to determine the antitumor effects of GEM in the early stages of treatment. We compared the enhancement patterns of pancreatic tumors visualized by contrast-enhanced ultrasonography (CE-US) using a microbubble contrast agent. We investigated whether CE-US is useful for determining treatment efficacy and outcome in the early stages of pancreatic cancer chemotherapy by assessing changes in intratumor hemodynamics using CE-US with a contrast agent.

Applications

CE-US revealed that the change in intratumor blood flow after GEM treatment was correlated with serum CA19-9 level and outcome. Patients with serum CA19-9 levels decreasing to less than half the baseline level, and patients with an abundant intratumor blood flow, tended to have a good outcome.

Peer review

It is an interesting paper. The study revealed that CE-US is potentially useful for evaluating treatment efficacy and outcome in the early stages of pancreatic cancer chemotherapy.

REFERENCES

1 **World Health Organization**. The world health report. *WHO* 2000
2 **Hansen R**, Quebbeman E, Ritch P, Chitambar C, Anderson T. Continuous 5-fluorouracil (5FU) infusion in carcinoma of the pancreas: a phase II study. *Am J Med Sci* 1988; **295**: 91-93
3 **Kovach JS**, Moertel CG, Schutt AJ, Hahn RG, Reitemeier RJ. Proceedings: A controlled study of combined 1,3-bis-(2-chloroethyl)-1-nitrosourea and 5-fluorouracil therapy for advanced gastric and pancreatic cancer. *Cancer* 1974; **33**: 563-567
4 **Wiggans RG**, Woolley PV, Macdonald JS, Smythe T, Ueno W, Schein PS. Phase II trial of streptozotocin, mitomycin-C and 5-fluorouracil (SMF) in the treatment of advanced pancreatic cancer. *Cancer* 1978; **41**: 387-391
5 **Evans TR**, Lofts FJ, Mansi JL, Glees JP, Dalgleish AG, Knight MJ. A phase II study of continuous-infusion 5-fluorouracil with cisplatin and epirubicin in inoperable pancreatic cancer. *Br J Cancer* 1996; **73**: 1260-1264
6 **Rothman H**, Cantrell JE Jr, Lokich J, Difino S, Harvey J, Ahlgren J, Fryer J. Continuous infusion 5-fluorouracil plus weekly cisplatin for pancreatic carcinoma. A Mid-Atlantic Oncology Program study. *Cancer* 1991; **68**: 264-268
7 **Crown J**, Casper ES, Botet J, Murray P, Kelsen DP. Lack of efficacy of high-dose leucovorin and fluorouracil in patients with advanced pancreatic adenocarcinoma. *J Clin Oncol* 1991; **9**: 1682-1986
8 **Moertel CG**, Frytak S, Hahn RG, O'Connell MJ, Reitemeier RJ, Rubin J, Schutt AJ, Weiland LH, Childs DS, Holbrook MA, Lavin PT, Livstone E, Spiro H, Knowlton A, Kalser M, Barkin J, Lessner H, Mann-Kaplan R, Ramming K, Douglas HO Jr, Thomas P, Nave H, Bateman J, Lokich J, Brooks J, Chaffey J, Corson JM, Zamcheck N, Novak JW. Therapy of locally unresectable pancreatic carcinoma: a randomized comparison of high dose (6000 rads) radiation alone, moderate dose radiation (4000 rads + 5-fluorouracil), and high dose radiation + 5-fluorouracil: The Gastrointestinal Tumor Study Group. *Cancer* 1981; **48**: 1705-1710
9 **Gastrointestinal Tumor Study Group.** Treatment of locally unresectable carcinoma of the pancreas: comparison of combined-modality therapy (chemotherapy plus radiotherapy) to chemotherapy alone. *J Natl Cancer Inst* 1988; **80**: 751-755
10 **Klaassen DJ**, MacIntyre JM, Catton GE, Engstrom PF, Moertel CG. Treatment of locally unresectable cancer of the stomach and pancreas: a randomized comparison of 5-fluorouracil alone with radiation plus concurrent and maintenance 5-fluorouracil--an Eastern Cooperative Oncology Group study. *J Clin Oncol* 1985; **3**: 373-378
11 **Storniolo AM**, Enas NH, Brown CA, Voi M, Rothenberg ML, Schilsky R. An investigational new drug treatment program for patients with gemcitabine: results for over 3000 patients with pancreatic carcinoma. *Cancer* 1999; **85**: 1261-1268
12 **Carmichael J**, Fink U, Russell RC, Spittle MF, Harris AL, Spiessi G, Blatter J. Phase II study of gemcitabine in patients with advanced pancreatic cancer. *Br J Cancer* 1996; **73**: 101-105
13 **Burris HA 3rd**, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403-2413
14 **Casper ES**, Green MR, Kelsen DP, Heelan RT, Brown TD, Flombaum CD, Trochanowski B, Tarassoff PG. Phase II trial of gemcitabine (2,2'-difluorodeoxycytidine) in patients with adenocarcinoma of the pancreas. *Invest New Drugs* 1994; **12**: 29-34
15 **Rothenberg ML**, Moore MJ, Cripps MC, Andersen JS,

Portenoy RK, Burris HA 3rd, Green MR, Tarassoff PG, Brown TD, Casper ES, Storniolo AM, Von Hoff DD. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. *Ann Oncol* 1996; **7**: 347-353

16 **Sofuni A**, Iijima H, Moriyasu F, Nakayama D, Shimizu M, Nakamura K, Itokawa F, Itoi T. Differential diagnosis of pancreatic tumors using ultrasound contrast imaging. *J Gastroenterol* 2005; **40**: 518-525

17 **Kitano M**, Kudo M, Maekawa K, Suetomi Y, Sakamoto H, Fukuta N, Nakaoka R, Kawasaki T. Dynamic imaging of pancreatic diseases by contrast enhanced coded phase inversion harmonic ultrasonography. *Gut* 2004; **53**: 854-859

18 **Itoi T**, Takei K, Sofuni A, Itokawa F, Tsuchiya T, Kurihara T, Nakamura K, Moriyasu F, Tsuchida A, Kasuya K. Immunohistochemical analysis of p53 and MIB-1 in tissue specimens obtained from endoscopic ultrasonography-guided fine needle aspiration biopsy for the diagnosis of solid pancreatic masses. *Oncol Rep* 2005; **13**: 229-234

19 **Halm U**, Schumann T, Schiefke I, Witzigmann H, Mössner J, Keim V. Decrease of CA 19-9 during chemotherapy with gemcitabine predicts survival time in patients with advanced pancreatic cancer. *Br J Cancer* 2000; **82**: 1013-1016

20 **Heinemann V**, Schermuly MM, Stieber P, Schulz L, Jüngst D, Wilkowski R, Schalhorn A. CA19-9: a pedictor of response in pancreatic cancer treated with gemcitabine and cisplatin. *Anticancer Res* 1999; **19**: 2433-2435

21 **Koprowski H**, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. *Somatic Cell Genet* 1979; **5**: 957-971

22 **Itoi T**, Sofuni A, Fukushima N, Itokawa F, Tsuchiya T, Kurihara T, Moriyasu F, Tsuchida A, Kasuya K. Ribonucleotide reductase subunit M2 mRNA expression in pretreatment biopsies obtained from unresectable pancreatic carcinomas. *J Gastroenterol* 2007; **42**: 389-394

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BASIC RESEARCH

Metformin induces apoptosis of pancreatic cancer cells

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Abstract

AIM: To assess the role and mechanism of metformin in inducing apoptosis of pancreatic cancer cells.

METHODS: The human pancreatic cancer cell lines ASPC-1, BxPc-3, PANC-1 and SW1990 were exposed to metformin. The inhibition of cell proliferation and colony formation *via* apoptosis induction and S phase arrest in pancreatic cancer cell lines of metformin was tested.

RESULTS: In each pancreatic cancer cell line tested, metformin inhibited cell proliferation in a dose dependent manner in MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays). Flow cytometric analysis showed that metformin reduced the number of cells in G1 and increased the percentage of cells in S phase as well as the apoptotic fraction. Enzymelinked immunosorbent assay (ELISA) showed that metformin induced apoptosis in all pancreatic cancer cell lines. In Western blot studies, metformin induced poly-ADP-ribose polymerase (PARP) cleavage (an indicator of caspase activation) in all pancreatic cancer cell lines. The general caspase inhibitor (VAD-fmk) completely abolished metformin-induced PARP cleavage and apoptosis in ASPC-1 BxPc-3 and PANC-1, the caspase-8 specific inhibitor (IETD-fmk) and the caspase-9 specific inhibitor (LEHD-fmk) only partially abrogated metformin-induced apoptosis and PARP cleavage in BxPc-3 and PANC-1 cells. We also observed that metformin treatment dramatically reduced epidermal growth factor receptor (EGFR) and phosphorylated mitogen activated protein kinase (P-MAPK) in both a time- and dose-dependent manner in all cell lines tested.

CONCLUSION: Metformin significantly inhibits cell proliferation and apoptosis in all pancreatic cell lines. And the metformin-induced apoptosis is associated with PARP cleavage, activation of caspase-3, -8, and -9 in a time- and dose-dependent manner. Hence, both caspase-8 and -9-initiated apoptotic signaling pathways contribute to metformin-induced apoptosis in pancreatic cell lines.

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Key words: Metformin; Pancreatic cancer; Molecular classification; Apoptosis

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INTRODUCTION

The incidence of pancreatic cancer has steadily increased year by year, however its prognosis is still dismal, despite of all efforts in early diagnosis and therapy. Even with a complete surgical resection, the 5-year survival rate is < 20%[1]. It is the fourth leading cause of cancer-related deaths in Western industrialized countries[2]. In 2006, it was estimated that more than 33 700 new cases of pancreatic cancer would be diagnosed in the United States, with virtually the same number of deaths (32 300) from this disease[3]. Conventional therapies associated with surgery and radiotherapy often in combination with chemotherapy show modest efficacy in local control and palliation and no real progress in patient survival[4-6]. Thus, novel approaches to human pancreatic carcinoma therapy are urgently needed.

Metformin (1,1-dimethylbiguanide hydrochloride) is the most widely prescribed anti-hyperglycemic agent in the world. It reduces blood glucose, is not associated with significant toxicity or hypoglycemia, increases insulin sensitivity and reduces serum insulin levels[7].

Population-based studies have shown that patients treated with metformin exhibit unexpected but beneficial reductions in both obesity and cancer of several subtypes[8]. We have studied the effects of metformin on pancreatic cancer and identified novel molecular mechanisms of metformin activity.

MATERIALS AND METHODS

Reagents

Metformin was purchased from Sigma Chemical Co., MO and dissolved in sterile water to make a 1M stock solution. Caspase-3 substrate, Ac-DEVD-pNA, caspase-8 substrate, Ac-IETD-pNA, and caspase-9 substrate, Ac-LEHD-pNA, were obtained from Alexis Biochemicals, San Diego, CA. Specific pan-caspase inhibitor, z-VAD-fmk, caspase-8 inhibitor, z-IETD-fmk, and caspase-9 inhibitor, z-LEHD-fmk, were obtained from BD Biosciences, San Jose, CA.

Antibodies for Western blot analysis were from following sources: caspase-8 mouse mAb (1C12), caspase-9 polyclonal antibody, and caspase-3 rabbit mAb (8G10), P-MAPK (Phospho-p44/42 MAP Kinase Thr202/Tyr204), Akt, and P-Akt (Phospho-Akt, Ser-473) (Cell Signaling Technology, Inc., Beverly, MA); MAPK (ERK2) (Santa Cruz, CA, USA); Poly (ADP-ribose) polymerase (PARP) mAb (C-2-10) (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA); EGFR mouse mAb (clone F4), β-actin mouse mAb (clone AC-75) (Sigma Chemical Co.). All other reagents were purchased from Sigma Chemical Co. unless otherwise specified.

Cells and cell culture

The human pancreatic cancer cell lines SW1990 ASPC-1 BxPc-3 PANC-1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 (Life Technologies). All cell lines were cultured at a 37℃ humidified atmosphere containing 95% air and 5% CO_2 and were split twice a week.

Cell proliferation assay

The CellTiter96™ AQ non-radioactive cell proliferation kit (Promega Corp., Madison, WI) was used to determine the cell viability. In brief, cells were plated onto 96-well plates with complete medium for 24 h incubation in a 37℃ humidified atmosphere containing 95% air and 5% CO_2. Cells were then grown in either 0.1 mL medium with 5% FBS as control, or 0.1 mL of the same medium containing a series of doses of metformin and incubated for another 72 h. After reading all wells at 490 nm with a micro-plate reader, the percentages of surviving cells from each group relative to controls, defined as 100% survival, were determined by reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt).

Clonogenic assay

In brief, cells were seeded into 6-well plates in triplicates at a density of 100-500 cells/well in 2 mL of medium containing 10% fetal bovine serum (FBS). After 24 h incubation, cells were then cultured with medium with 5% FBS as control, or the same medium containing a series of doses of metformin for 14 d in a 37℃ humidified atmosphere containing 95% air and 5% CO_2. The cell clones were stained for 15 min with a solution containing 0.5% crystal violet and 25% methanol, followed by three rinses with tap water to remove excess dye. The colony numbers were counted with gel documentation system EAGLE EYETM Ⅱ (Stratagene, La Jolla, CA).

Flow cytometric analysis

Flow cytometric analyses were performed to define the cell cycle distribution for metformin treated and untreated cells. In brief, cells grown in 100-mm culture dishes were harvested by trypsinization and fixed with 70% ethanol. Cells were stained for total DNA content with a solution containing 50 μg/mL propidium iodide and 100 μg/mL RNase I in phosphate buffered saline (PBS) for 30 min at 37℃. Cell cycle distribution was then analyzed at the Flow Cytometry Core Facility of UCDHSC with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Caspase enzymatic activity assay

Caspase enzymatic activities were measured using a modified colorimetric assay kit from Clontech Laboratories, Inc. (Palo Alto, CA). The assay was based on spectrophotometric detection of the chromophore p-nitroanilide (pNA), which is cleaved from caspase-specific substrates by activated caspases (DEVD-pNA by activated caspase-3, IETD-pNA by activated caspase-8, and LEHD-pNA by activated caspase-9).

Quantification of apoptosis

An apoptosis ELISA kit (Roche Diagnostics Corp.) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes). This photometric enzyme immunoassay was performed according to the manufacturer's instructions.

Western blotting analysis

Protein expression levels were determined by Western blot analysis. Briefly, cells were lysed in a buffer containing 50 mmol/L Tris, pH 7.4, 50 mmol/L NaCl, 0.5% NP-40, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 g/mL leupeptin, and 25 g/mL aprotinin. The lysates were centrifuged at full speed in a microcentrifuge for 20 min and the supernatants were collected for protein concentration determination by the Coomassie Plus protein assay reagent (Pierce Chemical Co., Rockford, IL). Equal amounts of cell lysates were boiled in Laemmli SDS-sample buffer, resolved by SDS-PAGE, and Western blot analysis with specific antibodies as described in the Figure legends.

RESULTS

Metformin inhibits cell proliferation/survival in pancreatic cancer cells

In each pancreatic cancer cell line tested, metformin

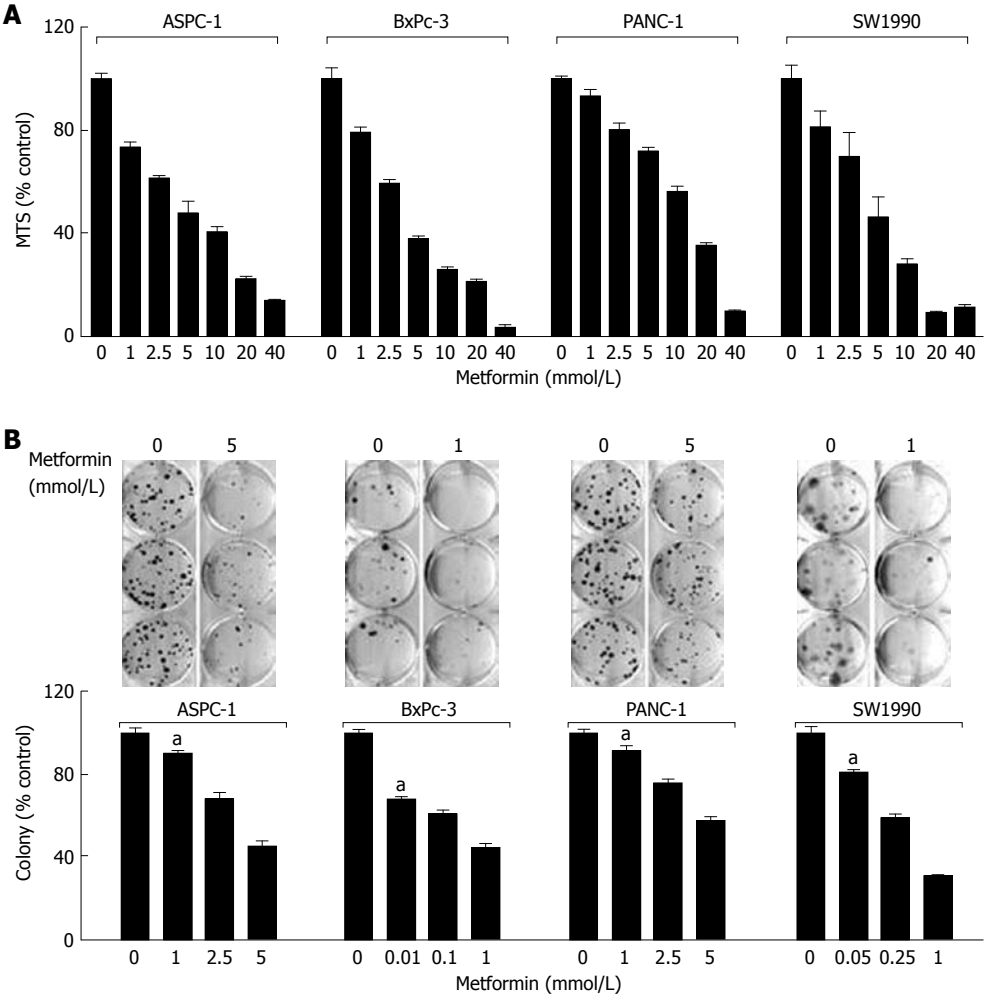

Figure 1 Metformin inhibits proliferation/survival in the basal-like subtype of pancreatic cancer cells. A: ASPC-1, BxPc-3, PANC-1 and SW1990 were plated onto 96-well plates with either complete medium or medium containing a series doses of metformin; B: ASPC-1, BxPc-3, PANC-1 and SW1990 were grown in triplicates in the absence or presence of metformin at different concentrations for 2-3 wk.

inhibited cell proliferation in a dose-dependent manner in MTS assays (Figure 1A). The MTS IC50 values for metformin activity were less than 5 mmol/L (in three of the four lines ASPC 1, BxPc 3, and PANC 1); while for SW1990 cells it was 10 mmol/L. To study the long- term effects of metformin on pancreatic cancer cells, we used clonogenicity assays. Metformin resulted in significantly fewer colonies at concentrations well below what was inhibitory using the 72 h MTS assay described above. The lowest inhibitory concentrations for the 4 cell lines ASPC 1, BxPc 3, and PANC 1, and SW1990 were 1 mmol/L, 0.01 mmol/L, 1 mmol/L, and 0.05 mmol/L, respectively (Figure 1B).

Metformin blocks cell cycle progression and induces apoptosis in pancreatic cancer cells

To study the effects of metformin on cell cycle distribution and progression, we used flow cytometric methods on metformin treated and untreated cells. Metformin reduced the number of cells in G1 and increased the percentage of cells in S phase as well as the apoptotic fraction (Figure 2). The percentages of apoptotic cells in all four pancreatic cancer cell lines were increased significantly in metformin-treated as compared with untreated cells (Figure 2). These data suggest that the effects of metformin on pancreatic cancer cells were distinct and, therefore, should occur through different molecular mechanisms.

Metformin selectively induces apoptosis via caspases activation in pancreatic cancer cells

ELISA specific assays for apoptosis were used to quantitatively evaluate metformin associated apoptosis in pancreatic cancer cell lines. Metformin induced apoptosis in all pancreatic cancer cell lines (Figure 3A). In Western blot studies, metformin also induced PARP cleavage (an indicator of caspase activation) pancreatic cancer cell lines (Figure 3A). Consistent with these data, protein levels of the pro-caspases-8, -9, and -3 were each reduced by metformin in the pancreatic cell lines (Figure 3A). Using these three assays (Western blot analyses of PARP cleavage, an apoptosis specific ELISA and caspase activity assays) the effects of metformin over a range of treatment intervals and drug concentrations were evaluated.

Activation of both caspase-8 and caspase-9 contributes to metformin-induced apoptosis

There are two well characterized caspase cascades in

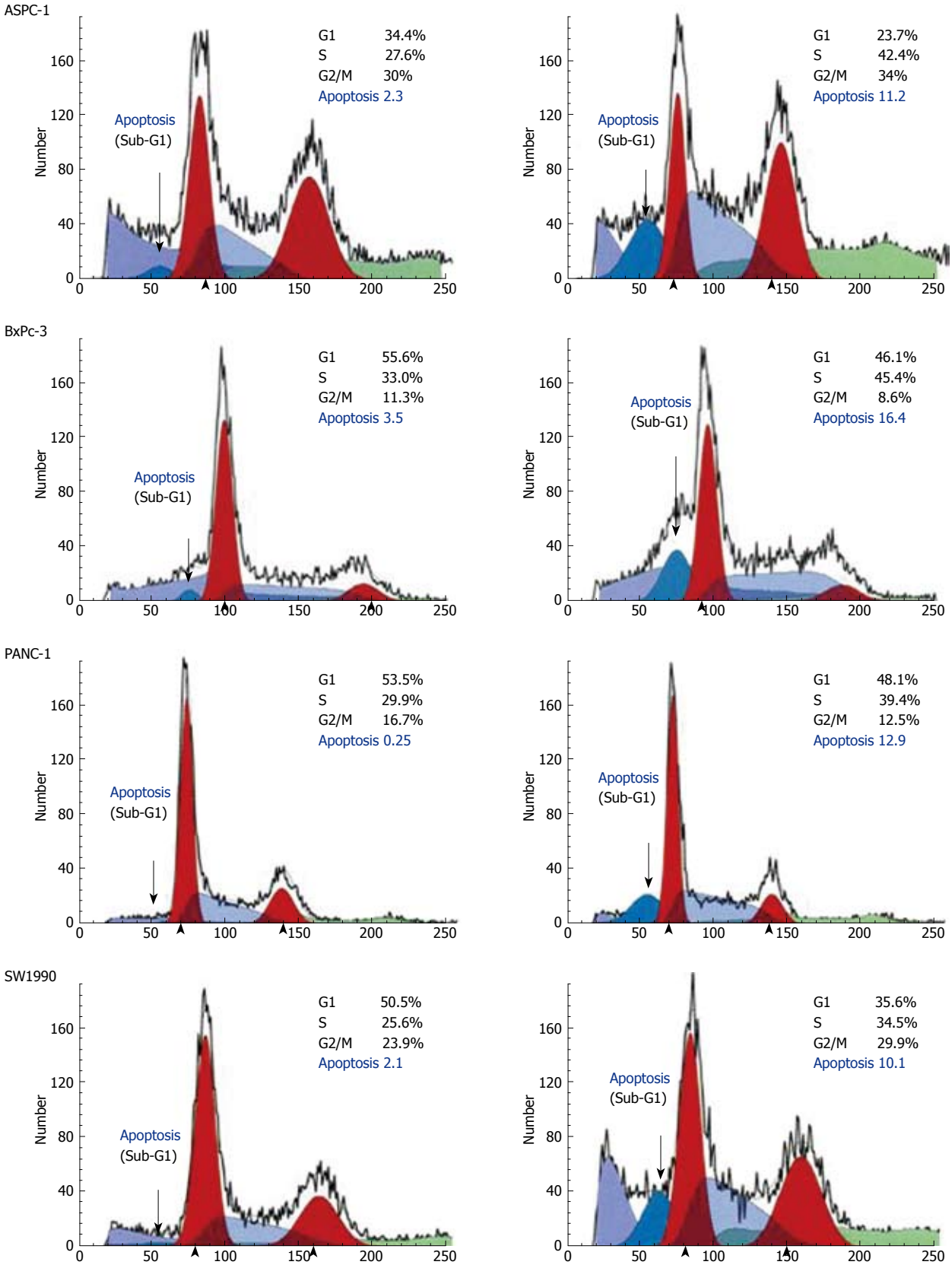

Figure 2 Metformin induces apoptosis and blocks cell cycle progression in the pancreatic cancer cells.

apoptosis: one initiated by cell surface death receptors (the so-called extrinsic pathway *via* caspase-8) and the other triggered by changes in mitochondrial integrity (caspase-9 activation, known as the intrinsic pathway)[9-11]. To further define the effects of metformin, we used specific caspase inhibitors to determine which might block metformin-induced apoptosis. The general caspase inhibitor, VAD-fmk completely abolished metformin-induced PARP cleavage and apoptosis in three cell lines (Figure 4). The caspase-8 specific inhibitor (IETD-fmk) and the caspase-9

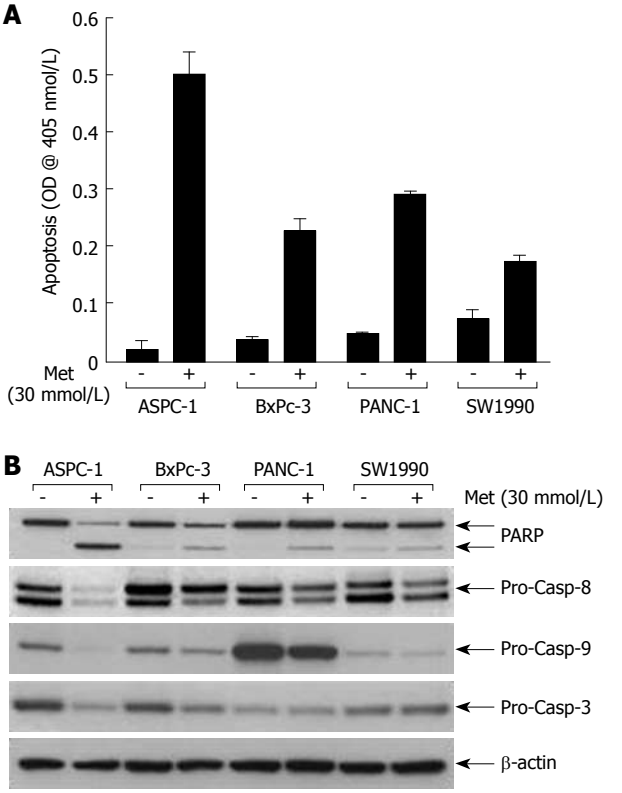

Figure 3 Metformin increases PARP cleavage, reduces levels of pro-caspase-8, -9, -3, and induces apoptosis in pancreatic cancer cells.

specific inhibitor (LEHD-fmk) only partially abrogated metformin-induced apoptosis and PARP cleavage in BxPc-3 and PANC-1 cells (Figure 4B and C), although IETD-fmk had a much greater effect than LEHD-fmk in ASPC-1 cells (Figure 4A).

Metformin reduces epidermal growth factor receptor (EGFR) and inhibits Akt and MAPK signaling

Pancreatic carcinomas were well known for EGFR overexpression, and they appear to utilize EGFR as an important pro-carcinogenic, pro-growth receptor. Using Western blot analyses, we observed that metformin treatment dramatically reduced EGFR and P-MAPK, in both a time- and dose-dependent manner in three cell lines tested (Figure 5). Of interest, short-term (8 h) treatment with metformin transiently raised P-MAPK levels. Early induction of MAPK signaling may reflect metformin's interaction with the insulin receptor[12]. P-Akt levels were also significantly lowered by metformin treatment in one cell line (ASPC-1; Figure 5A), although there were only minor changes of P-Akt in BxPc-3 cells (Figure 5B). P-Akt was undetectable and unchanged by metformin in PANC-1 cells (Figure 5C). This data suggests that apoptosis induced by metformin may be mechanistically driven by a reduced EGFR, with subsequent inactivation of downstream signaling involving MAPK and to a lesser extent Akt.

DISCUSSION

The development, continued growth and metastasis

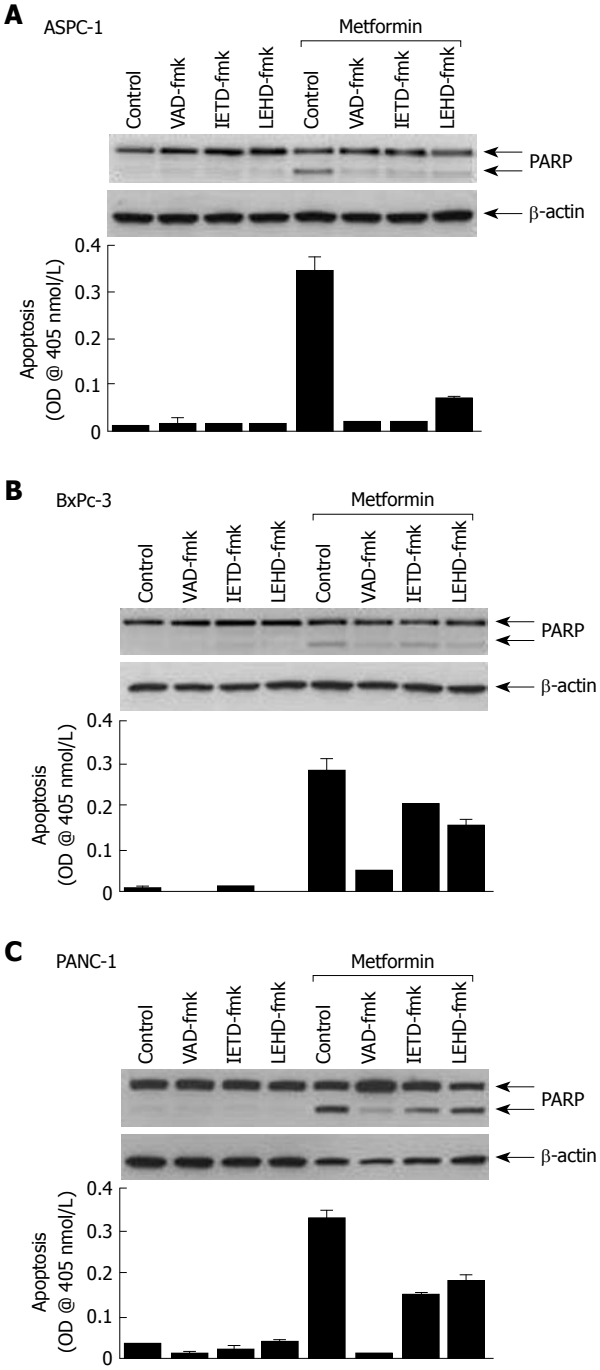

Figure 4 Activation of both caspase-8 and caspase-9 contributes to metformin-induced apoptosis in pancreatic cancer cells. A: ASPC-1; B: BxPc-3; C: PANC-1.

of pancreatic cancer are driven by multiple genetic and epigenetic changes, including inactivation of tumor suppressor genes and activation of proto-oncogenes[13]. Since the last decade, molecular biology and technology have contributed significantly to the development of therapeutic agents in medicine, and especially in oncology. The major areas include inhibition of tumor growth, inhibition of metastatic invasion, and inhibition of intercellular signal transduction and compensation of gene expression[14]. But it still needs a long time to treat pancreatic cancer patients. Up to now, only two combinations, Gem plus erlotinib and Gem plus

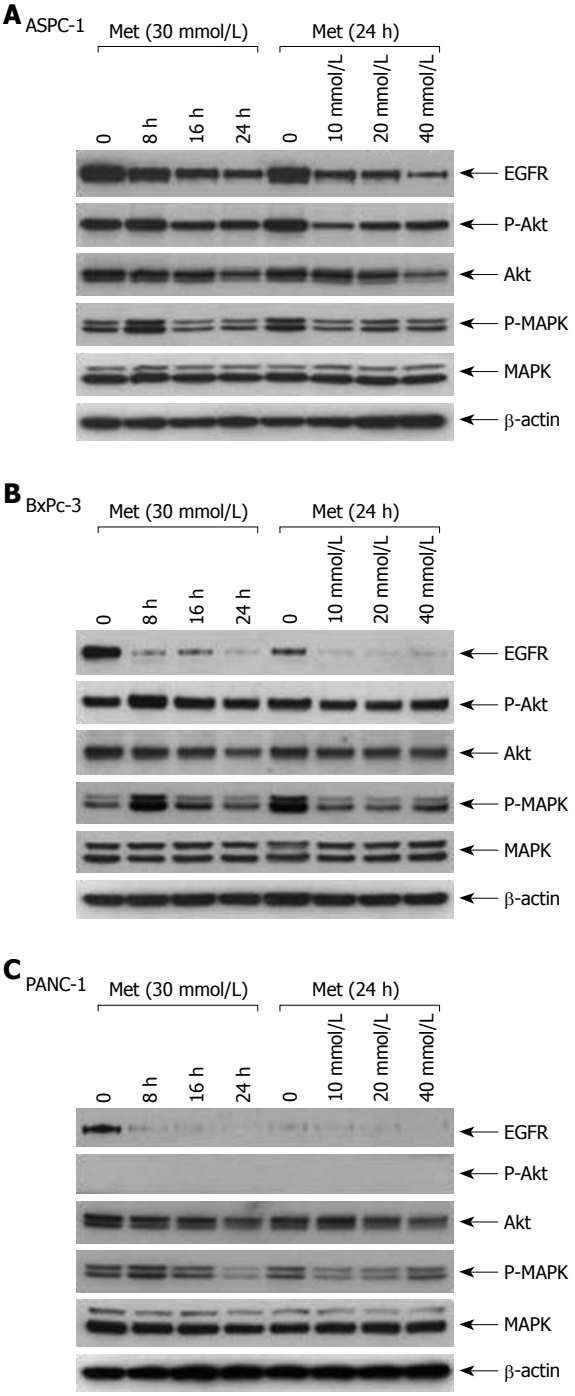

Figure 5 Metformin lowers EGFR expression levels and inhibits downstream signaling in pancreatic cancer cells. A: ASPC-1; B: BxPc-3; C: PANC-1.

capecitabine have achieved a slightly longer survival of the patients[15].

Metformin has a long track record of human use, with limited toxicity and it is relatively inexpensive. It might, therefore, be of great clinical benefit for pancreatic cancer treatment. According to our data, concomitant metformin therapy can enhance the response of patients to DNA damaging agents (chemotherapy and radiation therapy) because of their extended arrest in the S phase. Metformin should also enhance RTK inhibitor and anti-EGFR treatment response, because of its action on EGFR and P-MAPK effects. Finally, metformin might enhance treatment with apoptosis-inducing agents that inhibit PARP, for it induces PARP cleavage. Its usage in pancreatic cancer patients may also have additional benefits, including weight control, stabilization of pre-diabetic syndromes, regulation of glucose/insulin and adipogenesis pathways.

Two recent studies have indicated that EGFR was detected in more than 95% of patients with pancreatic cancer[16,17]. Also, co-expression of EGFR and its ligands is a common occurrence in pancreatic cancer and has been shown to function as an autocrine[13]. Real advancement towards individualized pancreatic cancer treatment will require understanding the molecular mechanisms underlying pancreatic cancer biology[18,19]. Early studies of basal carcinomas focused on EGFR as a molecular target, although EGFR inhibitors have not shown great efficacy in clinical trials[11,20]. The data we present here indicates that metformin may serve as a valuable treatment option for pancreatic cancer patients.

Metformin is widely used as a first-line treatment for pre-diabetic syndromes and type Ⅱ diabetes[21]. Of interest, some studies have shown that women treated with metformin have a lower overall incidence of cancer, including pancreatic cancer[8]. Metformin may therefore be useful as a preventative agent in selected patients to reduce, either directly through cell growth inhibition or indirectly through obesity and diabetes control. Further studies of metformin may provide more insights into its efficacy.

In summary, we have demonstrated that the anti-diabetic drug, metformin, selectively induces apoptosis through activation of the caspase cascade, abrogation of EGFR and downstream signaling in the basal subtype of pancreatic cancer cells. Metformin may be a low toxicity, novel therapeutic strategy for the difficult-to-treat pancreatic cancer patients.

ACKNOWLEDGMENTS

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COMMENTS

Background

Pancreatic carcinoma is one of the tumors with a high incidence rate. It is less sensitive to standard adjuvant chemotherapy. Metformin (1,1-dimethylbiguanide hydrochloride) is the most widely prescribed anti-hyperglycemic agent in the world. Population-based studies have shown that patients treated with metformin exhibit unexpected but beneficial reductions in both obesity and cancer. But no study about the therapeutic effect of metformin in pancreatic caner has been reported.

Research frontiers

There are many studies about the treatment of pancreatic cancer. Target therapy is the most popular part of pancreatic cancer research as well as the use of traditional medicine. Metformin is one of the traditional drugs which are thought to have anti-cancer effects.

Innovations and breakthroughs

This study showed that metformin significantly inhibited cell proliferation and induced apoptosis in all pancreatic cell lines, and the metformin-induced

apoptosis was associated with poly-ADP-ribose polymerase leavage, activation of caspase-3, -8, and -9 in a time- and dose-dependent manner.

Applications

Metformin is a common drug which has been used for many years. This study has suggested that it had a potential therapeutic effect for pancreatic cancer. Although further *in vivo* studies are needed, metformin may be a low toxicity, novel therapeutic drug for the difficult-to-treat pancreatic cancer patients.

Peer review

This is a very interesting study. Metformin, the commonly used anti-diabetic drug has been proven to have anti-cancer effect in pancreatic carcinoma in *in vitro* studies with pancreatic cancer cell line. The study was strictly done with standard molecular techniques. The result is of clinical implication in the treatment of pancreatic cancer.

REFERENCES

1 **Wagner M**, Redaelli C, Lietz M, Seiler CA, Friess H, Buchler MW. Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma. *Br J Surg* 2004; **91**: 586-594

2 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108

3 **Jemal A**, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA Cancer J Clin* 2006; **56**: 106-130

4 **Azria D**, Ychou M, Jacot W, Thezenas S, Lemanski C, Senesse P, Prost P, Delard R, Masson B, Dubois JB. Treatment of unresectable, locally advanced pancreatic adenocarcinoma with combined radiochemotherapy with 5-fluorouracil and cisplatin. *Pancreas* 2002; **25**: 360-365

5 **Li D**, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet* 2004; **363**: 1049-1057

6 **Czito BG**, Willett CG, Bendell JC, Morse MA, Tyler DS, Fernando NH, Mantyh CR, Blobe GC, Honeycutt W, Yu D, Clary BM, Pappas TN, Ludwig KA, Hurwitz HI. Increased toxicity with gefitinib, capecitabine, and radiation therapy in pancreatic and rectal cancer: phase I trial results. *J Clin Oncol* 2006; **24**: 656-662

7 **Hundal RS**, Inzucchi SE. Metformin: new understandings, new uses. *Drugs* 2003; **63**: 1879-1894

8 **Evans JM**, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 2005; **330**: 1304-1305

9 **Ashkenazi A**, Dixit VM. Death receptors: signaling and modulation. *Science* 1998; **281**: 1305-1308

10 **Thornberry NA**, Lazebnik Y. Caspases: enemies within. Science 1998; **281**: 1312-1316

11 **Green MR**. Targeting targeted therapy. *N Engl J Med* 2004; **350**: 2191-2193

12 **Holland W**, Morrison T, Chang Y, Wiernsperger N, Stith BJ. Metformin (Glucophage) inhibits tyrosine phosphatase activity to stimulate the insulin receptor tyrosine kinase. *Biochem Pharmacol* 2004; **67**: 2081-2091

13 **Papageorgio C**, Perry MC. Epidermal growth factor receptor-targeted therapy for pancreatic cancer. *Cancer Invest* 2007; **25**: 647-657

14 **Dhar A**, Mehta S, Banerjee S, Dhar K, Dhar G, Sengupta K, Ray G, Banerjee SK, Campbell DR. Epidermal growth factor receptor: is a novel therapeutic target for pancreatic cancer? *Front Biosci* 2005; **10**: 1763-1767

15 **Boeck S**, Hinke A, Wilkowski R, Heinemann V. Importance of performance status for treatment outcome in advanced pancreatic cancer. *World J Gastroenterol* 2007; **13**: 224-227

16 **Xiong HQ**, Rosenberg A, LoBuglio A, Schmidt W, Wolff RA, Deutsch J, Needle M, Abbruzzese JL. Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, in combination with gemcitabine for advanced pancreatic cancer: a multicenter phase II Trial. *J Clin Oncol* 2004; **22**: 2610-2616

17 **Bloomston M**, Bhardwaj A, Ellison EC, Frankel WL. Epidermal growth factor receptor expression in pancreatic carcinoma using tissue microarray technique. *Dig Surg* 2006; **23**: 74-79

18 **Baselga J**, Arteaga CL. Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. *J Clin Oncol* 2005; **23**: 2445-2459

19 **Krause DS**, Van Etten RA. Tyrosine kinases as targets for cancer therapy. *N Engl J Med* 2005; **353**: 172-187

20 **Spector NL**, Xia W, Burris H 3rd, Hurwitz H, Dees EC, Dowlati A, O'Neil B, Overmoyer B, Marcom PK, Blackwell KL, Smith DA, Koch KM, Stead A, Mangum S, Ellis MJ, Liu L, Man AK, Bremer TM, Harris J, Bacus S. Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. *J Clin Oncol* 2005; **23**: 2502-2512

21 **Kirpichnikov D**, McFarlane SI, Sowers JR. Metformin: an update. *Ann Intern Med* 2002; **137**: 25-33

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BASIC RESEARCH

Tissue array for *Tp53*, *C-myc*, *CCND1* gene over-expression in different tumors

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Abstract

AIM: To rapidly detect molecular alterations in different malignancies and investigate the possible role of *Tp53*, C-*myc*, and *CCND1* genes in development of tumors in human organs and their adjacent normal tissues, as well as the possible relation between well- and poorly-differentiated tumors.

METHODS: A tissue array consisting of seven different tumors was generated. The tissue array included 120 points of esophagus, 120 points of stomach, 80 points of rectum, 60 points of thyroid gland, 100 points of mammary gland, 80 points of liver, and 80 points of colon. Expressions of *Tp53*, *C-myc*, and *CCND1* were determined by RNA *in situ* hybridization. 3′ terminal digoxin-labeled anti-sense single stranded oligonucleotide and locked nucleic acid modifying probe were used.

RESULTS: The expression level of *Tp53* gene was higher in six different carcinoma tissue samples than in paracancerous tissue samples with the exception in colon carcinoma tissue samples ($P < 0.05$). The expression level of *CCND1* gene was significantly different in different carcinoma tissue samples with the exception in esophagus and colon carcinoma tissue samples. The expression level of *C-myc* gene was different in esophagus carcinoma tissue samples ($\chi^2 = 18.495$, $P = 0.000$), stomach carcinoma tissue samples ($\chi^2 = 23.750$, $P = 0.000$), and thyroid gland tissue samples ($\chi^2 = 10.999$, $P = 0.004$). The intensity of signals was also different in different carcinoma tissue samples and paracancerous tissue samples.

CONCLUSION: Over-expression of the *Tp53*, *CCND1*, and *C-myc* genes appears to play a role in development of human cancer by regulating the expression of mRNA. *Tp53*, *CCND1* and *C-myc* genes are significantly correlated with the development of different carcinomas.

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Key words: *Tp53*; *C-myc*; *CCND1*; Tissue microarray; RNA *in situ* hybridization

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INTRODUCTION

Over-expression or amplification of a particular oncogene was first described in a tumor. Subsequently, other tumors were evaluated, mostly in the order of their perceived importance with rare tumors neglected. Therefore, it may take several years from the discovery of a potentially important molecular alteration to the definition of different primary tumors where this specific alteration may play a role in the development of such tumors. Tissue microarray has the potential to greatly facilitate analysis of alterations in different

tumors. *Tp53* is a specific protein produced by the most commonly mutated gene in human cancer that suppresses the growth of tumors[1]. Like other tumor-suppressor genes, *Tp53* normally controls cell growth. If *Tp53* is physically lost or not in effect (because of its inactivation), it may permit cells to divide without restraint[2]. The level of *Tp53* has a prognostic (predictive) value for tumors. For example, breast cancer patients with a high level of *Tp53* after mastectomy are at a higher risk for cancer recurrence than those with a low level of *Tp53*[3]. The buildup of *Tp53* within cancer cells is a sign that *Tp53* is not working properly to suppress the growth of tumors[4]. *CCND1* forms a holoenzyme with a cyclin-dependent kinase (CDK), either CDK4 or CDK6 that phosphorylates the retinoblastoma gene product of pRb. Since the phosphorylation of pRb results in the release of E2F transcription factors, freeing them to stimulate transcription of growth-promoting target genes, over-expression of *CCND1* promotes tumor progression through the G1 phase of cell cycle in cells grown on a substratum[5,6]. Over-expression of *CCND1* has been reported in a variety of human tumors including cancers of the lung, head, neck, and bladder[7]. It was reported the over-expression rate of *CCND1* is 30%-73% in patients with breast carcinoma[8,9]. Whether the expression of *CCND1* can serve as a prognostic indicator of tumors has also been investigated, but the conclusions are contradictory in breast carcinoma[10]. *C-myc* gene is an important member of the *myc* gene family, can translocate and regulate a variety of substances, enable an unlimited cell proliferation, immortalize cell life, and is involved in tumor development[11,12].

At present, studies about *Tp53*, *CCND1* and *C-myc* are mainly focused on Caucasian (white race) patients but not on Asians. Since the carcinogenesis of some organ carcinomas might show discrepancies among different races (Caucasian and Asian), detailed information on over-expression and amplification of the three genes in Chinese patients with carcinoma and its correlation with pathological parameters is needed. To more clearly address the importance of over-expression and expression of *Tp53*, *CCND1* and *C-myc* genes in human cancer, we used *in situ* hybridation technique, which can clearly distinguish stromal from carcinoma components, and decrease the loss of such components in RNA extraction procedure. This approach to the specific location of genes on chromosomes is a technique for the hybridization of DNA and RNA "*in situ*". This procedure can isolate or synthesize "*in vitro*" specific radioactive RNA or DNA (known as probes), and then anneal them to chromosomes treated in such a manner that their basic double stranded DNA has been "melted" or dissociated. The relation between *Tp53* and *CCND1*, *C-myc* mRNA expressions was also discussed in this study.

MATERIALS AND METHODS

Materials and microarray construction

A total of 620 primary tumor tissue samples from 7 different tumors and 20 normal tissue samples were snap-frozen and stored at -70℃. All patients were Chinese and underwent operation at Xiamen University Hospital during 2000-2006. Tissue blocks measuring approximately 1.5 cm × 1.5 cm × 0.3 cm of grossly apparent carcinoma and non-pathologic organs were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (1‰ DEPC, pH 7.4) for 24 h, dehydrated through gradient ethanol, and embedded in paraffin. A hematoxylin and eosin (HE)-stained section was made from each block to define the representative tumor region. Representative areas in different lesions were carefully selected on HE-stained sections and marked on individual paraffin blocks. Tissue cylinders with a diameter of 1-mm were then punched from tumor areas in each "donor" tissue block and put into a recipient paraffin block using a custom-made precision instrument. Five-mm sections of the resulting multiple tumor tissue microarray blocks were transferred to glass slides using the paraffin sectioning aid system [adhesive-coated slides (PSA-CS4x), adhesive tape, and UV lamp; Instru-medics, Inc., Hackensack, NJ], supporting the cohesion of 0.6-mm array elements. The final TMA consisted of 640 1-mm diameter TMA cores each spaced at 0.8 mm between core centers. A section stained with HE was reviewed to confirm the presence of morphologically representative areas in the original lesions.

Preparation of digoxigenin-labeled probes for RNA in situ hybridization

Anti-sense probes matched the corresponding sequence. Locked nucleic acid (LNA) was modified to increase the stability and sensitivity of probes. The sequences of probes are 5'-CAGGACAGGCACAA ACACGCACCT*CAAAGCTGTTCCGTCCAGTA GATTAC-3Dig (*Tp53*), 5'-CCTCCTCGCACTTC TGTTCCTCGCAGACCT*CCAGCATCCAGGT GGCGACGATCTTCCG-3Dig (*CCND1*), 5'- CTTCCT CATCTTCTTGTTCCTCCTCAGAG T*CGCTGCTG GTGGTGGGCGGTGTC-3Dig (*C-myc*). The positive probe was 30T. Asterisk indicates that the LNA modifying site and 3' terminal were labeled with digoxigenin. All probes were synthesized by Sangon (Shanghai).

RNA *in situ* hybridization

Hybridization procedures were performed in this study based on the instructions of RISH kits (Cybrdi USA) with some modifications. The glassware was washed, rinsed in distilled deionized water, and autoclaved before use. Gloves were worn when the glassware and slides were handled to prevent RNase contamination on the tissue. Because of the differences in tissues and probes, we performed different pilot-experiments to achieve the best results (Table 1). Deparaffinized sections were mounted on Denhardt-coated glass slides and treated with pepsin (0.25 mg/mL in DEPC H2O-HCl) for 25-30 min in a 37℃ water bath. The treated sections were then processed for *in situ* hybridization at 42℃-45℃ for 36-48 h. The hybridization mixture contained the labeled

Table 1 Pilot-experiment data

	Probe concentration (ng/μL)		Digest time (min)	Incubation time (h)/temperature (℃)	Chromogenic time (min)
Tissue array	*Tp53*	10	30	44/48	110
Tissue array	*C-myc*	10	20	41.5/42	30
Tissue array	*CCND1*	10	20	36/45	50

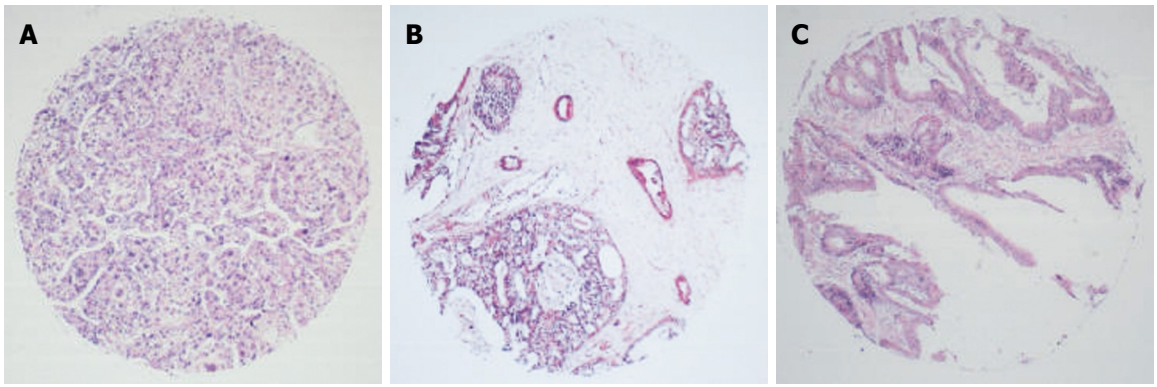

Figure 1 Over-expression of *CCND1* in hepatoma (A), breast cancer (B), and gastric cancer (C).

oligonucleotide probe, 50% formamide, 10 mmol/L Tris-HCl, 1 mmol/L vanadyl-ribonucleoside complex (Sigma 94740), 1 mmol/L CTAB (Sigma 855820, pH 7.0), 0.15 mol/L Nacl, 1 mmol/L EDTA (pH 7.0), 1 × Denhardt's mixture and 10% dextran sulfate. After hybridization, the slides were washed three times, 30 min each time, in 0.1 mol/L TBS at room temperature, then treated with TBS (100 mmol/L Tris, pH 7.5, 150 mmol/L NaCl) containing a 1% blocking reagent (Roche) and 0.03% Triton X-100 for 30 min at room temperature and incubated for 30 min with antidioxigenin alkaline phosphataseconjugated antibodies (Roche) diluted at 1:500 in TBS containing 0.03% Triton X-100 and a 1% blocking reagent. After washed three times, 15 min each time, in TBS and 0.05% Tween, the slides were rinsed in a DAP-buffer (100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$) and subsequently hybridization signals were visualized using nitroblue tetrazolium and 5-brom-4-chlor-3-indolyl phosphate as substrates [DAP-buffer in 10% PVA(Sigma 341584)].

Statistical analysis

All cases were first grouped to calculate the percentages of positive and negative cases. χ^2 contingency test was used to evaluate the differences among groups. Analyses were performed using the statistical package SPSS 10.0 (SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

Technical considerations

The tissue micro-array technology is substantially different from the traditional multi-tissue blocks, which are often used in pathology laboratories for antibody testing. The most important advantages of tissue micro-array technology include increased capacity, negligible damage to the original tissue blocks, precise positioning of tissue specimens and possibility of automatic construction and analysis of arrays. In this study, we chose 4% paraformaldehyde in phosphate-buffered saline (1‰ DEPC PBS) as a fixation agent, which can decrease degradation of RNA and result in a good morphology. RISH analysis showed that 80%-95% of tumor samples were interpretable. RISH-related weak hybridization, background, and tissue damage were responsible for about one-sixth of the non-informative cases.

Expression of Tp53, C-myc and CCND1 gene in different organs

RNA *in situ* hybridization (RNA-ISH) was used to detect specific RNAs *in situ*. Most protocols using 4% paraformaldehyde as a fixation agent increased the probe permeability, and hybridization was performed in a buffer containing 50% formamide. The typical results of ISH were observed as amethyst dots on arrays, locating in cytoplasm or cytoblasts (Figures 1-3). RNA analysis and quantification required completely intact, non-degraded RNA samples to produce optimal results. Vanadium oxide ions and formation of complex nucleoside could protect RNA degradation from RNase. Cetyltrimethyl ammonium bromide (CTAB) could stabilize the Oligo probe and target sequence formation of double-stranded structures, thus improving the re-annealing speed.

Two major factors, probe accessibility and affinity to the targeted RNA molecules, were found to affect the hybridization efficiency. Poor probe hybridization efficiency was found to be one of the major drawbacks of RNA-targeted *in situ* hybridization. The monomer containing LNA greatly improved the stability and sensitivity of RNA-targeted *in situ* hybridization. The six array elements resulting in 640 points are shown in Figures 1 and 2.

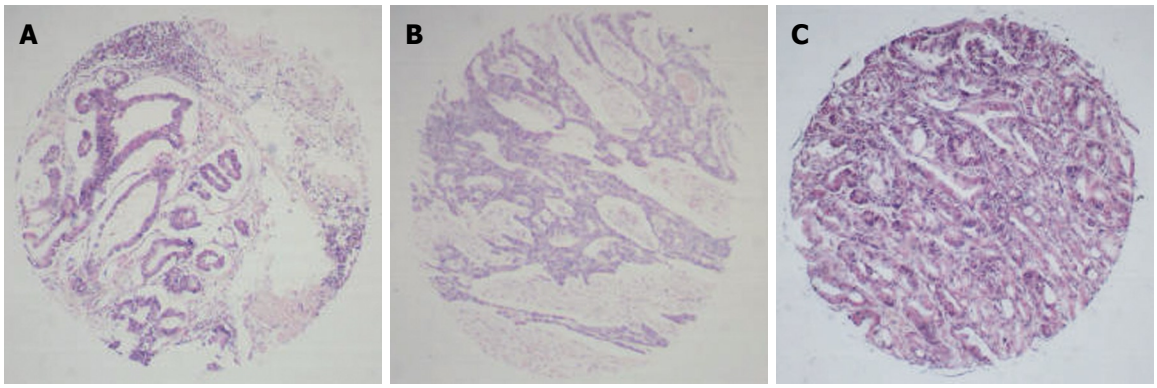

Figure 2 Over-expression of *C-myc* in esophageal carcinoma (A), esophageal squamous cell carcinoma (B), and colon cancer (C).

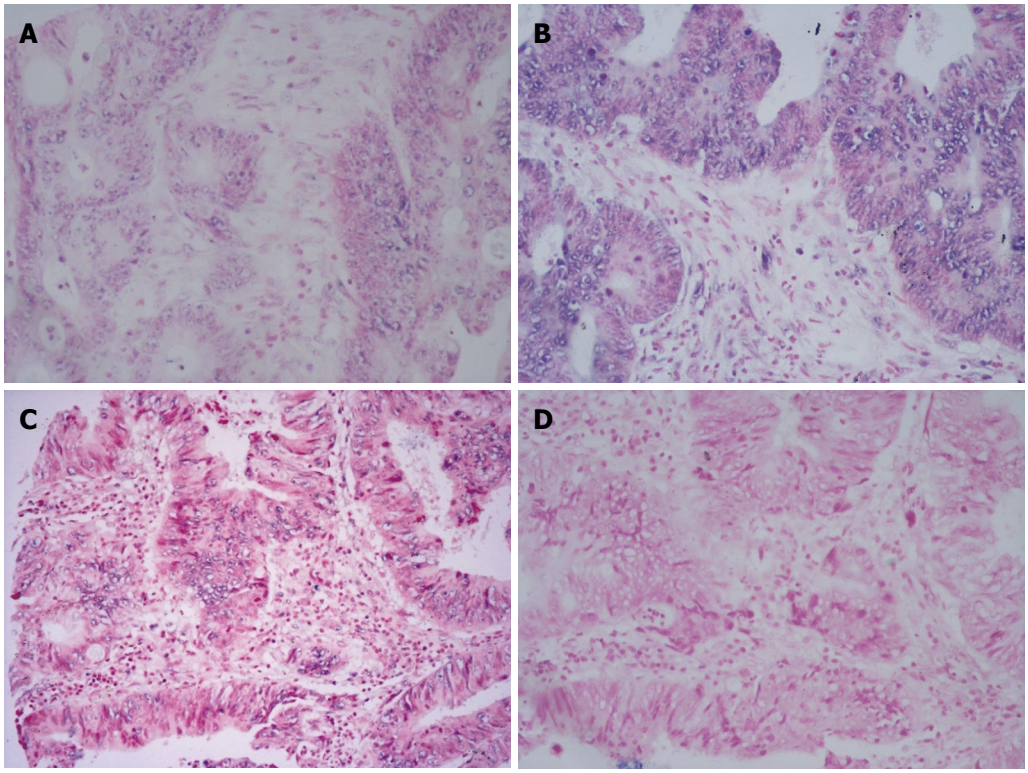

Figure 3 RISH showing expression of *CCND1* (A),*C-myc* (B),*Tp53* (C), and negative expression (D) in different tumor tissue samples. Deparaffinized sections mounted on denhardt-coated glass slides were treated with pepsin for 20, 25 and 30 min, respectively in a 37℃ water bath. The treated sections were then processed for *in situ* hybridization at 4℃, 42℃ and 44℃ for 36, 41.5 and 48 h, respectively. The hybridization mixture contained 10 ng of the labeled oligonucleotide probe. Post-hybridization, slides were treated with a 1% blocking reagent for 30 min at room temperature and then incubated for 45, 60 and 50 min, respectively, with antidioxigenin alkaline phosphatase conjugated antibodies diluted at 1:500 in TBS. After washed three times for 5 min in TBS, the slides were rinsed in a DAP-buffer and hybridization signals were visualized using NBT/BCIP as substrates (x 200).

Gene over-expression

A total of 640 samples were studied for *Tp53*, *CCND1* and *C-myc* mRNA expression, with non-radioactive *in situ* hybridization. ISH results were expressed as intensity and percentage based upon the signal intensity of positive staining and the number of stained cells within the sample, respectively. Tumor was graded according to the World Health Organization System. Normal tissues were also obtained from patients and a tumor free area in the same specimen served as a control. The presence of occasional tumor cells without detectable over-expression might be attributed to the truncated cells that had lost their genetic material during sectioning or tissue pretreatment before hybridization.

Tp53 was over-expressed in different tumors. The over-expression frequencies of *Tp53* in these tumors are shown in Table 2. A significant difference was observed in carcinomatous and paracancerous tissue samples, including those of esophagus, stomach, rectum, thyroid gland, liver, mammary gland. *Tp53* was over-expressed in almost all tumor cells within an array element. Our data indicate that expression of *Tp53* in tumor tissues may play a role in cell carcinomatous change. Intracellular levels of *Tp53* were elevated due to the increased stability and higher steady state of the protein, which may permit cells to divide without restraint. The positive

Table 2 Abnormal expression of *Tp53* mRNA and over-expression of *Tp53* gene in tissue array

Histological grade	*n*	*Tp53* positive	*P* value
Esophagus			
Paracancerous tissue	30	6	$P = 0.000$
Ⅰ	30	20	
Ⅱ	30	19	
Ⅲ	30	5	
Stomach			
Paracancerous tissue	30	15	$P = 0.000$
Ⅰ	30	20	
Ⅱ	30	29	
Ⅲ	30	15	
Rectum			
Paracancerous tissue	20	2	$P = 0.001$
Ⅰ	20	5	
Ⅱ	20	13	
Ⅲ	20	5	
Thyroid gland			
Paracancerous tissue	20	2	$P = 0.023$
Follicular adenoma	20	7	
Papillary carcinoma	20	10	
Hepar			
Paracancerous tissue	20	6	$P = 0.000$
Ⅰ	20	19	
Ⅱ	20	16	
Ⅲ	20	15	
Colon			
Paracancerous tissue	20	13	$P = 0.555$
Ⅰ	20	11	
Ⅱ	20	14	
Ⅲ	20	10	
Mammary gland			
Paracancerous tissue	20	3	$P = 0.000$
Lobular hyperplasia	20	4	
Fibroadenoma	20	4	
Lobular carcinoma	20	15	
DCIS	20	15	

Table 3 Abnormal expression of *CCND1* mRNA and over-expression of *CCND1* gene in tissue array

Histological grade	*n*	*CCND1* positive	*P* value
Esophagus			
Paracancerous tissue	30	19	
Ⅰ	30	25	$P = 0.058$
Ⅱ	30	27	
Ⅲ	30	25	
Stomach			
Paracancerous tissue	30	15	
Ⅰ	30	16	$P = 0.034$
Ⅱ	30	25	
Ⅲ	30	19	
Rectum			
Paracancerous tissue	20	1	
Ⅰ	20	5	$P = 0.000$
Ⅱ	20	15	
Ⅲ	20	10	
Thyroid gland			
Paracancerous tissue	20	3	
Follicular adenoma	20	10	$P = 0.000$
Papillary carcinoma	20	16	
Hepar			
Paracancerous tissue	20	11	
Ⅰ	20	19	$P = 0.037$
Ⅱ	20	15	
Ⅲ	20	14	
Colon			
Paracancerous tissue	20	13	
Ⅰ	20	19	$P = 0.064$
Ⅱ	20	14	
Ⅲ	20	12	
Mammary gland			
Paracancerous tissue	20	3	
Lobular hyperplasia	20	4	$P = 0.001$
Fibroadenoma	20	8	
Lobular carcinoma	20	10	
DCIS	20	15	

expression rate of *Tp53* was 48.9% (44/90) in carcinoma tissue samples and 20% (6/30) in normal adjacent tissue samples. In most cases, carcinomatous tissue samples had stronger signals than paracancerous tissue samples (Figures 4 and 5). Stronger positive dots (positive cells > 50%) were observed in carcinomatous tissue samples.

CCND1 ISH signals, located exclusively in nuclei, were variable in terms of staining intensity and proportion of positive nuclei among the cells in individual cases. The over-expression frequencies of all tumors are shown in Table 3. In this study, a significant difference was found in carcinomatous and paracancerous tissue samples, including those of stomach, rectum, thyroid gland, liver, and mammary gland. Our data indicate that *CCND1* expression was significantly associated with carcinomatous change. *CCND1* was expressed only in one paracancerous tissue sample of rectum, but in 4 carcinomatous tissue samples. In most cases, carcinomatous tissue samples had stronger signals than paracancerous tissue samples (Figures 4 and 5).

The over-expression frequencies of *C-myc* gene in all tumors are shown in Table 4. In this study, a significant difference was found in carcinomatous and paracancerous tissue samples, including those of esophagus, stomach, and thyroid gland. The expression of *C-myc* gene in carcinomatous and paracancerous tissues samples was not significantly associated with the histological grade of tumors. The expression of *C-myc* mRNA was heterogeneous in breast tumor tissue samples, with no predominant morphologic subtype in the high or low categories($\chi^2 = 7.062$, $P = 0.133$). However, breast carcinoma tissue samples had stronger signals (positive cells > 75 %) than normal adjacent tissue samples. The positive expression rate of *C-myc* mRNA was 62.5% (25/40) in carcinomatous tissue samples, 60% (12/20) in lobular hyperplasia samples, and 60% (12/20) in fibroadenoma tissue samples.

In our study, the positive expression rate of the three probes was not significantly different in colon carcinoma and paracancerous tissue samples, which was 58.3%, 75.0%, 66.7% and 65.0%, 65.0%, 50.0%, respectively (χ^2 =2.083, $P = 0.555$; $\chi^2 = 7.273$, $P = 0.064$; $\chi^2 = 3.627$, $P = 0.305$), suggesting that tumor grade is not related with the gene expression level. The signal intensity was different in colon. Hybridization signals (positive cells > 75%) were always observed both in *CCND1* and in *C-myc*. Carcinoma tissue was associated with weaker signals (positive cells < 50%). However, an opposite tendency was found in thyroid and mammary glands.

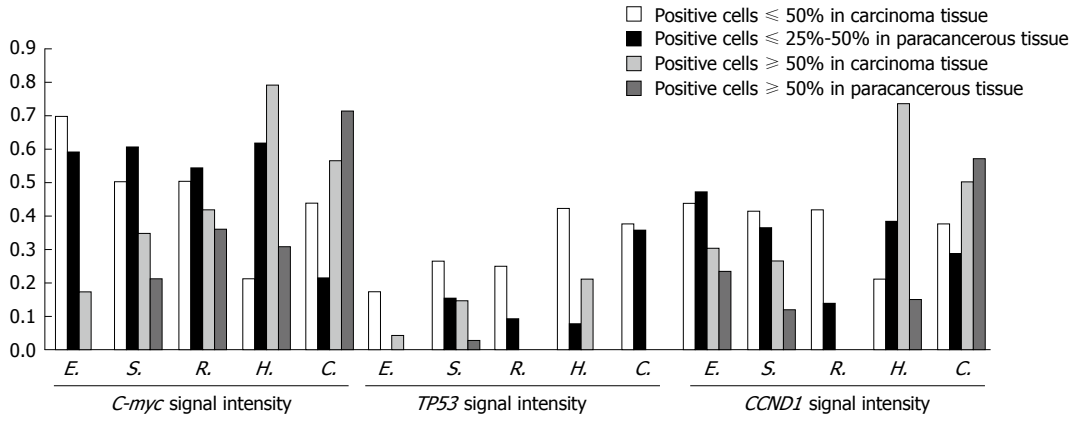

Figure 4 Signal intensity of *Tp53*, *CCND1*, and *C-myc* gene expression in different organs. E: Esophagus; S: Stomach; R: Rectum; H: Liver; C: Colon.

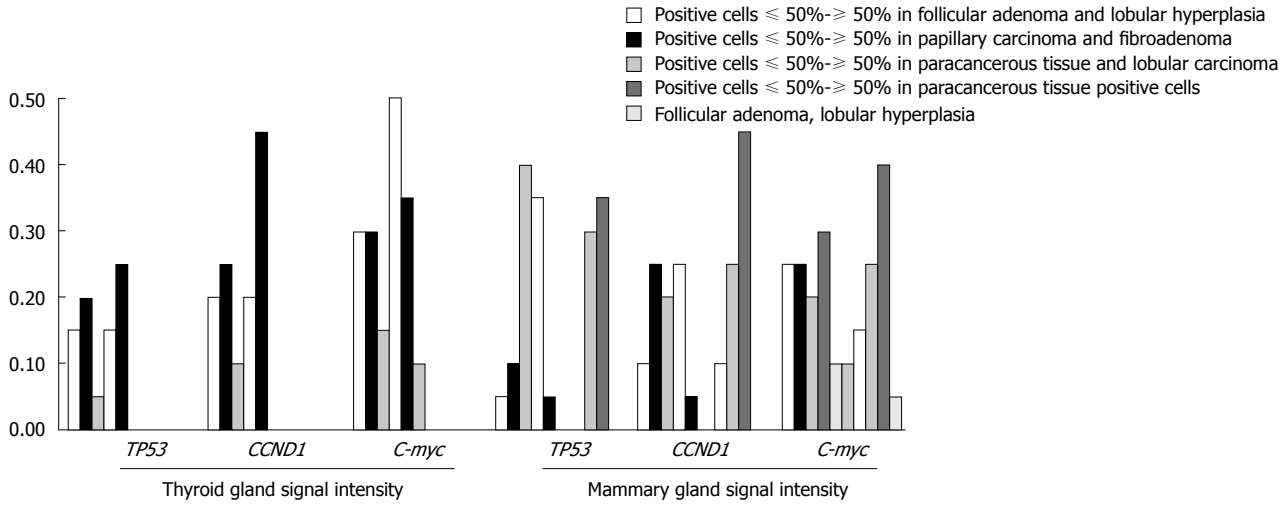

Figure 5 Signal intensity of *Tp53*, *CCND1*, and *C-myc* expression in thyroid gland and mammary gland.

The correlation coefficients of *Tp53-CCND1*, *CCND1-C-myc* and *Tp53-C-myc* were 0.653 (t = 3.76, P = 0.001), 0.737 (t = 4.753, P = 0.000) and 0.459 (t = 2.253, P = 0.036), respectively. If this finding was validated by an additional analysis in a larger population, these gene ratios could be used as prognostic markers in diagnostic biopsies.

DISCUSSION

In this study, we used the TMA technology because it allows analysis of a large number of samples and markers. A major concern for the TMA technique is the extent to which tumor heterogeneity may affect the validity of results. This issue has been addressed in TMA studies, which demonstrated that all previous findings from large sections could be fully reproduced[13,14]. The data on *Tp53*, *CCND1*, and *C-myc*, RNA *in situ* hybridization most commonly studied in associated tumors, are consistent with the reported findings[13,14]. In this study, the positive expression rates of *Tp53*, *CCND1*, and *C-myc* RNA were higher than those in previous reports[13,14], confirming the usefulness of the TMA approach. In our study, all tissue samples were fixed in phosphate-buffered saline (1‰ DEPC PBS) containing 4% paraformaldehyde that can prevent mRNA degradation from RNase and result in good morphology, indicating that tissue micro-array may be powerful in identification of different types of tumor with a particular molecular alteration.

Northern blot, dot blot or PCR-based approach has been used in detecting the expression of *Tp53*, *CCND1* and *C-myc* mRNA in different tumors, but just a few reports are available on *in situ* hybridization. Some normal tissuses are dominated by adipose cells, differing greatly from tumor tissue in its epithelial cellularity. Normal and tumor tissues cannot be rigorously compared using techniques involving RNA extraction from total tissue. Therefore, conclusions such as 'increased expression' may be more difficult to make from studies with Northern blot, dot blot and PCR-based techniques requiring RNA extraction from tissues not fastidiously microdissected for selection of tumor cells. In this study, a more sensitive hybridization mixture decreased RNA degradation, thus accelerating oligonucleotide probe-RNA annealing. The signal intensity can be increased and low abundance RNA can be detected using a locked nucleic acid modifier

Table 4 Abnormal expression of *C-myc* mRNA and over-expression of *C-myc* gene in tissue array

Histological grade	*n*	*C-myc* positive	*P* value
Esophagus			
Paracancerous tissue	30	17	
Ⅰ	30	29	$P = 0.000$
Ⅱ	30	27	
Ⅲ	30	20	
Stomach			
Paracancerous tissue	30	28	
Ⅰ	30	15	$P = 0.000$
Ⅱ	30	28	
Ⅲ	30	25	
Rectum			
Paracancerous tissue	20	16	
Ⅰ	20	11	$P = 0.214$
Ⅱ	20	10	
Ⅲ	20	13	
Thyroid gland			
Paracancerous tissue	20	9	
Follicular adenoma	20	18	$P = 0.004$
Papillary carcinoma	20	16	
Hepar			
Paracancerous tissue	20	10	
Ⅰ	20	11	$P = 0.813$
Ⅱ	20	10	
Ⅲ	20	8	
Colon			
Paracancerous tissue	20	10	
Ⅰ	20	15	$P = 0.305$
Ⅱ	20	14	
Ⅲ	20	11	
Mammary gland			
Paracancerous tissue	20	7	
Lobular hyperplasia	20	12	$P = 0.133$
Fibroadenoma	20	12	
Lobular carcinoma	20	10	
DCIS	20	15	

to increase its stability and sensitivity[15-17]. The non-specificity signal can be decreased and the specificity can be increased using a different temperature and probe concentration. A strong hybridization signal appears in the transmitting tissue of pistil a few hours after 10% PVA (MW, 70-100 kDa) is used[18-20].

Our results reveal that the expression of *Tp53* was higher in six different carcinoma samples than in their adjacent normal adjacent tissue samples with the exception in colon tissue samples. In this study, the expression of *Tp53* was observed in 4 cases of lobular hyperplasia and in 20 cases of fibroadenoma. However, the positive expression rate of *Tp53* was 75% (30/40) in breast carcinoma samples and 75% (30/40) in lobular carcinoma tissue samples, suggesting that determination of *Tp53* gene by RISH contributes to the diagnosis of carcinoma and distinguishes DCIS from atypical hyperplasia. Moreover, carcinoma tissue often has a stronger signal than paracancerous tissue. In the present study, the signal (positive cells > 50%) was stronger in liver and thyroid gland carcinoma tissue samples than in follicular adenoma tissue samples. Originally, *TP53* was thought to be an oncogene because over 50% of cancer cells tested showed a high level of *Tp53* protein. However, all of them are mutated forms of *Tp53*. It was reported that the *Tp53* gene acquires frequent mutations during the development of human malignancies including cancer of colon, breast, and lungs[21]. As described earlier, intracellular regulation of *Tp53* expression can occur at the level of mRNA or *Tp53* protein. A recent study indicated that even a brief reactivation of endogenous *Tp53* in *Tp53*-deficient tumors can lead to a complete tumor regression[22].

At present, the expression of *CCND1* has been investigated in several differences tumors, showing that patients with positive expression of *CCND1* usually have a poor prognosis compared to those with negative expression of *CCND1* in lung cancer, head and neck squamous cell carcinoma, and bladder cancer[23]. Although positive expression of *CCND1* can serve as a poor prognostic factor or is associated with a worse prognosis, the expression of *CCND1* is not correlated with the prognosis of cancer patients[24]. Furthermore, intensive investigations have been done on the correlation between *CCND1* expression and patient survival in Caucasian females with breast carcinoma, but systematic investigations on alteration of *CCND1* in Asian females are rare[23,24]. Whether *CCND1* expression, clinicopathological parameters, survival rate and other prognostic markers are associated with cell cycle is not clear. In our study, a significant difference was found in positive expression rate of *CCND1* between different tumors except for tumors of esophagus ($\chi^2 = 7.500$, $P = 0.058$) and colon ($\chi^2 = 7.273$, $P = 0.064$). These results agree with the conclusions of other studies[23,24].

The expression of *CCND1* plays an important role in the early staging of carcinogenesis in Caucasian females with breast carcinoma. In the present study, 4 patients had lobular hyperplasia, 25 had breast carcinoma, suggesting that expression of *CCND1* also plays an important role in Chinese patients with breast carcinoma. *CCND1* was over-expressed in gastric adenocarcinoma tissue samples.

The *C-myc* oncogene is amplified or over-expressed in different human cancers. Experiments *in vivo* have also causally linked aberrant expression of this gene to the development and progression of cancer in different body sites[25]. However, several critical issues regarding the significance of *C-myc* in human cancer still remain obscure. *C-myc* is essential for tumor development, since it regulates factors necessary for the growth of tumors lending a new potential target to anti-angiogenic cancer therapies. Our study showed that the expression of *C-myc* was significantly different in carcinoma and its adjacent normal tissue samples. In our study, 18 patients had follicular adenoma and 16 had papillary carcinoma. The signal intensity of *C-myc* was also similar in follicular adenoma and papillary carcinoma patients with no strong signal occurred in paracancerous tissue samples, indicating that determination of *C-myc* gene by RISH can contribute to the diagnosis of carcinoma and distinguish carcinoma from follicular adenoma.

C-myc gene over-expression is associated with a poor

prognosis of breast cancer patients[26]. The abnormal expression of *C-myc* mRNA and over-expression of *C-myc* in tissue array are listed in Table 4. The prognostic value for the over-expression of *C-myc* mRNA or protein is inconsistent and conflicting[26]. In our study, c-myc gene expression had no significant difference in breast lobular hyperplasia, fibroadenoma, lobular and ductal carcinoma tissue samples and their adjacent normal tissue samples ($\chi^2 = 7.062$, $P = 0.133$), and *C-myc* mRNA was highly expressed in 50% (75%) of high grade breast carcinoma tissue samples.

Our data show that the three genes were not differently expressed in colon carcinoma tissue samples, which is not consistent with the data reported by Deming[27] using the relatively insensitive Southern blot technique, suggesting that the three genes are not important factors for colon carcinogenesis and not significantly correlated with DCC deleted in colon cancer (DCC) and mutated in colon cancer (MCC) genes.

In normal cells, *Tp53* gene is activated due to DNA damage and increases the transcription of p21 inhibiting the activity of cyclin/CDK complex and preventing cells from entering S phase. Mutation of *Tp53* gene reflected by positive expression of mutated type Tp53 protein is one of the main causes for malignant transformation. In this study, *CCND1* was correlated with *Tp53* expression. We believe that although *Tp53* could induce the expression of p21 and inhibit the function of *CCND1*/CDK complex in normal cells, abnormal expression of *CCND1* does not necessarily respond to the dysfunction of wild-type *Tp53* protein or positive expression of mutated type *p53* in carcinoma cells. A pathway independent of *Tp53* mutation may exist in carcinoma, which could also result in *CCND1* over-expression. Over-expression of *CCND1* may play an important role in the carcinogenesis of tumors without cooperation of *Tp53* mutation[28].

In conclusion, it is necessary to analyze the lower grade tumors and premalignant lesions with the same tools to determine whether the expressions of *Tp53*, *CCND1* and *C-myc* are different, and to compare the development and progression of tumors. Only a subtle deregulation of the expression of *C-myc* is sufficient to allow genomic instability. Whether gene expression precedes or follows its over-expression in the course of cancer needs to be further studied.

COMMENTS

Background

The *Tp53*, *CCND1* and *C-myc* oncogenes are amplified or over-expressed in many types of human cancer. Experiments *in vivo* have also causally linked aberrant expression of these genes to the development and progression of cancer in different body sites. However, several critical issues regarding the significance of *Tp53*, *CCND1* and *C-myc* in human cancer still remain obscure. The frequency of amplification, over-expression of mRNA and protein in breast cancer is about 50%-100%. Whether the expression of these genes is altered at the cytogenetic level in different types of human carcinoma remains unclear.

Research frontiers

The development and progression of cancer in different body sites are very intricate and involve the expression of multiple cytokines and oncogenes such as *Tp53*, *CCND1* and *C-myc*.

Innovations and breakthroughs

Few studies are available on the correlation of *Tp53*, *CCND1*, *C-myc* with seven kinds of tissue. To study the expression of oncogene during the development of cancer, we constructed tissue microarrays consisting of samples from 7 types of tumor. Using scrupulous *R* & *ISH* and LNA modifying probe, we achieved high expressions of these genes in different tumor tissues. *C-myc* mRNA was highly expressed in 50% of high grade breast carcinomas, which was much higher than the reported data (22%) by real-time RT-PCR.

Applications

The relationship between oncogene and tumor clarifies the mechanism of tumor and provides an important molecular basis for closer observation of the nature of tumor, which can be widely applied in the diagnosis, treatment, and prognosis of cancer.

Peer review

The authors studied the expression of *Tp53*, *C-myc*, and *CCND1* in different tumors using tissue microarrays. The study was well designed. The findings are reliable and can be widely applied in the diagnosis, treatment, and prognosis of cancer.

REFERENCES

1 **Rogel A**, Popliker M, Webb CG, Oren M. p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors. *Mol Cell Biol* 1985; **5**: 2851-2855

2 **Mercer WE**, Avignolo C, Baserga R. Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies. *Mol Cell Biol* 1984; **4**: 276-281

3 **Davidoff AM**, Humphrey PA, Iglehart JD, Marks JR. Genetic basis for p53 overexpression in human breast cancer. *Proc Natl Acad Sci USA* 1991; **88**: 5006-5010

4 **Sasano H**, Goukon Y, Nishihira T, Nagura H. In situ hybridization and immunohistochemistry of p53 tumor suppressor gene in human esophageal carcinoma. *Am J Pathol* 1992; **141**: 545-550

5 **Ligueros M**, Jeoung D, Tang B, Hochhauser D, Reidenberg MM, Sonenberg M. Gossypol inhibition of mitosis, cyclin D1 and Rb protein in human mammary cancer cells and cyclin-D1 transfected human fibrosarcoma cells. *Br J Cancer* 1997; **76**: 21-28

6 **Zwijsen RM**, Wientjens E, Klompmaker R, van der Sman J, Bernards R, Michalides RJ. CDK-independent activation of estrogen receptor by cyclin D1. *Cell* 1997; **88**: 405-415

7 **Michalides RJ**, van Veelen NM, Kristel PM, Hart AA, Loftus BM, Hilgers FJ, Balm AJ. Overexpression of cyclin D1 indicates a poor prognosis in squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg* 1997; **123**: 497-502

8 **Zhang SY**, Caamano J, Cooper F, Guo X, Klein-Szanto AJ. Immunohistochemistry of cyclin D1 in human breast cancer. *Am J Clin Pathol* 1994; **102**: 695-698

9 **Barnes DM**. Cyclin D1 in mammary carcinoma. *J Pathol* 1997; **181**: 267-269

10 **Weinstein IB**. Relevance of cyclin D1 and other molecular markers to cancer chemoprevention. *J Cell Biochem Suppl* 1996; **25**: 23-28

11 **Augenlicht LH**, Wadler S, Corner G, Richards C, Ryan L, Multani AS, Pathak S, Benson A, Haller D, Heerdt BG. Low-level c-myc amplification in human colonic carcinoma cell lines and tumors: a frequent, p53-independent mutation associated with improved outcome in a randomized multi-institutional trial. *Cancer Res* 1997; **57**: 1769-1775

12 **Aulmann S**, Bentz M, Sinn HP. C-myc oncogene amplification in ductal carcinoma in situ of the breast. *Breast Cancer Res Treat* 2002; **74**: 25-31

13 **Kononen J**, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; **4**: 844-847

14 **Battifora H**. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. *Lab Invest* 1986; **55**: 244-248

15 **Castoldi M**, Schmidt S, Benes V, Noerholm M, Kulozik AE, Hentze MW, Muckenthaler MU. A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). *RNA* 2006; **12**: 913-920
16 **Kloosterman WP**, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RH. In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods* 2006; **3**: 27-29
17 **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838
18 **Pontius BW**, Berg P. Rapid renaturation of complementary DNA strands mediated by cationic detergents: a role for high-probability binding domains in enhancing the kinetics of molecular assembly processes. *Proc Natl Acad Sci USA* 1991; **88**: 8237-8241
19 **Pontius BW**, Berg P. Renaturation of complementary DNA strands mediated by purified mammalian heterogeneous nuclear ribonucleoprotein A1 protein: implications for a mechanism for rapid molecular assembly. *Proc Natl Acad Sci USA* 1990; **87**: 8403-8407
20 **De Block M**, Debrouwer D. RNA-RNA in situ hybridization using digoxigenin-labeled probes: the use of high-molecular-weight polyvinyl alcohol in the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction. *Anal Biochem* 1993; **215**: 86-89
21 **Ventura A**, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, Newman J, Reczek EE, Weissleder R, Jacks T. Restoration of p53 function leads to tumour regression in vivo. *Nature* 2007; **445**: 661-665
22 **Xue W**, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, Cordon-Cardo C, Lowe SW. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 2007; **445**: 656-660
23 **Lam WW**, Fielding R, Ho EY. Predicting psychological morbidity in Chinese women after surgery for breast carcinoma. *Cancer* 2005; **103**: 637-646
24 **Luini A**, Gatti G, Ballardini B, Zurrida S, Galimberti V, Veronesi P, Vento AR, Monti S, Viale G, Paganelli G, Veronesi U. Development of axillary surgery in breast cancer. *Ann Oncol* 2005; **16**: 259-262
25 **Chana JS**, Grover R, Wilson GD, Hudson DA, Forders M, Sanders R, Grobbelaar AO. The clinical significance of c-myc oncogene expression in melanomas of the scalp. *Br J Plast Surg* 1998; **51**: 191-194
26 **Balch CM**, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N, Urist M, McMasters KM, Ross MI, Kirkwood JM, Atkins MB, Thompson JA, Coit DG, Byrd D, Desmond R, Zhang Y, Liu PY, Lyman GH, Morabito A. Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. *J Clin Oncol* 2001; **19**: 3622-3634
27 **Deming SL**, Nass SJ, Dickson RB, Trock BJ. C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. *Br J Cancer* 2000; **83**: 1688-1695
28 **Jin M**, Inoue S, Umemura T, Moriya J, Arakawa M, Nagashima K, Kato H. Cyclin D1, p16 and retinoblastoma gene product expression as a predictor for prognosis in non-small cell lung cancer at stages I and II. *Lung Cancer* 2001; **34**: 207-218

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RAPID COMMUNICATION

Vitamin E treatment for children with chronic hepatitis B: A randomized placebo controlled trial

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Abstract

AIM: To evaluate the safety and efficacy of vitamin E in children with chronic hepatitis B.

METHODS: We randomly assigned patients with chronic hepatitis B, positive for hepatitis B e antigen (HBeAg), to receive either vitamin E or placebo once daily for 6 mo in a 3:1 ratio and double-blind manner. The primary end point was HBeAg seroconversion, defined as the loss of HBeAg, undetectable levels of serum hepatitis B virus DNA, and the appearance of antibodies against HBeAg 12 mo after therapy.

RESULTS: At baseline visit, 49 patients had normal and 43 had increased serum aminotransferase levels. Twenty-nine patients did not respond to previous treatment with interferon-α or lamivudine. Seventy-six children completed the study; 16 were non-compliant (n = 7), lost to follow-up (n = 7), or started another antiviral treatment (n = 3). Intention-to-treat analysis showed HBeAg seroconversion in 16 children (23.2%) treated with vitamin E and two (8.7%) in the placebo group (P = 0.13). Vitamin E was well tolerated.

CONCLUSION: There is only a tendency that vitamin E may promote HBeAg seroconversion. Therefore larger studies are needed to clarify the role of antioxidants in the therapy of chronic hepatitis B.

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Key words: Vertical transmission; Immune tolerance; Re-treatment; Infant

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INTRODUCTION

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV). Chronic HBV infection causes cirrhosis, hepatocellular carcinoma, and end-stage liver disease which accounts for approximately 1 million deaths each year. In most countries horizontal transmission of HBV is the main route of infection

during childhood[1-3]. In this age group the first stage of infection is characterized by the presence of hepatitis B s antigen (HBsAg) and hepatitis B e antigen (HBeAg) in serum, a high virus load, and low inflammatory activity. These parameters indicate a low or absent immune response against HBV which can persist for decades. The great majority of patients in this "immune tolerance" phase have no sustained response to current treatment[4]. However, it is known that chronic HBV carriers show spontaneous HBeAg seroconversion at approximately 10% per year[5]. Among patients with predictors of beneficial response such as high levels of serum transaminases and low levels of HBV DNA, only approximately one-third respond to interferon alpha[6-9] and about 20% respond to nucleoside analogues[10-12]. Furthermore, it has been shown that interferon alpha treatment simply accelerates anti-HBe seroconversion[13]. The question of whether it is worth treating a patient is further complicated by the significant side effects of interferon alpha treatment and the potential to develop resistance to nucleoside analogues. Therefore further treatment options are needed.

Since a defective immune response is likely to be one factor in the pathogenesis of liver damage caused by HBV, immunomodulatory substances have been tested for the treatment of chronic hepatitis B[14-19] but the results are conflicting. In a recent placebo controlled study, Andreone *et al*[20] tested vitamin E for chronic hepatitis B. In this pilot trial, the response rate was significantly higher in patients in the vitamin E group than in the placebo group.

Vitamin E is an antioxidant in the cell membrane which acts as a scavenger of free radicals. In patients suffering from various hepatopathies, it is able to protect against liver damage[21,22]. Vitamin E was also shown to enhance clinically relevant T-cell-mediated function[23].

The purpose of this study was to evaluate the safety and efficacy of a high-dose 6-mo course of vitamin E in a large number of chronically infected children. Our study included a 12-mo follow-up period, which allowed us to evaluate post-treatment effects and the durability of response.

MATERIALS AND METHODS

We conducted a prospective, randomized, double-blind, placebo-controlled study of patients with chronic hepatitis B in 22 German centers and one Austrian center. The dose of vitamin E depended on the patients' weight. Children below 20 kg received 200 IU of *RRR*-α-tocopheryl acetate concentrate once daily for 6 mo. Patients between 20 and 40 kg received 400 IU and patients of more than 40 kg received 600 IU once daily for 6 mo (Cognis, Nutrition&health, Düsseldorf, Germany). After the treatment period, patients were followed up for 12 mo. Placebo capsules were indistinguishable from vitamin E capsules and were produced by the same company. Eligible patients were randomized to receive either vitamin E or placebo in a 3:1 ratio. Patients were randomly assigned by a computer-generated program at the study center (Children's Hospital, Helios Klinikum Wuppertal, Germany).

The local ethics committee (Witten-Herdecke University, Germany) approved this study. Written consent was given from parents and also from the patient if older than 11 years.

Inclusion criteria

Inclusion criteria were: (1) age between 1 and 17 years; (2) presence of HBsAg and HBeAg for at least 6 mo; (3) presence of HBV DNA in serum for at least 6 mo (HBV-DNA > 10 000 copies/mL).

Exclusion criteria

Exclusion criteria were: (1) antiviral therapy within the 6 mo prior to the study; (2) concurrent participation in another clinical trial; (3) hepatic decompensation; (4) co-infection with hepatitis C, hepatitis D or human immunodeficiency virus; (5) pregnancy or breast feeding.

Monitoring

The following biochemical and virological data were examined at baseline visit and 2, 4, 6, 12 and 18 mo after starting therapy: vitamin E, aspartate aminotransferases, γ-glutamyl transferase, complete blood count, thromboplastin time, thyroid stimulating antibodies, HBV DNA, HBeAg, anti-HBe, HBsAg, anti-HBs. At each visit, the child underwent a physical examination and was interviewed regarding potential side effects and adverse reactions. Biochemical and serological markers of hepatitis B infection were tested in each center.

Evaluation of efficacy

The primary end point for efficacy was seroconversion to anti-HBe, loss of HBeAg and loss of HBV DNA at the end of follow-up (12 mo after treatment), defined as HBV-DNA level under 400 copies/mL or in other tests < 5 pg/mL.

Statistical analyses

The comparison of groups with respect to responses at 18 mo was done by Fisher's exact test.

RESULTS

Characteristics of patients

Ninety-two children and adolescents were enrolled in the study. Baseline characteristics of the patients are shown in Table 1. Vitamin E and placebo groups were similar with respect to demographic and clinical characteristics. Of the 76 patients completing the study, 18 (16 vitamin E and two placebo) responded to therapy as defined by HBeAg seroconversion, loss of HBeAg and HBV DNA (< 400 copies/mL) and normalization of alanine aminotransferase (ALT).

Exclusion of patients

Of the 92 patients enrolled, 16 were excluded (13 of the vitamin E and three of the placebo group). Seven patients

Table 1 Characteristics and results of 76 children who completed the study before and after treatment with vitamin E or placebo

	Vitamin E	Placebo
Total	56	20
Sex (%)		
Female	22 (39.3)	8 (40)
Male	34 (60.7)	12 (60)
Age (yr)	10.4	11.8
Alanine transaminase level (%)		
Normal	33 (58.9)	13 (65)
Elevated	23 (41.1)	7 (35)
More than double above normal level	8	2
HBV-DNA (%)		
< 1000 pg/mL	22 (39.3)	7 (35)
> 1000 pg/mL	35 (60.7)	13 (65)
Route of transmission (%)		
Parenteral	1 (1.8)	1 (5)
Vertical	34 (60.7)	10 (50)
Unknown	21 (37.5)	9 (45)
Interferon alpha pre-treatment (%)[1]	11 (19.6)	4 (20)
Lamivudine pre-treatment (%)[1]	5 (8.9)	3 (15)
HBeAg seroconversion (%)[2]	16 (28.6)	2 (10)

[1]Four patients were treated first with interferon alpha and re-treated with Lamivudine; [2]12 mo follow-up evaluation after end of treatment.

were non-compliant as medication was taken only for a reduced period or was taken irregularly and one patient administered additional self-medication with vitamin E. Seven patients were lost to follow-up and three patients started treatment with interferon alpha or lamivudine.

Dosage

Twenty-six of the 69 (37.7%) patients in the vitamin E group received 600 IU, 30 (43.5%) 400 IU and eight (11.6%) 200 IU.

Levels of transaminases before treatment

Patients were classified into two groups: one with normal transaminase levels and the second with elevated transaminase levels. In the children who responded to therapy, eight patients had normal transaminase levels before treatment and eight patients had elevated transaminases, while all children with response in the placebo group had elevated transaminases (Table 2). Eighteen children seroconverted to anti-HBe and 15 of them showed ALT elevation before seroconversion (mean 3.2 times normal range). In three of these patients transaminases increased slightly only before seroconversion while the rest of them had a mild transaminase flare immediately before and during anti-HBe seroconversion. None of them showed acute exacerbation of hepatitis B.

Levels of HBV DNA

Patients were classified into two groups: one with low HBV DNA titers of less than 1000 pg/mL and one with high HBV DNA of more than 1000 pg/mL. Twelve (75%) patients with low and four (25%) with high viral DNA responded to vitamin E. In the two responders in the placebo group, one patient had high HBV DNA titers and one low.

Table 2 Characteristics of 18 children with chronic hepatitis B who responded to therapy with either vitamin E or placebo. Response defined as anti-HBe seroconversion, loss of HBeAg and HBV-DNA < 400 copies/mL

	Vitamin E	Placebo
Total	16	2
Sex (%)		
Female	10 (62.5)	2 (100)
Male	6 (37.5)	0
Age (yr)	12.4	14.2
Alanine transaminase level (%)		
Normal	8 (50)	2 (100)
Elevated	8 (50)	0
HBV-DNA (%)		
< 1000 pg/mL	12 (75)	1 (50)
> 1000 pg/mL	4 (25)	1 (50)
Route of transmission (%)		
Parenteral	1 (6.3)	0
Vertical	8 (50)	0
Unknown	7 (43.7)	2 (50)
Interferon alpha pretreatment (%)	2 (11)	0
Lamivudine pretreatment (%)	0	1 (50)
Dosage (%)		
200 IU Vitamin E	0	
400 IU Vitamin E	4 (25)	
600 IU Vitamin E	12 (75)	
HBeAg seroconversion after initiation of treatment		
6 mo	6	0
12 mo	7	1
18 mo	3	1

Route of infection

Thirty-four patients (49.3%) in the vitamin E group and 10 patients (43.5%) in the placebo group were likely to be infected by her mother, either perinatally or later in childhood. Of these, nine children responded to vitamin E treatment; none of the children in the placebo group responded. The route of infection was unknown in 21 children (30.4%) in the vitamin E group and nine (39.1%) in the placebo group. Eight and two children responded to therapy, respectively. The only patient with parenteral infection in the vitamin E group responded to therapy while the child in the placebo group did not.

Side effects

The therapy was well tolerated. Three children treated with vitamin E experienced self-limited gastroenteritis of 1-3 wk duration. Monitoring of the above-mentioned biochemical and clinical data did not reveal any other side effects or adverse events. No child had before or during follow-up abnormal TSH or thromboplastin time. There was no significant increase in the GGT-level and no pathological change of the whole blood count.

Time of HBeAg seroconversion

In the vitamin E group, six patients responded during vitamin E treatment, seven within the first year and three at 18 mo after the start of therapy. In the placebo group, both patients responded only during the follow-up period at 12 and 18 mo. During anti-HBe seroconversion and loss of HBeAg no child developed acute exacerbation of hepatitis B.

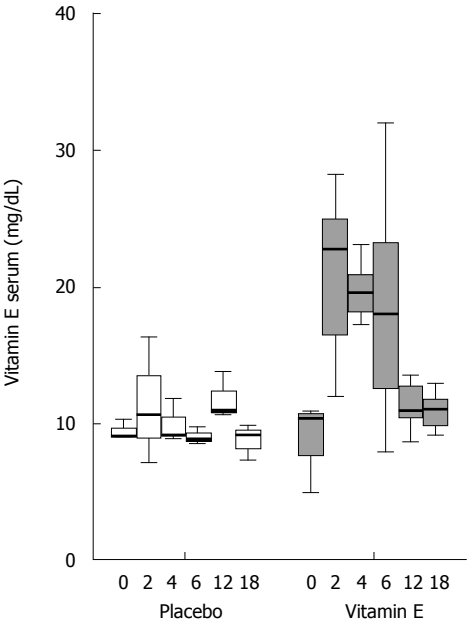

Figure 1 The significant vitamin E levels before, during and after treatment (mean ± SD) in patients treated with placebo or vitamin E.

Vitamin E levels

A significant increase in vitamin E levels was observed in the vitamin E group. Vitamin E levels doubled within 2 mo and fell to starting levels immediately after cessation of therapy. No significant change in vitamin E levels was found in the placebo group (Figure 1).

DISCUSSION

The results of this study suggest that vitamin E is well tolerated in children and adolescents with chronic hepatitis B and yields a higher HBeAg seroconversion rate than patients treated with placebo. Among 69 children treated with vitamin E, 23.2% became negative for serum markers of viral replication (HBeAg and HBV DNA). As expected, an 8.7% spontaneous rate of anti-HBe seroconversion and loss of HBeAg was found in the control group, in line with previous reports[5,8,24-26]. The response rate in vitamin E-treated-children was slightly worse than in patients treated with interferon alpha[5,8,24-26] and comparable with the treatment with nucleoside analogues[10-12]. This is surprising since the majority of children treated in our study had poor predictors of virological response. Among children who completed the study, approximately two-thirds had normal ALT levels and high levels of viremia, and one-third were resistant to one or two previous antiviral treatments. In the study of Andreone *et al*[20], 7 of 15 patients treated with vitamin E showed normalization of ALT levels, 8 of 15 became negative for HBV DNA, and 3 of 7 HBeAg positive patients achieved seroconversion which differed significantly from the placebo group. It is known that seroconversion of HBeAg may occur after years of treatment with interferon or lamivudine. In our study population, 19 children were treated with interferon or lamivudine before treatment with vitamin E (Table 1). Only one child was treated 7-12 mo before entering this study with interferon, and another three patients were treated 12-36 mo before starting this study. The remaining patients received antiviral therapy more than 2 years before the study. We therefore think that antiviral therapy prior to the study should not be a significant bias of the results of this study.

It is known that vitamin E improves the aminotransferase status in patients with various liver diseases such as hepatitis C, hemochromatosis and Wilson's disease[21,22]. This effect is probably based on its antioxidant properties, resulting in the protection of liver damage by oxidative stress. However, immunomodulatory effects are also documented. It was shown *in vivo* that vitamin E enhances T cell-mediated function, acts as a proliferative stimulator of lymphocytes and has natural killer cell activity[23,27,28]. Furthermore, T helper cells are polarized towards the T helper-1 phenotype which is known to be under-represented in chronic hepatitis B carriers[29]. It can be hypothesized that these effects of vitamin E may be a factor in the antiviral immune response seen in our patients.

According to current consensus statements on the treatment of HBeAg positive hepatitis B, only patients with ALT levels greater than double normal values, or moderate/severe hepatitis on biopsy should be considered for therapy[30-32]. In childhood, however, the majority of patients are immune tolerant to HBV, resulting in no significant liver inflammation. The results of this study suggest that vitamin E may be used for these patients; this is supported by the finding that half of the patients with HBeAg seroconversion had normal aminotransferases before treatment. Moreover, it may be useful for patients who have previously failed with interferon alpha or nucleoside analogues as re-treatment with the same drugs either as monotherapy or in combination[33,34]. The excellent safety of vitamin E found in this study and in many other reports, even in long-term treatment, further favors this therapy[35].

Although the HBeAg seroconversion rate was higher in the vitamin E group, our study population is too small to show a significant difference between the two groups. Our study is hampered by the relatively high number of patients who had to be excluded because of non-compliance or loss of follow-up. This may in part be due to the long follow-up period and the high number of treatment centers involved in this study. Furthermore, some patients may not have been convinced that vitamin E can be used as a "real" drug and can be effective against hepatitis B. The results of this study may, however, improve the motivation of such patients and lead to a bigger study population.

We conclude that treatment with high doses of vitamin E is well tolerated and may promote HBeAg seroconversion even in difficult to treat patients, but further studies are warranted to verify the effectiveness of vitamin E.

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COMMENTS

Background

With an anual mortality of around 800 000 patients per year, chronic hepatitis B is one of the most serious worldwide health problems.

Research frontiers

Chronic hepatitis B can be regarded as a difficult to treat disease. Despite treatment options like interferon-alpha and nucleos(t)ide analogues, the majority of patients fail to respond to treatment. Moreover during the so-called immune tolerant phase of HBV infection, no treatment can be offered to the patient as the chance to respond to treatment is minimal.

Innovations and breakthroughs

Recent reports have demonstrated that treatment with vitamin E can normalize liver transaminases and may also lead to HBeAg seroconversion. In this report, studied for the first time in children, the effect of vitamin E was evaluated in a placebo-controlled trial. The overall effect in the vitamin E group was higher than in the placebo group but this effect did not reach significance.

Applications

In particular, children are often in the immune tolerant phase of infection and therefore no treatement is indicated. As vitamin E has an excellent side-effect profile, it may be used in otherwise not treatable children. This has however been evaluated in larger studies.

Peer review

Authors investigated the putative role of vitamin E in chronic hepatitis B. The study was conducted carefully and the double-blinded placebo-controlled trial reaches a high standard.

REFERENCES

1 **Kiire CF**. The epidemiology and prophylaxis of hepatitis B in sub-Saharan Africa: a view from tropical and subtropical Africa. *Gut* 1996; **38** Suppl 2: S5-S12
2 **Hahne S**, Ramsay M, Balogun K, Edmunds WJ, Mortimer P. Incidence and routes of transmission of hepatitis B virus in England and Wales, 1995-2000: implications for immunisation policy. *J Clin Virol* 2004; **29**: 211-220
3 **Yao GB**. Importance of perinatal versus horizontal transmission of hepatitis B virus infection in China. *Gut* 1996; **38** Suppl 2: S39-S42
4 **Lai CL**, Lok AS, Lin HJ, Wu PC, Yeoh EK, Yeung CY. Placebo-controlled trial of recombinant alpha 2-interferon in Chinese HBsAg-carrier children. *Lancet* 1987; **2**: 877-880
5 **Hui CK**, Leung N, Shek TW, Yao H, Lee WK, Lai JY, Lai ST, Wong WM, Lai LS, Poon RT, Lo CM, Fan ST, Lau GK. Sustained disease remission after spontaneous HBeAg seroconversion is associated with reduction in fibrosis progression in chronic hepatitis B Chinese patients. *Hepatology* 2007; **46**: 690-698
6 **Korenman J**, Baker B, Waggoner J, Everhart JE, Di Bisceglie AM, Hoofnagle JH. Long-term remission of chronic hepatitis B after alpha-interferon therapy. *Ann Intern Med* 1991; **114**: 629-634
7 **Niederau C**, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, Haussinger D. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. *N Engl J Med* 1996; **334**: 1422-1427
8 **Sokal EM**, Conjeevaram HS, Roberts EA, Alvarez F, Bern EM, Goyens P, Rosenthal P, Lachaux A, Shelton M, Sarles J, Hoofnagle J. Interferon alfa therapy for chronic hepatitis B in children: a multinational randomized controlled trial. *Gastroenterology* 1998; **114**: 988-995
9 **van Zonneveld M**, Honkoop P, Hansen BE, Niesters HG, Murad SD, de Man RA, Schalm SW, Janssen HL. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. *Hepatology* 2004; **39**: 804-810
10 **Dienstag JL**, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256-1263
11 **Lai CL**, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61-68
12 **Jonas MM**, Mizerski J, Badia IB, Areias JA, Schwarz KB, Little NR, Greensmith MJ, Gardner SD, Bell MS, Sokal EM. Clinical trial of lamivudine in children with chronic hepatitis B. *N Engl J Med* 2002; **346**: 1706-1713
13 **Bortolotti F**, Jara P, Barbera C, Gregorio GV, Vegnente A, Zancan L, Hierro L, Crivellaro C, Vergani GM, Iorio R, Pace M, Con P, Gatta A. Long term effect of alpha interferon in children with chronic hepatitis B. *Gut* 2000; **46**: 715-718
14 **Fattovich G**, Brollo L, Pontisso P, Pornaro E, Rugge M, Alberti A, Realdi G. Levamisole therapy in chronic type B hepatitis. Results of a double-blind randomized trial. *Gastroenterology* 1986; **91**: 692-696
15 **Fattovich G**, Giustina G, Alberti A, Guido M, Pontisso P, Favarato S, Benvegno L, Ruol A. A randomized controlled trial of thymopentin therapy in patients with chronic hepatitis B. *J Hepatol* 1994; **21**: 361-366
16 **Mutchnick MG**, Lindsay KL, Schiff ER, Cummings GD, Appelman HD, Peleman RR, Silva M, Roach KC, Simmons F, Milstein S, Gordon SC, Ehrinpreis MN. Thymosin alpha1 treatment of chronic hepatitis B: results of a phase III multicentre, randomized, double-blind and placebo-controlled study. *J Viral Hepat* 1999; **6**: 397-403
17 **Farhat BA**, Marinos G, Daniels HM, Naoumov NV, Williams R. Evaluation of efficacy and safety of thymus humoral factor-gamma 2 in the management of chronic hepatitis B. *J Hepatol* 1995; **23**: 21-27
18 **Arase Y**, Tsubota A, Suzuki Y, Suzuki F, Kobayashi M, Someya T, Akuta N, Hosaka T, Saitoh S, Ikeda K, Kobayashi M, Kumada H. A pilot study of thymosin alpha1 therapy for chronic hepatitis B patients. *Intern Med* 2003; **42**: 941-946
19 **Amarapurkar D**, Das HS. Thymosin alpha in the treatment of chronic hepatitis B: an uncontrolled open-label trial. *Indian J Gastroenterol* 2002; **21**: 59-61
20 **Andreone P**, Fiorino S, Cursaro C, Gramenzi A, Margotti M, Di Giammarino L, Biselli M, Miniero R, Gasbarrini G, Bernardi M. Vitamin E as treatment for chronic hepatitis B: results of a randomized controlled pilot trial. *Antiviral Res* 2001; **49**: 75-81
21 **von Herbay A**, Stahl W, Niederau C, Sies H. Vitamin E improves the aminotransferase status of patients suffering from viral hepatitis C: a randomized, double-blind, placebo-controlled study. *Free Radic Res* 1997; **27**: 599-605
22 **von Herbay A**, Stahl W, Niederau C, von Laar J, Strohmeyer G, Sies H. Diminished plasma levels of vitamin E in patients with severe viral hepatitis. *Free Radic Res* 1996; **25**: 461-466
23 **Meydani SN**, Meydani M, Blumberg JB, Leka LS, Siber G, Loszewski R, Thompson C, Pedrosa MC, Diamond RD, Stollar BD. Vitamin E supplementation and in vivo immune response in healthy elderly subjects. A randomized controlled trial. *JAMA* 1997; **277**: 1380-1386
24 **Bortolotti F**, Cadrobbi P, Crivellaro C, Guido M, Rugge

M, Noventa F, Calzia R, Realdi G. Long-term outcome of chronic type B hepatitis in patients who acquire hepatitis B virus infection in childhood. *Gastroenterology* 1990; **99**: 805-810

25 **Gregorio GV**, Jara P, Hierro L, Diaz C, de la Vega A, Vegnente A, Iorio R, Bortolotti F, Crivellaro C, Zancan L, Daniels H, Portmann B, Mieli-Vergani G. Lymphoblastoid interferon alfa with or without steroid pretreatment in children with chronic hepatitis B: a multicenter controlled trial. *Hepatology* 1996; **23**: 700-707

26 **Chang MH**, Sung JL, Lee CY, Chen CJ, Chen JS, Hsu HY, Lee PI, Chen DS. Factors affecting clearance of hepatitis B e antigen in hepatitis B surface antigen carrier children. *J Pediatr* 1989; **115**: 385-390

27 **Meydani SN**, Barklund MP, Liu S, Meydani M, Miller RA, Cannon JG, Morrow FD, Rocklin R, Blumberg JB. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. *Am J Clin Nutr* 1990; **52**: 557-563

28 **Wang Y**, Huang DS, Liang B, Watson RR. Nutritional status and immune responses in mice with murine AIDS are normalized by vitamin E supplementation. *J Nutr* 1994; **124**: 2024-2032

29 **Bertoletti A**, D'Elios MM, Boni C, De Carli M, Zignego AL, Durazzo M, Missale G, Penna A, Fiaccadori F, Del Prete G, Ferrari C. Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. *Gastroenterology* 1997; **112**: 193-199

30 **Wong DK**, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med* 1993; **119**: 312-323

31 **Lok AS**, McMahon BJ. Chronic hepatitis B: update of recommendations. *Hepatology* 2004; **39**: 857-861

32 **EASL International Consensus Conference on Hepatitis B**. 13-14 September, 2002: Geneva, Switzerland. Consensus statement (short version). *J Hepatol* 2003; **38**: 533-540

33 **Mutimer D**, Naoumov N, Honkoop P, Marinos G, Ahmed M, de Man R, McPhillips P, Johnson M, Williams R, Elias E, Schalm S. Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic hepatitis B infection: results of a pilot study. *J Hepatol* 1998; **28**: 923-929

34 **Ballauff A**, Schneider T, Gerner P, Habermehl P, Behrens R, Wirth S. Safety and efficacy of interferon retreatment in children with chronic hepatitis B. *Eur J Pediatr* 1998; **157**: 382-385

35 **Kappus H**, Diplock AT. Tolerance and safety of vitamin E: a toxicological position report. *Free Radic Biol Med* 1992; **13**: 55-74

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RAPID COMMUNICATION

Transjugular intrahepatic portosystemic shunt-placement increases arginine/asymmetric dimethylarginine ratio in cirrhotic patients

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Abstract

AIM: To analyze the change of dimethylarginine plasma levels in cirrhotic patients receiving transjugular intrahepatic portosystemic shunt (TIPS).

METHODS: To determine arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and nitric oxide (NO) plasma levels, blood samples were collected from the superior cava, hepatic, and portal vein just before, directly after, and 3 mo after TIPS-placement.

RESULTS: A significant increase in the arginine/ADMA ratio after TIPS placement was shown. Moreover, TIPS placement enhanced renal function and thereby decreased systemic SDMA levels. In patients with renal dysfunction before TIPS placement, both the arginine/ADMA ratio and creatinine clearance rate increased significantly, while this was not the case in patients with normal renal function before TIPS placement. Hepatic function did not change significantly after TIPS placement and no significant decline in ADMA plasma levels was measured.

CONCLUSION: The increase of the arginine/ADMA ratio after TIPS placement suggests an increase in intracellular NO bioavailability. In addition, this study suggests that TIPS placement does not alter dimethylarginine dimethylaminohydrolase (DDAH) activity and confirms the major role of the liver as an ADMA clearing organ.

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Key words: Asymmetric dimethylarginine; Symmetric dimethylarginine; Arginine; Liver cirrhosis; Transjugular intrahepatic portosystemic shunt

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INTRODUCTION

In 1977, Carnegie and co-workers[1] pointed out the potential role of the liver in the metabolism of asymmetric dimethylarginine (ADMA) by reporting an increased urinary excretion of ADMA in patients with liver disease. Later, it was shown in an organ balance study in rats that the liver takes up substantial amounts of ADMA, thereby suggesting a crucial role for the liver in regulating systemic ADMA concentrations[2]. These results were confirmed in patients undergoing hepatic surgery in whom it was also shown that the clearing of symmetric dimethylarginine (SDMA) was not only confined to the kidney, but the human liver also took up small amounts of SDMA from the portosystemic

circulation[3]. Elevated ADMA levels have been reported in patients eligible for liver transplantation[4,5], in postoperative patients undergoing major liver resection[6], in patients suffering from decompensated alcoholic cirrhosis[7], and in critically ill patients with hepatic dysfunction[8]. ADMA plays a regulatory role in the arginine-nitric oxide (NO) pathway by inhibiting the enzyme NO synthase[9] and by competing with arginine and SDMA for cellular transport across cationic amino acid transporters (CAT) of system y+[10]. Both ADMA and SDMA are removed from the body by urinary excretion. However, the main eliminatory route for ADMA is degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) which is highly expressed in the liver, but is also present in the kidney, and in endothelial cells[11,12].

Dimethylarginines may play an important pathophysiological role in liver cirrhosis because this disease is characterised by endothelial dysfunction and NO deficiency in the intrahepatic circulation (review article[13]). In fact, increased intrahepatic vascular resistance in cirrhosis is not only due to structural changes, but also due to a dynamic component. The latter has largely been attributed to a reduced intrahepatic endothelial NO-synthase activity in liver cirrhosis[14]. Indeed, it has been suggested that an alteration in hepatic DDAH expression and/or activity in liver disease leads to high intrahepatic ADMA levels along with resultant endothelial dysfunction and increased intrahepatic resistance[15]. In cirrhotic patients, elevated peripheral ADMA and NO levels have been reported[7,16] and it has been suggested that ADMA might oppose the peripheral vasodilation caused by excessive systemic NO production during liver cirrhosis[7]. In order to analyze the change of dimethylarginine plasma levels in cirrhotic humans, we studied patients receiving transjugular intrahepatic portosystemic shunt (TIPS).

MATERIALS AND METHODS

The study was approved by the institutional review board and medical ethical review committee of the University Hospital Regensburg in Germany. Before study entry, patients were informed on the purpose of the study and informed consent was obtained from all patients.

Patients

The study population consisted of 25 patients suffering from liver cirrhosis and undergoing TIPS-placement mainly because of refractory ascites or recurrent esophageal variceal bleeding. The etiology of liver cirrhosis was: alcoholic hepatitis (20), viral hepatitis (2), cryptogenic hepatitis (2), and myeloproliferative disease (1). All patients had severe portal hypertension (portal pressure > 12 mmHg) which was determined during TIPS-placement. The diagnosis of liver cirrhosis was based on clinical, biochemical, and ultrasonographic data. Severity of hepatic failure was scored according to the Child-Pugh Classification[17].

Blood sampling and analysis

Blood samples were collected just before TIPS-placement from the superior caval vein, hepatic vein, and portal vein. Directly after TIPS-implantation and -dilation to its final lumen (before ending the procedure and dismissing the patient to the ward), and 3 mo after placement of the stent (to investigate patency of the shunt), blood was drawn again from the superior caval vein, portal vein and from another hepatic vein to prevent sampling from the extended portal venous tract.

ADMA, SDMA, and arginine plasma concentrations were measured by high-performance liquid chromatography with fluorescence detection using monomethylarginine as internal standard, as previously described[18]. After sample cleanup by solid-phase extraction, the analytes were derivatized with ortho-phthaldialdehyde reagent containing 3-mercaptopropionic acid. Chromatographic separation of the fluorescent derivatives was performed on a monolithic column as recently described[19]. Intra-assay coefficients of variation (CVs) were < 1.2% for all analytes. Inter-assay CVs were < 3.0% for ADMA and arginine and < 4% for SDMA. Reference values for ADMA have been obtained from plasma of healthy laboratory personnel and medical students[18]. The concentration of ADMA in these individuals is normally distributed. These patients did not have liver or kidney diseases and had no diseases that influence ADMA levels.

NO_x concentrations were measured using the Nitric Oxide Analyzer from Sievers Instruments (Boulder, Colorado, USA), as described previously[20,21]. Briefly, this assay is based upon spectrophotometric analysis after chemiluminescent reaction between NO and ozone (detection limit < 1 μmol/L). Creatinine clearance rate was calculated from plasma and urinary concentrations in 24 h collected urine. Other biochemical parameters were determined by standard laboratory methods.

Statistical analysis

Differences between timepoints were tested with Wilcoxon signed ranks test. For each comparison, the overall α-level was set at 0.05. Relations between variables were investigated by Spearman's rho. Data are presented as medians and interquartile ranges (IQR). Statistical analyses were performed using SPSS (SPSS 11.0 for Windows).

RESULTS

Patients

Patient characteristics are presented in Table 1. Hepatic synthetic and clearing functions were slightly impaired as reflected by decreased factor V, antithrombin Ⅲ, albumin, and cholinesterase concentrations and slightly increased bilirubin levels. Three months after TIPS-placement, no significant improvement was seen in either laboratory parameters of hepatic function, hepatic enzyme concentrations, or Child-Pugh score (data not shown).

Although creatinine and urea levels were within the

Table 1 Demographic data and parameters of hepatic and renal function

	Demographic data		
Number of patients	25		
Gender: Male/Female	18/7		
Age: Median (range)	55 (40-81)		
TIPS indication			
Ascites	20		
Esophageal varices	2		
Others	3		
Child-Pugh classification			
A	5		
B	14		
C	6		
Biochemical markers of hepatic function	Median	IQR	Reference range
Bilirubin (mmol/L)	20	13-35	< 17
Aspartate aminotransferase (U/L)	19	13-34	< 50
Alanine aminotransferase (U/L)	15	7-27	< 50
Alkaline phosphatase (U/L)	135	99-185	< 124
Prothrombin time (%)	72	59-81	> 70
Factor V (%)	52	40-80	> 75
Antithrombin (%)	67	48-79	> 75
Fibrinogen (mg/dL)	299	232-408	180-350
Albumin (g/L)	35	31-41	37-53
Cholinesterase (U/L)	2049	1461-2800	5320-12920
Alpha-fetoprotein (ng/mL)	3.1	2.5-4.0	< 8.1
Biochemical markers of renal function			
Urea (mmol/L)	13	8-18	4-18
Creatinine (μmol/L)	77	59-113	< 97
Creatinine clearance (mL/min)	80	38-115	97-160

Table 2 Biochemical markers of renal function

	Just before TIPS		Directly after TIPS		3 mo after TIPS	
	Median	IQR	Median	IQR	Median	IQR
Urea (mmol/L)	13	8-18	12	5-33	11[a]	5-16
Creatinine (μmol/L)	77	59-113	63	54-107	70[a]	57-89
Creatinine clearance (mL/min)	80	38-115	110	75-138	128[a]	97-161

[a]$P < 0.05$ *vs* just before TIPS-placement.

Table 3 Pressure (mmHg) in portal vein and right atrium

	Just before TIPS		Directly after TIPS		3 mo after TIPS	
	Median	IQR	Median	IQR	Median	IQR
Portal vein	30	20-35	23[a]	16-27	17[a]	10-23
Right atrium	9	1-12	13[a]	5-17	6[a]	2-15
Gradient (pv-ra)	21	19-24	10[a]	9-11	11[a]	7-15

Pv: Portal vein; ra: Right atrium. [a]$P < 0.05$ *vs* just before TIPS-placement.

normal range, creatinine clearance rate was decreased at baseline. Placement of TIPS enhanced renal function as illustrated in Table 2.

Portal and systemic pressures

Portal hypertension was present in all patients. TIPS-placement caused an immediate decrease in both portal pressure and in portosystemic pressure gradient (Table 3). After 3 mo, these pressures were still decreased in comparison to baseline values.

Table 4 Plasma concentrations (μmol/L) of ADMA, SDMA, and arginine

	Just before TIPS		Directly after TIPS		3 mo after TIPS	
	Median	IQR	Median	IQR	Median	IQR
ADMA						
Caval vein	0.64	0.58-0.72	0.69	0.61-0.78	0.59	0.53-0.74
Portal vein	0.67	0.60-0.79	0.7	0.62-0.80	0.58	0.52-0.77
Hepatic vein	0.62	0.59-0.71	0.66	0.57-0.84	0.57	0.52-0.74
SDMA						
Caval vein	0.74	0.60-1.16	0.81	0.64-1.11	0.61[a]	0.46-0.86
Portal vein	0.79	0.62-1.13	0.72	0.64-1.06	0.62[a]	0.46-0.89
Hepatic vein	0.79	0.61-1.09	0.77	0.64-1.09	0.60[a]	0.44-0.89
Arginine						
Caval vein	64	56-85	76	68-102	77[a]	65-104
Portal vein	69	62-93	79	64-102	90[a]	70-106
Hepatic vein	54	46-75	67	59-88	72[a]	57-101
Arginine/ADMA						
Caval vein	95	83-132	117	89-141	128[a]	110-175
Portal vein	83	68-123	98	80-130	123[a]	92-152
Hepatic vein	108	88-138	114	88-137	141[a]	112-185

[a]$P < 0.05$ *vs* just before TIPS-placement.

Concentrations of ADMA, SDMA, and arginine

The changes in ADMA, SDMA, and arginine concentrations in the systemic, portal, and hepatic vein are shown in Table 4. At all 3 three timepoints, median systemic ADMA and SDMA plasma levels were higher in cirrhotic patients compared to healthy volunteers[18] (ADMA: 0.42 μmol/L (0.37-0.47); $P < 0.05$, SDMA: 0.46 μmol/L (0.42-0.52); $P < 0.05$, respectively). In contrast, arginine concentrations were lower at baseline compared to healthy individuals (arginine: 88 μmol/L (76-113); $P < 0.05$), but did not differ anymore after TIPS-placement.

Although ADMA levels did not show a significant change due to TIPS-placement, SDMA concentrations were significantly lower 3 mo after TIPS-placement in comparison to baseline values. Placement of TIPS caused a significant increment of both arginine levels and arginine/ADMA ratios.

When an additional analysis was performed between patients with (creatinine clearance rate < 97 mL/min) and patients without (creatinine clearance rate > 97 mL/min) additional renal dysfunction before TIPS placement, arginine/ADMA ratio increased significantly ($P = 0.021$) in patients with renal dysfunction; before TIPS placement: 94 (81-135) and 3 mo after TIPS placement: 124 (99-174). This was also the case for creatinine clearance rate which improved significantly ($P = 0.036$) from 44 mL/min (27-70) before TIPS placement to 105 mL/min (73-146) 3 mo after TIPS placement. In patients with normal renal function, no significant changes in either creatinine clearance rate or arginine/ADMA ratio was seen.

NO_x plasma concentrations

Although NO_x plasma levels showed a decreasing

Table 5 Plasma concentrations of NO_x (μmol/L)

NO_x	Just before TIPS		Directly after TIPS		3 mo after TIPS	
	Median	IQR	Median	IQR	Median	IQR
Systemic vein	112	37-243	107	33-212	44	26-103
Portal vein	83	35-241	85	45-223	57	21-96
Hepatic vein	85	35-304	86	32-220	39	21-76

tendency after TIPS placement, changes did not reach statistical significance (Table 5).

SDMA concentrations were positively related to NO_x before and 3 mo after TIPS placement ($r = 0.53$; $P = 0.027$, and $r = 0.60$; $P = 0.025$, respectively). ADMA was also related to NO_x plasma levels 3 mo after TIPS placement ($r = 0.67$; $P = 0.009$). Neither significant relations were present between arginine/ADMA ratios and NO_x plasma levels nor between NO_x concentrations and portosystemic pressure gradient.

Relations between dimethylarginines and hepatic and renal function

ADMA was positively related to Child-Pugh score before and 3 mo after TIPS placement ($r = 0.44$; $P = 0.034$ and $r = 0.59$; $P = 0.028$, respectively). SDMA was positively related to Child-Pugh score 3 mo after TIPS placement ($r = 0.66$; $P = 0.011$).

SDMA was related to creatinine clearance rate before and 3 mo after TIPS placement ($r = -0.70$; $P < 0.001$, $r = -0.85$; $P < 0.001$, respectively). ADMA was also related to creatinine clearance rate 3 mo after TIPS placement ($r = -0.70$; $P = 0.007$).

Neither ADMA nor SDMA was related to porto-systemic pressure gradient.

DISCUSSION

The main finding in the present study was an increase of the arginine/ADMA ratio three months after placement of TIPS in cirrhotic patients. In addition, TIPS placement caused a decrease in SDMA levels which may be partially explained by an advantageous effect on renal function as reflected by an increase in creatinine clearance rate due to TIPS placement. Interestingly, when patients with and patients without renal dysfunction before TIPS placement were studied separately, both the arginine/ADMA ratio and the creatinine clearance rate improved significantly in patients with renal dysfunction before TIPS placement, while these changes were not seen in patients with normal renal function. The advantageous effect of TIPS placement on renal function is also reflected by the strong and significant correlation between SDMA plasma levels and creatinine clearance before and particularly after TIPS placement. The relation between dimethylarginines and creatinine clearance rate has already been shown in patients with renal insufficiency[22]. Interestingly, the clearing of SDMA is not only confined to the kidney, but the human liver also takes up substantial amounts of SDMA[3]. Indeed, we also found a relation between SDMA and Child-Pugh score, thereby underlining the reported SDMA clearing capacity of the liver. Thus, increased SDMA levels in our studied cirrhotic patients may be caused by a combination of renal and hepatic dysfunction. Also for ADMA, we observed increased plasma concentrations at baseline being also closely related to the severity of liver dysfunction. This is in accordance with the findings of Lluch and coworkers[7] who likewise reported a direct relationship with the Child-Pugh score of patients being evaluated.

The main metabolic route for ADMA is degradation *via* DDAH[23] and the liver, which has a high DDAH activity, has been shown to be an important regulator of plasma ADMA levels in both animals and humans[2,3]. In portal hypertensive conditions, a recent study of Mookerjee and coworkers[24] showed reduced DDAH expression and increased ADMA levels in liver tissue of patients with severe alcoholic hepatitis. In addition, they suggested that elevated dimethylarginines may serve as a marker of a deleterious outcome in patients with alcoholic hepatitis. Also, in patients undergoing liver transplantation, ADMA has been shown to be a potential marker of acute allograft rejection[4]. Moreover, in cirrhotic animals, significantly decreased hepatic clearance of ADMA has been demonstrated[25].

In our study population, systemic ADMA concentrations did not decrease after TIPS placement. This is not surprising considering the well-known TIPS-induced decrease in hepatic extraction capacity. In other words, we assumed ADMA levels to increase after TIPS placement due to shunting and thus less degradation by hepatic DDAH. However, this increasing effect on ADMA serum levels induced by the TIPS implantation may be offset by the observed increase in renal function and the most likely improvement in renal ADMA clearance. In fact, ADMA plasma levels strongly correlated with creatinine clearance 3 mo after TIPS placement. Moreover, these data are in accordance with the observation of unaltered liver function represented by the lack of significant changes in biochemical laboratory parameters indicating hepatic function nor in Child-Pugh score after TIPS placement. Because we did not measure arterial dimethylarginine concentrations nor organ blood flow of the liver and kidney, no definite conclusions can be drawn about hepatic and renal elimination of dimethylarginines. Nonetheless, renal dysfunction often develops in patients with severe liver disease and a causal role for ADMA has been proposed in the development of the hepatorenal syndrome[26]. Recently, Lluch and co-workers[16] studied dimethylarginine concentrations in cirrhotic patients with hepatorenal syndrome and confirmed this hypothesis. Moreover, they suggested that SDMA may be a marker of renal dysfunction in cirrhotic patients. Therefore, the lack of an increase in ADMA and actual decrease in SDMA levels may well contribute to the well-known beneficial effects of TIPS with respect to neurohormonal status and kidney function in liver cirrhosis.

In liver cirrhosis, Laleman and co-workers[25] substantiated the potential role of ADMA in the

pathogenesis of impaired intrahepatic NO production. The known decrease in intrahepatic NO synthase activity in rats with biliary cirrhosis was found to be associated with an increase in circulating ADMA concentrations. In addition, endothelium-dependent vasorelaxation, measured in a liver perfusion model, was reduced in bile-duct ligated rats and addition of ADMA to the perfusate further blunted this vasodilatory response. However, in our study no association between ADMA levels and the severity of portal hypertension could be detected. A potential explanation may be the mode of action by which TIPS lowers portal pressure being independent from ADMA. Moreover, also SDMA has been reported to interfere with NO synthesis by competing with arginine for transport across cell membranes[10]. Especially high levels of SDMA in combination with low arginine concentrations may decrease NO synthesis significantly and hemodynamical consequences may be the same as reported for ADMA[23,27].

The gut produces citrulline which is used by the kidneys to synthesize arginine. It can be hypothesized that a decrease in portal pressure will have an advantageous effect on blood flow and function of the intestines, thereby increasing citrulline production by the gut and possibly enhancing renal arginine synthesis. Arginine is degraded in the liver that contains large amounts of arginase which breaks down arginine into urea and ornithine. TIPS placement may lead to a decreased capacity to eliminate arginine in the liver because blood does not enter the hepatocyte, but shunts directly from a portal branch into the hepatic vein. This may explain the increase in arginine plasma levels after TIPS placement. As a precursor of NO, this increase in arginine concentrations may enhance renal blood flow, thereby stimulating glomerular filtration rate and clearing a larger amount of dimethylarginines from the systemic circulation. This compensatory increase in renal excretion of dimethylarginines in fact will prevent a rise in ADMA levels induced by TIPS-induced portal decompression.

Liver cirrhosis is characterized by excessive systemic and particularly splanchnic NO production representing the pathophysiological hallmark in the development of the hyperdynamic circulatory syndrome. This vascular NO overproduction is stimulated by an in increase in portal pressure[28] and is an attempt to open the portal circulation and to enhance collateral blood flow in the systemic circulation bypassing the hepatic circulation. Besides a rise in arginine levels, the arginine/ADMA ratio increased significantly after TIPS placement. Theoretically, an increase in the arginine/ADMA ratio leads to an elevation in NO bioavailability. In our study, nonetheless, NO_x plasma levels showed a decreasing tendency after TIPS placement. While no relationship was found between NO_x and the arginine/ADMA ratio, both ADMA and SDMA were positively related to NO_x levels. This finding substantiates the hypothesis that dimethylarginines might oppose the peripheral vasodilation caused by excessive systemic NO production during liver cirrhosis[7]. It can be hypothesized that after TIPS placement, portal pressure drops and thus the main stimulus for splanchnic NO overproduction is greatly attenuated. In addition, renal and probably also gut function improve, thereby causing arginine synthesis (*via* gut-derived citrulline) by the kidney to increase. Furthermore, ADMA slightly decreases due to enhanced function of the kidney causing the arginine/ADMA ratio to increase. This is advantageous for the y^+ pump that is now able to transport more arginine into the cell, where it is converted to NO. The increased arginine/ADMA ratio after TIPS may result in a better NO-availability on tissue level, while systemic (plasma) NO is decreased due to less splanchnic NO release.

In conclusion, the main finding of the present study was a significant increase in the arginine/ADMA ratio in cirrhotic patients 3 mo after TIPS placement. In addition, TIPS enhanced renal function and concomitantly significantly lowered systemic SDMA levels, but did not change hepatic function. In line with this unaltered liver function, no significant decline in ADMA plasma levels could be detected, thereby confirming the major role of the liver as ADMA clearing organ.

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COMMENTS

Background

Asymmetric dimethylarginine (ADMA) plays a regulatory role in the arginine-nitric oxide (NO) pathway by inhibiting the enzyme NO synthase. In addition, dimethylarginines may play an important pathophysiological role in liver cirrhosis because this disease is characterised by endothelial dysfunction and NO deficiency in the intrahepatic circulation. In cirrhotic patients, elevated peripheral ADMA and NO levels have been reported and it has been suggested that ADMA might oppose the peripheral vasodilation caused by excessive systemic NO production during liver cirrhosis. In order to analyse dimethylarginine plasma levels in cirrhotic humans, authors studied patients receiving transjugular intrahepatic portosystemic shunt (TIPS).

Research frontiers

Elevated ADMA levels have been reported in patients eligible for liver transplantation in postoperative patients undergoing major liver resection, in patients suffering from decompensated alcoholic cirrhosis, and in critically ill patients with hepatic dysfunction. In fact, increased intrahepatic vascular resistance in cirrhosis is not only due to structural changes, but also due to a dynamic component. It has been suggested that an alteration in intrahepatic ADMA levels may lead to endothelial dysfunction and increased intrahepatic resistance. In cirrhotic patients, elevated peripheral ADMA and NO levels have been reported and it has been suggested that ADMA might oppose the peripheral vasodilation caused by excessive systemic NO production during liver cirrhosis.

Innovations and breakthroughs

This study shows an increase of the arginine/ADMA ratio after TIPS placement suggesting an increase in intracellular NO bioavailability. In addition, this study suggests that TIPS placement does not alter dimethylarginine dimethylaminohydrolase (DDAH) activity and confirms the major role of the liver as ADMA clearing organ.

Applications

TIPS placement is associated with an increased arginine/ADMA ratio suggesting an increased NO bioavailability which could have a positive effect on the microcirculation of important organs.

Peer review

In this study, the levels of ADMA, symmetric dimethylarginine (SDMA) and arginine in caval/portal and hepatic vein as well as the NO_x plasma levels in patients with cirrhosis were analyzed. Most of patients enrolled in this study suffered from alcohol-induced liver disease. The key finding of the manuscript is that arginine levels increase after TIPS-placement. The study is interesting and well performed.

REFERENCES

1 **Carnegie PR**, Fellows FC, Symington GR. Urinary excretion of methylarginine in human disease. *Metabolism* 1977; **26**: 531-537

2 **Nijveldt RJ**, Teerlink T, Siroen MP, van Lambalgen AA, Rauwerda JA, van Leeuwen PA. The liver is an important organ in the metabolism of asymmetrical dimethylarginine (ADMA). *Clin Nutr* 2003; **22**: 17-22

3 **Siroen MP**, van der Sijp JR, Teerlink T, van Schaik C, Nijveldt RJ, van Leeuwen PA. The human liver clears both asymmetric and symmetric dimethylarginine. *Hepatology* 2005; **41**: 559-565

4 **Siroen MP**, Warlé MC, Teerlink T, Nijveldt RJ, Kuipers EJ, Metselaar HJ, Tilanus HW, Kuik DJ, van der Sijp JR, Meijer S, van der Hoven B, van Leeuwen PA. The transplanted liver graft is capable of clearing asymmetric dimethylarginine. *Liver Transpl* 2004; **10**: 1524-1530

5 **Tsikas D**, Rode I, Becker T, Nashan B, Klempnauer J, Frolich JC. Elevated plasma and urine levels of ADMA and 15(S)-8-iso-PGF2alpha in end-stage liver disease. *Hepatology* 2003; **38**: 1063-1064

6 **Nijveldt RJ**, Teerlink T, Siroen MP, van der Hoven B, Prins HA, Wiezer MJ, Meijer C, van der Sijp JR, Cuesta MA, Meijer S, van Leeuwen PA. Elevation of asymmetric dimethylarginine (ADMA) in patients developing hepatic failure after major hepatectomy. *JPEN J Parenter Enteral Nutr* 2004; **28**: 382-387

7 **Lluch P**, Torondel B, Medina P, Segarra G, Del Olmo JA, Serra MA, Rodrigo JM. Plasma concentrations of nitric oxide and asymmetric dimethylarginine in human alcoholic cirrhosis. *J Hepatol* 2004; **41**: 55-59

8 **Nijveldt RJ**, Teerlink T, van der Hoven B, Siroen MP, Kuik DJ, Rauwerda JA, van Leeuwen PA. Asymmetrical dimethylarginine (ADMA) in critically ill patients: high plasma ADMA concentration is an independent risk factor of ICU mortality. *Clin Nutr* 2003; **22**: 23-30

9 **Vallance P**, Leone A, Calver A, Collier J, Moncada S. Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *J Cardiovasc Pharmacol* 1992; **20** Suppl 12: S60-S62

10 **Closs EI**, Basha FZ, Habermeier A, Forstermann U. Interference of L-arginine analogues with L-arginine transport mediated by the y+ carrier hCAT-2B. *Nitric Oxide* 1997; **1**: 65-73

11 **Kimoto M**, Tsuji H, Ogawa T, Sasaoka K. Detection of NG,NG-dimethylarginine dimethylaminohydrolase in the nitric oxide-generating systems of rats using monoclonal antibody. *Arch Biochem Biophys* 1993; **300**: 657-662

12 **Kimoto M**, Whitley GS, Tsuji H, Ogawa T. Detection of NG,NG-dimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. *J Biochem* 1995; **117**: 237-238

13 **Wiest R**, Groszmann RJ. The paradox of nitric oxide in cirrhosis and portal hypertension: too much, not enough. *Hepatology* 2002; **35**: 478-491

14 **Shah V**, Toruner M, Haddad F, Cadelina G, Papapetropoulos A, Choo K, Sessa WC, Groszmann RJ. Impaired endothelial nitric oxide synthase activity associated with enhanced caveolin binding in experimental cirrhosis in the rat. *Gastroenterology* 1999; **117**: 1222-1228

15 **Mookerjee RP**, Vairappan B, Jalan R. The puzzle of endothelial nitric oxide synthase dysfunction in portal hypertension: The missing piece? *Hepatology* 2007; **46**: 943-946

16 **Lluch P**, Mauricio MD, Vila JM, Segarra G, Medina P, Del Olmo JA, Rodrigo JM, Serra MA. Accumulation of symmetric dimethylarginine in hepatorenal syndrome. *Exp Biol Med* (Maywood) 2006; **231**: 70-75

17 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649

18 **Teerlink T**, Nijveldt RJ, de Jong S, van Leeuwen PA. Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. *Anal Biochem* 2002; **303**: 131-137

19 **de Jong S**, Teerlink T. Analysis of asymmetric dimethylarginine in plasma by HPLC using a monolithic column. *Anal Biochem* 2006; **353**: 287-289

20 **Hori N**, Wiest R, Groszmann RJ. Enhanced release of nitric oxide in response to changes in flow and shear stress in the superior mesenteric arteries of portal hypertensive rats. *Hepatology* 1998; **28**: 1467-1473

21 **Wiest R**, Shah V, Sessa WC, Groszmann RJ. NO overproduction by eNOS precedes hyperdynamic splanchnic circulation in portal hypertensive rats. *Am J Physiol* 1999; **276**: G1043-G1051

22 **Al Banchaabouchi M**, Marescau B, Possemiers I, D'Hooge R, Levillain O, De Deyn PP. NG, NG-dimethylarginine and NG, NG-dimethylarginine in renal insufficiency. *Pflugers Arch* 2000; **439**: 524-531

23 **Achan V**, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P. Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. *Arterioscler Thromb Vasc Biol* 2003; **23**: 1455-1459

24 **Mookerjee RP**, Malaki M, Davies NA, Hodges SJ, Dalton RN, Turner C, Sen S, Williams R, Leiper J, Vallance P, Jalan R. Increasing dimethylarginine levels are associated with adverse clinical outcome in severe alcoholic hepatitis. *Hepatology* 2007; **45**: 62-71

25 **Laleman W**, Omasta A, Van de Casteele M, Zeegers M, Vander Elst I, Van Landeghem L, Severi T, van Pelt J, Roskams T, Fevery J, Nevens F. A role for asymmetric dimethylarginine in the pathophysiology of portal hypertension in rats with biliary cirrhosis. *Hepatology* 2005; **42**: 1382-1390

26 **Nijveldt RJ**, Teerlink T, van Leeuwen PA. The asymmetrical dimethylarginine (ADMA)-multiple organ failure hypothesis. *Clin Nutr* 2003; **22**: 99-104

27 **Kielstein JT**, Impraim B, Simmel S, Bode-Boger SM, Tsikas D, Frolich JC, Hoeper MM, Haller H, Fliser D. Cardiovascular effects of systemic nitric oxide synthase inhibition with asymmetrical dimethylarginine in humans. *Circulation* 2004; **109**: 172-177

28 **Abraldes JG**, Iwakiri Y, Loureiro-Silva M, Haq O, Sessa WC, Groszmann RJ. Mild increases in portal pressure upregulate vascular endothelial growth factor and endothelial nitric oxide synthase in the intestinal microcirculatory bed, leading to a hyperdynamic state. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G980-G987

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RAPID COMMUNICATION

Four-week pegylated interferon α-2a monotherapy for chronic hepatitis C with genotype 2 and low viral load: A pilot, randomized study

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Abstract

AIM: To assess the efficacy and advantages of 4-wk pegylated interferon α-2a (peg-IFN-α2a) monotherapy for chronic hepatitis C patients with strong predictors of sustained virologic response (SVR).

METHODS: Patients (n = 33) with genotype 2 and low viral load (< 100 KIU/mL), who became HCV RNA negative after 1 wk of IFN treatment, were randomly allocated to receive a 4- or 12-wk treatment course at a ratio of 2:1, respectively, with a subsequent 24-wk follow-up period. Peg-IFN-α2a was administered subcutaneously at a dose of 180 μg or 90 μg once weekly. SVR was defined as absence of serum HCV RNA at the end of the follow-up period.

RESULTS: All patients completed the treatment schedule, and more than half were symptom-free during the treatment. In the 4-wk treatment group, 20 of 22 (91%) patients achieved SVR. Two patients relapsed, but achieved SVR following re-treatment with peg-IFN-α2a alone. In the 12-wk treatment group, 11 of 11 (100%) patients attained SVR.

CONCLUSION: Our results show that a 4-wk course of peg-IFN-α2a monotherapy can achieve a high SVR rate in "IFN-sensitive" patients, without negatively affecting outcome.

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Key words: Chronic hepatitis C; Pegylated interferon alpha-2a monotherapy; Genotype 2; Low viral load; Randomized pilot study

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INTRODUCTION

Pegylated interferon alpha (peg-IFN-α) in combination with ribavirin for 24 wk is currently recommended as a standard treatment for patients with hepatitis C virus (HCV) genotypes 2 and 3, because prolongation of treatment duration to 48 wk does not always provide a substantial gain in the sustained virologic response (SVR) rate[1-5]. In contrast, short-term combination treatment (i.e. less than 24 wk of duration) has been evaluated in genotype 2 and 3 patients with or without initial virological response[6-11]. Shorter treatment may lead to a substantial reduction in patients' burden and to the avoidance of premature termination of treatment without adversely affecting the outcome. Still, the pros and cons of a shorter treatment have not been conclusively defined and treating patients for less than 24 wk is still controversial.

A recent trend in the treatment strategy for chronic hepatitis C is the development of tailored or

individualized treatment regimens based on major strong predictors of SVR to IFN-based treatment, such as HCV genotype[2,3,12-17], pretreatment viral load[2,4,12,14,15,18], and initial virologic response to treatment[15,19-24]. Further subdivision or stratification of patients by combining these predictors may allow the development of more rational and optimal regimen without adversely affecting the outcome: e.g. treatment duration or combination with or without ribavirin. Specifically, a subgroup of genotype 2 patients with low viral load showed an excellent response even to conventional IFN monotherapy[12-14,18]. Furthermore, if initial virologic response is also taken into consideration in determining treatment regimen for the "IFN-sensitive" patient subgroup, even a shorter course of peg-IFN-α2a monotherapy might result in a high SVR rate without adversely affecting the outcome.

In the present study, we evaluated the efficacy and tolerability of a 4-wk peg-IFN-α2a monotherapy for "IFN-sensitive" patients with genotype 2 and low viral load, who became HCV RNA negative after 1 wk of treatment, in a pilot, randomized trial comparing a 4-wk *versus* a 12-wk treatment schedule. This study may allow us to substantially reduce the total dose and duration of peg-IFN-α treatment and to spare the use of ribavirin in the "IFN-sensitive" patient subgroup.

MATERIALS AND METHODS

Study population

Thirty-seven patients chronically infected with HCV genotype 2 and low viral load were consecutively enrolled in this pilot randomized study. They received short-term peg-IFN-α2a monotherapy at the Department of Gastroenterology and Hepatology, Kashiwa Hospital, the Jikei University School of Medicine, between August 2006 and July 2007. Eligible patients had anti-HCV antibodies, infection with HCV genotype 2 confirmed by polymerase chain reaction (PCR)-based method[25], serum HCV RNA levels < 100 kilo-international units (KIU)/mL by a quantitative PCR assay (Amplicor HCV Monitor Version 2.0, Roche Diagnostics, Tokyo, Japan; lower detection limit, 0.5 KIU/mL) (defined as 'low' viral load) at the enrolment, persistently abnormal serum alanine transaminase (ALT) concentrations (> 30 IU/L) during the preceding 24 wk, platelet counts ⩾ 50 × 10^3/μL, neutrophil counts ⩾ 750 /μL, and hemoglobin values ⩾ 10 g/dL. All patients were ⩾ 20 years of age and none had received prior IFN-containing treatment. Exclusion criteria were: liver cancer or decompensated liver cirrhosis; other forms of liver disease; coexisting serious psychiatric or medical illness; treatment with any other antiviral or immunomodulatory agent administered within the preceding 12 wk; hepatitis B surface antigen or hepatitis B core antibody; and pregnancy or lactation. The Local Ethics Committee of Jikei University School of Medicine approved the study. All patients provided informed consent before entry into the trial. Liver biopsy was performed between the enrolment and the beginning of treatment, and histopathologic evaluation was carried out using the ranking system for grading of necroinflammation activity and staging of fibrosis[26]. Steatosis was graded as mild (< 30% of hepatocytes contained lipid droplets in the cytoplasm), moderate (30%-60%), or severe (> 60%).

Study protocol

When patients showed virological response (VR) after 1 wk of treatment, patients were randomly allocated to receive a 4- or 12-wk treatment course at a ratio of 2:1 using a central randomization system, respectively, with a subsequent 24-wk follow-up period. Randomization was performed by means of sealed, opaque, numbered envelopes, each containing a sheet of paper assigning to either group, which were prepared independently by a medical statistician. The total sample size was estimated statistically (two-sided $\alpha = 0.10$, $\beta = 0.20$). Patients without VR after 1 wk were excluded from the trial, and all received a 12-wk treatment course. VR was defined as undetectable serum HCV RNA, using a qualitative PCR assay (Amplicor HCV version 2.0, Roche Diagnostics) with a lower detection limit of 50 IU/mL. Peg-IFN-alpha-2a (Roche, Nutley, NJ) at a dose of 180 μg (platelet count > 90 × 10^3/μL and neutrophil count > 1500/μL) or 90 μg (< 90 × 10^3/μL and < 1500/μL, respectively) was administered subcutaneously once weekly. During the treatment, the dose of peg-IFN was adjusted based on platelet and/or neutrophil counts determined before each administration: Peg-IFN was reduced from 180 to 90 μg when platelet or neutrophil counts fell below 90 × 10^3/μL and 1500/μL, respectively, and from 90 to 45 or 30 μg when platelet or neutrophil counts fell below 70% of the values observed the previous week. Peg-IFN was discontinued when the platelet count, the neutrophil count, or the hemoglobin value dropped below 25 × 10^3/μL, 500/μL, and 8.5 g/dL, respectively.

Clinical, laboratory and hematological data were assessed once weekly during the treatment and every 4 wk during the 24-wk follow-up period. Virological assessment was performed after 1 wk from the initiation of treatment, at the end of treatment, and every 4 wk during the follow-up period. Sustained virologic response (SVR) was defined as undetectable serum HCV RNA at the end of follow-up period. Safety was monitored clinically by careful interview and medical examination throughout the study.

Statistical analysis

The χ^2 test, Fisher's exact two-tail test, or Mann-Whitney test were used for statistical comparisons between groups, where appropriate. Treatment outcomes were analyzed on an intention-to-treat basis. All *P* values for statistical tests were two-tailed and values less than 0.05 were considered statistically significant. All calculations were performed using the SPSS 15.0 statistical package (SPSS, Chicago, IL).

RESULTS

Patient characteristics

Of 37 patients, 33 (89%) showed VR after 1 wk

Table 1 Characteristics of treatment-naïve chronic hepatitis C patients with genotype 2 and low viral load at the start of treatment (mean ± SD)

	4-wk treatment group (n = 22)	12-wk treatment group (n = 11)
Gender (M/F)	10/12	6/5
Age (yr)	57 ± 10	56 ± 12
Body weight (kg)	58.5 ± 10.7	60.6 ± 10.4
Body mass index (kg/m^2)	23.0 ± 2.9	21.8 ± 2.2
History of transfusion (yes/no)	10/12	3/8
Initial dosage (180 μg/90 μg)	20/2	9/2
Aspartate transaminase (IU/L)	53 ± 27	55 ± 32
Alanine transaminase (IU/L)	75 ± 35	77 ± 36
γ-glutamyl transpeptidase (IU/L)	52 ± 24	48 ± 30
Albumin (g/dL)	4.3 ± 0.3	4.2 ± 0.3
Total cholesterol (mg/dL)	169 ± 22	182 ± 35
Platelet count (× 10^3/μL)	157 ± 58	165 ± 62
Hemoglobin (g/dL)	13.3 ± 1.6	13.6 ± 1.8
Leukocyte count (/μL)	4700 ± 1500	5300 ± 900
Neutrophil count (/μL)	2490 ± 1140	2550 ± 613
Liver histology		
Stage (1/2/3/4)	9/5/5/0	5/4/2/0
Grade (mild/moderate/severe)	11/8/0	8/3/0
Steatosis (mild/moderate/severe)	15/4/0	9/2/0
Viral load (KIU/mL)	24 ± 21	24 ± 22

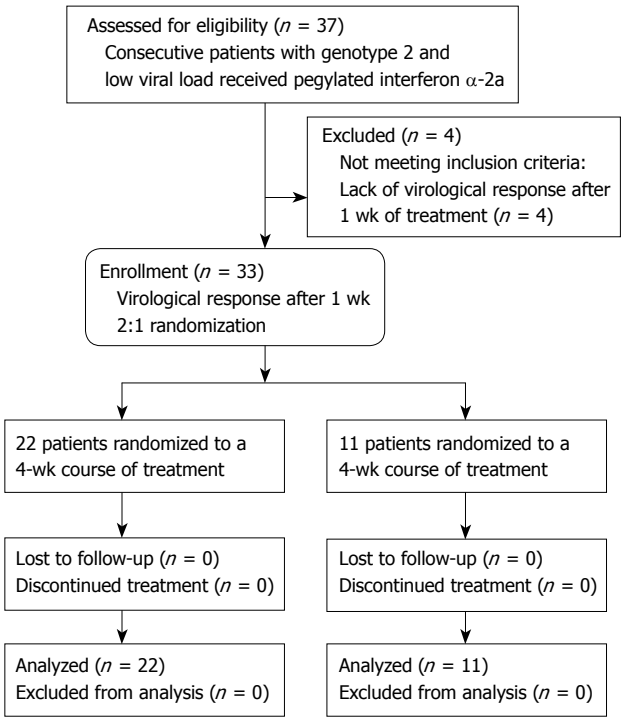

Figure 1 Flow diagram of patients included in the pilot study.

of treatment. The 33 patients were subsequently randomized to the 4-wk (n = 22) or the 12-wk (n = 11) treatment arms (Table 1, Figure 1). There was no statistically significant difference between the two groups as far as the baseline characteristics features were concerned. Irrespective of the treatment course, all 33 patients completed the treatment and were closely followed up as scheduled. None of the patients was lost to follow-up. None of the patients received growth factors for cytopenias, such as granulocyte-colony stimulating factor and erythropoietin. A liver biopsy was not available in 3 patients because of a bleeding disorder (n = 1, 4-wk treatment group) or because of patients' refusal (n = 2, both for the 4-wk treatment group).

Sustained virological response rates

In the 4-wk treatment group, 20 of 22 (91%) patients achieved SVR. Two patients relapsed 8 wk after the end of treatment. In the 12-wk treatment group, 11 of 11 (100%) patients attained SVR. No statistical difference was observed between both groups as to the SVR rates. The two patients who relapsed were offered re-treatment (4-wk and 12-wk regimens, respectively): both agreed and achieved SVR.

Predictors of sustained virological response or relapse

Univariate analyses failed to identify factors (including patients' age, body weight, and the presence of steatosis) associated with SVR or relapse.

Safety

Six patients in the 4-wk treatment group and five in the 12-wk treatment group complained of low-grade fever, headache, or malaise, but these symptoms were well tolerated during treatment. The dose of peg-IFN-α2a was reduced in three (all for thrombocytopenia) and four (two for neutropenia and two for thrombocytopenia) patients, respectively. Consequently, the cumulative dose of peg-IFN-α2a was 180 to 720 μg and 1350 to 2160 μg, respectively. All patients recovered completely from the adverse events after the completion of treatment.

Medication price

On the basis of current prices in Japan, the cost of medication (peg-IFN-α2a of 90 or 180 μg weekly) for 4-wk treatment course is between 536 and 1044 USD, and that for 12-wk treatment course is between 1608 and 3131 USD.

DISCUSSION

The results of this randomized pilot study suggest that a subgroup of patients could achieve a sufficiently high rate of SVR after only a 4-wk course of peg-IFN-α2a monotherapy. One of the most IFN-sensitive subgroups appears to include patients with genotype 2 and low viral load who exhibit VR after 1 wk of treatment.

Patients with genotype 2 or 3 are more sensitive to peg-IFN-α plus ribavirin treatment than those with genotype 1, and the current recommendation advocates a 24-wk treatment course, because more than 80% of the former group will attain SVR[3-5,7,11,27]. Recently, several studies suggested that the treatment duration could be shortened from 24 to between 12 and 16 wk without adversely affecting outcome in patients with genotype 2 or 3 who achieve VR after 4 wk of treatment[6-8,11]. In contrast, large trials indicated that shortening the treatment duration to 16 or 14 wk lessened the SVR rates[9,10]. It remains controversial whether the optimal

treatment duration is 24 wk or less than 24 wk for patients with genotype 2 or 3. A shorter treatment may not to be suitable for every patient with genotype 2 or 3. There may be a need to investigate whether the 4 wk timepoint from the beginning of treatment is appropriate to predict the therapy outcome after 24-wk treatment course.

There is ample evidence that peg-IFN-α plus ribavirin treatment is more beneficial in patients with genotype 2 than those with genotype 3[4,7-10]. This suggests that treatment regimens should be tailored or individualized for each genotype, with a special emphasis on the duration of treatment. As for patients with genotype 2 alone, even high-dose conventional IFN-α monotherapy for 24 wk produced an SVR rate of 78%[14]. Furthermore, the inclusion of baseline viral load and initial virologic response, as strong predictors of SVR independent of genotype[4,6,7,10,11,15,16,19-24,27], may identify patients suitable for shorter treatment in a more accurate way. In a small size study, an SVR rate of 100% was reported in patients with genotype 2a and low viral load (< 100 KIU/mL) treated with IFN-α for 6 wk[28]. In another study, 89.5% and 100% of patients, without genotype 1 and high viral load (> 100 KIU/mL), who showed VR after 2 wk of treatment, achieved SVR with 8-wk and 24-wk peg-IFN-α2a monotherapy, respectively[29]. Although the sample size was very small, this pilot randomized trial suggested that the duration of peg-IFN-α2a monotherapy may be further shortened while maintaining a high SVR rate by dividing patients according to the presence or absence of strong predictors of response.

Common or characteristic adverse events are more severe and frequent in the combination treatment with ribavirin than in IFN-α or peg-IFN-α monotherapy, especially with prolonged treatment duration, leading to frequent withdrawals from treatment, because ribavirin accumulates in various tissues as well as in erythrocytes[2,15,24]. Therefore, only a minority of patients in need of therapy actually receives peg-IFN-α plus ribavirin treatment. Before participation in this trial, IFN-based treatment has been withheld from some patients for a variety of reasons such as advanced age, or the relatively low hemoglobin level and/or platelet count. Symptoms recorded in this study were mild or few, supporting the view that a short treatment course is safer and associated with few adverse events and less frequent withdrawal from treatment[1,2,7,8,11,30]. Our trial demonstrated that a 4-wk peg-IFN-α2a monotherapy was safe for such patients, and suggested that the indication for treatment could be extended to include patients considered otherwise unsuitable for the peg-IFN-α plus ribavirin combination therapy.

The combination treatment is costly, and the longer the treatment duration, the higher the cost of treatment. Currently, the cost of treatment of a person weighing 65 kg who receives peg-IFN-α2a at 180 μg weekly and ribavirin at 800 mg daily for 24 wk is approximately 11253 USD in Japan. Thus, a 4-wk peg-IFN-α2a monotherapy would achieve a > 90% reduction in cost and drug exposure. When one adds the costs of medical consultation and laboratory tests, the 4-wk peg-IFN-α2a monotherapy provides a substantial saving in costs and inconvenience compared to the extended treatment.

In conclusion, the present study showed high SVR rates in genotype 2 patients with low viral load who had undetectable serum HCV RNA after 1 wk of treatment, treated with a 4-wk course of peg-IFN-α2a monotherapy. Tailoring or individualizing treatment to individual patients would considerably reduce both patients' and society burdens, without adversely affecting the clinical outcome.

COMMENTS

Background

Hepatitis C virus (HCV) genotype, pretreatment viral load and initial virologic response are major predictors of the treatment outcome in interferon (IFN)-based treatment for chronic hepatitis C. Patients infected with genotype 2 or 3 are currently recommended to receive a 24-wk course of pegylated IFN alpha (peg-IFN-α) plus ribavirin combination therapy. However, treatment regimens should be modified for those subgroups of genotype 2 patients with favorable predictors.

Research frontiers

To improve the treatment outcome while reducing the adverse effects and treatment costs, peg-IFN-α plus ribavirin combination treatment regimens for chronic hepatitis C are rationally tailored or reasonably individualized based on major predictors of response. The duration of combination therapy for genotype 1 or 4 patients is modified from 24 wk to 48 or 72 wk according to virological response after 4 wk, 12swk and 24 wk after treatment. In contrast, it remains controversial whether the treatment duration for genotype 2 or 3 patients could be shortened from 24 wk to 12, 14 or 16 wk according to the virological response after 4 wk of therapy.

Innovations and breakthroughs

This pilot, randomized study demonstrates that further stratification of patients by combining strong predictors may shorten the treatment duration and allow peg-IFN-α monotherapy without adversely affecting the outcome, leading to the substantial reduction in both patients' and society burdens. Moreover, this study questions whether the virological response at 4 wk after treatment is appropriate for efficiently discriminating the treatment outcome in a 24-wk treatment course for genotype 2 patients.

Applications

This randomized pilot study suggests that further stratification by combining strong predictors may be extended to individuals infected with other genotypes in tailoring or individualizing more rational and optimal regimens.

Terminology

Virological response is defined as undetectable serum HCV RNA (< 50 IU/mL). Treatment outcome means sustained virologic response, defined as undetectable serum HCV RNA at the end of the 24-wk follow-up period.

Peer review

This is an interesting and well-written manuscript describing an important issue in hepatitis C treatment.

REFERENCES

1 **McHutchison JG**, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492

2 **Poynard T**, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional

Therapy Group (IHIT) *Lancet* 1998; **352**: 1426-1432
3 **Hadziyannis SJ**, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355
4 **Zeuzem S**, Hultcrantz R, Bourliere M, Goeser T, Marcellin P, Sanchez-Tapias J, Sarrazin C, Harvey J, Brass C, Albrecht J. Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J Hepatol* 2004; **40**: 993-999
5 **Strader DB**, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; **39**: 1147-1171
6 **Dalgard O**, Bjoro K, Hellum KB, Myrvang B, Ritland S, Skaug K, Raknerud N, Bell H. Treatment with pegylated interferon and ribavarin in HCV infection with genotype 2 or 3 for 14 weeks: a pilot study. *Hepatology* 2004; **40**: 1260-1265
7 **von Wagner M**, Huber M, Berg T, Hinrichsen H, Rasenack J, Heintges T, Bergk A, Bernsmeier C, Haussinger D, Herrmann E, Zeuzem S. Peginterferon-alpha-2a (40KD) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. *Gastroenterology* 2005; **129**: 522-527
8 **Mangia A**, Santoro R, Minerva N, Ricci GL, Carretta V, Persico M, Vinelli F, Scotto G, Bacca D, Annese M, Romano M, Zechini F, Sogari F, Spirito F, Andriulli A. Peginterferon alfa-2b and ribavirin for 12 vs. 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2005; **352**: 2609-2617
9 **Shiffman ML**, Suter F, Bacon BR, Nelson D, Harley H, Sola R, Shafran SD, Barange K, Lin A, Soman A, Zeuzem S. Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2007; **357**: 124-134
10 **Dalgard O**, Bjoro K, Ring-Larsen H, Bjornsson E, Holberg-Petersen M, Skovlund E, Reichard O, Myrvang B, Sundelof B, Ritland S, Hellum K, Fryden A, Florholmen J, Verbaan H. Pegylated interferon alfa and ribavirin for 14 versus 24 weeks in patients with hepatitis C virus genotype 2 or 3 and rapid virological response. *Hepatology* 2008; **47**: 35-42
11 **Yu ML**, Dai CY, Huang JF, Hou NJ, Lee LP, Hsieh MY, Chiu CF, Lin ZY, Chen SC, Hsieh MY, Wang LY, Chang WY, Chuang WL. A randomised study of peginterferon and ribavirin for 16 versus 24 weeks in patients with genotype 2 chronic hepatitis C. *Gut* 2007; **56**: 553-559
12 **Yoshioka K**, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T. Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of hepatitis C virus. *Hepatology* 1992; **16**: 293-299
13 **Tsubota A**, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Hiromitsu K. Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. *Hepatology* 1994; **19**: 1088-1094
14 **Tsubota A**, Kumada H, Chayama K, Arase Y, Saitoh S, Koida I, Murashima N, Suzuki Y, Kobayashi M, Takagi K, Kobayashi M, Ikeda K. Relationship between pretreatment viremia level and response to interferon-alpha therapy in chronic hepatitis C differs in viral type 1 and 2 infections. *Dig Dis Sci* 1996; **41**: 1925-1932
15 **Davis GL**, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1493-1499
16 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
17 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
18 **Lau JY**, Simmonds P, Urdea MS. Implications of variations of "conserved" regions of hepatitis C virus genome. *Lancet* 1995; **346**: 425-426
19 **Neumann AU**, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998; **282**: 103-107
20 **Zeuzem S**, Lee JH, Franke A, Ruster B, Prummer O, Herrmann G, Roth WK. Quantification of the initial decline of serum hepatitis C virus RNA and response to interferon alfa. *Hepatology* 1998; **27**: 1149-1156
21 **Lee WM**, Reddy KR, Tong MJ, Black M, van Leeuwen DJ, Hollinger FB, Mullen KD, Pimstone N, Albert D, Gardner S. Early hepatitis C virus-RNA responses predict interferon treatment outcomes in chronic hepatitis C. The Consensus Interferon Study Group. *Hepatology* 1998; **28**: 1411-1415
22 **Neumann AU**, Lam NP, Dahari H, Davidian M, Wiley TE, Mika BP, Perelson AS, Layden TJ. Differences in viral dynamics between genotypes 1 and 2 of hepatitis C virus. *J Infect Dis* 2000; **182**: 28-35
23 **Bekkering FC**, Stalgis C, McHutchison JG, Brouwer JT, Perelson AS. Estimation of early hepatitis C viral clearance in patients receiving daily interferon and ribavirin therapy using a mathematical model. *Hepatology* 2001; **33**: 419-423
24 **Bjoro K**, Bell H, Hellum KB, Skaug K, Raknerud N, Sandvei P, Doskeland B, Maeland A, Lund-Tonnesen S, Myrvang B. Effect of combined interferon-alpha induction therapy and ribavirin on chronic hepatitis C virus infection: a randomized multicentre study. *Scand J Gastroenterol* 2002; **37**: 226-232
25 **Okamoto H**, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J Gen Virol* 1992; **73** (Pt 3): 673-679
26 **Desmet VJ**, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513-1520
27 **Davis GL**, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645-652
28 **Tabaru A**, Narita R, Hiura M, Abe S, Otsuki M. Efficacy of short-term interferon therapy for patients infected with hepatitis C virus genotype 2a. *Am J Gastroenterol* 2005; **100**: 862-867
29 **Jeong S**, Kawakami Y, Kitamoto M, Ishihara H, Tsuji K, Aimitsu S, Kawakami H, Uka K, Takaki S, Kodama H, Waki K, Imamura M, Aikata H, Takahashi S, Chayama K. Prospective study of short-term peginterferon-alpha-2a monotherapy in patients who had a virological response at 2 weeks after initiation of interferon therapy. *J Gastroenterol Hepatol* 2008; **23**: 541-545
30 **McHutchison JG**, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, Dienstag J, Lee WM, Mak C, Garaud JJ, Albrecht JK. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061-1069

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RAPID COMMUNICATION

Pegylated interferon plus ribavirin for genotype Ib chronic hepatitis C in Japan

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Abstract

AIM: To evaluate the efficacy of pegylated interferon α-2b (peg-IFNα-2b) plus ribavirin (RBV) therapy in Japanese patients with chronic hepatitis C (CHC) genotype Ib and a high viral load.

METHODS: One hundred and twenty CHC patients (58.3% male) who received peg-IFNα-2b plus RBV therapy for 48 wk were enrolled. Sustained virological response (SVR) and clinical parameters were evaluated.

RESULTS: One hundred (83.3%) of 120 patients completed 48 wk of treatment. 53 patients (44.3%) achieved SVR. Early virological response (EVR) and end of treatment response (ETR) rates were 50% and 73.3%, respectively. The clinical parameters (SVR *vs* non-SVR) associated with SVR, ALT (108.4 IU/L *vs* 74.5 IU/L, $P = 0.063$), EVR (76.4% *vs* 16.4%, $P < 0.0001$), adherence to peg-IFN (≥ 80% of planned dose) at week 12 (48.1% *vs* 13.6%, $P = 0.00036$), adherence to peg-IFN at week 48 (54.7% *vs* 16.2%, $P < 0.0001$) and adherence to RBV at week 48 (56.1% *vs* 32.1%, $P = 0.0102$) were determined using univariate analysis, and EVR and adherence to peg-IFN at week 48 were determined using multivariate analysis. In the older patient group (> 56 years), SVR in females was significantly lower than that in males (17% *vs* 50%, $P = 0.0262$). EVR and adherence to Peg-IFN were demonstrated to be the main factors associated with SVR.

CONCLUSION: Peg-IFNα-2b plus RBV combination therapy demonstrated good tolerability in Japanese patients with CHC and resulted in a SVR rate of 44.3%. Treatment of elderly female patients is still challenging and maintenance of adherence to peg-IFNα-2b is important in improving the SVR rate.

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Key words: Chronic hepatitis C; Pegylated interferon; Ribavirin

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INTRODUCTION

In Japan, annual mortality due to liver cancer exceeds 30 000 and 75% of liver cancer is associated with hepatitis C virus (HCV) infection[1]. The combination of pegylated interferon (peg-IFN) plus ribavirin (RBV) is one of the most effective therapies for chronic hepatitis C (CHC), and the effect of this combination is reported to be higher than conventional interferon[2,3]. However, the majority of Japanese CHC patients are infected with HCV genotype Ib and have a high viral load, and treatment with conventional interferon has its difficulties[4]. CHC patients in Japan tend to be older than CHC patients in other countries therefore, problems such as a higher incidence of liver cancer and lower tolerability to treatment have been observed[4,5]. The HCV strain and the efficacy of interferon treatment vary between races and countries[6,7]. Identification of the factors associated with treatment efficacy is extremely important, however, few studies involving large populations have reported on the treatment of Japanese CHC patients with pegylated interferon alpha-2b (peg-IFNα-2b) plus RBV[8,9]. In this study, we evaluated the efficacy and safety of peg-IFNα-2b plus RBV therapy in CHC genotype Ib patients with a high viral load. This treatment became available in Japan for health insurance approved treatment from December 2004. In addition, we attempted to identify predictive factors for treatment outcome.

MATERIALS AND METHODS

Study population

One hundred and thirty CHC genotype Ib patients with a high viral load, who received peg-IFNα-2b plus RBV therapy in our hospital or our affiliated institutions between December 2005 and November 2006 were enrolled in this study. The diagnosis of CHC was based on the following criteria; HCV antibody positive, HCV-RNA positive and elevation of serum alanine aminotransferase (ALT) activity (> 35 IU/L) within 6 mo of screening. Exclusion criteria were leucopenia [white blood cell (WBC) count < 3000/μL], neutropenia [neutrophil (ne) count <1500/μL], thrombocytopenia [platelet (PLT) count < 90 000/μL], anemia [hemoglobin (Hb) < 12 g/dL], cirrhosis, creatinine clearance < 50 mL/min, uncontrolled mental disorder, severe heart or lung disease, or autoimmune disease. The study was approved by the ethical committee of Tohoku University according to the Declaration of Helsinki. All patients gave written informed consent before enrollment.

Treatment regimen

The patients received peg-IFNα-2b (Pegintron®; Schering-Plough, Kenilworth, NJ, USA) at a dosage of 1.5 mg/kg every week subcutaneously for 48 wk. Daily RBV (Rebetol®, Schering-Plough) was given orally for 48 wk and the dosage was adjusted according to weight (600 mg for ⩽ 60 kg, 800 mg for 60 to 80 kg, 1000 mg for > 80 kg). Blood samples were obtained every four weeks and were analyzed for biochemical parameters including ALT and HCV RNA levels. The HCV genotype was determined using a kit. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of the NS5 region. HCV RNA levels were measured by quantitative RT-PCR (Amplicor, Roche Diagnostic Systems, CA, USA). HCV RNA negativity was evaluated by qualitative RT-PCR (Amplicor, Roche), which has a higher sensitivity than the quantitative method. The lower limit of the assay in the quantitative method was 5 KIU/mL (equivalent to 5000 copies/mL) and was 50 IU/mL (equivalent to 50 copies/mL) in the qualitative method. Early virological response (EVR) was defined as undetectable HCV RNA after 12 wk. Sustained virological response (SVR) was defined as undetectable HCV RNA at 24 wk after completion of treatment.

Statistical analysis

Fisher's exact test and the Mann-Whitney *U* test were used to evaluate the parameters [age, sex, weight, body mass index (BMI), EVR, peg-IFN adherence, RBV adherence, HCV RNA, ALT, WBC, Hb, and PLT] to determine SVR. Quantitative data were divided into two groups using the median to examine the differences. We conducted multivariate analysis using binary logistic regression on the parameters which achieved statistical significance ($P < 0.05$) using univariate analysis. All analyses were performed using a statistical software package (StatView-J version 5.0, SAS Institute Inc. Cary, NC, USA).

RESULTS

Patient characteristics

The details of clinical background, blood biochemistry and virological data on the CHC patients who received peg-IFNα-2b plus RBV therapy are shown in Table 1. Seventy of 120 patients (58.3%) were male, and 50 patients (41.7%) were female. The mean age was 54.8 years, and the median age was 56 years. The mean age of males was 54.1 years, and the mean age of females was 55.8 years. The median BMI was 23.6. Seventy seven patients (64.2%) had no previous history of IFN treatment and 41 patients (34.2%) had been treated with IFN previously. Of these previously treated patients, 8 were null-responders (patients who did not achieve a virological or biochemical response during IFN treatment), 15 were relapsers, and 18 patients had no available virological response.

Treatment efficacy

One hundred of 120 patients (83.3%) completed 48 wk of treatment and 24 wk of follow up. Using intention to treat (ITT) analysis, 53 patients (44.3%) achieved SVR. The rate of EVR was 50%. Response rate at the end of treatment was 73.3%. The transition rate of HCV RNA negativity with time is shown in Figure 1. Patients discontinued treatment due to depression in 3, neutropenia in 3, retinopathy in 2, anemia in 1,

Table 1 Clinical characteristics of patients at baseline (mean ± SE)

No. of patients	120
Sex, *n* (%)	
Male	70 (58.3)
Female	50 (41.7)
Age (median, range, yr)	54.8 ± 0.98 (56, 27-75)
Male	54.1 ± 1.39 (55.5, 29-72)
Female	55.8 ± 1.33 (56, 27-75)
Weight	62.1 ± 1.09 (61.4, 35.0-99.8)
Body mass index (median, range, kg)	23.7 ± 0.32 (23.6, 14.6-34.1)
Viral load (kIU kirocopies/mL)	1510 (120->5000)
ALT (median, range, IU/L)	89.4 ± 7.39 (67, 18-636)
WBC (median, range, /μL)	5083 ± 136.6 (4900, 2400-9000)
Hemoglobin (median, range, g/dL)	14.4 ± 0.12 (14.1, 11.8-17.2)
Platelet (median, range, × 10^3/μL)	163.1 ± 4.71 (162.5, 8.1-33.2)
Interferon treatment history, *n* (%)	
Present	41 (34.2)
Null-responder/relapser/unknown	8/15/18
Absent	77 (64.2)
Unknown	2 (1.6)

ALT: Alanine aminotransferase; WBC: White blood cell.

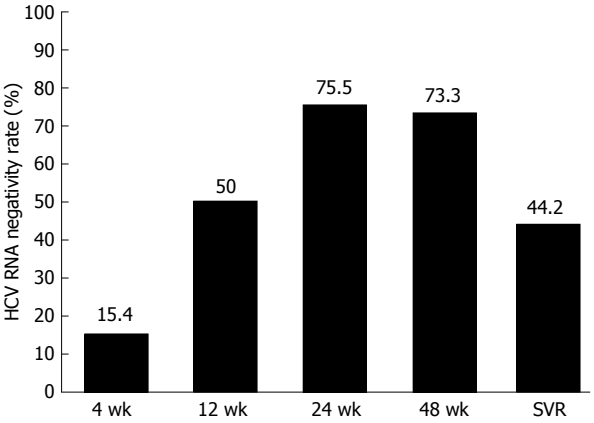

Figure 1 The transition rate of HCV RNA negativity with time.

cutaneous reaction in 1, palsy in 1, HSV infection in 1, and no response to treatment in 7.

Relationship between clinical parameters and SVR

The association between SVR rate and the baseline clinical parameters before treatment or treatment-related factors was examined using univariate analysis. The following baseline factors were analyzed: age, sex, BMI, HCV RNA level, ALT, WBC, Hb, and PLT. A summary of these results is shown in Table 2. The mean ALT level in patients who achieved SVR was 108.4 IU/L, which was significantly higher than the ALT level of 74.5 IU/L in the non-SVR group ($P = 0.0478$). The PLT level in patients in the SVR group was 1.73 ×10^5/μL, which was higher than the PLT level of 1.55 ×10^5/μL in the non-SVR group ($P = 0.063$). To determine the factors associated with treatment outcome, we examined the relationship between the SVR ratio and achievement of EVR or adherence to peg-IFN and RBV. A summary of these results is shown in Table 2 and Figure 2. As shown in Table 2, the ratio of patients who achieved EVR was

Table 2 Univariate analysis of association between sustained virological response (SVR) and influential factors (mean ± SE)

Factor	SVR patients (*n* = 53)	Non-SVR patients (*n* = 67)	*P*
Parameters before interferon treatment			
Age (yr)	52.5 ± 1.50	56.5 ± 1.26	0.0481
Sex (Male:Female)	35:18	35:32	0.1402
Body mass index	23.6 ± 0.48	23.8 ± 0.44	0.3611
Viral load (kirocopies/mL, median)	1500	1800	0.1963
ALT (IU/L)	108.4 ± 13.8	74.5 ± 7.04	0.0478
WBC (/μL)	5227 ± 201	4967 ± 186	0.2880
Hemoglobin (g/dL)	14.5 ± 0.18	14.3 ± 0.16	0.2352
Platelet (× 10^3/μL)	173 ± 7.7	155 ± 5.7	0.0630
Parameters associated with treatment			
EVR	42/51 (82.4%)	13/59 (22.0%)	< 0.0001
Cumulative exposure to peg-IFN			
12 wk (≥ 80%/< 80%)	38/41 (92.7%)	41/69 (68.3%)	0.0034
Overall (≥ 80%/< 80%)	35/41 (85.4%)	29/60 (48.3%)	0.0001
Cumulative exposure to RBV			
12 wk (≥ 80%/< 80%)	41/50 (82%)	44/63 (69.8%)	0.1882
Overall (≥ 80%/< 80%)	32/50 (64%)	25/63 (39.7%)	0.0138

ALT: Alanine aminotransferase; WBC: White blood cell; EVR: Early virological response; Peg-IFN: Pegylated interferon; RBV: Ribavirin.

Table 3 Multivariate analysis of association between sustained virological response and influential factors

Factor	Coefficient	χ^2	Odds ratio (95% CI)	*P*
EVR (not achieved)	-2.725	19.325	0.066 (0.019-0.221)	< 0.0001
Cumulative exposure to peg-IFN				
Overall (≥ 80%)	2.392	6.600	10.934 (1.763-67.82)	0.0102
Constant	1.294			

EVR: Early virological response; Peg-IFN: Pegylated interferon.

significantly higher in the SVR group than in the non-SVR group ($P < 0.0001$). The SVR rate in patients who achieved EVR was 76.4%, and this was significantly higher than the SVR rate in the non-EVR group which was 16.4% ($P < 0.0001$). The ratio of patients who received 80% or more of the scheduled dose of peg-IFN or RBV was significantly higher in the SVR group than in the non-SVR group. The SVR rate in patients who received 80% or more of the scheduled dose of peg-IFN was 48.1% (12th wk) and 54.7% (overall). The SVR rate in patients who did not receive sufficient peg-IFN was 13.6% (12th wk) and 16.2% (overall), and these were significantly lower than the group who had good adherence. The group with adequate adherence to RBV (overall) showed an SVR rate of 56.1%, which was significantly higher than the SVR rate of 32.1% in the poor adherence group ($P = 0.0102$). For the factors which were determined as statistically significant by univariate analysis, we subsequently conducted multivariate analysis. The results of this analysis are shown in Table 3. Using binary logistic analysis, EVR and adherence to peg-IFN were determined to be independent predictive factors for SVR.

We examined a group of patients who were older than the median age (56 years). From the baseline factors obtained before treatment, sex was determined

Table 4 Univariate analysis of association between sustained virological response (SVR) and influential factors (mean ± SE)

Factor	SVR patients (n = 20)	Non-SVR patients (n = 37)	P
Parameters before interferon treatment			
Age (yr)	64.0 ± 0.71	63.8 ± 0.73	0.6637
Sex (male:female)	16:4	18:19	0.0262
Body mass index	23.3 ± 0.56	23.6 ± 0.53	0.3973
Viral load (kirocopies/mL)	1500	1800	0.3616
ALT (IU/L)	94.5 ± 31.1	76.6 ± 11.0	0.3038
WBC (/μL)	5119 ± 313	4832 ± 223	0.3798
Hemoglobin (g/dL)	14.1 ± 0.23	14.1 ± 0.19	0.8473
Platelet (× 10^3/μL)	173 ± 12.4	151 ± 7.9	0.1434
Parameters associated with treatment			
EVR	13/18 (72.2%)	7/32 (21.9%)	0.0008
Cumulative exposure to peg-IFN			
12 wk (≥ 80%/< 80%)	16/17 (94.1%)	20/33 (60.6%)	0.0183
Overall (≥ 80%/< 80%)	15/17 (88.2%)	14/33 (42.4%)	0.0023
Cumulative exposure to RBV			
12 wk (≥ 80%/< 80%)	14/20 (70%)	20/34 (58.8%)	0.5612
Overall (≥ 80%/< 80%)	8/20 (40%)	13/34 (38.2%)	> 0.9999

ALT: Alanine aminotransferase; WBC: White blood cell; EVR: Early virological response; Peg-IFN: Pegylated interferon; RBV: Ribavirin.

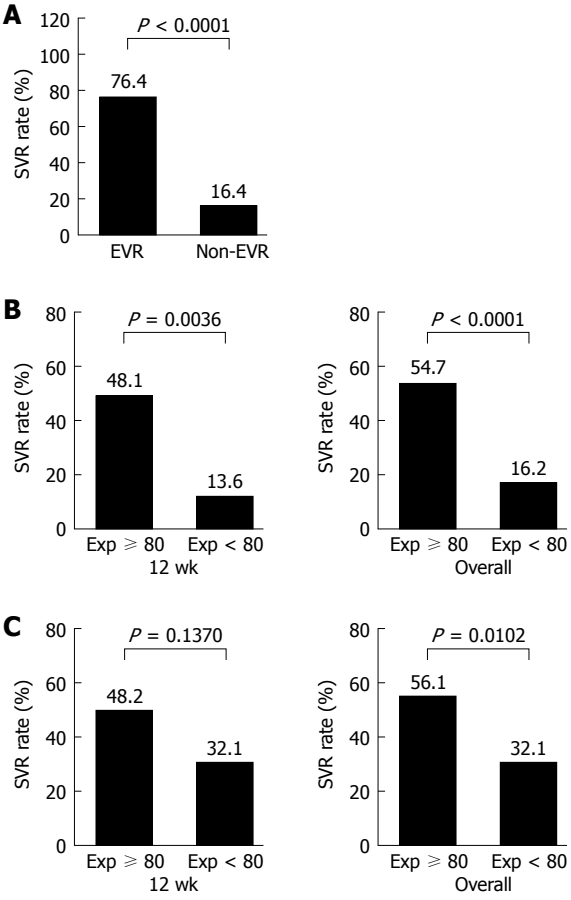

Figure 2 The clinical parameters associated with SVR rate using univariate analysis. A: The relationship between EVR and SVR rate; B: The relationship between cumulative exposure to peg-IFN and SVR rate; C: The relationship between cumulative exposure to RBV and SVR rate.

to be a parameter which may be associated with SVR (Table 4). The SVR rate in females was 17%, which was significantly lower than the SVR rate of 50% in males

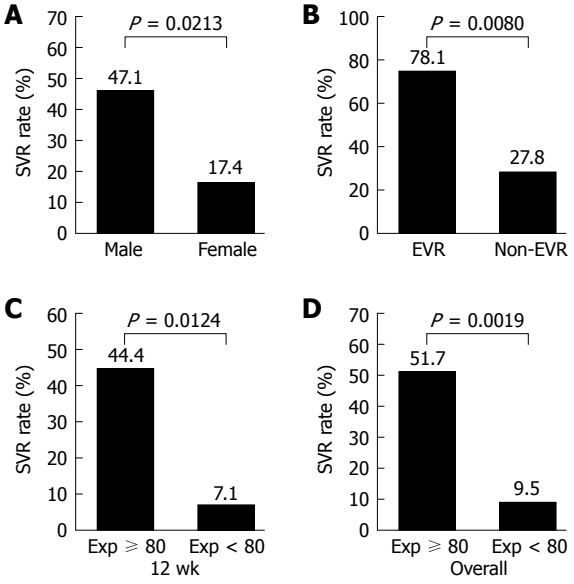

Figure 3 Clinical parameters in the older patient group (> 56 yr) associated with SVR rate using univariate analysis. A: The relationship between sex and SVR rate; B: The relationship between EVR and SVR. C and D: The relationship between cumulative exposure to peg-IFN and SVR.

(*P* = 0.0262) (Figure 3A). From the factors associated with treatment outcome, EVR and adherence to peg-IFN were demonstrated to be significant (Figure 3B). In particular, the SVR rate in the group with poor adherence to peg-IFN was 7.1% (1 of 14) at the 12th wk and was 9.5% (2 of 21) at the end of treatment. These rates were extremely low compared with the SVR rate of 44.4% (12th wk) and 51.7% (overall) in the group with good adherence to peg-IFN (Figure 3C and D).

DISCUSSION

One hundred of 130 patients completed peg-IFNα-2b plus RBV combination therapy in our hospital and related institutions. Treatment was discontinued in 13 patients (10.8%) due to adverse effects. The treatment showed good tolerability in Japanese patients. A study of peg-IFNα-2b plus RBV combination therapy in Caucasian and African American CHC patients reported a discontinuation rate of 21%[10]. Another study on Japanese CHC patients reported a 21% discontinuation rate[9]. Although we cannot compare these studies directly, it seems that tolerability in this study was satisfactory. At least in a clinical setting, peg-IFNα-2b plus RBV was well tolerated in our study of Japanese patients.

In this study, age, ALT level, EVR achievement, and adherence to Peg-IFN and RBV were associated with a high SVR rate using univariate analysis. After multivariate analysis, EVR and adherence to peg-IFN were demonstrated to be associated with SVR. Of the baseline factors assessed before treatment, age, sex, WBC, α-feto protein level, γ-glutamyl transpeptidase, and LDL-cholesterol have been reported to be associated with a high SVR rate following peg-IFNα-2b plus RBV therapy in CHC Japanese patients[8,11,12]. The results of this study were very similar to those of our

study. Davis *et al*[13] reported that EVR was considered to be associated with SVR in patients with CHC treated with IFN. As a result of this study, EVR was found to be one of the factors which most influenced SVR rate in Japanese patients treated with peg-IFNα-2b plus RBV combination therapy. In our study of older patients (older than the median), sex, EVR, and adherence to peg-IFN were associated with SVR rate. The SVR rate in older females was remarkably low at 17.4% compared to the SVR rate in all females included in the study which was 36.0% (data not shown).

Adherence to peg-IFN was found to influence the SVR rate as a treatment-related factor in this study. SVR rates were low in patients who did not receive 80% or more of the intended dose of peg-IFN. The effect of adherence to IFN on SVR has been reported previously[13-15]. In a study on peg-IFNα-2a/RBV therapy in patients with HCV genotype I, it was reported that the SVR rate in cases who had a reduction in RBV dosage before the 20th wk was remarkably low[15]. Furthermore, a reduction in RBV dosage and/or peg-IFNα-2a dosage after the 24th wk did not influence the SVR rate[15]. On the other hand, a study on African American patients with HCV genotype I reported that a reduction in peg-IFNα-2b dosage influenced the SVR rate more than a reduction in RBV dosage[14]. In the current study, adherence to RBV up to the 12th wk did not significantly influence the SVR rate, but overall adherence to RBV significantly influenced the SVR rate. Unlike the reports on Caucasian and African American patients, it may be that overall adherence to RBV is important in Japanese patients.

It was notable that adherence to peg-IFNα-2b significantly influenced SVR in this study. In the patients who did not receive 80% or more of the intended dose by the 12th wk, the SVR rate decreased markedly. Adherence to peg-IFNα-2b at the 12th wk may be critical in determining whether the treatment should be continued. It is often difficult to maintain adherence to peg-IFNα-2b simply to improve the SVR rate, because IFN dosage and the hematologic adverse effects of this drug are problematic[16,17]. Recently, a 72-wk treatment protocol for late virological responders was reported[18,19]. Further examination of the impact of prolonged administration in patients with poor adherence to peg-IFNα-2b is needed.

In conclusion, peg-IFNα-2b plus RBV combination therapy demonstrated good tolerability in Japanese patients with CHC, and resulted in a SVR rate of 44.3%. Treatment of older female patients and maintenance of adherence to peg-IFNα-2b are important factors in improving SVR rate.

COMMENTS

Background

Pegylated interferon α-2b (peg-IFNα-2b) plus ribavirin (RBV) is a standard treatment of chronic hepatitis C globally. However, the impact of this treatment in an ordinary clinical setting in Asian patients is still unclear.

Research frontiers

It is well documented that data from clinical practice is not comparable to those of clinical trials. This is believed to be derived from differences in recruited patients in phase Ⅱ and Ⅲ clinical trials and usual clinical settings (e.g. young *vs* elderly, highly motivated *vs* reluctant, *etc*).

Innovations and breakthroughs

The current study demonstrated that outcome is dependent on therapeutic adherence (> 80% of expected peg-IFN dosage). The overall treatment success [sustained virological response (SVR)] was 44.3%, almost equivalent to those in phase III clinical trials.

Applications

The total SVR rate was equivalent to clinical trials. The elderly, especially female patients showed a lower response to treatment. The reason for this is still unclear and future investigations are feasible in order to understand this observation.

Terminology

SVR indicates sustained virological response, which means sustained (more than 24 wk after treatment) viral clearance from the infected host.

Peer review

It is very important to describe the true clinical impact of global standard treatment in Asian races. Fortunately, the results were almost equivalent to those of other global regions. Although female patients seem to have a disadvantage with this treatment, these patients could have comparable results if adherence to both drugs is maintained.

REFERENCES

1 **Umemura T**, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *Hepatol Res* 2007; **37** Suppl 2: S95-S100

2 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

3 **McHutchison JG**, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492

4 **Higuchi M**, Tanaka E, Kiyosawa K. Epidemiology and clinical aspects on hepatitis C. *Jpn J Infect Dis* 2002; **55**: 69-77

5 **Kiyosawa K**, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, Tanaka E. Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; **127**: S17-S26

6 **He XS**, Ji X, Hale MB, Cheung R, Ahmed A, Guo Y, Nolan GP, Pfeffer LM, Wright TL, Risch N, Tibshirani R, Greenberg HB. Global transcriptional response to interferon is a determinant of HCV treatment outcome and is modified by race. *Hepatology* 2006; **44**: 352-359

7 **Luo S**, Cassidy W, Jeffers L, Reddy KR, Bruno C, Howell CD. Interferon-stimulated gene expression in black and white hepatitis C patients during peginterferon alfa-2a combination therapy. *Clin Gastroenterol Hepatol* 2005; **3**: 499-506

8 **Akuta N**, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Miyakawa Y, Kumada H. Prediction of response to pegylated interferon and ribavirin in hepatitis C by polymorphisms in the viral core protein and very early dynamics of viremia. *Intervirology* 2007; **50**: 361-368

9 **Hiramatsu N**, Kurashige N, Oze T, Takehara T, Tamura S, Kasahara A, Oshita M, Katayama K, Yoshihara H, Imai Y, Kato M, Kawata S, Tsubouchi H, Kumada H, Okanoue T, Kakumu S, Hayashi N. Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C. *Hepatol Res* 2008; **38**: 52-59

10 **Muir AJ**, Bornstein JD, Killenberg PG. Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. *N Engl J Med* 2004; **350**: 2265-2271

11 **Akuta N**, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. *J Med Virol* 2007; **79**: 1686-1695
12 **Akuta N**, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. *Hepatology* 2007; **46**: 1357-1364
13 **Davis GL**, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645-652
14 **McHutchison JG**, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, Dienstag J, Lee WM, Mak C, Garaud JJ, Albrecht JK. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061-1069
15 **Shiffman ML**, Di Bisceglie AM, Lindsay KL, Morishima C, Wright EC, Everson GT, Lok AS, Morgan TR, Bonkovsky HL, Lee WM, Dienstag JL, Ghany MG, Goodman ZD, Everhart JE. Peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C who have failed prior treatment. *Gastroenterology* 2004; **126**: 1015-1023; discussion 947
16 **Russo MW**, Fried MW. Side effects of therapy for chronic hepatitis C. *Gastroenterology* 2003; **124**: 1711-1719
17 **Soza A**, Everhart JE, Ghany MG, Doo E, Heller T, Promrat K, Park Y, Liang TJ, Hoofnagle JH. Neutropenia during combination therapy of interferon alfa and ribavirin for chronic hepatitis C. *Hepatology* 2002; **36**: 1273-1279
18 **Arase Y**, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Kobayashi M, Kobayashi M, Suzuki F, Akuta N, Someya T, Kumada H. Efficacy of prolonged interferon therapy for patients with chronic hepatitis C with HCV-genotype 1b and high virus load. *J Gastroenterol* 2003; **38**: 158-163
19 **Arase Y**, Suzuki F, Tsubota A, Suzuki Y, Saitoh S, Kobayashi M, Akuta N, Someya T, Hosaka T, Kobayashi M, Sezaki H, Ikeda K, Kumada H. Sustained negativity for HCV-RNA over 24 or more months by long-term interferon therapy correlates with eradication of HCV in patients with hepatitis C virus genotype 1b and high viral load. *Intervirology* 2004; **47**: 19-25

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RAPID COMMUNICATION

Hepatitis B virus genotypes and lamivudine resistance mutations in Jordan

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Abstract

AIM: To investigate and identify prevalent hepatitis B virus (HBV) genotypes and to explore lamivudine-resistant mutations among treated and untreated patients in Jordan.

METHODS: A total of 107 cases with chronic hepatitis B were recruited from different medical centers in Jordan. Serological tests were preformed for all cases using a microparticle enzyme immunoassay. HBV Genotyping was performed for 70 cases using Line probe genotyping assay. The YMDD mutations were explored for 20 cases (4 were lamivudine naive) using the INNO-LiPA HBV DR assay.

RESULTS: Genotype D was the only detected genotype. A total of 6 YMDD mutations were detected in 5 treated patients (31%) while one mutation was detected in the naive patients. Seventeen percent of cases were positive for HBeAg and had statistically significant higher levels of serum aminotransferases.

CONCLUSION: HBV genotype D appears to be the only circulating type in Jordanian patients. The YMDD mutations were detected in 31% of lamivudine-treated cases with similar patterns to those found in the literature. We also found a relatively low prevalence of HBeAg expression among examined cases (17%). Awareness of these serologic, genotypic and resistance patterns might help in the formulation of management plans and for predicting clinical outcomes. Further larger scale studies are needed to confirm our results and to examine possible associations among clinical, serologic, and genetic patterns of HBV infections in Jordan.

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Key words: Hepatitis B virus; Genotypes; Lamivudine; YMDD mutation; Jordan

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INTRODUCTION

Infections with hepatitis B virus (HBV) continue to be a worldwide problem[1] and a considerable proportion of these infections usually progress to chronic infection and hepatocellular carcinoma[2]. In view of this significant diseases burden, vaccination against hepatitis B emerged as the most cost-effective prevention method[3]. The available treatments of HBV infections include interferon-alpha (IFN-α) and nucleoside analogue agents (lamivudine, adefovir, entecavir, telbivudine, and others)[4]. However, responses to these treatment regimens are variable and are still a long way from being perfect. Besides, treatment protocols are associated with considerable risks of evolving resistant mutants[5]. These hepatitis B mutants can appear in patients as a consequence of the constant selection pressure from either the immune response or treatment choice. In the meantime, classification of HBV has changed from the serologic subtype classification to a more precise genotype genetic classifi-

cation. Hepatitis B virus has been classified into the eight genotypes (A-H) on the basis of nucleotide sequence differences, and these genotypes have typical geographic distributions and may have different pathogenicity and epidemiology[6]. These genotypes are generated during replication of HBV DNA through an RNA reverse transcriptase intermediary step that lacks proofreading functions[7,8]. Lamivudine, as a potent nucleoside analogue, has been used for chronic HBV infections therapy. However, the need for long-term courses is associated with emergence of lamivudine-resistant mutations. The most common of these mutations affect HBV polymerase-reverse transcriptase. The most commonly reported mutations are the substitution of either valine (M204V) or isoleucine (M204I) for methionine in the tyr-met-asp-asp (YMDD) motif located in the polymerase active site (domain C), and substitution of methionine for leucine (L180M) in the active site (domain B)[9,10]. The present study aimed to identify prevalent hepatitis B genotypes and lamivudine-resistant mutations among treated and untreated HBV patients in Jordan.

MATERIALS AND METHODS

Patients and setting

In this cross-sectional study, we recruited 107 patients with chronic hepatitis B from different departments of King Abdullah University Hospital, Jordan University Hospital, and Princess Badea'h Hospital. All were positive for HBsAg. Genotyping and lamivudine-resistance mutation analysis were performed for 70, and 20 patients, respectively (budgetary restrictions meant we couldn't genotype all the patients). There was no recruitment discrimination made in respect to active or previous lamivudine treatment.

HBV serological markers testing

Twenty ml of peripheral blood were taken and serum samples were aliquoted and stored at -70℃ until used. Sera were tested for HBsAg, anti-HBc, anti-HBs, HBeAg, and anti-HBe using the microparticle enzyme immunoassay (Abbott diagnostic laboratories/AXSYM system, v2, 200, Ireland).

HBV DNA extraction

HBV DNA was extracted from the serum using a DNA-sorb-B kit (Sacace Sr biotechnologies company, Italy). DNA was re-suspended in a final volume of 30 μL of sterile, nuclease-free water and then stored at -20℃ till used.

HBV genotypes detection

HBV Genotyping was performed using the Line probe genotyping assay (INNO-LiPA HBV Genotyping assay; Innogenetics, Ghent, Belgium). It is a line probe assay designed to identify hepatitis B genotypes A to G by detection of type-specific sequences (328-619 nucleotides) in the HBV polymerase gene (domains B to C), which are overlapped with specific sequences in the HBV surface gene. In summary, the biotinylated PCR product (after amplification) was denatured by adding denaturation solution at room temperature. It was then hybridized with specific oligonucleotides probes immobilized as parallel lines on membrane based-strips in hybridization solution at 50℃ using a water bath. Stringency solution was then added to strengthen the binding between the probe and single stranded denatured DNA. Unhybridized DNA was washed from the strips by addition of rinse solution. Conjugate solution was then added to the strips at room temperature. Finally, a chromogenic substrate was added to the strips. A purple precipitate was observed in the lines, identifying stable hybrids formed between the DNA and the probes.

Amplification of HBV DNA and YMDD mutations detection

Amplification of HBV DNA was as described previously[9]. YMDD mutations were detected using a lamivudine resistance assay (INNO-LiPA HBV DR; Innogenetics, Ghent, Belgium). The amplified region of the HBV genome is common between the HBsAg gene and the polymerase gene, therefore the same protocol was followed for both amplification and hybridization processes. This assay detects mutations or polymorphisms at codons 180, 204 and 207 of the HBV polymerase gene, in addition to HBsAg-specific codons, due to the overlapping reading frame.

Statistical analysis

Data were processed using the statistical package for statistical science software (SPSS, version 10, Chicago. Inc). Statistical analysis was carried out using Fisher's exact test, Chi-square test, and *t*-test wherever appropriate. A P value of < 0.05 was considered significant.

RESULTS

Patients' demographics

We recruited 107 patients (31 female and 76 male). Mean age of cases was 34.1 years (SD = 13). Chronicity was confirmed in all cases with positive results for HBsAg, anti-HBcAg-IgG and negative results for anti-HBsAg-IgM. Sixteen patients had a history of receiving lamivudine (Twelve were actively receiving it, while 4 were off treatment at time of enrolment). None of HBeAg negative cases had hepatitis-related symptoms (jaundice, liver enlargement, and Ascites), while 33% of the HBeAg positive cases had such symptoms (Table 1).

Serological patterns

Most patients did not express HBeAg (83%). However, the mean levels of alanine aminotransferase (ALT), and aspartate aminotransferases (AST) were significantly higher in the HBeAg positive group (121 *vs* 38 and 143 *vs* 37, respectively) (Table 1) and these ALT and AST values were statistically significantly abnormal.

HBV genotyping

Surprisingly and interestingly, the 70 selected samples (positive-HBsAg) were all positive for genotype D. We

Table 1 Clinical and serological patterns of all 107 HBsAg positive patients *n* (%)

Criteria	HBeAg positive	HBeAg negative	*P*
Number of cases	18 (17)	89 (83)	< 0.0001
Hepatitis-related symptoms	6 (330)	0 (0)	< 0.0001
Positive HBeAb	0 (0)	89 (100)	< 0.0001
Abnormal ALT	18 (100)	35 (39)	< 0.0001
Abnormal AST	16 (89)	12 (13)	< 0.0001
Abnormal ALP	4 (22)	18 (20)	0.85

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.

did not detect any other genotypes. Detecting no other genotypes in our cohort made it impossible to compare between HBV genotypes and their effects on clinical course of disease or on response to antiviral drugs.

Lamivudine resistance mutations

Lamivudine resistant mutations were tested for 20 cases (4 were lamivudine naive and 16 with a history of lamivudine treatment) (Table 2). Six mutations were detected in 5 different patients with a history of lamivudine treatment (31%). Two mutations (M204Ile and L180M) were detected in one patient. On the other hand, one mutation only (M204V) was detected in a lamivudine naive patient. The mutation M204Ile was the most prevalent and durations of lamivudine treatment ranged from 1 to 13 years.

DISCUSSION

This is the first report from Jordan that investigates HBV genotypes and lamivudine resistance mutations. Our results showed that genotype D is at least the most (if not the only) prevalent genotype in Jordan. This genotype is also the most prevalent genotype worldwide, with more concentration in the Middle East and the regions around as Turkey, Egypt, and Gulf region. Recent studies have found that genotype D accounts for 81%-85% of all genotypes in Saudi Arabia and almost all genotypes in Egypt[11-13]. This finding might have potential impact on selection of antiviral drugs, prediction of disease courses and clinical responses. There is accumulating evidence that patients with genotype D might achieve higher sustained viral response rate than patients with genotype A[14], despite being less responsive to interferon treatment when compared to genotypes A and B[5]. It is also known that that genotype D has a higher likelihood of developing advanced cirrhosis compared to genotype A[15]. Furthermore, there is evidence that the rate of resistance to lamivudine is lower in patients infected with genotype D than in patients with genotype A[5]. In our study, the prevalence of lamivudine resistant mutations was 31%. It is difficult to compare this prevalence to other studies because our cases received variable durations of lamivudine treatment. However, this prevalence seems broadly similar to those in other reports showing that there is a 20% yearly chance for resistance

Table 2 Profile of detected YMDD mutations among tested cases

Mutation	Patients with history of lamivudine treatment	lamivudine naive patients
Patients tested	16	4
HBeAg Positive	3	1
M204Ile	4	0
M204V	1	1
L180M	1	0
Total	6	1

to emerge in lamivudine-treated patients. The mutation M204Ile was the most prevalent, followed by the M204V mutation, which is similar to cases reported previously[16]. We also detected a mutation in an apparently lamivudine-naive case. This might be due to transmission of the virus from a lamivudine-treated index case or, less likely, to a spontaneous mutation. In general, HBV mutations conferring drug resistance to lamivudine are rare in lamivudine naive patients but do exist[17]. In one of our patients, the M204V mutation was accompanied by the mutation L180M. This combination is also common and has been described before[18]. Our study has also found a relatively low rate of HBeAg positive patients (17%). This might be explained by the rapid clearance of HBeAg among patients with genotype D demonstrated in previous reports[12].

In conclusion, HBV genotype D appears to be the only circulating type in Jordanian patients. The YMDD mutations were detected in 31% of lamivudine-treated cases with similar patterns to those in the literature. We also found a relatively low prevalence of HBeAg expression among examined cases (17%). Awareness of these serologic, genotypic and resistance patterns might help in the formulation of management plans and in predicting clinical outcomes. Further larger scale studies are needed to confirm our results and to examine possible associations among clinical, serologic, and genetic patterns of HBV infections in Jordan.

COMMENTS

Background

Hepatitis B virus (HBV) infections continue to impose huge medical, social, and economic burdens on patients and countries all over the world. Chronic liver disease, cirrhosis, and hepatocellular carcinoma are serious consequences of such infections. These infections are possibly transmitted through transfusion of blood products, unprotected sex, vertical transmission from mothers, or other different risky behaviours. As of today, and despite tremendous medical advances, there is permanent cure for these infections.

Research frontiers

Individualizing the management of patients with hepatitis B infections is based on understanding the local prevalence of different genotypes of this virus. It is also affected by the patterns of resistance manifested by local strains. We addressed these issues in this article. We studied the genotype profiles and resistance patterns of hepatitis B strains among Jordanian patients.

Innovations and breakthroughs

Even though we expected genotype D to be the most prevalent one among Jordanian patients, it was surprising that no other genotypes were detected. With a small number of strains tested for YMDD resistance mutations, it was apparent that our YMDD lamivudine resistance profiles were similar to those encountered

in other surrounding countries and other different parts of the world. During the twentieth century, huge advancements in understanding the basics of this disease have been achieved. Researchers have focused on different frontiers including pathogenesis, diagnostic approaches, genetic profiling, antiviral therapy, resistance patterns, and vaccines development.

Applications

This study may give certain insights to Jordanian physicians managing hepatitis B patients. Genotypic profiles and resistance patterns might help in decisions regarding selection of antiviral therapy, duration of treatments, and expected responses to treatment. Knowing the genetic profiling of hepatitis B strains might reduce the cost of diagnostic testing.

Terminology

Lamivudine is a well-known antiviral drug used for treatment of hepatitis B and other viral infections. YMDD is a motif that is commonly mutated in hepatitis B virus lamivudine resistant mutants.

Peer review

In this article, the authors have described HBV molecular epidemiology in Jordan. The objectives were clearly stated and a respectable sample size (107 subjects) was studied. All subjects were HBsAg positive. Only 17% of cases were positive for HBeAg. 100% of cases were genotyped as HBV genotype D. YMDD mutations were observed in 31% of cases analyzed. Overall, the manuscript is well written and results are clearly presented. Interpretation of the findings is appropriate.

REFERENCES

1 **McMahon BJ**. Natural history of chronic hepatitis B - clinical implications. *Medscape J Med* 2008; **10**: 91
2 **Kazim SN**, Chauhan R, Das BC, Sarin SK. Association of core promoter mutations with viral breakthrough in chronic hepatitis B patients on long-term lamivudine therapy. *J Gastroenterol Hepatol* 2006; **21**: 1525-1532
3 **Lok AS**, McMahon BJ. Chronic hepatitis B: update of recommendations. *Hepatology* 2004; **39**: 857-861
4 **Connor BA**, Jacobs RJ, Meyerhoff AS. Hepatitis B risks and immunization coverage among American travelers. *J Travel Med* 2006; **13**: 273-280
5 **Palumbo E**. Hepatitis B genotypes and response to antiviral therapy: a review. *Am J Ther* 2007; **14**: 306-309
6 **Wiegand J**, Hasenclever D, Tillmann HL. Should treatment of hepatitis B depend on hepatitis B virus genotypes? A hypothesis generated from an explorative analysis of published evidence. *Antivir Ther* 2008; **13**: 211-220
7 **Nassal M**. Hepatitis B viruses: reverse transcription a different way. *Virus Res* 2008; **134**: 235-249
8 **Hunt CM**, McGill JM, Allen MI, Condreay LD. Clinical relevance of hepatitis B viral mutations. *Hepatology* 2000; **31**: 1037-1044
9 **Yalcin K**, Degertekin H, Alp MN, Tekes S, Satici O, Budak T. Determination of serum hepatitis B virus DNA in chronic HBsAg carriers: clinical significance and correlation with serological markers. *Turk J Gastroenterol* 2003; **14**: 157-163
10 **Chen CH**, Lee CM, Lu SN, Changchien CS, Eng HL, Huang CM, Wang JH, Hung CH, Hu TH. Clinical significance of hepatitis B virus (HBV) genotypes and precore and core promoter mutations affecting HBV e antigen expression in Taiwan. *J Clin Microbiol* 2005; **43**: 6000-6006
11 **Al Ashgar HI**, Imambaccus H, Peedikayil MC, Al Thawadi S, Al Quaiz M, Al Fadda M, Al Kahtani K, Kagevi I, Khan MQ. Prevalence of hepatitis B virus genotype in Saudi Arabia: a preliminary report. *Indian J Gastroenterol* 2008; **27**: 81-82
12 **Abdo AA**, Al-Jarallah BM, Sanai FM, Hersi AS, Al-Swat K, Azzam NA, Al-Dukhayil M, Al-Maarik A, Al-Faleh FZ. Hepatitis B genotypes: relation to clinical outcome in patients with chronic hepatitis B in Saudi Arabia. *World J Gastroenterol* 2006; **12**: 7019-7024
13 **Saudy N**, Sugauchi F, Tanaka Y, Suzuki S, Aal AA, Zaid MA, Agha S, Mizokami M. Genotypes and phylogenetic characterization of hepatitis B and delta viruses in Egypt. *J Med Virol* 2003; **70**: 529-536
14 **Thakur V**, Sarin SK, Rehman S, Guptan RC, Kazim SN, Kumar S. Role of HBV genotype in predicting response to lamivudine therapy in patients with chronic hepatitis B. *Indian J Gastroenterol* 2005; **24**: 12-15
15 **Thakur V**, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *J Gastroenterol Hepatol* 2002; **17**: 165-170
16 **Tillmann HL**. Antiviral therapy and resistance with hepatitis B virus infection. *World J Gastroenterol* 2007; **13**: 125-140
17 **Alvarado-Esquivel C**, de la Ascension Carrera-Gracia M, Conde-Gonzalez CJ, Juarez-Figueroa L, Ruiz-Maya L, Aguilar-Benavides S, Torres-Valenzuela A, Sablon E. Genotypic resistance to lamivudine among hepatitis B virus isolates in Mexico. *J Antimicrob Chemother* 2006; **57**: 221-223
18 **Delaney WE 4th**, Locarnini S, Shaw T. Resistance of hepatitis B virus to antiviral drugs: current aspects and directions for future investigation. *Antivir Chem Chemother* 2001; **12**: 1-35

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RAPID COMMUNICATION

Tuberculous peritonitis in children: Report of nine patients and review of the literature

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Abstract

AIM: To present our experience with tuberculous peritonitis treated in our hospital from 2002-2007.

METHODS: We reviewed the medical records of 9 children with tuberculous peritonitis.

RESULTS: Nine patients (5 boys, 4 girls) of mean age 14.2 years were diagnosed with peritoneal tuberculosis. All patients presented with abdominal distention. Abdominal pain was seen in 55.5% and fever in 44.4% of the patients. Four cases had coexisting pleural effusion and two had pulmonary tuberculosis with parenchymal consolidation. Ultrasonography found ascites with septation in 7 patients. Two patients had only ascites without septation. Ascitic fluid analysis of 8 patients yielded serum-ascite albumin gradients of less than 1.1 gr/dL. Laparoscopy and laparotomy showed that whitish tuberculi were the most common appearance. Adhesions were also seen in three cases. The diagnosis of peritoneal tuberculosis was confirmed histo-pathologically in 7 patients and microbiologically in two. Two patients had been diagnosed by ascitic fluid diagnostic features and a positive response to antituberculous treatment. All patients completed the antituberculous therapy without any complications.

CONCLUSION: Tuberculous peritonitis has to be clinically suspected in all patients with slowly progressive abdominal distension, particularly when it is accompanied by fever and pain. Laparoscopy and peritoneal biopsy are still the most reliable, quick and safe methods for the diagnosis of tuberculous peritonitis.

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Key words: Child; Clinical presentation; Diagnosis; Tuberculous peritonitis

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INTRODUCTION

Tuberculous peritonitis (TBP) is an uncommon presentation of tuberculosis (TB) especially in children without any other debilitating disease such as cirrhosis, diabetes and chronic renal failure on continuous ambulatory peritoneal dialysis[1]. It is estimated that TBP occurs in 0.1%-3.5 % of all patients with pulmonary TB and represents 4%-10% of all extrapulmonary TB[2,3]. Most of the cases are in their 30's or 40's and it is rarely seen in children[1,4]. It results from hematogenous spread or contagious spread from an abdominal focus or mesenteric lymph node[4,5]. Most patients have chronic abdominal complaints. Due to the protean nature of the manifestations, diagnosis is often delayed and the rate of complications and mortality increases. Therefore, clinicians should be aware of the disease for the early diagnosis. We aimed to review the clinical features of the TBP in children that were followed up in our center.

MATERIALS AND METHODS

In this retrospective study, we reviewed the medical records of 9 children with TBP followed by our Pediatric Department from 2002-2007. The presentation symptoms, history of TB exposure, biochemical tests, clinical and histological features of the patients were recorded. Other causes of ascite and chronic liver

Table 1 Summary of patients' details, clinical presentations and outcome

Patients	1	2	3	4	5	6	7	8	9
Age/sex (Yr)	14/M	14/F	16/M	16/M	16/F	11/M	14/M	12/F	15/F
Clinical presentation	Abdominal distention Abdominal Pain Night sweats Cough	Abdominal distention	Abdominal distention Abdominal Pain Night sweats Weight loss	Abdominal distention Weight loss Night sweats Fever Cough	Abdominal distention Weight loss Fever	Abdominal distention	Abdominal distention Abdominal Pain	Abdominal distention Abdominal Pain Fever Cough	Abdominal distention Abdominal Pain Fever
Thoracic involvement	Consolidation	None	Pleural effusion	Pleural effusion Consolidation	Pleural effusion	None	None	Pleural effusion	None
Contact history	Father Brother	None	Mother	Uncle	Mother Grandmother	Grandfather	Father	None	None
BCG /TST (scar/mm)	Neg/22	Neg/17	Neg/6	Neg/10	Pos/0	Pos/20	Neg/15	Pos/13	Pos/16
ADA (IU/dL)	-	121	-	-	-	-	94	-	102
SAAG	-	1.0	0.5	0.7	0.3	0.8	0.4	0.5	0.6
Ascitic fluid AFB/culture	-/-	Neg/Pos	Neg/Neg	Neg/Neg	Neg/Pos	Neg/Neg	Neg/Neg	Pos/Neg	Neg/Neg
Abdominal USG	Minimal ascite	Ascites with septation	Ascites with septation	Ascites with septation LAP	Ascites with septation	Ascites	Ascites with septation	Ascites with septation Hepatomegaly	Ascites with septation LAP
Laparoscopic/ laparotomic appearance	Whitish tuberculi Adhesions	Whitish tuberculi	-	-	Whitish tuberculi Adhesions	Whitish tuberculi	Whitish tuberculi Adhesions	Whitish tuberculi	Whitish tuberculi
Peritoneal histopathology	Caseating granuloma	Caseating granuloma	-	-	Caseating granuloma	Caseating granuloma	Caseating granuloma	Caseating granuloma	Caseating granuloma
Outcome	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive

-: Not obtained; BCG: Bacillus calmette-guerin vaccination; TST: Tuberculin skin test; SAAG: Serum-ascite albumin gradient; ADA: Adenosine deaminase activity; AFB: Acid fast bacilli; USG: Ultrasonography; LAP: Lymphadenopathy.

diseases were ruled out in all patients. None of the patients had any other chronic disease.

The tuberculin skin test (TST) was evaluated 48-72 h after intradermal injection of 5 tuberculin units of purified protein derivative. It was considered to be positive when the induration was greater than or equal to 15 mm in previously vaccinated patients and greater than or equal to 10 mm in patients who had never been vaccinated.

Diagnosis of TBP was based on either typical laparoscopic appearance with tubercles and histological presence of caseating granuloma or diagnostic features of the ascitic fluid and response of the antituberculous therapy (in the absence of tissue samples being available). Patients were treated with isoniazide (10 mg/kg per day) and rifampin (15 mg/kg per day) for 9 mo and pyrazinamide (30 mg/kg per day) and streptomycin (20-40 mg/kg per day) for the first 2 mo. Metilprednisolone (2 mg/kg per day) was also given to four patients (patients 1-3 and 5) for the first 6 wk. The data were expressed as mean ± SD.

RESULTS

Nine patients were diagnosed with peritoneal tuberculosis. They were 5 boys and 4 girls with a mean age of 14.2 years (range 11-16 years). At presentation, abdominal distention was a common complaint in all patients (100%). In addition, five had abdominal pain (55.5%), four had fever (44.4%), three had coughing, three had weight loss, and three had night sweating (33.3%). The mean duration of symptoms was 41.5 ± 30.2 (7-90) d. Six patients had a history of tuberculosis within the family (for details see Table 1).

At admission, only four of the patients had two Bacillus Calmette-Guerin (BCG) scars. The tuberculin skin tests (TST) were positive in 6 patients with an induration ranging from 10-22 mm. Five cases had concomitant thoracic involvement (Table 1). Three patients had pleural effusion (Figure 1), one had parenchymal consolidation, and one (patient 4) had both of them. The most common abdominal ultrasonography (USG) findings were ascites with septation (found in 7 patients). Two had only ascites without septation (patients 1 and 6), one had hepatomegaly (patient 8) and two had intra-abdominal lymphadenopathies (patients 4 and 9) as well as ascites. In one patient (patient 1), peritoneal fluid could not be obtained. This patient had been previously operated on because of intestinal obstruction in another center, where they had seen multiple adhesions and whitish tuberculi on peritoneum. The patient was then referred to our center.

Ascitic fluid analysis was performed in 8 patients. All of the ascitic fluids were exudative, and the serum-ascite albumin gradient was less than 1.1 g/dL in all of them. Laboratory findings of serum and ascitic fluid are shown in Table 2. Direct examination of ascitic fluids revealed a predominance of lymphocytes. Acid fast bacilli (AFB) only found in one patient (patient 8). Positive cultures for M. tuberculosis were present in two patients. Three patients showed high adenosine deaminase activity (ADA)

Table 2 Laboratory findings of the patients at presentation

Parameter	Mean ± SD (range)
Hemoglobin (g/dL)	11.2 ± 1.3 (9.9-13)
White cell count (10^{12}/L)	6.4 ± 1.9 (4.1-9.0)
Erythrocyte sedimentation rate (mm/h)	42.3 ± 21.0 (10-72)
C-reactive protein (g/dL)	79.6 ± 63.7 (21-203)
Serum total protein (g/dL)	7.4 ± 0.7 (6.5-8.5)
Serum albumin (g/dL)	3.2 ± 0.5 (2.4-3.9)
Serum/ascites albumin gradient (g/dL)	0.6 ± 0.2 (0.3-1.0)
Ascites LDH (IU/L)	746.8 ± 327.1 (366-1331)
Ascites total protein (g/dL)	5.2 ± 0.6 (4.7-6.0)
Ascites albumin (g/dL)	2.6 ± 0.5 (1.9-3.4)
Ascites glucose (mg/dL)	71.7 ± 15.1 (49-89)
Ascites polymorphonuclear leukocyte (per mm^3)	1915 ± 1578 (520-5200)
Ascites lymphocyte (per mm^3)	3135 ± 3165 (602-9400)

LDH: Lactate dehydrogenase.

in their ascitic fluid. The clinical features of patients are shown in Table 1.

Laparoscopy was performed on 6 patients and laparotomy on one, and whitish tubercles were the most common findings in all. In three of the cases, adhesions were also seen. All biopsy samples obtained from 7 patients revealed caseating granuloma (Figure 2). The diagnosis of peritoneal tuberculosis was confirmed histopathologically in 7 patients, and two of these patients were also proved microbiologically. The remaining 2 patients had been diagnosed by ascitic fluid diagnostic features and a positive response to antituberculous treatment.

All patients completed the 9 mo course of therapy and recovered without any complications. Patients were followed for 6 to 15 mo after the end of therapy and all were in good health.

DISCUSSION

Tuberculous infection is still a significant cause of morbidity and mortality in the world. It is estimated that the incidence of TBP among all forms of TB varies from 0.1 % to 0.7 % worldwide and it is seen most commonly in patients between 35 and 45 years of age[4]. It is seen in children with a lower frequency. Recently, Forssbohm *et al*[6] reported that in Germany, only 5% of peritoneal TB cases were children under 14 years old. In the US, peritoneal TB accounts 0.3% of all TB cases in children less than 20 years and median age in children was 13 years[7]. Our review showed that TBP is also seen in lower frequency in our local area, the Black Sea region of Turkey. We have followed up only 9 children with TBP in a 5 year period. Mean age in this study was 14.2 years, in accordance with the literature. There was only one patient of 11 years old, the others were between 14-16 years. However, younger children with TBP have been reported in two studies from Turkey[8,9].

The initial symptoms of TBP cases are nonspecific, such as abdominal distention, pain, fever and weight loss and the occurrence of these events take a long time[5,8-10]. The clinical presentation of our patients was similar

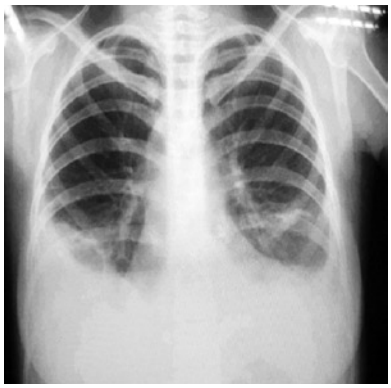

Figure 1 Chest roentgenogram showing pleural effusion on both sides (patient 5).

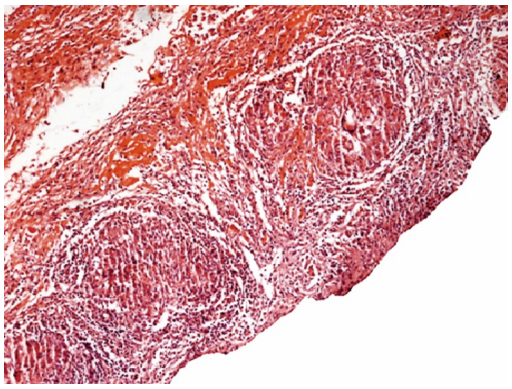

Figure 2 Histological appearance of caseating granuloma (patient 5, haematoxylin and eosin stain, original magnification x 10).

and ascites, causing abdominal distention was present in all cases. Constitutional symptoms of abdominal pain was seen in 55.5% of the patients, fever in 44.4%, and weight loss, night sweating and coughing were seen in 33.3% of the patients. One patient (patient 1) presented with small-bowel obstruction due to chronic peritoneal inflammation and adhesions. Intestinal obstruction developed in patients with TBP is also reported in the other studies[4,11-13].

Due to the nonspecific symptoms and physical findings, diagnosis is often delayed. The gold standard of diagnosis of TB in children is the triad of contact with a patient with active TB, positive TST and compatible physical and radiological findings. Clinicians must be aware of this serious health problem and inquire of familial history of TB. In our series, 66.7 % of patients have a contact history within the family and therefore we consider that family history is an important factor to take into account when making the diagnosis. In addition, TST is a helpful tool in diagnosis of TB and applied frequently. However, its diagnostic value in TBP can be variable. The frequency of TST positivity was higher (66.7%) in our study population than reported as 18%-27% in the other studies[8,14,15].

Chest radiographs are abnormal in 50-75% of patients with TBP[5]. It has been reported that 12%-63% of patients with TBP also had pleural effusion in different studies[8,16,17]. Similarly, in our study, 5 patients

(55.5%) had thoracic involvement, 4 of them had pleural effusion and 2 had consolidation. In most studies, direct examination of AFB and culture positivity of the peritoneal fluid are rarely seen[2,9,14,18]. In the present study, ARB were seen in only one case (patient 8) and culture of peritoneal fluid was positive in two cases (patients 2 and 5).

Analysis of ascitic fluid often shows exudative features with lymphocytic predominance and serum-ascite albumin gradient lesser than 1.1 gr/dL[18]. The ascite samples acquired from eight patients in our study had lymphocytic predominance and low serum-ascite albumin gradient, therefore we also suggest that the ascitic fluid features mentioned above might be a good indicator for diagnosis.

High levels of ADA in the ascitic fluid has been shown to be compatible with the diagnosis of TBP with high sensitivity (100%) and specificity (97%), but the analysis of ADA activity is expensive and may not be available everywhere[18-20]. We were able to assess ADA activity in ascitic fluid in only three patients and their values were high.

Bacillus can reach the peritoneum through the gastrointestinal tract *via* mesenteric lymph nodes or directly from the blood[5]. The most common form of disease is wet peritonitis, both visceral and parietal peritoneal layers are affected with the formation of multiple tuberculous nodules and ascites[21]. Abdominal USG is a non-invasive and easy available method of detecting abdominal fluid and lymphadenopathy. So it can be used for the diagnosis of TBP as a first step investigation. The most specific sonographic findings of TBP are ascites with fine septations and lymphadenopathy with hypoechogenic centers indicating caseating necrosis[18]. Abdominal USG of the present patients revealed ascites as the most common findings in all patients, also with fine septations in 7 of them, intra-abdominal LAP was seen in two patients, and hepatomegaly in one.

A thickened peritoneum, whitish tubercles and adhesions are the most common appearance of TBP in laparoscopy or laparotomy. Besides specific appearance, these procedures allow us to take a peritoneal biopsy, which is the gold standard for diagnosis[22]. In our series, 6 patients had undergone laparoscopy and one had a laparotomy and whitish tubercles on peritoneum were seen in all and adhesions in two. This pathognomonic appearance was proved by the observation of caseating granuloma on the histology. No complications were encountered in our patients.

Some authors suggest that corticosteroid administration combined with antituberculosis treatment can reduce the complications and morbidity rate, however, there is a controversy about the benefit[23-25]. In the present study, we added metilprednisolone to antituberculosis therapy in four patients, with positive results.

In conclusion, TB is still a major cause of mortality and morbidity worldwide. Although TBP is uncommon in childhood, it needs to be considered in all patients presenting with ascites, particularly when it is accompanied by fever and abdominal pain. Due to its high fatality rate, if not diagnosed in time, early diagnosis is very important. Laparoscopy and peritoneal biopsy are still the most reliable, quick and safe methods for the diagnosis of TBP.

COMMENTS

Background

Tuberculous peritonitis is an uncommon presentation of tuberculosis in children. Its diagnosis is more difficult in children because of nonspecificity of symptoms and difficulty in confirming the diagnosis.

Research frontiers

Tuberculous peritonitis is seen most commonly in adults and seen in children with a lower frequency. Due to the insidious nature of the manifestations, diagnosis is often delayed and the rate of complications and mortality increases. Chronic abdominal complaints, ascidic fluid investigations and laparoscopy are important in diagnosis.

Innovations and breakthroughs

Abdominal distention due to ascites was present in all of our cases and fever and abdominal pain were seen in almost half of them. In addition, serum-ascite albumin gradient less than 1.1 gr/dL, laparoscopic and histological findings were important in the diagnosis.

Applications

Although the clinical signs and symptoms suggest the disease, the laparoscopy and biopsy are still the most reliable methods for the definite diagnosis of tuberculous peritonitis and they are easy to apply.

Terminology

Low serum-ascite albumin gradient (< 1.1 gr/dL) is a better distinguishing marker for separating ascites related to non-portal hypertension origin from ascites with portal hypertension.

Peer review

Tuberculosis peritonitis (TP) even if it is rare in developed countries; it remains frequent in developing countries. This retrospective study includes 9 cases. Even if the number of cases is small, it is necessary to stay in touch with this disease since it still affects children. This study is well done even though is retrospective. The different observations are well documented. This study confirms that, regardless of enormous progress in imagery, laparoscopy is still the most reliable method for the diagnosis of TP.

REFERENCES

1 **Lazarus AA**, Thilagar B. Abdominal tuberculosis. *Dis Mon* 2007; **53**: 32-38

2 **Demir K**, Okten A, Kaymakoglu S, Dincer D, Besisik F, Cevikbas U, Ozdil S, Bostas G, Mungan Z, Cakaloglu Y. Tuberculous peritonitis--reports of 26 cases, detailing diagnostic and therapeutic problems. *Eur J Gastroenterol Hepatol* 2001; **13**: 581-585

3 **Sochocky S**. Tuberculous peritonitis. A review of 100 cases. *Am Rev Respir Dis* 1967; **95**: 398-401

4 **Sanai FM**, Bzeizi KI. Systematic review: tuberculous peritonitis--presenting features, diagnostic strategies and treatment. *Aliment Pharmacol Ther* 2005; **22**: 685-700

5 **Cruz AT**, Starke JR. Clinical manifestations of tuberculosis in children. *Paediatr Respir Rev* 2007; **8**: 107-117

6 **Forssbohm M**, Zwahlen M, Loddenkemper R, Rieder HL. Demographic characteristics of patients with extrapulmonary tuberculosis in Germany. *Eur Respir J* 2008; **31**: 99-105

7 **Starke JS**, Smith KC. Tuberculosis. In: Feigin RD, Cherry JD, Demmler GD, Kaplan SL, eds. Textbook of Pediatric Infectious Diseases. 5th ed. Philadelphia: Saunders, 2004: 1337-1379

8 **Tanrikulu AC**, Aldemir M, Gurkan F, Suner A, Dagli CE, Ece A. Clinical review of tuberculous peritonitis in 39 patients in Diyarbakir, Turkey. *J Gastroenterol Hepatol* 2005;

20: 906-909
9 **Gurkan F**, Ozates M, Bosnak M, Dikici B, Bosnak V, TasMA, Haspolat K. Tuberculous peritonitis in 11 children: clinical features and diagnostic approach. *Pediatr Int* 1999; **41**: 510-513
10 **Maltezou HC**, Spyridis P, Kafetzis DA. Extra-pulmonary tuberculosis in children. *Arch Dis Child* 2000; **83**: 342-346
11 **Ozbey H**, Tireli GA, Salman T. Abdominal tuberculosis in children. *Eur J Pediatr Surg* 2003; **13**: 116-119
12 **Akcakaya A**, Sahin M, Coskun A, Demiray S. Comparison of mechanical bowel obstruction cases of intra-abdominal tumor and non-tumoral origin. *World J Surg* 2006; **30**: 1295-1299
13 **Saczek KB**, Schaaf HS, Voss M, Cotton MF, Moore SW. Diagnostic dilemmas in abdominal tuberculosis in children. *Pediatr Surg Int* 2001; **17**: 111-115
14 **Muneef MA**, Memish Z, Mahmoud SA, Sadoon SA, Bannatyne R, Khan Y. Tuberculosis in the belly: a review of forty-six cases involving the gastrointestinal tract and peritoneum. *Scand J Gastroenterol* 2001; **36**: 528-532
15 **Sotoudehmanesh R**, Shirazian N, Asgari AA, Malekzadeh R. Tuberculous peritonitis in an endemic area. *Dig Liver Dis* 2003; **35**: 37-40
16 **Wang HK**, Hsueh PR, Hung CC, Chang SC, Luh KT, Hsieh WC. Tuberculous peritonitis: analysis of 35 cases. *J Microbiol Immunol Infect* 1998; **31**: 113-118
17 **Uygur-Bayramicli O**, Dabak G, Dabak R. A clinical dilemma: abdominal tuberculosis. *World J Gastroenterol* 2003; **9**: 1098-1101
18 **Rasheed S**, Zinicola R, Watson D, Bajwa A, McDonald PJ. Intra-abdominal and gastrointestinal tuberculosis. *Colorectal Dis* 2007; **9**: 773-783
19 **Riquelme A**, Calvo M, Salech F, Valderrama S, Pattillo A, Arellano M, Arrese M, Soza A, Viviani P, Letelier LM. Value of adenosine deaminase (ADA) in ascitic fluid for the diagnosis of tuberculous peritonitis: a meta-analysis. *J Clin Gastroenterol* 2006; **40**: 705-710
20 **Hillebrand DJ**, Runyon BA, Yasmineh WG, Rynders GP. Ascitic fluid adenosine deaminase insensitivity in detecting tuberculous peritonitis in the United States. *Hepatology* 1996; **24**: 1408-1412
21 **Aston NO**. Abdominal tuberculosis. *World J Surg* 1997; **21**: 492-499
22 **Bedioui H**, Ksantini R, Nouira K, Mekni A, Daghfous A, Chebbi F, Rebai W, Fteriche F, Jouini M, Kacem M, Ben Mami N, Filali A, Bensafta Z. Role of laparoscopic surgery in the etiologic diagnosis of exsudative ascites: a prospective study of 90 cases. *Gastroenterol Clin Biol* 2007; **31**: 1146-1149
23 **Alrajhi AA**, Halim MA, al-Hokail A, Alrabiah F, al-Omran K. Corticosteroid treatment of peritoneal tuberculosis. *Clin Infect Dis* 1998; **27**: 52-56
24 **Haas DW**. Is adjunctive corticosteroid therapy indicated during tuberculous peritonitis? *Clin Infect Dis* 1998; **27**: 57-58
25 **Bukharie H**. Paradoxical response to anti-tuberculous drugs: resolution with corticosteroid therapy. *Scand J Infect Dis* 2000; **32**: 96-97

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RAPID COMMUNICATION

Inhibition of hepatic interleukin-18 production by rosiglitazone in a rat model of nonalcoholic fatty liver disease

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Abstract

AIM: To investigate the effects of rosiglitazone (RGZ) on expression of interleukin-18 (IL-18) and caspase-1 in liver of non-alcoholic fatty liver disease (NAFLD) rats.

METHODS: Twenty-eight Sprague-Dawley (SD) rats were randomly divided into control, NAFLD, and RGZ treated NAFLD groups. A NAFLD rat model of NAFLD was established by feeding the animals with a high-fat diet for 12 wk. The NAFLD animals were treated with RGZ or vehicle for the last 4 wk (week 9-12) and then sacrificed to obtain liver tissues. Histological changes were analyzed with HE, oil red O and Masson's trichrome staining. Expressions of IL-18 and caspase-1 were detected using immunohistochemical staining and semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis.

RESULTS: The expression levels of both IL-18 and caspase-1 were higher in the liver of NAFLD group than in the control group. Steatosis, inflammation and fibrosis, found in the liver of NAFLD rats, were significantly improved 4 wk after RGZ treatment. The elevated hepatic IL-18 and caspase-1 expressions in NAFLD group were also significantly attenuated after RGZ treatment.

CONCLUSION: RGZ treatment can ameliorate increased hepatic IL-18 production and histological changes in liver of NAFLD rats. The beneficial effects of RGZ on NAFLD may be partly due to its inhibitory effect on hepatic IL-18 production.

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Key words: Insulin resistance; Interleukin-18; Non-alcoholic fatty liver; Rosiglitazone

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INTRODUCTION

Interleukin-18 (IL-18), previously called interferon-gamma (IFN-γ) inducing factor, is originally identified as a pro-inflammatory cytokine derived from Kupffer cells in animals with acute liver injury induced by endotoxin[1]. IL-18 is closely related to and acts synergistically with IL-12. However, its amino acid sequence and structure motifs resemble the IL-1 family. IL-18 expression has been demonstrated in a variety of cell types originated from both immune and non-immune systems, suggesting that IL-18 may have a wide range of cellular sources and functions apart from being a macrophage-derived inducer of IFN-γ production from type 1 T helper cells[2]. IL-18 is intracellularly synthesized as a non-functional precursor protein, pro-IL-18. Like pro-IL-1β, pro-IL-18 is then processed by caspase-1 into a bioactive mature form[3].

Non-alcoholic fatty liver disease (NAFLD), one of the most common causes of chronic liver diseases, represents a spectrum of liver disease extending from simple fatty liver through steatohepatitis to cirrhosis

in the absence of a history of significant alcohol use. NAFLD is considered one of the clinical features of metabolic syndrome in which insulin resistance plays a central role[4]. Several lines of evidence show that IL-18 may be important in the pathogenesis of inflammatory processes, which contribute to the development of insulin resistance. It has been shown that elevated serum IL-18 levels are associated with insulin resistance in obese subjects, women with polycystic ovary syndrome, and patients with type 2 diabetes mellitus[5-7]. Furthermore, hepatic IL-18 level is elevated in insulin resistance-related obese mice with NAFLD[8].

Rosiglitazone (RGZ), a selective ligand of peroxisome proliferator-activated receptor gamma (PPAR-γ), is an insulin sensitizer that has been used in a number of insulin-resistant conditions, including NAFLD. Several clinical studies showed that RGZ could improve liver enzyme levels and histological changes in NAFLD patients by increasing insulin sensitivity[9-11]. However, whether the beneficial effect of RGZ on NAFLD is associated with reduced IL-18 expression in the liver remains unclear. This study analyzed the expression of IL-18 and caspase-1 in the liver of NAFLD rats, and investigated the effects of RGZ on hepatic IL-18 production and liver histology.

MATERIALS AND METHODS

Animal and experimental protocol

Twenty-eight male Sprague-Dawley (SD) rats, weighing 140-160 g, were housed in individual cages at 22℃ with free access to food (standard chow diet) and water for 1 wk before initiation of the experiment. The study protocol was approved by the Animal Care and Use Committee of Peking University Health Science Center.

The rats were randomly divided into control group (n = 6), NAFLD group (n = 11), and RGZ-treated NAFLD group (n = 11). Rats in the control group were maintained on the standard chow diet for 12 wk. A rat model of NAFLD was induced by a high-fat diet (standard chow diet + 10% lard + 2% cholesterol) for 8 wk as previously described[12]. Subsequently, rats in the RGZ-treated NAFLD group were treated with RGZ maleate (Avandia®, 4 mg/kg per day) *via* gavage, whereas rats in the NAFLD group were given normal saline for another 4 wk. At the end of study, all rats were sacrificed after 12 h of fasting. Blood samples were collected for biochemical assays. The liver was removed and weighted after rinsed with ice-cold saline, and sampled for histological study and RNA extraction.

Biochemical analyses

Serum insulin concentrations were determined with a radioimmunoassay kit (Beijing Atom HighTech Co., Ltd., Beijing, China). Serum leptin and adiponectin levels were measured with an ELISA kit (Invitrogen, Carlsbad, CA, USA). Free fatty acid (FFA) concentrations were analyzed using a commercially available kit (Randox, Antrim, UK). Additional blood biochemical parameters, including glucose, triglycerides, total cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), were assayed using an automatic biochemical analyzer. Homeostasis model assessment (HOMA) was employed to estimate the insulin resistance index (HOMA-IR), which reflects both peripheral and hepatic insulin resistance[13]. Liver weight index (%) was calculated as liver weight/body weight × 100.

Histological studies

The sections of liver tissues from the center of the largest hepatic lobes were fixed in 10% buffered formaldehyde, and then embedded in paraffin. A 5 μm-thick section cut from a paraffin-embedded block was stained with HE and Masson's trichrome. To visualize the fat droplet accumulation, frozen liver tissue sections were stained with oil red O. Steatosis, necro-inflammatory grade and stage of fibrosis were assessed as previously described[14]. Liver histology was evaluated blindly.

Immunohistochemical staining

Goat IL-18 polyclonal antibody was obtained from Santa Cruz (Santa Cruz, CA, USA). Rabbit caspase-1 polyclonal antibody was purchased from Lab Vision (Fremont, CA, USA). Immunohistochemical staining of 5 μm-thick paraffin-embedded liver tissue sections was performed according to its manufacturer's protocol (Vectastain Elite ABC kit; Vector, Burlingame, CA, USA). Briefly, liver sections were deparaffinized in xylene and rehydrated in graded ethanol. After endogenous peroxidase and biotin were blocked, the tissues were pre-incubated with 3% horse serum for 30 min to prevent non-specific reactions. The sections were then incubated with primary antibodies diluted at 1:150 for 60 min. On negative control sections, the step with primary antibodies was omitted. Polyclonal antibodies were detected using a biotinylated anti-goat or rabbit IgG diluted at 1:200 in 5% bovine serum albumin for 30 min. The sections were incubated with R.T.U. Vectastain Elite ABC reagent for 30 min, stained with diaminobenzidine for 5 min and counterstained with hematoxylin before they were mounted.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the liver tissues using a TRIzol reagent (Gibco, Carlsbad, CA, USA) according to its manufacturer's instructions. cDNA was prepared with the SuperScription First-Strand Synthesis System (Invitrogen) as previously described[15] and amplified by polymerase chain reaction (PCR). The sequences of primers used for PCR are 5'-GGCTCTTGTGTCAACT TCAAA-3' and 5'-TTATCAGTCTGGTCTGGGATT-3' (232 bp) for *IL-18*, 5'-TCCTGAGGGCAAAGAGGAA GC-3' and 5'-GGCAAGACGTGTACGAGTGGGT-3' (479 bp) for *caspase-1*, 5'-GCTCGTCGTCGACAACG GCTC-3' and 5'-CAAACATGATCTGGGTCATCT TCTC-3' (353 bp) for *β-acti*, respectively. Each semi-quantitative RT-PCR analysis was performed with a set

of *IL-18* or caspase-1 primers in combination with a set of primers for house-keeping gene *β-actin* as an internal standard. The conditions of PCR were as follows: 1 cycle at 94℃ for 5 min; 30 cycles at 94℃ for 30 s, at 60℃ for 30 s, at 72℃ for 45 s; 1 cycle at 72℃ for 5 min. The PCR products were separated on a 2% agarose gel which was dried and then scanned using an ultraviolet gel imaging system (BioRad, Hercules, CA, USA). Gene expression levels were represented as ratios of target gene to co-amplified internal standard.

Statistical analysis

Data are expressed as mean ± SE. Analysis of variance was used to compare the means of three groups, followed by Newman-Keuls test to determine the statistical significance between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of RGZ on hepatic histology

The liver tissues from all groups were stained with HE and analyzed (Figure 1A-C). In contrast to the control group, typical steatosis and portal and lobular inflammation were observed in the NAFLD group after 12 wk of feeding with a high-fat diet (Figure 1A and B). In the NAFLD group, steatosis was observed in about 65% of hepatocytes (mean score: 3 *vs* 0 in the control group). Moderate infiltration of mononuclear and polymorphonuclear cells was also found (mean score: 2 *vs* 0). Fat droplet accumulation and fibrosis (mean score: 1 *vs* 0) were observed in sections stained with oil red O (Figure 1D-F) and Masson's trichrome (Figure 1G-I) in the NAFLD group (Figure 1E and H), but not in the control group (Figure 1D and G). These findings suggest that the animal model of NAFLD was successfully established. Furthermore, markedly attenuated steatosis (mean score: 0.6), inflammation (mean score: 0.5) and fibrosis (mean score: 0.2) were observed 4 wk after treatment with RGZ (Figure 1C and I).

Effects of RGZ on hepatic metrology and biochemistry

Increased liver weight and liver weight index observed in the NAFLD group were significantly improved after RGZ treatment (Table 1). The hepatic surface was smooth, red-brown, and soft in the control group, whereas the liver in the NAFLD group was enlarged in dimmer color with a moderate texture. The liver feature of the RGZ-treated NAFLD group was in between the above two groups (data not shown). Moreover, serum ALT, AST and ALP levels were significantly elevated in the NAFLD group and significantly reduced in the RGZ-treated NAFLD group compared with the untreated NAFLD group (Table 1).

Effects of RGZ on insulin resistance and metabolic parameters

Increased serum insulin, leptin, FFA, and HOMA-IR levels were observed in the NAFLD group, which were attenuated in the RGZ-treated NAFLD group. In contrast, decreased serum adiponectin levels were found in the NAFLD group, which were ameliorated in the RGZ-treated NAFLD group. Fasting blood glucose concentration was significantly elevated in the NAFLD group and decreased in the RGZ-treated NAFLD group. The levels of serum triglycerides were significantly higher in the NAFLD group, and reduced after RGZ treatment. Serum cholesterol levels were significantly higher in the NAFLD group, and tended to become lower in the RGZ-treated NAFLD group although the difference was not statistically significant (Table 1).

Table 1 Effects of RGZ on blood biochemistry and hepatic parameters (mean ± SE)

Parameters	Control (n = 6)	NAFLD (n = 11)	RGZ-treated NAFLD (n = 11)
Glucose (mmol/L)	6.48 ± 0.32	7.31 ± 0.20[a]	6.26 ± 0.16[c]
Insulin (mU/L)	20.41 ± 1.85	27.03 ± 1.48[a]	21.07 ± 1.19[c]
HOMA-IR	5.90 ± 0.58	8.93 ± 0.48[a]	5.95 ± 0.41[c]
Leptin (μg/L)	2.63 ± 0.13	4.21 ± 0.09[a]	3.75 ± 0.05[a,c]
Adiponectin (mg/L)	2.22 ± 0.05	1.64 ± 0.07[a]	1.91 ± 0.10[a,c]
Free fatty acid (μmol/L)	361.0 ± 42.6	539.1 ± 32.1[a]	401.0 ± 32.9[c]
Triglycerides (mmol/L)	0.51 ± 0.04	1.21 ± 0.04[a]	0.82 ± 0.06[a,c]
Cholesterol (mmol/L)	1.45 ± 0.14	2.69 ± 0.11[a]	2.38 ± 0.11[a]
Body weight (g)	563.2 ± 9.6	581.6 ± 9.7	571.4 ± 9.1
Liver weight (g)	13.90 ± 0.53	26.51 ± 1.96[a]	15.86 ± 1.06[a,c]
Liver weight index (%)	2.48 ± 0.05	4.51 ± 0.17[a]	3.00 ± 0.16[c]
Alanine aminotransferase (U/L)	57.2 ± 5.8	100.9 ± 7.2[a]	53.6 ± 5.8[c]
Aspartate aminotransferase (U/L)	164.4 ± 11.8	197.5 ± 9.1[a]	151.2 ± 9.5[c]
Alkaline phosphatase (U/L)	84.8 ± 6.1	174.6 ± 11.6[a]	87.3 ± 4.9[c]

[a]$P < 0.05$ *vs* control group, [c]$P < 0.05$ *vs* NAFLD group. HOMA-IR: Homeostasis model assessment-insulin resistance index.

Effects of RGZ on IL-18 and caspase-1 expression in liver tissues

Immunohistochemical staining was used to analyze the expression of IL-18 (Figure 1J-L) and caspase-1 (Figure 1M-O) proteins in the liver tissues. Positive expression of either IL-18 or caspase-1 was rarely detected in liver tissues from the control group with very week staining in some Kupffer cells (Figure 1J and M). However, the NAFLD group exhibited a strong expression of both IL-18 and caspase-1 in the liver lobules. Hepatocytes and/or infiltrating inflammatory cells within the lobules were the major cell types expressing IL-18 and caspase-1 (Figure 1K and N). Compared to the untreated NAFLD group, the expression of hepatic IL-18 and caspase-1 was significantly inhibited 4 wk after treatment with RGZ (Figure 1L and O).

RT-PCR analysis further showed that constitutive *IL-18* or *caspase-1* mRNA expression was found in liver tissues from the control group. The mRNA levels of *IL-18* and *caspase-1* were significantly higher in liver tissues from the NAFLD group than in those from the control group. The mRNA expression of *IL-18* and *caspase-1* was significantly reduced in the RGZ-treated

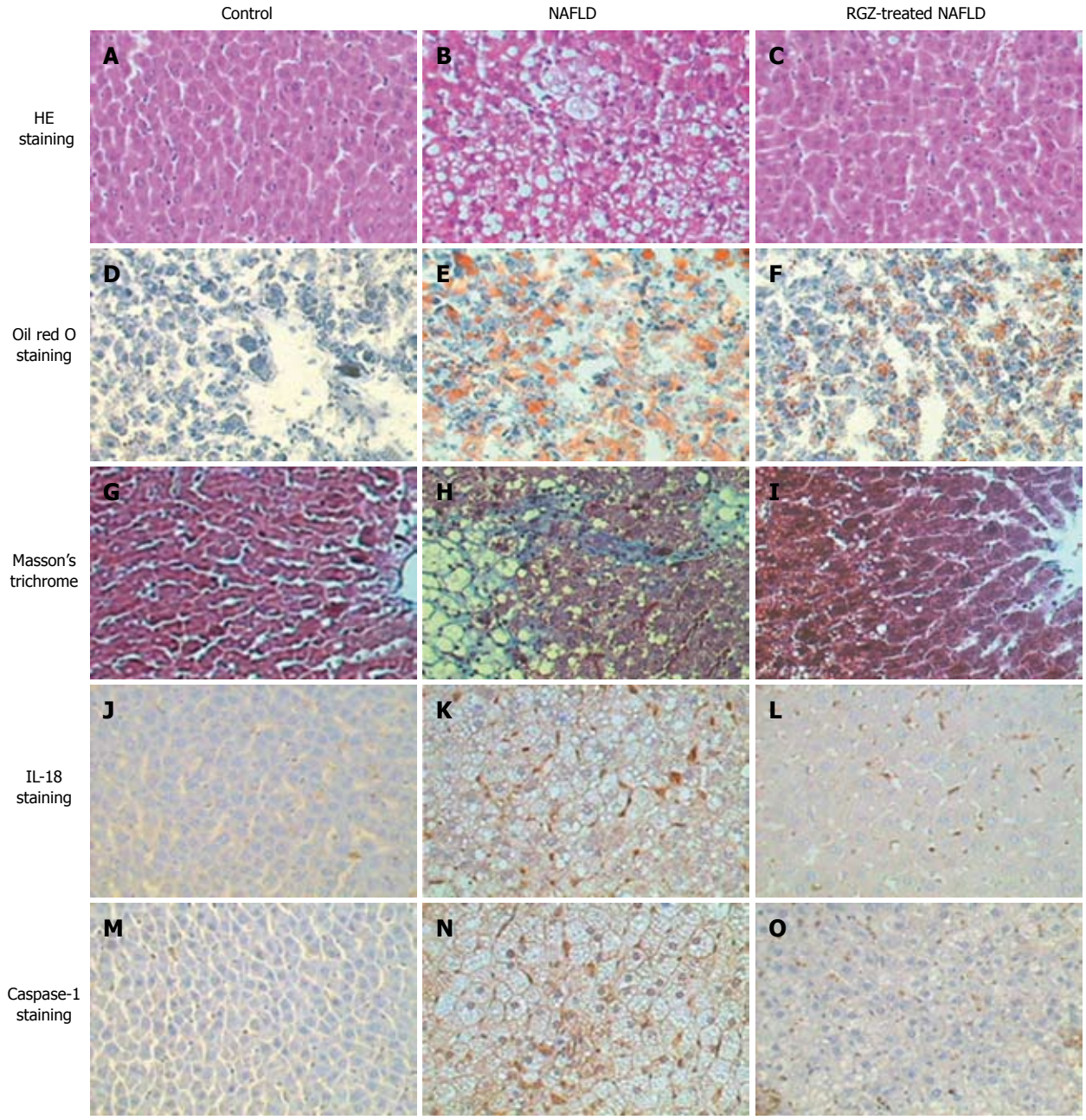

Figure 1 Histological studies of rat livers in normal control, NAFLD and RGZ-treated NAFLD groups (x 400). Liver tissue sections were stained with HE, A: Normal control group; B: NAFLD group; C: RGZ-treated NAFLD group. Liver tissue sections were stained with oil red O, D: normal control group; E: NAFLD group; F: RGZ-treated NAFLD group. Liver tissue sections were stained with Masson's trichrome, G: Normal control group; H: NAFLD group; I: RGZ-treated NAFLD group. Liver tissue sections were stained with immunohistochemistry for IL-18, J: Normal control group; K: NAFLD group; L: RGZ-treated NAFLD group. Liver tissue sections were stained with immunohistochemistry for caspase-1, M: Normal control group; N: NAFLD group; O: RGZ-treated NAFLD group. Histological changes in fatty liver disease and IL-18- or caspase-1-positive staining cells were rarely detectable in the control group. NAFLD rat liver showed steatosis and moderate inflammatory changes, fat droplet accumulation, mild fibrosis, strong IL-18- and caspase-1-positive staining. A significant improvement of steatosis, inflammation, fibrosis, and IL-18 and caspase-1 staining was observed in liver of the RGZ-treated NAFLD group.

NAFLD group compared to the NAFLD group, but remained higher in the RGZ-treated NAFLD group than in the control group (Figure 2).

DISCUSSION

Insulin resistance is closely associated with NAFLD, typically known as a part of the metabolic syndrome, and has been implicated as a contributing factor for the pathogenesis of NAFLD[4,16-18]. Treatment with RGZ, an oral anti-diabetic agent of the thiazolidinediones, leads to the improvement in insulin resistance with ameliorated histological and biochemical changes of liver injury in diabetic and non-diabetic patients with NAFLD[9-11]. Pioglitazone[19], another thiazolidinedione insulin-sensitizer, and metformin[20] exert similar effects in non-diabetic patients with NAFLD. These findings suggest that insulin resistance contributes to the development of NAFLD and that insulin sensitizers may represent important agents for the treatment of NAFLD. Furthermore, the beneficial effects of insulin-sensitizing agents, RGZ[21,22], pioglitazone[12] and

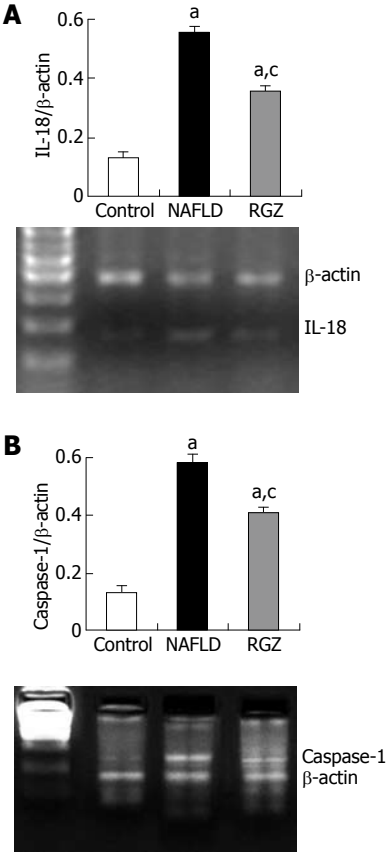

Figure 2 RT-PCR analysis of mRNA expression of *IL-18* (A) and caspase-1 (B) in liver tissues of the three groups. The histograms show the ratio of target gene expression to *β-actin*. Data are presented as mean ± SE from six independent experiments in the control group and eleven in the NAFLD and RGZ-treated NAFLD groups. $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* NAFLD group.

metformin[21], on hepatic steatosis and inflammation have been confirmed in various animal models of NAFLD. Compared to metformin, RGZ appears to be a better drug for improving hepatic steatosis[21]. In line with the above reports[12,21,22], our study showed that increased liver weight and liver weight index, elevated serum liver enzyme levels and altered liver histological conditions including steatosis, inflammation and fibrosis were observed in the NAFLD rats, which were significantly improved 4 wk after RGZ treatment. In agreement with previous reports[12,21], the present study also showed that the levels of serum insulin, leptin, FFA and triglycerides as well as HOMA-IR were significantly increased, whereas the levels of serum adiponectin remarkably decreased in the NAFLD group. Four weeks after RGZ treatment, these abnormalities were significantly improved, suggesting that insulin resistance may play an important role in the pathogenesis of NAFLD.

Overnutrition-induced chronic inflammation is a key component in the pathogenesis of insulin resistance and metabolic syndrome. Pro-inflammatory cytokines can cause insulin resistance in adipose tissue, skeletal muscle and liver by inhibiting the insulin signal transduction[23]. A role of IL-18 has been recently postulated in the development of insulin resistance based on the observation that elevated serum IL-18 levels are associated with insulin resistance and hypoadiponectinemia[5-7,24]. In obese women, circulating levels of IL-18 are increased and positively associated with body weight and visceral fat, which can be ameliorated by caloric restriction-induced weight loss over 1 year[25]. A recent report showed that plasma IL-18 is associated with changes in insulin resistance and reduced after weight loss with a 15-wk life-style intervention. In addition, the expression of IL-18 in adipose tissue is increased in obese subjects but not affected by weight loss[26], indicating that changes in plasma IL-18 are related to insulin resistance rather than to obesity. Notably, the fact that increased serum IL-18 levels are associated with increased serum liver enzyme concentrations suggests that IL-18 might contribute to the development of liver disease associated with insulin resistance[27,28]. The role of IL-18 in the development of insulin resistance-related NAFLD is further supported by a report showing that both serum IL-18 concentration and hepatic *IL-18* mRNA expression are elevated in lipopolysaccharide-treated *ob/ob* mice, an animal model of fatty liver disease[8]. Moreover, IL-18 may play an important role in liver injury caused by hepatitis B virus infection[29], hepatic ischemia/reperfusion[30], and endotoxin exposure[31] since the liver injury can be reversed by blockage of IL-18 *via* either gene knockout[31] or neutralizing antibody[30]. Importantly, exogenous administration of IL-18 with IL-12 to BALB/c mice induces fatty liver in an IFN-γ and nitric oxide dependent manner[32], suggesting that IL-18 plays a pivotal role in the inflammatory cascade leading to NAFLD associated with insulin resistance.

In the present study, the expression of IL-18 and caspase-1 was extremely low in liver tissues from the control animals. However, the expression of IL-18 and caspase-1 was significantly increased in liver lobules from NAFLD rats. Hepatocytes and inflammatory cells within the lobules are the major cell types expressing IL-18 and caspase-1. IL-18 is synthesized as a precursor molecule without biological activity and requires caspase-1 for cleavage into a mature peptide and subsequent release[3]. Increased expression of caspase-1 in hepatocytes and infiltrating inflammatory cells indicates that both cell types may produce and secrete mature IL-18. This finding is generally consistent with a previous report showing that IL-18 production can be originated from Kupffer cells as well as injured hepatocytes[29]. As noted above, RGZ improves the histological and biochemical changes in NAFLD along with the improvement in insulin resistance[9-11]. In addition, increased circulating levels of IL-18 are significantly decreased after RGZ therapy for patients with metabolic syndrome[33] or type 2 diabetes mellitus[34]. The present study showed that increased expression of IL-18 and caspase-1 in livers of NAFLD rats was reduced 4 wk after treatment with RGZ. Therefore, it is conceivable that hepatic IL-18 production may have a critical role in the development of NAFLD and that the beneficial effects of RGZ on NAFLD may be mediated by inhibiting IL-18 expression possibly *via* PPAR-γ activation[35].

The mechanism by which hepatic IL-18 production

contributes to the development of NAFLD remains to be elucidated. IL-18 is a pro-inflammatory cytokine with multiple functions including stimulation of IFN-γ production[1-3], enhancement of IL-1β, and IL-8 production *via* direct stimulation of tumor necrosis factor-α (TNF-α) production[36]. A previous study showed that intraperitoneal administration of IL-18 with IL-12 induces mouse fatty liver in an IFN-γ dependent manner[32]. Macrophage-derived TNF-α contributes to insulin resistance and development of hepatic steatosis in diet-induced obesity[37]. It was also reported that either IFN-γ or TNF-α up-regulates gene expression or posttranslational processing of *IL-18* in several tissue cell types[15,38,39], indicating that IL-18 may contribute to the development of insulin resistance-related NAFLD *via* a complex cytokine network of IL-18 and many other cytokines such as TNF-α, IFN-γ and IL-1β.

In summary, increased hepatic IL-18 production along with liver histological and biochemical changes can be ameliorated after RGZ treatment. The beneficial effects of RGZ on NAFLD may be due to its direct anti-inflammatory properties, possibly *via* PPAR-γ activation, or secondary to improved insulin resistance.

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COMMENTS

Background

Interleukin-18 (IL-18), originally identified as a pro-inflammatory cytokine in endotoxin-induced liver injury, is an important mediator of innate and adaptive immunity. Recently, IL-18 has been reported to be associated with insulin resistance-related non-alcoholic fatty liver disease (NAFLD).

Research frontiers

Treatment with thiazolidinedione (TZD) leads to improvement in insulin resistance and ameliorates histological and biochemical changes of liver injury in patients with NAFLD. However, whether the beneficial effect of TZD on NAFLD is associated with reduced IL-18 expression in the liver remains unknown.

Innovations and breakthroughs

The data show that increased hepatic IL-18 and caspase-1 expression along with liver histological and biochemical changes can be ameliorated after rosiglitazone (RGZ) treatment in an animal model of NAFLD. Therefore, the beneficial effects of RGZ on NAFLD may be due to its direct anti-inflammatory properties or secondary to improved insulin resistance, *via* peroxisome proliferator-activated receptor gamma (PPAR-γ) activation.

Applications

IL-18 plays a pivotal role in the inflammatory cascade leading to NAFLD associated with insulin resistance. Therefore, blockage of hepatic IL-18 action may be a novel target for treatment of NAFLD.

Terminology

NAFLD represents a spectrum of liver disease extending from simple fatty liver through steatohepatitis to cirrhosis in the absence of a history of significant alcohol use. Low-grade inflammation, characterized by abnormal cytokine production, increased synthesis of acute-phase reactants and activation of inflammatory signaling pathways, is a key component in the pathogenesis of insulin resistance, obesity and steatohepatitis.

Peer review

This is an interesting paper. The authors showed that RGZ treatment can improve the increased hepatic IL-18 expression along with liver histological changes in a rat model of NAFLD.

REFERENCES

1 **Okamura H**, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, Torigoe K, Okura T, Nukada Y, Hattori K. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 1995; **378**: 88-91
2 **Boraschi D**, Dinarello CA. IL-18 in autoimmunity: review. *Eur Cytokine Netw* 2006; **17**: 224-252
3 **Ghayur T**, Banerjee S, Hugunin M, Butler D, Herzog L, Carter A, Quintal L, Sekut L, Talanian R, Paskind M, Wong W, Kamen R, Tracey D, Allen H. Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. *Nature* 1997; **386**: 619-623
4 **Jiang J**, Torok N. Nonalcoholic steatohepatitis and the metabolic syndrome. *Metab Syndr Relat Disord* 2008; **6**: 1-7
5 **Escobar-Morreale HF**, Botella-Carretero JI, Villuendas G, Sancho J, San Millan JL. Serum interleukin-18 concentrations are increased in the polycystic ovary syndrome: relationship to insulin resistance and to obesity. *J Clin Endocrinol Metab* 2004; **89**: 806-811
6 **Fischer CP**, Perstrup LB, Berntsen A, Eskildsen P, Pedersen BK. Elevated plasma interleukin-18 is a marker of insulin-resistance in type 2 diabetic and non-diabetic humans. *Clin Immunol* 2005; **117**: 152-160
7 **Zhang YF**, Yang YS, Hong J, Gu WQ, Shen CF, Xu M, Du PF, Li XY, Ning G. Elevated serum levels of interleukin-18 are associated with insulin resistance in women with polycystic ovary syndrome. *Endocrine* 2006; **29**: 419-423
8 **Guebre-Xabier M**, Yang S, Lin HZ, Schwenk R, Krzych U, Diehl AM. Altered hepatic lymphocyte subpopulations in obesity-related murine fatty livers: potential mechanism for sensitization to liver damage. *Hepatology* 2000; **31**: 633-640
9 **Neuschwander-Tetri BA**, Brunt EM, Wehmeier KR, Oliver D, Bacon BR. Improved nonalcoholic steatohepatitis after 48 weeks of treatment with the PPAR-gamma ligand rosiglitazone. *Hepatology* 2003; **38**: 1008-1017
10 **Wang CH**, Leung CH, Liu SC, Chung CH. Safety and effectiveness of rosiglitazone in type 2 diabetes patients with nonalcoholic Fatty liver disease. *J Formos Med Assoc* 2006; **105**: 743-752
11 **Caldwell SH**, Patrie JT, Brunt EM, Redick JA, Davis CA, Park SH, Neuschwander-Tetri BA. The effects of 48 weeks of rosiglitazone on hepatocyte mitochondria in human nonalcoholic steatohepatitis. *Hepatology* 2007; **46**: 1101-1107
12 **Xu P**, Zhang XG, Li YM, Yu CH, Xu L, Xu GY. Research on the protection effect of pioglitazone for non-alcoholic fatty liver disease (NAFLD) in rats. *J Zhejiang Univ Sci B* 2006; **7**: 627-633
13 **Bonora E**, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, Muggeo M. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* 2000; **23**: 57-63
14 **Brunt EM**, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467-2474
15 **Hong TP**, Andersen NA, Nielsen K, Karlsen AE, Fantuzzi G, Eizirik DL, Dinarello CA, Mandrup-Poulsen T. Interleukin-18 mRNA, but not interleukin-18 receptor mRNA, is constitutively expressed in islet beta-cells and up-regulated by interferon-gamma. *Eur Cytokine Netw* 2000; **11**: 193-205
16 **Hsiao PJ**, Kuo KK, Shin SJ, Yang YH, Lin WY, Yang JF, Chiu CC, Chuang WL, Tsai TR, Yu ML. Significant correlations between severe fatty liver and risk factors for metabolic syndrome. *J Gastroenterol Hepatol* 2007; **22**: 2118-2123
17 **Zelber-Sagi S**, Nitzan-Kaluski D, Halpern Z, Oren R. Prevalence of primary non-alcoholic fatty liver disease in a population-based study and its association with biochemical

and anthropometric measures. *Liver Int* 2006; **26**: 856-863
18 **Sanyal AJ**, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183-1192
19 **Promrat K**, Lutchman G, Uwaifo GI, Freedman RJ, Soza A, Heller T, Doo E, Ghany M, Premkumar A, Park Y, Liang TJ, Yanovski JA, Kleiner DE, Hoofnagle JH. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. *Hepatology* 2004; **39**: 188-196
20 **Marchesini G**, Brizi M, Bianchi G, Tomassetti S, Zoli M, Melchionda N. Metformin in non-alcoholic steatohepatitis. *Lancet* 2001; **358**: 893-894
21 **Assy N**, Grozovski M, Bersudsky I, Szvalb S, Hussein O. Effect of insulin-sensitizing agents in combination with ezetimibe, and valsartan in rats with non-alcoholic fatty liver disease. *World J Gastroenterol* 2006; **12**: 4369-4376
22 **Seo YS**, Kim JH, Jo NY, Choi KM, Baik SH, Park JJ, Kim JS, Byun KS, Bak YT, Lee CH, Kim A, Yeon JE. PPAR agonists treatment is effective in a nonalcoholic fatty liver disease animal model by modulating fatty-acid metabolic enzymes. *J Gastroenterol Hepatol* 2008; **23**: 102-109
23 **de Luca C**, Olefsky JM. Inflammation and insulin resistance. *FEBS Lett* 2008; **582**: 97-105
24 **Straczkowski M**, Kowalska I, Nikolajuk A, Otziomek E, Adamska A, Karolczuk-Zarachowicz M, Gorska M. Increased serum interleukin-18 concentration is associated with hypoadiponectinemia in obesity, independently of insulin resistance. *Int J Obes* (Lond) 2007; **31**: 221-225
25 **Esposito K**, Pontillo A, Ciotola M, Di Palo C, Grella E, Nicoletti G, Giugliano D. Weight loss reduces interleukin-18 levels in obese women. *J Clin Endocrinol Metab* 2002; **87**: 3864-3866
26 **Bruun JM**, Stallknecht B, Helge JW, Richelsen B. Interleukin-18 in plasma and adipose tissue: effects of obesity, insulin resistance, and weight loss. *Eur J Endocrinol* 2007; **157**: 465-471
27 **Lopez-Bermejo A**, Bosch M, Recasens M, Biarnes J, Esteve E, Casamitjana R, Vendrell J, Ricart W, Fernandez-Real JM. Potential role of interleukin-18 in liver disease associated with insulin resistance. *Obes Res* 2005; **13**: 1925-1931
28 **Vecchiet J**, Falasca K, Cacciatore P, Zingariello P, Dalessandro M, Marinopiccoli M, D'Amico E, Palazzi C, Petrarca C, Conti P, Pizzigallo E, Guagnano MT. Association between plasma interleukin-18 levels and liver injury in chronic hepatitis C virus infection and non-alcoholic fatty liver disease. *Ann Clin Lab Sci* 2005; **35**: 415-422
29 **Lee MO**, Choi YH, Shin EC, Kang HJ, Kim YM, Jeong SY, Seong JK, Yu DY, Cho H, Park JH, Kim SJ. Hepatitis B virus X protein induced expression of interleukin 18 (IL-18): a potential mechanism for liver injury caused by hepatitis B virus (HBV) infection. *J Hepatol* 2002; **37**: 380-386
30 **Takeuchi D**, Yoshidome H, Kato A, Ito H, Kimura F, Shimizu H, Ohtsuka M, Morita Y, Miyazaki M. Interleukin 18 causes hepatic ischemia/reperfusion injury by suppressing anti-inflammatory cytokine expression in mice. *Hepatology* 2004; **39**: 699-710
31 **Sakao Y**, Takeda K, Tsutsui H, Kaisho T, Nomura F, Okamura H, Nakanishi K, Akira S. IL-18-deficient mice are resistant to endotoxin-induced liver injury but highly susceptible to endotoxin shock. *Int Immunol* 1999; **11**: 471-480
32 **Chikano S**, Sawada K, Shimoyama T, Kashiwamura SI, Sugihara A, Sekikawa K, Terada N, Nakanishi K, Okamura H. IL-18 and IL-12 induce intestinal inflammation and fatty liver in mice in an IFN-gamma dependent manner. *Gut* 2000; **47**: 779-786
33 **Esposito K**, Ciotola M, Carleo D, Schisano B, Saccomanno F, Sasso FC, Cozzolino D, Assaloni R, Merante D, Ceriello A, Giugliano D. Effect of rosiglitazone on endothelial function and inflammatory markers in patients with the metabolic syndrome. *Diabetes Care* 2006; **29**: 1071-1076
34 **Kim HJ**, Kang ES, Kim DJ, Kim SH, Ahn CW, Cha BS, Nam M, Chung CH, Lee KW, Nam CM, Lee HC. Effects of rosiglitazone and metformin on inflammatory markers and adipokines: decrease in interleukin-18 is an independent factor for the improvement of homeostasis model assessment-beta in type 2 diabetes mellitus. *Clin Endocrinol* (Oxf) 2007; **66**: 282-289
35 **Fortunato F**, Berger I, Gross ML, Rieger P, Buechler MW, Werner J. Immune-compromised state in the rat pancreas after chronic alcohol exposure: the role of peroxisome proliferator-activated receptor gamma. *J Pathol* 2007; **213**: 441-452
36 **Puren AJ**, Fantuzzi G, Gu Y, Su MS, Dinarello CA. Interleukin-18 (IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14+ human blood mononuclear cells. *J Clin Invest* 1998; **101**: 711-721
37 **De Taeye BM**, Novitskaya T, McGuinness OP, Gleaves L, Medda M, Covington JW, Vaughan DE. Macrophage TNF-alpha contributes to insulin resistance and hepatic steatosis in diet-induced obesity. *Am J Physiol Endocrinol Metab* 2007; **293**: E713-E725
38 **Striz I**, Krasna E, Honsova E, Lacha J, Petrickova K, Jaresova M, Lodererova A, Bohmova R, Valhova S, Slavcev A, Vitko S. Interleukin 18 (IL-18) upregulation in acute rejection of kidney allograft. *Immunol Lett* 2005; **99**: 30-35
39 **Chandrasekar B**, Colston JT, de la Rosa SD, Rao PP, Freeman GL. TNF-alpha and H2O2 induce IL-18 and IL-18R beta expression in cardiomyocytes via NF-kappa B activation. *Biochem Biophys Res Commun* 2003; **303**: 1152-1158

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RAPID COMMUNICATION

Liver resection for benign hepatic lesions: A retrospective analysis of 827 consecutive cases

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Abstract

AIM: To analyze the operative and perioperative factors associated with hepatectomy of benign hepatic lesions.

METHODS: A total of 827 condecutive cases of benign hepatic lesion undergoing hepatectomy from January 1986 to December 2005 in the Chinese PLA General Hospital were investigated retrospectively according to their medical documentation.

RESULTS: The effect of operative and perioperative factors on the outcome of patients were analyzed. Of the 827 cases undergoing hepatectomy for more than 3 liver segments accounted for 22.1%, 316 (38.21%) required transfusion of blood products during operation. The average operating time was 220.59 ± 109.13 min, the average hospital stay after operation was 13.55 ± 9.38 d. Child-Pugh A accounted for 98.13%. The postoperative complication rate was 13.54% and the in-hospital mortality rate was 0.24%. Multivariate analysis showed that operating time (P = 0.004, OR = 1.003) and albumin value (P = 0.040, OR = 0.938) were the independent predictors of morbidity and indicated that operating time, blood transfusion, complication rate, and LOS had a trend to decrease.

CONCLUSION: Hepatectomy for benign hepatic lesions can be performed safely with a low morbidity and mortality, provided that it is carried out with optimized perioperative management and an innovative surgical technique.

INTRODUCTION

Hepatectomy is a dangerous and complex operation[1-6]. The choice of surgical treatment for benign hepatic lesion is controversial. Erdogan *et al*[7] reported that the indications for surgical resection of liver hemangioma are progressive abdominal pain and > 5 cm in diameter, which have been justified in patients with minimal or no symptoms, even in patients with giant hemangiomas. Yoon *et al*[8] performed 52 operations for 115 hepatic hemangiomas, enucleation was performed for 31 (60%) of the 52 patients who underwent surgical resection. Postoperative complications were found in 13 patients (25%) with no peri-operative death occurred. Of the 52 patients with symptoms before resection, 96% had resolution of their symptoms after operation. Chaib *et al*[9] suggested that hepatic adenoma should be removed in all cases, especially in female patients on oral contraceptives when their tumor was greater than 3 cm in diameter or when malignancy cannot be excluded. No postoperative death occurred in 24 patients with hepatic adenoma. Bile leakage occurred in 1 patient, intraperitoneal abscess in 1 patient, pleural effusion in 2 patients, venous thrombosis in 1 patient and wound infection in 1 patient. Ibrahim *et al*[10] suggested that indications for intervention in cases of benign liver tumors should include symptoms, suspicion of malignancy, or risk of malignant change. Liver resection for benign liver tumor is safe, but indications for

intervention must be evaluated carefully. The presence of chronic parenchymal liver disease does not increase the morbidity of benign hepatic lesions in these patients. This study retrospectively investigated the perioperative predictors by reviewing 827 consecutive cases of benign hepatic lesion undergoing hepatectomy from January 1986 to December 2005 in Chinese PLA General Hospital.

MATERIALS AND METHODS

Patients

A total of 827 consecutive cases (334 males, 493 females, the male and female ratio = 1:1.48) of benign hepatic lesion undergoing hepatectomy from January 1986 to December 2005 in the Chinese PLA General Hospital were included in this study. Their age was 5-79 years and mean age was 43.88 years. Of the 827 cases, 345 had hepatic hemangiomas, 245 had intrahepatic bile duct stones, 35 had hepatic cysts, 35 had hepatic focal nodular hyperplasia, 17 had strictures of intrahepatic bile duct, 17 had hepatic adenoma, 16 had hepatic angioleiomyolipoma, 16 had hepatic echinococcosis, 13 had hepatic inflammatory pseudotumor, 11 had hepatic trauma, 11 had congenital biliary duct cystic dilatation, 10 had other inflammatory diseases (including 4 cases of extrahepatic disease and hepatic adherence disease, 3 cases of hepatic focal necrosis, 1 case of liver calcification, 1 case of hepatic mycotic chronic granuloma, 1 case of sclerosing cholangitis), 7 had hepatic cystadenoma, 4 had hepatic atypical hyperplasia, 4 had hepatic parasitic diseases, 4 had hepatic phthisis, 4 had hepatic granuloma, 4 had hepatic spontaneous haematoma, 3 had hepatic hamartoma, 3 had hepatapostema, and 23 had other diseases (including 6 cases of chronic cholecystitis with suspected preoperative malignant diseases, 4 cases of nodular cirrhosis with suspected preoperative malignant diseases, 2 cases of hepatic hemangioma and intrahepatic bile duct stone, 2 cases of portal hypertension requiring partial liver resection, 1 case of hepatic purpura, 1 case of hepatic lipoma, 1 case of hepatic adenomatous hyperplasia nodus, 1 case of hepatic fibroangiolipoma, 1 case of hepatic leiomyoma, 1 case of benign hepatic occupying lesion with suspected preoperative malignant diseases, 1 case of giant hepatic regenerative nodus with suspected preoperative malignant diseases, 1 case of hepatic artery-portal vein fistula requiring partial liver resection, 1 case of Budd-Chiari syndrome requiring partial liver resection).

Diagnostic methods and preoperative hepatic functional evaluation

All the results were obtained based on pathological diagnosis. Child-Pugh A was scored in 736 out of 750 cases, accounting for 98.13% (Table 1).

Table 1 Preoperative Child-Pugh score of patients

Child-Pugh score	*n*	%
5	635	84.7
6	62	8.3
7	39	5.2
8	9	1.2
9	5	0.7

Surgical methods

Incisions below the right costal margin were routinely used and the lesions were resected after liver was liberated from the left or right side. Regular hepatectomy, focal hepatectomy, and enucleation were performed. Hepatic hemangioma was enucleated after the liver blood flow was occluded. The liver sections were sutured face-to-face to reduce errhysis. Hepatic lobectomy or segmental resection was performed to remove intrahepatic bile duct stones since irregular focal hepatectomy could not accurately remove them, usually leading to remnant pathological tissue and stone. Fifty lesions were resected under laparoscope, accounting for 6.0%. Fifty-one lesions underwent line precoagulation liver resection with a WBD-2 equipment, accounting for 6.2%.

Statistical analysis

SPSS10.0 statistical software was used to analyze the 827 cases of benign hepatic lesions. $P < 0.05$ was considered statistically significant.

RESULTS

Extent of hepatectomy

Hepatectomy for more than 3 liver segments was performed in 183 cases, accounting for 22.1%, including 10 Ⅱ segmentectomies, 19 Ⅲ segmentectomies, 81 Ⅳ segmentectomies, 34 Ⅴ segmentectomies, 42 Ⅵ segmentectomies, 37 Ⅶ segmentectomies, 27 Ⅷ segmentectomies, 22 Ⅰ segmentectomies, 2 extended right hemibepatectomies, 3 extended left hemibepatectomies, 5 multiple suffusions, 129 right hemibepatectomies, 94 right posterior lobectomies, 49 right anterior lobectomies, 5 right trilobectomies, 4 central hepatectomies, 134 left hemibepatectomies, 1 left trilobectomy, and 255 left external lobectomies. If the lesions were located in multiple discontiguous segments (lobes), the locations were separately described. If the lesions were suffused in several segments (lobes), the locations were described as multiple suffusions.

Perioperative outcome

Perioperative results: Of the 827 cases, 517 (62.52%) had a blood loss of less than 200 mL, 99 (11.97%) had a blood loss of 200-400 mL, 146 (17.65%) had a blood loss of 400-1000 mL, 65 (7.86%) had a blood loss of more than 1000 mL , 316 (38.21%) required transfusion of blood products during operation (range 150-8090 mL, mean 879.33 ± 834.64 mL), including whole blood transfusion in 210 cases, red blood cell transfusion in 38 cases, plasma transfusion in 7 cases, mixed transfusion in the remaining cases, auto-blood transfusion in 63 (6.62%) cases (ranged 200-7079 mL, mean 870.45 ± 862.31 mL).

The operating time was 20 to 975 min (mean 220.59 ± 109.13 min). Hospital stay ranged 1-174 d (mean 25.24 ± 15.59 d). Postoperative hospital stay ranged 1-151 d (mean 13.55 ± 9.38 d).

Complications: Postoperative complications occurred in 112 cases (13.54%). The most common complications were epigastric complications related to liver cirrhosis: pleural effusion in 53 cases, hydroperitonia in 20 cases and perihepatic hydrops in 17 cases, which resolved without any sequelae after transfusion of condensed human serum albumin, adjustment of water and electrolyte balance, and administration of diuretics. The main procedure-related complications were perihepatic abscess in 7 cases, cholangitis in 4 cases, incisional infection or liquification in 13 cases. Bile leakage was the most severe complication in 10 cases (1.21%).

Logistic regression analysis of complication-related risk predictor was performed. Multivariate analysis showed that the independent predictors were gender, laparoscope, microwave in line precoagulation liver resection, abdominal operation history, simple anatomical hepatectomy, type B hepatitis, blood loss, the number of segments resected, operating time, albumin value, Child-Pugh score, and age. Operating time (P = 0.004, OR = 1.003) and albumin value (P = 0.040, OR = 0.938) were the independent predictors of complication. Operating time was the risk predictor and increased albumin value was the protection predictor.

Mortality: Two patients died with a mortality of 0.24%. One was a cases of liver trauma combined splenic rupture, hemorrhagic shock, and pelvic fracture. The blood pressure was too low to be measured during emergency operation although tremendous blood products and liquid were transfused. The patient died of blood loss and DIC eventually. The other was a case of intrahepatic bile duct stone undergoing left external lobectomy, exploration of the common bile duct, and T-tube drainage. Postoperative procedure was uneventful. Unfortunately, 15 d after operation, the patient died due to a sudden short breath, chest distress, and ventricular fibrillation.

Changes of perioperative predictors for hepatectomy in the last 20 years

The past 20 years were stratified into 4 stages with 5 years as a stage. The predictors were compared. Multivariate analysis indicated that operating time, blood transfusion, complication rate, and hospital stay had a trend to decrease (Table 2).

Table 2 Changes of perioperative predictors for hepatectomy in the last 20 years

Predictors	Yr	95% confidence interval (mean)	*P*
Age (yr)	86-90	42.676-48.375 (45.525)	0.004
	91-95	41.298-45.938 (43.618)	
	96-00	40.298-43.499 (41.898)	
	01-05	44.288-46.570 (45.429)	
Operating time (min)	86-90	236.649-290.639 (263.644)	< 0.001
	91-95	240.043-284.002 (262.022)	
	96-00	186.591-216.917 (201.754)	
	01-05	203.194-224.812 (214.003)	
Blood transfusion	86-90	67.1% (53/79)	< 0.001
	91-95	68.2% (75/110)	
	96-00	46.9% (99/211)	
	01-05	28.6% (122/427)	
Abdominal operation history	86-90	21.5% (17/79)	0.059
	91-95	30.0% (33/110)	
	96-00	19.0% (40/211)	
	01-05	20.8% (89/427)	
Blood loss (≥ 1000 mL/ < 1000 mL)	86-90	7.6% (6/79)	0.018
	91-95	8.2% (9/110)	
	96-00	8.5% (19/211)	
	01-05	11.7% (50/427)	
Albumin (g)	86-90	38.587-40.837 (39.712)	0.027
	91-95	40.444-42.275 (41.360)	
	96-00	40.694-41.958 (41.326)	
	01-05	40.060-40.961 (40.511)	
Child-Pugh score	86-90	5.188-5.524 (5.356)	0.544
	91-95	5.121-5.396 (5.258)	
	96-00	5.157-5.346 (5.251)	
	01-05	5.155-5.290 (5.223)	
Resected segments	86-90	2.047-2.631 (2.339)	0.026
	91-95	1.942-2.418 (2.180)	
	96-00	2.194-2.522 (2.358)	
	01-05	2.432-2.666 (2.549)	
Complications	86-90	20.3% (16/79)	0.004
	91-95	25.5% (28/110)	
	96-00	14.2% (30/211)	
	01-05	8.9% (38/427)	
Postoperative hospital stay (d)	86-90	17.016-21.560 (19.288)	< 0.001
	91-95	16.443-20.142 (18.292)	
	96-00	12.842-15.394 (14.118)	
	01-05	10.131-11.950 (11.041)	
Simple anatomical hepatectomy (yes/no)	86-90	37.5% (27/72)	0.265
	91-95	37.6% (38/101)	
	96-00	47.8% (98/205)	
	01-05	41.8% (165/395)	

DISCUSSION

Liver is the largest parenchymatous organ in the body which is deep seated in the epigastrium and protected by the bony thorax[11-14]. Liver has abundant blood flow and is a blest and indispensable organ, known as the "forbidden zone" or a "noli me tangere-do not touch me" organ. Liver surgery became possible in the second half of the 20th century and a rapid progress in liver surgery has been achieved in the latest 20-30 years[15,16]. Liver surgery for benign and malignant lesions mainly involves partial liver resection[17].

In the initial stage of liver surgery, the mortality is rather high due to the wide excision of liver and hepatectomy is only suitable for selective malignant lesions and the expected outcome is not good. However, the current surgical techniques make it possible to perform operations on liver. The safety is not inferior to other large intraabdominal operations. It was reported that the mortality of patients with liver tumors could be lowered to the minimum, even to zero[17,18]. Nowadays, surgery is a routine treatment modality for benign hepatic lesions. Zhi-Qiang Huang has reported two cases of intrahepatic bile duct stone (the secondary common benign hepatic lesion in China) treated with left external

lobectomy or right hepatectomy since 1958, leading to wide application of hepatectomy for benign hepatic lesions in clinical practice[19-23].

In this study, retrospectively investigated 827 consecutive cases of benign hepatic lesions were retrospectively investigated in the Chinese PLA General Hospital, which provides some evidence-based data about the improved hepatectomy techniques.

In this series, the most common diseases were hemangioma and intrahepatic bile duct stone, accounting for 41.7 % (345/827) and 29.6% (245/827), respectively. It was reported that the incidence of intrahepatic bile duct stone is higher than hemangioma in China[24].

Hepatectomy can be divided into two categories: hepatectomy involving bile duct and hepatectomy not involving bile duct. Usually, hepatectomy involving bile duct has more complications. The operation procedure, mainly using regular segmental resection or lobectomy, therefore, has different characteristics. Intrahepatic bile duct stone is the representative of this kind of lesion. In 1958, our operation team first reported regular lobectomy for intrahepatic bile duct stone based on the theory that intrahepatic bile duct stone is a kind of segmental lesion with a strict distribution within the liver[25,26]. After more than 50 years, this conception has been well established and developed gradually[27]. In the present series, 202 cases of intrahepatic bile duct stone underwent anatomical hepatectomy, accounting for 82.4% of the total cases, with a complication rate of 16.3% (data not shown). Hepatectomy not involving bile duct is mainly for liver tumors. Hepatic hemangioma is the main representative of benign hepatic lesions[10,19,21,28], which grows slowly and recurs occasionally and can be radically resected. Because of the well-demarked border between the tumor and its normal liver tissue, enucleation is usually performed. In this series, the complication rate was 11.3%, lower than that of intrahepatic bile duct stone.

The postoperative complication rate of hepatectomy was 13.54% in this series. Multivariate analysis showed that operating time (P = 0.004, OR = 1.003) and albumin value (P = 0.040, OR = 0.938) were the independent predictors of morbidity of benign hepatic lesion. Operating time was the risk predictor and increased albumin value was the protection predictor. The study indicated that some surgical technical refining, for instance, optimizing surgical instruments, increasing the surgeon's proficiency and co-operation level, should be taken into account to decrease the operating time so as to reduce complications.

The albumin value should be emphasized on the preoperative liver functional reserve evaluation. In this series, two patients died with a perioperative mortality of 0.24%. One was a case of liver trauma complicated by splenic rupture, hemorrhagic shock, and pelvic fracture, and died of blood loss and DIC. The other case had a sudden death because of ventricular fibrillation 15 d after operation.

The Chinese PLA General Hospital have performed, 2008 hepatectomies for benign and malignant lesions with a total perioperative complication rate of 14.44% and a mortality rate of 0.55%[29]. In this series, the perioperative complication rate and mortality were lower than the total perioperative complication rate and mortality, indicating that hepatectomy is safer for benign hepatic lesion than for malignant lesions.

In addition, multivariate analysis revealed that operating time, blood transfusion, complication rate, and hospital stay had a trend to decrease, indicating that surgical quality is improved.

In conclusion, hepatectomy is a safe procedure for benign hepatic lesions. Benign hepatic lesions should undergo aggressive surgical management when clinical presentations, diagnosis, society factors, and progress in liver surgery are considered. Reducing operating time and preoperative liver functional reserve evaluation play an important role in improving the operation quality.

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COMMENTS

Background

Hepatectomy is a dangerous and complex surgical procedure. The choice of surgical procedure for benign hepatic lesion is controversial.

Research frontiers

Operative safety and indication of hepatectomy for benign hepatic lesions at different sites are the key research frontier.

Innovations and breakthroughs

We analyzed the operative and perioperative factors associated with hepatectomy for benign hepatic lesions. Multivariate analysis showed that operating time (P = 0.004, OR = 1.003) and albumin value (P = 0.040, OR = 0.938) were the independent predictors of morbidity.

Applications

The postoperative complication rate of hepatectomy was 13.54% and in-hospital mortality rate was 0.24%, indicating that hepatectomy is a safe procedure for benign hepatic lesions.

Peer review

This is a very large series of hepatectomy for benign liver lesions. It is a publishable manuscript and very informative. The manuscript should provide more strong messages than "hepatectomy is a safe procedure for benign hepatic lesions". The authors advocated that benign hepatic lesions should undergo aggressive surgical management when clinical presentations, diagnosis, society factors, and progress in liver surgery are considered. Reducing operating time and preoperative liver functional reserve evaluation play an important role in improving the operation quality.

REFERENCES

1 **Tanabe KK**. The past 60 years in liver surgery. *Cancer* 2008; **113**: 1888-1896

2 **Ris F**, Majno P, Morel P, Terraz S, Andres A, Mentha G. [Complex liver resections: where are the limits?] *Rev Med Suisse* 2008; **4**: 1558-1562

3 **Fortner JG**, MacLean BJ, Kim DK, Howland WS, Turnbull AD, Goldiner P, Carlon G, Beattie EJ Jr. The seventies evolution in liver surgery for cancer. *Cancer* 1981; **47**: 2162-2166

4 **Mentha G**, Morel P, Giostra E, Grossholz M, Rubbia L, Buhler L, Rohner A. [Risk in major hepatectomy. A consecutive series of 113 extensive hepatectomies] *Schweiz*

Med Wochenschr 1995; **125**: 1820-1824
5 **Catalano OA**, Singh AH, Uppot RN, Hahn PF, Ferrone CR, Sahani DV. Vascular and biliary variants in the liver: implications for liver surgery. *Radiographics* 2008; **28**: 359-378
6 **Asiyanbola B**, Chang D, Gleisner AL, Nathan H, Choti MA, Schulick RD, Pawlik TM. Operative mortality after hepatic resection: are literature-based rates broadly applicable? *J Gastrointest Surg* 2008; **12**: 842-851
7 **Erdogan D**, Busch OR, van Delden OM, Bennink RJ, ten Kate FJ, Gouma DJ, van Gulik TM. Management of liver hemangiomas according to size and symptoms. *J Gastroenterol Hepatol* 2007; **22**: 1953-1958
8 **Yoon SS**, Charny CK, Fong Y, Jarnagin WR, Schwartz LH, Blumgart LH, DeMatteo RP. Diagnosis, management, and outcomes of 115 patients with hepatic hemangioma. *J Am Coll Surg* 2003; **197**: 392-402
9 **Chaib E**, Gama-Rodrigues J, Ribeiro MA Jr, Herman P, Saad WA. Hepatic adenoma. Timing for surgery. *Hepatogastroenterology* 2007; **54**: 1382-1387
10 **Ibrahim S**, Chen CL, Wang SH, Lin CC, Yang CH, Yong CC, Jawan B, Cheng YF. Liver resection for benign liver tumors: indications and outcome. *Am J Surg* 2007; **193**: 5-9
11 **McClusky DA 3rd**, Skandalakis LJ, Colborn GL, Skandalakis JE. Hepatic surgery and hepatic surgical anatomy: historical partners in progress. *World J Surg* 1997; **21**: 330-342
12 **Bonnichon P**. [The liver and surgeons] *Hist Sci Med* 2007; **41**: 95-104
13 **Foster JH**. History of liver surgery. *Arch Surg* 1991; **126**: 381-387
14 **Pappalardo G**, Frattaroli FM, Illomei G, Guadalaxara A, Ravo B. Historical evolution of the surgical anatomy of the liver. *Med Secoli* 1993; **5**: 263-278
15 **Fortner JG**, Blumgart LH. A historic perspective of liver surgery for tumors at the end of the millennium. *J Am Coll Surg* 2001; **193**: 210-222
16 **Tang ZY**. Hepatocellular carcinoma surgery--review of the past and prospects for the 21st century. *J Surg Oncol* 2005; **91**: 95-96
17 **Huang ZQ**, Huang XQ. Liver operative surgery. 2nd ed. Beijing: People's Military Medical Press, 2007: 1-4, 178-180
18 **Huang ZQ**. The surgical treatment of liver benign occuping lesions. Focus on hepatobiliary & pancreatic surgery. 1st ed. Beijing: People's Military Medical Press, 2005: 70-83
19 **Loss M**, Zulke C, Obed A, Stoltzing O, Schlitt HJ. [Surgical therapy of benign liver tumors] *Chirurg* 2008; **79**: 722-728
20 **Shortell CK**, Schwartz SI. Hepatic adenoma and focal nodular hyperplasia. *Surg Gynecol Obstet* 1991; **173**: 426-431
21 **Cherqui D**. [Benign liver tumors] *J Chir* (Paris) 2001; **138**: 19-26
22 **Kammula US**, Buell JF, Labow DM, Rosen S, Millis JM, Posner MC. Surgical management of benign tumors of the liver. *Int J Gastrointest Cancer* 2001; **30**: 141-146
23 **Rohner A**. [Treatment of benign tumors of the liver] *Schweiz Med Wochenschr* 1986; **116**: 1044-1050
24 **Huang ZQ**. The present status of surgical treatment of intrahepatic lithiasis in a nation-wide survey in China of 4197 operative cases 1981-1985. *Zhonghua Waike Zazhi* 1988; **26**: 513-522, 572
25 **Huang CC**. Partial resection of the liver in treatment of intrahepatic stones. *Chin Med J* 1959; **79**: 40-45
26 **Cai JX**, Huang ZQ. Clinical analysis of the surgical therapy for 749 cases of intrahepatic lithiasis. *Disan Junyi Daxue Xuebao* 1996; **18**: 59-61
27 **Dong JH**, Huang ZQ, Cai JX, Han BL, He ZP, Bie P, Wang SG, Li ZH, Chen P, Ma KS, Feng XB. Anatomic hepatectomy for the treatment of hepatolithiasis. *Zhonghua Putong Waike Zazhi* 2002; **17**: 418-420
28 **Bahirwani R**, Reddy KR. Review article: the evaluation of solitary liver masses. *Aliment Pharmacol Ther* 2008; **28**: 953-965
29 **Huang ZQ**, Xu LN, Yang T, Zhang WZ, Huang XQ, Liu R, Cai SW, Zhang AQ, Feng YQ, Zhou NX, Dong JH. The review of hepatectomy in the last two decades: the experience of unicentral 2008 connective hepatectomies. *Zhonghua Waike Zazhi* 2008; **46**: 1314-1321

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CASE REPORT

Large mucinous cystic neoplasm of the pancreas associated with pregnancy

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Abstract

Mucinous cystic neoplasms (MCNs) of the pancreas occur mostly in females and are potentially sex hormone-sensitive. However, a MCN occurring during pregnancy is quite rare. A 30-year-old woman in the tenth week of pregnancy was referred to us because of a rapid increase in left hypochondrial distending pain. On ultrasound, the patient had a large intra-abdominal cystic lesion. She was thereafter diagnosed with missed abortion and a computed tomography scan showed that the lesion was a cystic tumor 18 cm in diameter originating from the pancreatic tail. The patient subsequently underwent tumor resection with distal pancreatectomy, sparing the spleen. Histopathological analysis of the specimen revealed a pancreatic MCN with moderate dysplasia. Immunohistochemically, the tumor was positive for both estrogen and progesterone receptors. To our knowledge, this is the first reported case of pancreatic MCN with moderate dysplasia in association with pregnancy. Our case strongly indicates that pancreatic MCN is female-hormone dependent.

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Key words: Mucinous cystic neoplasm; Pancreatic tumor; Pregnancy

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INTRODUCTION

Mucinous cystic neoplasm (MCN) of the pancreas is an uncommon tumor characterized by a proliferation of mucin-producing columnar epithelium supported by ovarian-type stroma[1,2]. MCNs are typically located in the pancreatic body or tail and rarely communicate with the pancreatic duct[1,2]. Pancreatic MCNs occur almost exclusively in females, and are potentially sex hormone-sensitive, according to the studies reported to date[2]. However, MCNs encountered in pregnancy are rare; only eight such cases have been reported in the English literature[3-10]. In this report, we describe a 30-year-old woman with a large pancreatic MCN that grew rapidly during the early stage of pregnancy.

CASE REPORT

A 30-year-old woman in the tenth week of pregnancy was referred to our hospital due to increasing distending pain in the left hypochondrial region over the last month. She had no notable medical history, but had complained of vague epigastric discomfort over the past several years. Physical examination suggested the presence of an elastic hard mass, approximately 20 cm in diameter, in the left upper quadrant. Laboratory tests showed slight anemia and normal levels of amylase, liver enzymes, and tumor markers. An abdominal ultrasound demonstrated a huge cystic lesion in the left upper abdomen. Soon after hospitalization, her pregnancy ended in missed abortion at 10 wk gestation, which was confirmed by clinical and ultrasound findings. Further evaluation by computed tomography (CT) scanning revealed a well-demarcated cystic mass, measuring 18 cm × 14 cm, probably originating from the distal pancreas (Figure 1). There were

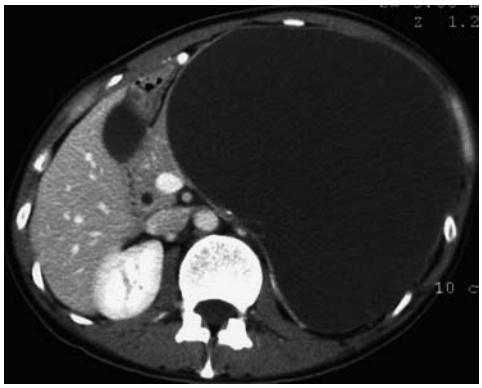

Figure 1 Abdominal computed tomography scan showing a huge cyst measuring 18 cm in diameter.

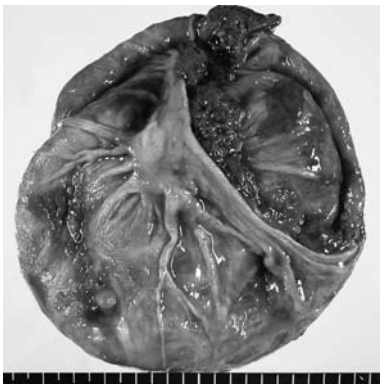

Figure 2 Macroscopic view of the cystic tumor.

no septa or protruding lesions inside the cystic mass.

Ultrasound-guided percutaneous cyst aspiration was then performed to reduce the pain, and 2100 mL of dark-brownish mucinous fluid was aspirated, without spillage into the peritoneal cavity. The aspirated fluid showed an amylase level of 59 IU/L, a carcinoembryonic antigen (CEA) level of 32100 ng/mL, and a carbohydrate-associated antigen (CA) 19-9 level of 34500 U/mL. Cytological examination of the fluid was class Ⅱ with no malignant findings. Thus, a diagnosis of possible pancreatic MCN was made. After dilatation and curettage, the cystic mass was resected with distal pancreatectomy. Since invasive carcinoma was not suspected by preoperative images and intraoperative findings, the spleen was preserved. The tumor was unilocular, had thick walls, and was filled with tenacious mucoid material and necrotic debris (Figure 2). Histopathological analysis showed a mucin-producing columnar epithelium lining the inner wall of the cyst, with ovarian-type stromal tissue (Figure 3A). The epithelium had a focal papillary architecture without significant cytologic atypia, giving a diagnosis of pancreatic MCN with moderate dysplasia (Figure 3B). Immunohistochemical studies showed positive staining for both progesterone and estrogen receptors in the stromal cell nuclei (Figure 3C and D). The patient had an uneventful post-operative course, and she remained disease-free for 12 mo after surgery.

DISCUSSION

To our knowledge, eight cases of pancreatic MCN associated with pregnancy have been reported since the first report in 1968 by Smithers *et al*[7]. Four cases were histologically diagnosed as benign mucinous cystadenoma[3-6], while the other four were diagnosed as mucinous cystadenocarcinoma[7-10]. Our case might be the first case of pancreatic MCN with moderate dysplasia (also referred to as "borderline tumor"), in association with pregnancy.

Although cystic tumors of the pancreas often grow slowly and can remain indolent for many years[5,10], MCNs which develop during pregnancy tend to grow more rapidly and achieve extraordinary size. Kato *et al*[4] reported that a pancreatic MCN in a pregnant patient rapidly increased in volume from 2619 to 4950 mL over 46 d. Ganepola *et al*[3] also reported a case whose cyst increased in size from 5.5 cm to more than 12 cm within 4 mo. In a recent review, 344 MCNs defined by ovarian-type stroma had a mean size of 8.7 cm (range, 0.6-35 cm)[11], whereas in the review of reported cases and in our case, pregnancy associated-MCNs had a mean size of 15 cm (range, 10-22 cm).

These findings are consistent with the concept that pancreatic MCNs may be responsive to female sex hormones. One series reported that stromal cell nuclei were positive for estrogen receptors in 15 of 65 MCN cases (23%) and for progesterone receptors in 46 cases (71%)[2]. The predominantly female occurrence and the expression of female hormone receptors in MCNs has been the subject of much speculation. One possibility is that MCNs originate from primitive ovarian cells, which are incorporated into the embryonic pancreas when the left primordial gonad is in close proximity to the dorsal pancreatic anlage during embryogenesis[1]. The dorsal pancreatic anlage gives rise to the body and tail of the pancreas, and this hypothesis could explain the predilection of MCNs for the distal pancreas. Another possibility is that the neoplastic epithelial cells of MCNs might induce ovarian stromal differentiation in cells that normally reside in the pancreas. This concept is based on the fact that the stroma in the fetal pancreas is morphologically similar to that of MCNs[12]. However, direct evidence for these hypotheses has yet to be found.

Pancreatic MCN presents a clinical problem in that it always carries a malignant potential. Mucinous cystadenocarcinoma can be considered not only as a neoplasm arising de novo but also as an evolution of the benign form[8]. As large tumor size was reported to be one of the predictive factors of malignant MCNs[13], it is prudent to assume that the increased growth of MCNs during pregnancy brings with it some degree of increased risk for malignant transformation[9]. However, it remains unclear whether high levels of estrogen and progesterone in pregnancy accelerate the malignant transformation of a benign tumor into a malignant tumor. In fact, there is evidence that a decrease in progesterone receptor immunoreactivity was correlated with histological or cytological atypicality and a worse prognosis[2].

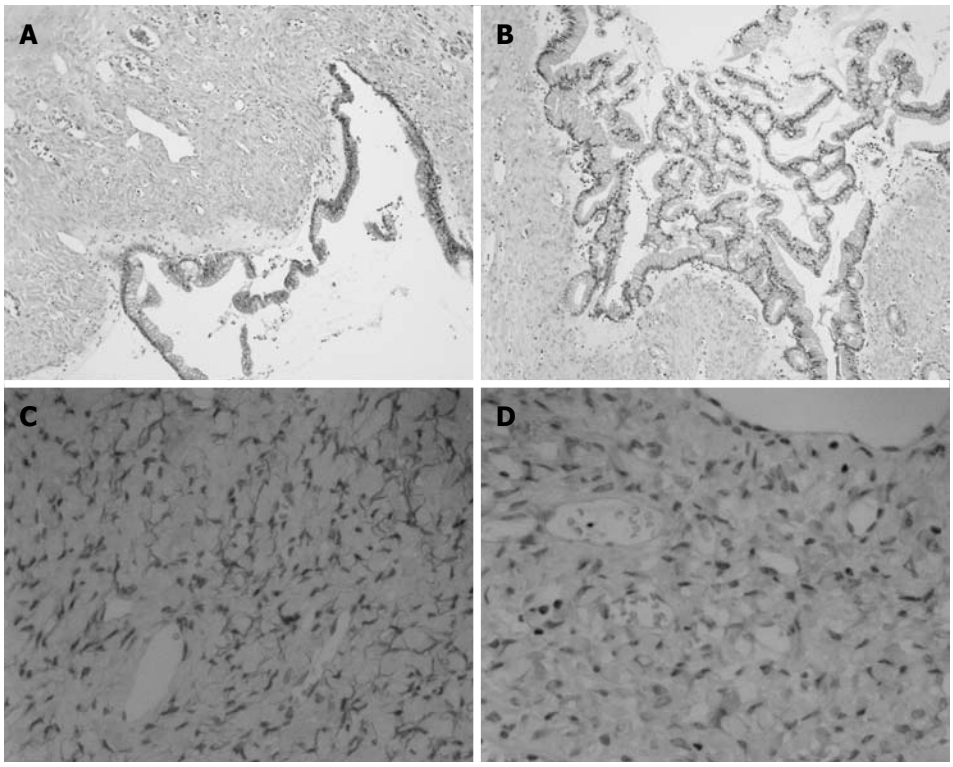

Figure 3 Microscopic findings of the cystic tumor. A: Columnar, mucin-producing epithelium with underlying ovarian-type stroma (HE, x 100); B: The epithelium had a focal papillary architecture (HE, x 100); C: Positive staining in the stromal cell nuclei for progesterone receptor (x 400); D: The estrogen receptor of stromal cell nuclei (x 400).

Pancreatic MCN detected during pregnancy requires special management considerations due to its large size and resultant risk for fetal growth restriction. So far, there have been four cases of successful antepartum resection of pancreatic MCN during pregnancy that later resulted in healthy infants[3-5,9]. These authors have recommended the second trimester as a safe period for surgery because this period provides enough time for fetal maturity. Since the tumor in the present case did not compress the uterus, there may be no direct correlation between tumor progression and miscarriage in our patient.

In the present case, successful cyst aspiration provided a basis for accurate diagnosis as well as symptom relief for the patient. Cyst fluid analysis has recently been utilized to differentiate pancreatic cystic neoplasms. van der Waaij *et al*[14] found that an amylase concentration below 250 U/L effectively ruled out pseudocyst. In addition, it has been reported that cyst fluid CA 19-9 level greater than 50000 U/mL had a sensitivity of 75% and a specificity of 90% for distinguishing mucinous tumors from other cystic lesions[15]. Furthermore, a recent series[16] revealed that cyst fluid CEA greater than 800 ng/mL had a sensitivity of 42.9% and a specificity of 95.2% for diagnosing pancreatic MCNs. When performing cyst aspiration, however, care must be taken to avoid spillage of cyst contents, which may result in the development of pseudomyxoma peritonei[4].

In summary, our report describes the ninth case of pancreatic MCN associated with pregnancy and the first such case of MCN with moderate dysplasia. Our case strongly indicates that pancreatic MCN is a hormone-dependent tumor that can grow dramatically during pregnancy. Considering the malignant potential of this tumor and adverse effects on the fetus, complete resection should be performed without delay.

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REFERENCES

1 **Zamboni G**, Scarpa A, Bogina G, Iacono C, Bassi C, Talamini G, Sessa F, Capella C, Solcia E, Rickaert F, Mariuzzi GM, Kloppel G. Mucinous cystic tumors of the pancreas: clinicopathological features, prognosis, and relationship to other mucinous cystic tumors. *Am J Surg Pathol* 1999; **23**: 410-422

2 **Thompson LD**, Becker RC, Przygodzki RM, Adair CF, Heffess CS. Mucinous cystic neoplasm (mucinous cystadenocarcinoma of low-grade malignant potential) of the pancreas: a clinicopathologic study of 130 cases. *Am J Surg Pathol* 1999; **23**: 1-16

3 **Ganepola GA**, Gritsman AY, Asimakopulos N, Yiengpruksawan A. Are pancreatic tumors hormone dependent?: A case report of unusual, rapidly growing pancreatic tumor during pregnancy, its possible relationship to female sex hormones, and review of the literature. *Am Surg* 1999; **65**: 105-111

4 **Kato M**, Kubota K, Kita J, Shimoda M, Rokkaku K, Inaba N, Fukasawa I, Honma K. Huge mucinous cystadenoma of the pancreas developing during pregnancy: a case report. *Pancreas* 2005; **30**: 186-188

5 **Lopez-Tomassetti Fernandez EM**, Martin Malagon A, Arteaga Gonzalez I, Muniz Montes JR, Diaz Luis H, Gonzalez Hermoso F, Carrillo Pallares A. Mucinous cystic neoplasm of the pancreas during pregnancy: the importance of proper management. *J Hepatobiliary Pancreat Surg* 2005; **12**: 494-497

6 **Ishikawa K**, Hirashita T, Kinoshita H, Kitano M, Matsuo S, Matsumata T, Kitano S. Large mucinous cystadenoma of the pancreas during pregnancy: report of a case. *Surg Today* 2007; **37**: 1013-1017

7 **Smithers BM**, Welch C, Goodall P. Cystadenocarcinoma of the pancreas presenting in pregnancy. *Br J Surg* 1986; **73**: 591

8 **Baiocchi C**, Landonio G, Majno M, Minola E, Scanzi F,

Ghislandi E. Pancreatic cystadenocarcinoma and pregnancy: a case report. *Tumori* 1990; **76**: 294-295
9 **Herring AA**, Graubard MB, Gan SI, Schwaitzberg SD. Mucinous cystadenocarcinoma of the pancreas during pregnancy. *Pancreas* 2007; **34**: 470-473
10 **Ozden S**, Haliloglu B, Ilter E, Akin FT, Kebudi A, Peker O. An extremely rare cause of acute abdomen in pregnancy: ruptured pancreatic mucinous cystadenocarcinoma. *Pancreas* 2007; **34**: 474-476
11 **Goh BK**, Tan YM, Chung YF, Chow PK, Cheow PC, Wong WK, Ooi LL. A review of mucinous cystic neoplasms of the pancreas defined by ovarian-type stroma: clinicopathological features of 344 patients. *World J Surg* 2006; **30**: 2236-2245
12 **Volkan Adsay N**. Cystic lesions of the pancreas. *Mod Pathol* 2007; **20** Suppl 1: S71-S93
13 **Crippa S**, Salvia R, Warshaw AL, Dominguez I, Bassi C, Falconi M, Thayer SP, Zamboni G, Lauwers GY, Mino-Kenudson M, Capelli P, Pederzoli P, Castillo CF. Mucinous cystic neoplasm of the pancreas is not an aggressive entity: lessons from 163 resected patients. *Ann Surg* 2008; **247**: 571-579
14 **van der Waaij LA**, van Dullemen HM, Porte RJ. Cyst fluid analysis in the differential diagnosis of pancreatic cystic lesions: a pooled analysis. *Gastrointest Endosc* 2005; **62**: 383-389
15 **Hammel P**, Levy P, Voitot H, Levy M, Vilgrain V, Zins M, Flejou JF, Molas G, Ruszniewski P, Bernades P. Preoperative cyst fluid analysis is useful for the differential diagnosis of cystic lesions of the pancreas. *Gastroenterology* 1995; **108**: 1230-1235
16 **Attasaranya S**, Pais S, LeBlanc J, McHenry L, Sherman S, DeWitt JM. Endoscopic ultrasound-guided fine needle aspiration and cyst fluid analysis for pancreatic cysts. *JOP* 2007; **8**: 553-563

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CASE REPORT

Primary multiple extragastrointestinal stromal tumors of the omentum with different mutations of *c-kit* gene

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Abstract

The author reports a very rare case of sporadic primary multiple extragastrointestinal stromal tumors (EGISTs) of the omentum associated with different mutations of the exon 11 of the *c-kit* gene in a 75-year-old man with gastric cancer. During an operation for the cancer, two solid tumors (10 mm and 8 mm) were found in the omentum. Both tumors consisted of cellular spindle cells. Mitotic figures were two and three per 50 high power fields. The tumor cells were positive for KIT, CD34 and vimentin, but negative for desmin, S100 protein, α-smooth muscle actin and p53 protein. Ki67 labeling was 2% and 3%. The larger EGIST showed a deletion of codons 552-558 of exon 11 of the *c-kit* gene, while the smaller EGIST had a point mutation at codon 559 (GTT→GAT) in exon 11 of the *c-kit* gene. Exons 9, 13, and 17 of the *c-kit* gene, and exons 12 and 18 of the platelet derived growth factor receptor α genes showed no mutations. The case shows that sporadic multiple EGISTs can occur in the omentum.

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Key words: Omentum; Extragastrointestinal stromal tumors; *c-kit*; Platelet derived growth factor receptor; CD34

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INTRODUCTION

Gastrointestinal stromal tumors (GIST) were once thought to be smooth muscle or neurogenic tumors, but recent advances in the study of the *c-kit* gene and platelet derived growth factor receptor α (*PDGFRA*) gene have revealed that GIST are associated with gain-of-function mutations of the *c-kit* gene and the *PDGFRA* gene[1,2]. KIT is a transmembrane receptor tyrosine kinase whose ligand is stem cell factor[3]. GIST is believed to be derived from interstitial cells of Cajal (ICC) (pacemaker cells) which are present in the muscular layer of the gastrointestinal walls[3]. ICC expresses KIT protein (CD117) and CD34[3]. Immunohistochemical demonstration of KIT and/or CD34 is a hallmark in the pathological diagnosis of GIST[3].

Mesenchymal tumors resembling GIST and positive for KIT have been found in the soft tissue[4] and less frequently in abdominal organs such as the liver[5], gall bladder[6,7], pancreas[8,9], and serosa[10]. Such tumors are called extragastrointestinal stromal tumors (EGIST). The present study presents a case having two EGISTs in the greater omentum. According to Todoroki *et al*[11], 28 cases of EGISTs in the omentum have been reported in the literature. However, primary multiple GISTs in the omentum have been reported only once, by Kim *et al*[12]. The author here reports this case with a comparison with normal omentum.

CASE REPORT

A 75-year-old Japanese man complained of epigastralgia. The patient did not have neurofibromatosis, and denied any family history of mesenchymal tumors of the gastrointestinal organs. An endoscopic examination showed a depressed lesion of the stomach, and a biopsy showed well-differentiated adenocarcinoma. No tumor formations were noted by various imaging modalities including CT. A gastrectomy was performed, and during the operation two small solid tumors (10 mm and 8 mm in diameter, respectively) were found in the greater omentum. The both tumors were not attached to the gastrointestinal organs. Both tumors were diagnosed as EGISTs as described below, and treated by imatinib. No recurrence was seen one year after the operation.

Cases of EGIST were reviewed from 27659 surgical and biopsy specimens performed from 2000 to 2007, in my laboratory. As a result, thee cases of EGIST were found. One was a uterine EGIST, one was an omental

Table 1 Primer sequences

Forward	Reverse
c-kit exon 9	
5′-TCCTAGAGTAAGCCAGGGCTT-3′	5′-TGGTAGACAGAGCCTAAACATCC-3′
c-kit exon11	
5′-GATCTATTTTTCCCTTTCTC-3′	5′-AGCCCCTGTTTCATACTGAC-3′
c-kit exon 13	
5′-GCTTGACATCAGTTTGCCAG-3′	5′-AAAGGCAGCTTGGACACGGCTTTA-3′
c-kit exon 17	
5′-CTCCTCCAACCTAATAGTGT-3′	5′-GTCAAGCAGAGAATGGGTAC-3′
PDGFRA exon12	
5′-TTGGATATTCACCAGTTACCTGTC-3′	5′-CAAGGGAAAAGCTCTTGG-3′
PDGFRA exon 18	
5′-ACCATGGATCAGCCAGTCTT-3′	5′-TGAAGGAGGATGAGCCTGACC-3′

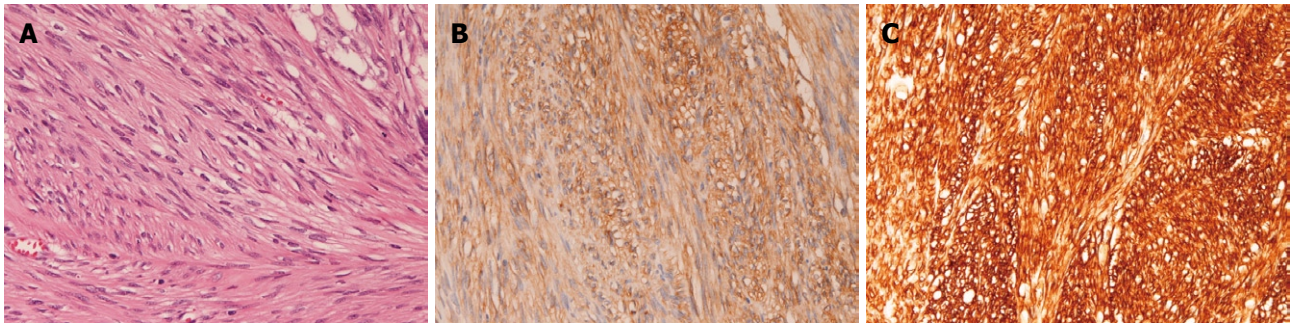

Figure 1 Histological and immunohistochemical findings of the tumors. A: Histology of the tumor. The tumor is composed of cellular spindle cells. Hematoxylin and eosin (x 200). B: KIT immunostaining of the tumor. KIT is diffusely and strongly positive in the tumor cells (x 200). C: CD34 immunostaining of the tumor. CD34 is diffusely and strongly positive in the tumor cells (x 200).

EGIST, and one was a mesenteric EGIST. In this case report, the author shows a case of omental EGIST. The patient's clinical records were obtained. As controls, three cases of normal omentum, which were resected in cases of ovarian cancer, were used. The materials were fixed in 10% formalin and embedded in paraffin. Several 3-μm sections were cut from each paraffin block, and stained with hematoxylin and eosin.

Immunohistochemical studies were performed by the DAKO's envision method as previously described[13]. The antibodies used were KIT (polyclonal, Dako Corp, Glustrup, Denmark), CD34 (QBEND10, Dako), vimentin (Vim 3B4, Dako), desmin (D33, Dako), α smooth muscle actin (1A4, Dako), S100 protein (polyclonal, Dako), p53 protein (DO7, Dako), and Ki-67 antigen (MIB1, Dako).

Genetic analyses for the *c-kit* gene (exons 9, 11, 13 and 17) and the *PDGFRA* gene (exons 12 and 18) were performed by direct sequencing of PCR products. The exons of both genes were selected because they are frequent mutation sites[3]. The primers are shown in Table 1. In brief, genomic DNA was extracted from the paraffin blocks by proteinase K digestion and phenol/chloroform extraction, and subjected to PCR for 40 cycles (94℃ for one minute, 52℃ for one minute, and 72℃ for one minute), using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, ABI, CA). The annealing temperature was 53℃. The PCR products were then subjected to electrophoresis in a 2% agarose gel with ethidium bromide, and the PCR products were diluted 1:1 in loading buffer (94% formamide, 10mg bromphenol blue), denatured by heating at 98℃ for three minutes, and snap frozen, before being electrophoresed on a polyacrylamide gel. The products were extracted and sequenced on an ABI PRIZM 3100 Genetic analyzer (Applied Biosystems, ABI, CA).

Histologically, the both tumors showed almost the same morphologies. Both tumors consisted of cellular spindle cells (Figure 1A). Mitotic figures were two in the smaller tumor and three in larger tumor, per 50 high power field (HPF). No epithelioid pattern was recognized and no necrotic areas were present. Immunohistochemically, the tumor cells of both tumors were strongly positive for KIT (Figure 1B), CD34 (Figure 1C) and vimentin, but negative for desmin, S100 protein, α-smooth muscle actin and p53 protein. Ki67 labeling was 2% in the smaller tumor and 3% in the larger tumor. Both tumors were diagnosed as EGIST. The normal omentum showed scattered mesenchymal cells positive for KIT and CD34 in the surface of the omentum. The stomach lesion was an early well-differentiated adenocarcinoma confined to the mucosa. The stomach showed no GISTs.

Genetic analyses for the *c-kit* gene (exons 9, 11, 13 and 17) and the *PDGFRA* (exons 12 and 18) showed a deletion at codon 552-558 of exon 11 of the *c-kit* gene in the larger tumor (Figure 2). The small tumor showed a point mutation (GTT→GAT) at codon 559 of exon 11 of the *c-kit* gene (Figure 3). Other exons of the *c-kit* gene showed no abnormalities. Exons 12 and 18 of the

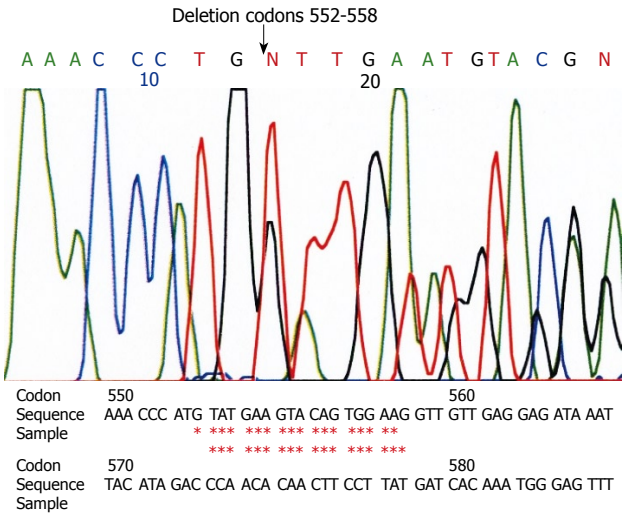

Figure 2 Computer analysis of a part of exon 11 of the *c-kit* gene in the larger tumor. Deletion of codons 552-558 is noted. Asterisks show the deletion.

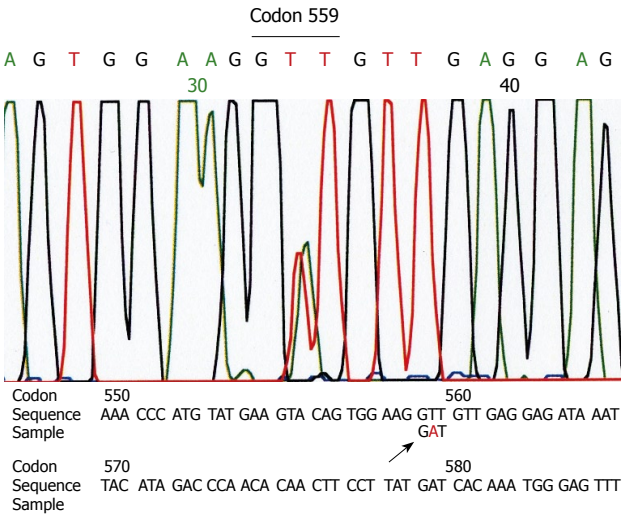

Figure 3 Computer analysis of a part of exon 11 of the *c-kit* gene in the smaller tumor. A mutation at codon 559 (GTT→GAT) is recognized (arrow).

PDGFRA gene also showed no abnormalities in both tumors. The normal omentum showed no abnormalities in either of the two genes.

DISCUSSION

According to Todoroki *et al*[11], there are 28 cases of EGIST of the omentum in the literature. Most of them are single EGIST[10,11], and multiple EGISTs of the omentum have been reported only once[12]. The present study reports a second case of primary multiple EGIST of the omentum. The diagnosis of EGIST of the present case was conclusive. The present study showed scattered KIT-positive ICC-like cells in surface of the omentum. Sakurai *et al*[14] also reported KIT-positive ICC-like mesenchymal cells in the omentum. Therefore, the present case may be derived from the ICC-like cells physiologically present in the omentum.

GIST is thought to be a potentially malignant tumor[3]. The malignant potential has been accessed by various parameters, such as tumor size, mitotic figures, necrosis, cell type, and Ki-67 labeling[15-17]. However, the parameters and the results varied from one observer to another. For example, Hasegawa *et al*[17] reported that tumor size of less than 5 cm was low risk, 5-10 cm posed an intermediate risk, and more than 10 cm posed a high risk. The present case is probably low risk due to the tumor size. GIST with mitotic figures less than 5 per 50 high power fields are low risk, 5-10 are intermediate risk, and more than 10 are high risk. The present case is low risk according to the mitotic count. The presence of necrosis is a high risk for malignancy, therefore the absence of necrosis in the present case also suggests it is low risk. Ki-67 labeling is also related to the GIST risk. In the present study, the Ki-67 was 2% and 3%, respectively, indicating low risk. Taken together, these data suggest that the present EGIST case has a low risk of malignancy.

The present study examined the *c-kit* and *PDGFRA* genes and found that the two EGISTs of the omentum had different mutations in exon 11 of the *c-kit* gene. It has been found that mutations ratio of the *c-kit* gene is 80% in exon 11, 15 % in exon 9, and less than 2% in exons 13 and 17[3]. Mutations ratio of the *PDGFRA* gene is 10% in exon 18 and less than 2% in exons 12 and 14[3]. Therefore, the present case showed a common mutation pattern. In addition, the present case shows that the two EGISTs are not identical tumors, but individual tumors with different pathogenesis.

Imatinib has been developed to treat GIST[3]. The present case also used Imanitib. Imanitib is effective in GIST cases with c-kit mutations in exon 11[3] and in present case it may have been be effective, because neither recurrence nor metastasis has been seen one year after the operation.

In summary, the author showed a very rare case of primary multiple EGISTs of the omentum with different mutations in exon 11 of the *c-kit* gene.

REFERENCES

1 **Hirota S**, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* 1998; **279**: 577-580

2 **Hirota S**, Ohashi A, Nishida T, Isozaki K, Kinoshita K, Shinomura Y, Kitamura Y. Gain-of-function mutations of platelet-derived growth factor receptor alpha gene in gastrointestinal stromal tumors. *Gastroenterology* 2003; **125**: 660-667

3 **Hirota S,** Isozaki K. Pathology of gastrointestinal stromal tumors. *Pathol Int* 2006; **56**: 1-9

4 **Yamamoto H**, Oda Y, Kawaguchi K, Nakamura N, Takahira T, Tamiya S, Saito T, Oshiro Y, Ohta M, Yao T, Tsuneyoshi M. c-kit and PDGFRA mutations in extragastrointestinal stromal tumor (gastrointestinal stromal tumor of the soft tissue). *Am J Surg Pathol* 2004; **28**: 479-488

5 **Hu X**, Forster J, Damjanov I. Primary malignant gastrointestinal stromal tumor of the liver. *Arch Pathol Lab Med* 2003; **127**: 1606-1608

6 **Ortiz-Hidalgo C**, de Leon Bojorge B, Albores-Saavedra J. Stromal tumor of the gallbladder with phenotype

of interstitial cells of Cajal: a previously unrecognized neoplasm. *Am J Surg Pathol* 2000; **24**: 1420-1423
7 **Mendoza-Marin M**, Hoang MP, Albores-Saavedra J. Malignant stromal tumor of the gallbladder with interstitial cells of Cajal phenotype. *Arch Pathol Lab Med* 2002; **126**: 481-483
8 **Yamaura K**, Kato K, Miyazawa M, Haba Y, Muramatsu A, Miyata K, Koide N. Stromal tumor of the pancreas with expression of c-kit protein: report of a case. *J Gastroenterol Hepatol* 2004; **19**: 467-470
9 **Daum O**, Klecka J, Ferda J, Treska V, Vanecek T, Sima R, Mukensnabl P, Michal M. Gastrointestinal stromal tumor of the pancreas: case report with documentation of KIT gene mutation. *Virchows Arch* 2005; **446**: 470-472
10 **Miettinen M**, Monihan JM, Sarlomo-Rikala M, Kovatich AJ, Carr NJ, Emory TS, Sobin LH. Gastrointestinal stromal tumors/smooth muscle tumors (GISTs) primary in the omentum and mesentery: clinicopathologic and immunohistochemical study of 26 cases. *Am J Surg Pathol* 1999; **23**: 1109-1118
11 **Todoroki T**, Sano T, Yamada S, Hirahara N, Toda N, Tsukada K, Motojima R, Motojima T. Clear cell carcinoid tumor of the distal common bile duct. *World J Surg Oncol* 2007; **5**: 6
12 **Kim JH**, Boo YJ, Jung CW, Park SS, Kim SJ, Mok YJ, Kim SD, Chae YS, Kim CS. Multiple malignant extragastrointestinal stromal tumors of the greater omentum and results of immunohistochemistry and mutation analysis: a case report. *World J Gastroenterol* 2007; **13**: 3392-3395
13 **Terada T**, Kawaguchi M, Furukawa K, Sekido Y, Osamura Y. Minute mixed ductal-endocrine carcinoma of the pancreas with predominant intraductal growth. *Pathol Int* 2002; **52**: 740-746
14 **Sakurai S**, Hishima T, Takazawa Y, Sano T, Nakajima T, Saito K, Morinaga S, Fukayama M. Gastrointestinal stromal tumors and KIT-positive mesenchymal cells in the omentum. *Pathol Int* 2001; **51**: 524-531
15 **Miettinen M**, Sobin LH, Lasota J. Gastrointestinal stromal tumors of the stomach: a clinicopathologic, immunohistochemical, and molecular genetic study of 1765 cases with long-term follow-up. *Am J Surg Pathol* 2005; **29**: 52-68
16 **Singer S**, Rubin BP, Lux ML, Chen CJ, Demetri GD, Fletcher CD, Fletcher JA. Prognostic value of KIT mutation type, mitotic activity, and histologic subtype in gastrointestinal stromal tumors. *J Clin Oncol* 2002; **20**: 3898-3905
17 **Hasegawa T**, Matsuno Y, Shimoda T, Hirohashi S. Gastrointestinal stromal tumor: consistent CD117 immunostaining for diagnosis, and prognostic classification based on tumor size and MIB-1 grade. *Hum Pathol* 2002; **33**: 669-676

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CASE REPORT

Intraoperative pulmonary hypertension occurred in an asymptomatic patient with pre-existent liver cirrhotic and portal hypertension

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Abstract

Portopulmonary hypertension (PPH) is clinically defined as the development of pulmonary arterial hypertension complicated by portal hypertension, with or without advanced hepatic disease. Physical signs may be absent in mild to moderate PPH and only appear in a hyperdynamic circulatory state. Similar signs of advanced liver disease can be observed in severe PPH, with ascites and lower extremity edema. Pulmonary hypertension is usually diagnosed after anesthetic induction during liver transplantation (LT). We present intraoperative pulmonary hypertension in a 41-year-old male patient with hepatic cirrhosis. Since this patient had no preoperation laboratory data supporting the diagnosises of pulmonary hypertension and was asymptomatic for a number of years, it was necessary to send him to the intensive care unit after operation. Further study should be focued on the diagnosis and treatment of pulmonary arterial hypertension in order to reduce its mortality.

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Key words: Pulmonary hypertension; Liver transplantation; Portal hypertension; Cirrhosis; Hepatitis

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INTRODUCTION

Patients with liver disease are predisposed to develop histological changes in pulmonary vessels, which will cause pulmonary vascular disease, particularly pulmonary hypertension. Portopulmonary hypertension (PPH) is usually regarded as a severe complication of chronic liver disease with portal hypertension and occurs in 5%-10% of patients with advanced liver disease[1]. Direct measurement of pulmonary artery pressure (PAP) by right-heart catheterization (RHC) remains the gold standard for detecting pulmonary hypertension. Doppler echocardiography using trans-tricuspid valve gradient allows noninvasive estimation of pulmonary artery systolic pressure, and is mostly used for liver transplant candidates. However, echocardiography and electrocardiography are seldom performed in patients with mild or moderate PPH[2].

Patients with PPH may be asymptomatic at the time of diagnosis, and symptoms associated with advanced liver disease and portal hypertension may be indistinguishable from those of PPH of any causes[3]. The recognition of PPH requires a high degree of clinical suspicion. Treatment options for patients with PPH are few. Previous studies have shown that cirrhotic patients with moderate to severe pulmonary hypertension have a very high mortality. Patients with severe PPH who undergo liver transplant without vasodilator therapy have an extremely poor survival[4]. However, recent reports indicate that preoperative therapy can reduce pulmonary hypertension and right ventricle dysfunction, thus improving clinical status and making liver transplantation (LT) feasible[5-7]. However, pro-operative therapy is not efficient for portal hypertension (PHT). There

is no doubt that the occurrence of non-pulmonary manifestations of perioperative pulmonary hypertension presents management challenges. This report describes our new experience with the management of portal and pulmonary hypertension.

CASE REPORT

A 41-year-old male patient with end-stage liver disease secondary to alcohol and hepatitis B was referred to our department for LT. Over the previous 10 mo, the patient had ariceal bleeding, increasing jaundice, and ascites. Cutaneous spider naevi were present and his fingertips were clubbed. He refrained from smoke five years ago due to chronic active hepatitis B and denied to have prior pulmonary problems. Pulmonary and cardiac examinations were unremarkable. Chest radiography showed normal main and central pulmonary arteries. Electrocardiogram was within the normal range with no evident signs of pressure overload of the right atrium and ventricle. Lung function tests showed a mild restrictive pattern that was ascribed to ascites. Total lung capacity, FEV1 and FVC were in normal range. Urinalysis was negative. His liver function tests were as follows: 101 μmol/L total bilirubin with a direct fraction of 50 μmol/L, 113 U/L alkaline phosphatase, 112 U/L ALT and 83 U/L AST. The end-stage liver disease score was 24. PCR showed that His viral profile was HBsAg (+), anti-HBs (-), HBeAg (-), anti-HBe (+), anti-HBc (+) and HBV DNA < 10^3 copies/mL. He received 4 wk of lamivudine therapy.

Following our usual protocol for monitoring LT recipients, routine intraoperative transesophageal echocardiography was performed during LT[8]. The pulmonary artery was a moderately enlarged. His systolic PAP was higher than 53 mmHg, pulmonary capillary wedge pressure (PCWP) was 10 mmHg, cardiac output (CO) was 7.4 L/min), pulmonary vascular resistance (PVR, 201 dyn·s·cm^{-5}) was higher than the usual values observed in cirrhotic patients[9]. To reduce the increased PAP, the patient administrated nitric oxide (40 ppm) and epoprostenol (40 μg). However, no notable reduction was found in the mean PAP (mPAP). Epoprostenol therapy was continued with its dose increased from 6 to 20 ng/kg per min. One hundred percent of oxygen was then administered before liver dissection. Then the PAP came down slowly. LT was performed using the "piggyback technique", a standard procedure without venovenous bypass[8]. The anhepatic phase lasted 78 min and reperfusion was tolerated without any significant incident. Immediately after the liver was reperfused, PAP increased to a maximum of 75 mm Hg (60% of systemic level) and terribly reflow was present in the graft. About 10 min later, PAP decreased to less than 40% of systemic level (Table 1).The speed of dobutamine infusion was increased to 3-5 mg/kg per min for continuous inotropic and pulmonary vasodilator effect. The color of graft became normal after 30 min. PVR was only slightly increased, but the mean PAP was markedly increased due to a profound increase in cardiac output. After haemostasis, further surgery was completed uneventfully. At the end of surgery, haemodynamics of the patient was stable. The total operative time was 8 h. The estimated blood loss was 3346 mL, so that the patient was transfused 10 U of packed red blood cells and 30 U of fresh-frozen plasma. Immunosuppression was initiated with cyclosporin, mycophenolate mofetil, and steroid was withdrawn followed by maintenance of immunosuppression with cyclosporin and mycophenolate mofetil.

During the following 24 h in the intensive care unit (ICU), haemodynamics was mildly increased in PVR which was reduced when pulmonary vasodilators (nitroglycerin, isoprenaline, epoprostenol) were used. At this time, mPAP was 35 mmHg. The patient was extubated with dobutamine discontinued approximately 20 h after surgery. PaO_2 was 9.2 kPa, $PaCO_2$ was 6.2 kPa, and HCO_3^{-2} was 8 kPa (pH 7.36) after extubation. Graft function was remarkable following a continuous decrease in transaminases and prothrombin time (PT 50%). The post-transplant viral prophylaxis included HBIG (10 000 U/d) during the anhepatic period and lamivudine (100 mg/d) in the morning of postoperative day 1[10]. On the 7th postoperative day, the patient was transferred to the normal ward. Three weeks later, the liver function was recovered with normal transaminases and PT. In the follow-up, pulmonary function, chest X-ray and pulmonary angiography showed no evidence of pulmonary hypertension.

In January 2008, almost 2 years after LT, the patient was still in good condition and had no signs of pulmonary hypertension. A Doppler echocardiogram showed that the size right and left ventricles was normal. The viral profile of the patient was not good after transplantation with persistent positive HBsAg and negative anti-HBs and HBV DNA.

DISCUSSION

PPH is defined as mPAP ⩾ 25 mmHg and PCWP ⩽ 15 mmHg with evidence of portal hypertension. It was reported that 60% of patients with PPH are asymptomatic at the time of diagnosis, and symptoms associated with advanced liver disease and portal hypertension may be indistinguishable from those of PPH[3]. Therefore, the diagnosis of PPH is frequently established in the operating room before the transplantation procedure. Some of these patients may have mPAP exceeding 25 mmHg, but they do not have the associated pathological changes in the pulmonary vasculature that increase the mortality[11]. Some right heart catheterization information is derived from the operating room during general anesthesia[12]. Pulmonary pressure tends to increase during the initial stage of anesthesia. Our patient's pulmonary hypertension was atypical before operation and revealed pulmonary hypertension after inhaling anesthesia. Since inhalational anesthetic agents can independently increase venous return, central filling and cardiac output can be achieved in patients with portal hypertension[13].

Table 1 Intra- and post-transplantation cardiopulmonary hemodynamic data

Time	mPAP (mmHg)	PVR (dyn·s·cm^{-5})	Therapeutic agents
Before clamp	35	254	Epoprostenol, NO, 100% O_2
Anhepatic	40	340	Epoprostenol, 100% O_2
Reperfusion (15 min)	40	NA	Epoprostenol and milrinone, adrenaline
Enter ICU	30	NA	Epoprostenol
24 h-pro	35	240	Epoprostenol and isoprenaline
48 h-pro	30	211	Epoprostenol and isoprenaline

LT: Liver transplantation; mPAP: Mean pulmonary artery pressure; PCWP: Pulmonary capillary wedge pressure; PVR: Pulmonary vascular resistance; NO: Nitric oxide; NA: Not applicable.

Until now, the treatment options for patients with PPH are few, and the reported mean survival time of PPH patients after diagnosis is approximately 15 mo[14]. Nitric oxide, prostacyclin and isosorbide-5-mononitrate have been administered to improve PAP before and after LT[15]. Because of the association of portosystemic shunting with pulmonary hypertension, LT cannot cure both portal hypertension and PHT. LT is well tolerated in patients with only mild or moderate PPH. The mortality rates of patients with or without PPH after operation are comparable[16]. Since the risks in patients with severe PHT appear to be unacceptably high, and the affected patients deny LT, the marked reduction in PAP after epoprostenol therapy for patients with severe PPH may result in a marked decrease in perioperative mortality after LT[17]. PAP in this patient was steadily increased vasodilator therapy. This patient had a significant response to intravenous epoprostenol and the nitric oxide was also inhaled to test the reversibility of mPAP during operation. Continuous use of pulmonary vasodilators (isoprenaline, epoprostenol) reduced his PVR. Pulmonary hypertension may exacerbate after operation when patients receive no epoprostenol, and the patients may die of right ventricle failure[4]. Improvement in and de novo development of PPH may occur following LT[18-20]. The reason why transplant patients develop pulmonary hypertension following transplantation is unknown[13].

It was reported that transplant mortality is 36% (13/36) in patients with PPH[21]. Evidence from two studies suggests that preoperative mPAP is an independent predictor of mortality[6,22]. Another study showed that mPAP is not an accurate predictor of mortality and that pressure measurement does not serve as an independent surrogate of the severity of disease or outcome[23]. The marked difference in outcome suggests that single hemodynamic variables are not related with mortality in patients with PPH. In the literature, death of patients with PPH undergoing LT often occurs in the postoperative period due to cardiocirculatory stress or infectious complications[1,24,25]. Alternatively, defective hepatic metabolism and portosystemic shunting may expose the pulmonary vasculature to proliferating, vasoconstrictive, or inflammatory compounds that accelerate the progression of pulmonary arteriopathy or right ventricular failure[25]. Safer strategies for LT in patients with asymptomatic PPH can reduce the mortality rate. Our current strategies are as follows. (1) Right heart catheterization should be done pre-operation when PPH is suspected; (2) pulmonary vascular resistance should be decreased with intravenous epoprostenol therapy after diagnosis of PPH; (3) nitric oxide, epoprostenol, calcium channel blockers and other agents should be used during perioperation[26]; (4) venovenous bypass or piggy back technique that allows a more restrictive volume replacement during the anhepatic phase should be used.

In conclusion, asymptomatic PHT is not a contraindication to LT, and some of PHT patients may survive many years after LT. Patients with asymptomatic PHT who might benefit from LT need to be better characterized, and reliable screening methods to identify these patients need to be developed.

REFERENCES

1 **Kuo PC**, Plotkin JS, Gaine S, Schroeder RA, Rustgi VK, Rubin LJ, Johnson LB. Portopulmonary hypertension and the liver transplant candidate. *Transplantation* 1999; **67**: 1087-1093

2 **Himelman RB**, Stulbarg M, Kircher B, Lee E, Kee L, Dean NC, Golden J, Wolfe CL, Schiller NB. Noninvasive evaluation of pulmonary artery pressure during exercise by saline-enhanced Doppler echocardiography in chronic pulmonary disease. *Circulation* 1989; **79**: 863-871

3 **Robalino BD**, Moodie DS. Association between primary pulmonary hypertension and portal hypertension: analysis of its pathophysiology and clinical, laboratory and hemodynamic manifestations. *J Am Coll Cardiol* 1991; **17**: 492-498

4 **Ramsay MA**, Simpson BR, Nguyen AT, Ramsay KJ, East C, Klintmalm GB. Severe pulmonary hypertension in liver transplant candidates. *Liver Transpl Surg* 1997; **3**: 494-500

5 **Halank M**, Miehlke S, Hoeffken G, Schmeisser A, Schulze M, Strasser RH. Use of oral endothelin-receptor antagonist bosentan in the treatment of portopulmonary hypertension. *Transplantation* 2004; **77**: 1775-1776

6 **Kuntzen C**, Gulberg V, Gerbes AL. Use of a mixed endothelin receptor antagonist in portopulmonary hypertension: a safe and effective therapy? *Gastroenterology* 2005; **128**: 164-168

7 **Makisalo H**, Koivusalo A, Vakkuri A, Hockerstedt K. Sildenafil for portopulmonary hypertension in a patient undergoing liver transplantation. *Liver Transpl* 2004; **10**: 945-950

8 **Zhang F**, Lu L, Qian X, Pu LY, Li GQ, Wang XH. Liver transplantation for erythropoietic protoporphyria with hepatic failure: a case report. *Transplant Proc* 2008; **40**: 1774-1776

9 **Martini GA**, Baltzer G, Arndt H. Some aspects of circulatory

disturbances in cirrhosis of the liver. *Prog Liver Dis* 1972; **4**: 231-250
10 **Zhang F**, Wang XH, Li XC, Kong LB, Sun BC, Li GQ, Qian XF, Chen F, Wang K, Lu S, Pu LY, Lu L. Emergency adult living donor right lobe liver transplantation for fulminant hepatic failure. *Front Med China* 2007; **1**: 282-286
11 **Kim WR**, Krowka MJ, Plevak DJ, Lee J, Rettke SR, Frantz RP, Wiesner RH. Accuracy of Doppler echocardiography in the assessment of pulmonary hypertension in liver transplant candidates. *Liver Transpl* 2000; **6**: 453-458
12 **Colle IO**, Moreau R, Godinho E, Belghiti J, Ettori F, Cohen-Solal A, Mal H, Bernuau J, Marty J, Lebrec D, Valla D, Durand F. Diagnosis of portopulmonary hypertension in candidates for liver transplantation: a prospective study. *Hepatology* 2003; **37**: 401-409
13 **Mandell MS**, Durham J, Kumpe D, Trotter JF, Everson GT, Niemann CU. The effects of desflurane and propofol on portosystemic pressure in patients with portal hypertension. *Anesth Analg* 2003; **97**: 1573-1577
14 **Mandell MS**, Groves BM. Pulmonary hypertension in chronic liver disease. *Clin Chest Med* 1996; **17**: 17-33
15 **Ricci GL**, Melgosa MT, Burgos F, Valera JL, Pizarro S, Roca J, Rodriguez-Roisin R, Barbera JA. Assessment of acute pulmonary vascular reactivity in portopulmonary hypertension. *Liver Transpl* 2007; **13**: 1506-1514
16 **Plevak D**, Krowka M, Rettke S, Dunn W, Southorn P. Successful liver transplantation in patients with mild to moderate pulmonary hypertension. *Transplant Proc* 1993; **25**: 1840
17 **Mair P**, Kaehler CH, Pomaroli A, Schwarz B, Vogel W, Margreiter R. Orthotopic liver transplantation in a patient with severe portopulmonary hypertension. *Acta Anaesthesiol Scand* 2001; **45**: 513-518
18 **Koneru B**, Ahmed S, Weisse AB, Grant GP, McKim KA. Resolution of pulmonary hypertension of cirrhosis after liver transplantation. *Transplantation* 1994; **58**: 1133-1135
19 **Rafanan AL**, Maurer J, Mehta AC, Schilz R. Progressive portopulmonary hypertension after liver transplantation treated with epoprostenol. *Chest* 2000; **118**: 1497-1500
20 **Koneru B**, Fisher A, Wilson DJ, Klein KM, delaTorre AN, Seguel J. De novo diagnosis of portopulmonary hypertension following liver transplantation. *Am J Transplant* 2002; **2**: 883-886
21 **Krowka MJ**, Mandell MS, Ramsay MA, Kawut SM, Fallon MB, Manzarbeitia C, Pardo M Jr, Marotta P, Uemoto S, Stoffel MP, Benson JT. Hepatopulmonary syndrome and portopulmonary hypertension: a report of the multicenter liver transplant database. *Liver Transpl* 2004; **10**: 174-182
22 **Krowka MJ**, Plevak DJ, Findlay JY, Rosen CB, Wiesner RH, Krom RA. Pulmonary hemodynamics and perioperative cardiopulmonary-related mortality in patients with portopulmonary hypertension undergoing liver transplantation. *Liver Transpl* 2000; **6**: 443-450
23 **Starkel P**, Vera A, Gunson B, Mutimer D. Outcome of liver transplantation for patients with pulmonary hypertension. *Liver Transpl* 2002; **8**: 382-388
24 **Kuo PC**, Plotkin JS, Johnson LB, Howell CD, Laurin JM, Bartlett ST, Rubin LJ. Distinctive clinical features of portopulmonary hypertension. *Chest* 1997; **112**: 980-986
25 **Kawut SM**, Horn EM, Berekashvili KK, Garofano RP, Goldsmith RL, Widlitz AC, Rosenzweig EB, Kerstein D, Barst RJ. New predictors of outcome in idiopathic pulmonary arterial hypertension. *Am J Cardiol* 2005; **95**: 199-203
26 **Austin MJ**, McDougall NI, Wendon JA, Sizer E, Knisely AS, Rela M, Wilson C, Callender ME, O'Grady JG, Heneghan MA. Safety and efficacy of combined use of sildenafil, bosentan, and iloprost before and after liver transplantation in severe portopulmonary hypertension. *Liver Transpl* 2008; **14**: 287-291

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CASE REPORT

Pregnancy after liver transplantation: Four-year follow-up of the first case in mainland China

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Abstract

The safety and feasibility of pregnancy following liver transplantation (LT) have been accredited in a series of LT center. The first case in mainland China is reported. The follow-up data of a 22-year-old pregnant patient with end-stage liver disease undergone orthotopic liver transplantation were analyzed retrospectively. After surgery, the patient was uneventfully recovered and became pregnant 33 mo after LT. The patient was closely monitored and treated with a standard and individualized triple-drug immunosuppressive therapy throughout her pregnancy. Caesarean section was performed in March 18, 2004, and a health live-born infant was delivered. After the delivery, a 4-year follow-up period indicated that the patient was satisfactory with her condition and her baby was healthy. Our case shows that a successful pregnancy following LT is possible and safe in women with end-stage liver diseases under close monitoring. Three factors including mother, baby, and transplanted liver function must be considered for the safety of high-risk pregnancies.

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Key words: Pregnancy; Liver transplantation; Follow-up

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INTRODUCTION

Liver transplantation (LT) is now an acceptable therapy for patients with end-stage liver disease. Among patients undergoing LT, approximately 11% are women at the reproductive age[1]. Within one year after a successful LT, 97% of female patients at the childbearing age recover menstrual function owing to the restored hepatic estrogen metabolism, and the likelihood of conception and successful pregnancy is high[2]. The first known post-transplantation pregnancy in a LT recipient was reported in 1978. Since then, a series of cases have been reported worldwide[3,4]. We report the first case in mainland China.

CASE REPORT

A 22-year-old nulliparous woman, with a 4-year-history of persistent right upper quadrant pain, intermittent jaundice, and fever, was admitted to our hospital in September 2000. She had no particular family or genetic history, and underwent a cholecystostomy for acute cholangitis four months ago. The serum levels of total bilirubin (TB), direct bilirubin (DB), γ-glutamyl transferase (γ-GT) and alkaline phosphatase (ALP), and white blood cell (WBC) count were markedly increased. Microbiologic culture of blood demonstrated that *Escherichia coli* were the responsible bacteria in the patient. Alpha-fetoprotein (AFP) and viral markers for hepatitis A, B, and C were negative. B-type ultrasonography and computerized tomography (CT) showed a diffuse abscess and cirrhotic nodules in the shrunken liver. Therefore, she was diagnosed having primary biliary cirrhosis, sclerosing cholangitis, bacteremia, and bacterial liver abscess.

On September 28, 2000, after having been treated effectively, she underwent a successful orthotopic LT using conventional techniques with a veno-venous bypass. The donor was a 19-year-old brain-dead healthy female caused by head trauma and her family consented to her

organ donation. The overall length of the OLT procedure was 530 min. The duration of anhepatic phase and venous bypass was 97 min and 73 min, respectively. The anastomosis time for suprahepatic vena cava, infrahepatic vena cava, and portal vein was 23, 13, and 15 min, respectively. Standard proximal end-to-end anastomosis between donor's common hepatic artery and recipient's proper hepatic artery was performed under magnification, which lasted 22 min. Choledochojejunostomy was carried out with a 40-cm-long Roux-en-Y jejunal limb. A total of 3600 mL blood was transfused and the volume of blood loss was 1400 mL. Intraoperative findings correlated well with CT and B ultrasound findings. The postoperative pathological diagnoses were biliary cirrhosis, sclerosing cholangitis, and liver abscess.

The recipient had an uneventful recovery in the postoperative period. She gained complete consciousness 1 h after LT, received extubation 10 h later, and was discharged on post-LT day 22. Normal menstrual cycles were observed (restored) 3 mo later. After operation, the patient received a standard triple-drug immunosuppressive therapy, including cyclosporine A (CsA), mycophenolate mofetil (MMF), and prednisone. The dose of each agent was individualized according to the plasma drug concentration. Three months after operation, MMF was discontinued, and prednisone and CsA were maintained. One year later, prednisone was discontinued, and CsA was maintained alone. Twenty-six months later, she had a pregnancy and also an unstable graft. By agreement between the patient and physicians, pregnancy was terminated at 22 wk of gestation. Thirty months after LT, she became pregnant for the second time. During the first trimester of this pregnancy, she presumed to discontinue CsA because of serious pregnancy reaction without physicians' permission. The sharply increased serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), TB, DB, and ALP were suggestive of rejection. She was hospitalized then and treated with a daily regimen of 1 g of MMF, in addition to 450 mg of CsA. Despite the argument for MMF use during pregnancy, the patient and physicians decided to continue the pregnancy with regular ultrasound examinations. Two months later, she recovered with normal liver function and the fetus developed well. Her serum level of CsA remained within the normal limit (100-150 mg/L).

On March 18, 2004, the mother patient at 37 weeks' gestation gave birth by Cesarean section to a boy weighing 2000 g and measuring 42 cm in length. The patient's postpartum course was uneventful with normal liver and renal functions and she was discharged on the 12th d after Caesarean section. However, the newborn's 1- and 5-min agar scores were 5 and 7, respectively. Although without any congenital malformation, he was diagnosed having hypoxic ischemic encephalopathy and neonatal pneumonia. His immune functions were examined on the first and seventh days after birth, showing that the baby was in a severe immunosuppressive state. After proper and careful treatment, the newborn was discharged in good health on the 22nd d after birth. The child demonstrated normal growth and development during the 4-year follow-up period.

Once a major surgery like LT is performed, a lifelong follow-up is required. After the postpartum course, a series of investigations were followed for the mother patient and her male baby routinely according to the tentative protocol of West China LT center. Subsequent visits were scheduled at each month (the first six months after LT), every 2 mo (6-12 mo after LT), every 3 mo (the 2nd year) and every 3-6 mo (the 3rd year and thereafter). During these visits, routine blood, liver and kidney function, blood concentration of immunosuppressant, hepatitis B or C, and ultrasound scans and X-rays would be regularly undertaken. As an outpatient, she was recommended for a series of notices including self-examination of appetite, abdomen, stool, urine, skin, strength, energy, *etc*, self-protection from infection, house environment, working environment, keeping fine spirit and proper exercise, relation with the family members, sexual intercourse with her husband and use of contraception, self-examination of her baby's growth and development, necessary communications with doctors, and regular follow-up. The major content of follow-up at the late-stage post-LT included the monitoring of immunosuppressant concentration, new graft function, discovery of biliary and vascular complications, primary disease recurrency, rejection, and side effects of drugs. Currently, at 49 mo follow-up, the recipient and her son lived well with normal liver allograft function during the follow-up at 49 mo.

DISCUSSION

LT is considered an ultimate life-saving measure for patients with severe liver dysfunction and may provide patients with the best chance for long-term disease-free survival. Successful grafting offers the patient gross improvement in life quality, maximized rehabilitation, and restores the chance for parenthood to most recipients at their reproductive age. Reproductive function and sexuality, which are important components of quality of life, are commonly affected by end-stage liver disease. Shortly after successful LT, most female recipients at childbearing age normalize their menstrual function, thus leading to pregnancy[5].

The number of pregnancies following LT is increasing due to the greater survivals associated with constantly improved management and immunosuppressive regimens. Although pregnancy after LT does not seem to have obviously deleterious effects on allograft function or survival, complications can occur both in mothers and infants. Pregnancies in transplant recipients appear to be associated with increased health risks, including ectopic pregnancy, hypertension and pre-eclampsia, infection, and need for delivery by Cesarean section. The incidence of prematurity, intrauterine growth retardation and malformation is relatively high in newborns at the gestationalage age (SGA), irrespective of the type of immunosuppressive therapy used. The severe risks are mainly related to the fact that transplant recipients must continue immunosuppressive therapy throughout their pregnancy[6].

Most immunosuppressive agents (e.g. azathioprine, cyclosporine, and MMF) have been found to be teratogenic in animals. However, the frequency of birth defects in infants born to women receiving immunosuppressive agents is not statistically different from that in the general population[7]. Long-term or late-onset medical complications of immunosuppressive therapy for diseases (e.g. autoimmune disease, cancer, and infertility) are as yet unknown. Immunosuppressive agents can cross the placenta and result in a severe immunosuppressive state in the newborn. Right on the day of delivery, the concentration of CsA in the umbilical blood is 14.1 μg/L (75 -175 μg/L in adults). Cyclosporine is known to cause endothelial cell dysfunction and reduced nitric oxide production, which may be associated with cell dysfunction and hypertension. Tacrolimus-based immunosuppressive regimens are associated with a much lower incidence of new onset of hypertension and toxemia of pregnancy. Report from the National Transplantation Pregnancy Registry (NTPR) demonstrated that there was not any congenital malformation observed in the 5 off springs born to women receiving MMF[2]. So, during pregnancy, female recipients may continue their immunosuppressive medications to stabilize the allograft function. On the other hand, during pregnancy, increased plasma volume and adipose tissue and higher hormone concentrations lead to great changes in drug metabolism. Thus, the levels of immunosuppressive agents must be carefully monitored to individually establish therapeutic doses[3]. Our results are consistent with these findings.

Few data are available on breast-feeding by mothers taking immunosuppressive drugs. Whether the risks of exposure to immunosuppression through breast milk outweigh, the benefits of breast-feeding remain unknown. Our patient did not breast feed her baby because she was on CsA at a dose of 150 mg one day.

Pregnancy does not appear to increase the risk of graft rejection or impairment. In this case, a rejection episode occurred once at 8 wk gestation, and was believed to be related to the patient's willful discontinuation of immunosuppressive therapy. Reduction of immunosuppressive medications during pregnancy, based on the premise of natural nonspecific maternal immunosuppression, may lead to rejection of transplanted organs, which can be successfully managed with adjustment of immunosuppressive medications.

It was reported that the rate of Cesarean deliveries among LT recipients is higher than that of the general population[8]. In this case, Cesarean section was performed just for obstetric reasons, because we had not any experience with the management of such a patient.

All pregnancies in solid-organ-transplant recipients should be considered at high risk associated with hypertension, preeclampsia, intrauterine growth retardation and prematurity, and managed by a multidisciplinary team. The expectant mother should be closely monitored (at least every 2 wk) by her transplantation physicians and her prenatal care should preferentially be managed by a specialist in high-risk obstetrics (maternal-fetal medicine). Nagy *et al*[2] reported that the interval from transplantation to pregnancy was shorter in the group that had abortions and terminations (24.4 ± 24.3 mo) than in the group that had live births (47.8 ± 28.7 mo). NTPR has advised female organ transplant recipients to wait 1 or 2 years after transplantation before attempting to conceive to insure that the transplanted organ is functioning adequately and to allow for stabilization of the immunosuppressive regimen[7,9]. We strongly advocate delaying conception for at least 24 mo after LT. In reproductive-age women, who have undergone a liver transplant, physicians need to be proactive in discussing fertility issues and should counsel their patients regarding the timing of pregnancy, as well as the risks and alternatives. Moreover, physicians should respect the decision that each recipient makes about the risks and benefits. In this case, till now, the excellent maternal and neonatal outcome was a result of perfect communication and cooperation between the patient and the multidisciplinary team of our LT center. To the present, two hundred and two pregnancies and 205 outcomes have been reported in 121 LT recipients in NTPR[4]. To our knowledge, this present case is the first successful pregnancy of woman after LT in mainland China.

In conclusion, pregnancies among liver-transplanted female recipients are generally regarded at high risk, but do not seem to have a deleterious effect on graft function or survival. Mother, baby, and transplanted liver function must be considered for the safety of these high-risk pregnancies.

REFERENCES

1 **Jain AB**, Reyes J, Marcos A, Mazariegos G, Eghtesad B, Fontes PA, Cacciarelli TV, Marsh JW, de Vera ME, Rafail A, Starzl TE, Fung JJ. Pregnancy after liver transplantation with tacrolimus immunosuppression: a single center's experience update at 13 years. *Transplantation* 2003; **76**: 827-832

2 **Nagy S**, Bush MC, Berkowitz R, Fishbein TM, Gomez-Lobo V. Pregnancy outcome in liver transplant recipients. *Obstet Gynecol* 2003; **102**: 121-128

3 **Armenti VT**, Radomski JS, Moritz MJ, Philips LZ, McGrory CH, Coscia LA. Report from the National Transplantation Pregnancy Registry (NTPR): outcomes of pregnancy after transplantation. *Clin Transpl* 2000; 123-134

4 **Surti B**, Tan J, Saab S. Pregnancy and liver transplantation. *Liver Int* 2008; **28**: 1200-1206

5 **Armenti V**T, Herrine SK, Radomski JS, Moritz MJ. Pregnancy after liver transplantation. *Liver Transpl* 2000; **6**: 671-685

6 **Ourahma S**, Sylla C, Barrou B, Mouquet C, Luciani J, Tallier M, Coriat P, Bitker MO. Future of children of transplanted mothers: results of a multicenter study. *Transplant Proc* 1998; **30**: 2808

7 **Armenti VT**, Radomski JS, Moritz MJ, Gaughan WJ, Philips LZ, McGrory CH, Coscia LA. Report from the National Transplantation Pregnancy Registry (NTPR): outcomes of pregnancy after transplantation. *Clin Transpl* 2002; 121-130

8 **Jabiry-Zieniewicz Z**, Bobrowska K, Pietrzak B, Kaminski P, Wielgos M, Durlik M, Zieniewicz K. Mode of delivery in women after liver transplantation. *Transplant Proc* 2007; **39**: 2796-2799

9 **Riely CA**. Contraception and pregnancy after liver transplantation. *Liver Transpl* 2001; **7**: S74-S76

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CASE REPORT

Undifferentiated embryonal sarcoma of liver in an old female: Case report and review of the literature

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Abstract

The clinical characteristics of undifferentiated (embryonal) sarcoma of the liver (UESL) were investigated and the best treatment modalities were recommended. Both histology and immuno-histochemistry demonstrated the cellular features of this peculiar tumor. The tumor size was 12 cm × 9 cm × 8 cm in the right liver lobe. The patient underwent surgical resection of the tumor. The postoperative recovery was uneventful and she died eight months after diagnosis. The tumor showed mixed spindle and polygonal cells within the myxoid matrix. Some tumor cells contained eosinophilic hyaline globules that were positive for resistant diastase. Immunohistochemistry showed positive vimentin. Stellate and spindle cells were positively stained with alpha-1-antichymotrypsin (AACT) and CD68. This case indicates that UESL is not obviously differentiated in old-aged adults.

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Key words: Undifferentiated (embryonal) sarcoma; Old-aged adult; Immunohistochemistry; Liver tumor

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Ma L, Liu YP, Geng CZ, Tian ZH, Wu GX, Wang XL. Undifferentiated embryonal sarcoma of liver in an old female: Case report and review of the literature. *World J Gastroenterol* 2008; 14(47): 7267-7270 Available from: URL: http://www.wjgnet.com/1007-9327/14/7267.asp DOI: http://dx.doi.org/10.3748/wjg.14.7267

INTRODUCTION

Undifferentiated (embryonal) sarcoma of the liver (UESL), first documented in 1978, is a rare and highly malignant hepatic neoplasm of mesenchymal origin and shows a divergent differentiation[1,2]. Although UESL is considered a relatively major entity in pediatric liver malignancies, its frequency in the adult population is extremely low. In fact, few reports have focused on the general features of adult cases[3]. Given that the majority of the populations are under the age of 30 years, adult cases over the age of 40 years are quite exceptional. Furthermore, the detailed pathological characteristics of adult cases based on particular immunohistochemistry are not yet clear. To our knowledge, no study has described the systemic pathology features of UESL. In the present study, we describe a UESL patient at the age of 61 years with no obvious mesenchymal differentiation such as smooth muscle phenotype.

CASE REPORT

A 61-year-old woman was admitted in November 2007 to the Department of Hepatobiliary Surgery because of a huge abdominal mass, located in the right hypochondrium. Her major complaint was abdominal pain. An abdominal ultrasound showed a lesion in the right liver lobe, which was avascular on angiographic examination, and subsequently confirmed by CT scan (Figure 1). However, her serum alpha-fetoprotein (AFP) level was normal. No pulmonary metastases were detected by chest X-ray examination. Medical and family histories were unremarkable, and the patient reported

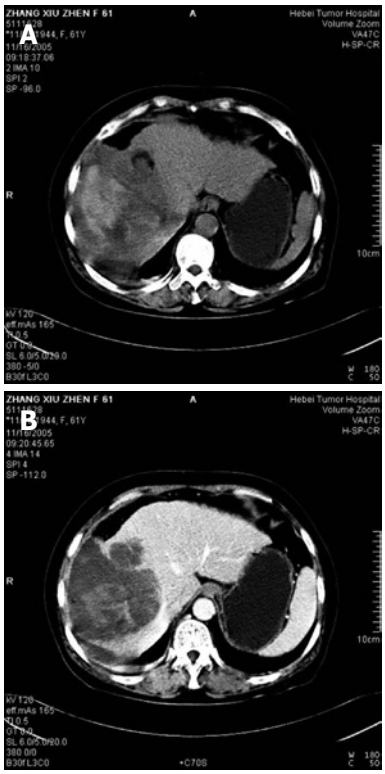

Figure 1 CT scan befor surgery showing a large mass (A) and a well-circumscribed and lowly-attenuated mass (B) in the right liver lobe.

no contact with any hepatitis virus carrier. Right hepatic lobectomy and cholecystectomy were performed at a laparatomy. The postoperative period was uneventful, no adjuvant irradiation or chemotherapy was administered. The patient died after 8 mo.

A mass measuring 12 cm × 9 cm × 8 cm was found in the right liver lobe. The tumor was well circumscribed and demarcated from the normal liver tissue. The cut surface revealed a white or yellowish soft mass with massive necrosis, hemorrhage and degeneration.

For light microscopy, fresh tissue was fixed in 10% formalin and embedded in paraffin. The tissue was cut into 4-μm thick sections which were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) with and without diastase. The histopathological features of the neoplasm are presented in Figures 2 and 3. The primary tumor showed proliferation of atypical spindle cells mixed with stellated cells. These cells had obvious heteromorphism and were irregularly arranged. In some areas, the cells were differentiated into epithelium and had light eosinophilic cytoplasm. Cell membrane was fuzzy (Figure 2A). Some tumor cells contained eosinophilic hyaline globules, which were resistant to diastase and positive for PAS (Figure 2D, Figure 3B). Some ducts lined by a single layer of cuboidal cells resembling normal bile ducts were found in the tumor periphery (Figure 2B and C). Lymphocytes and plasma cells infiltrated around many thin-walled vessels in the background component of the tumor.

Immunohistochemical and alkaline phosphatase staining was performed using a labeled streptavidin biotin system. Primary antibodies such as broad-spectrum vimentin, desmin, alpha-smooth muscle actin (SMA), muscle-specific actin, neuronspecific enolase (NSE), HMB-45, CD68, CD34, factor Ⅷ, epithelial membrane antigen (EMA) and cytokeratins AE1/AE3 (Dako, Kyoto, Japan) were used. Immunostained sections were graded using a sliding scale of 1+ to 4+ according to the percentage of reactive cells (-: negative; 1+: < 10%; 2+: 10%-25%; 3+: > 25%-50%; 4+: > 50%). The immunohistochemical findings are summarized in Table 1. Most tumor cells were strongly reactive with vimentin (Figure 3A). Some stellate and spindle cells showed a granular cytoplasmic positivity for AACT and CD68. Lysozyme, alpha-sarcomeric actin, myoglobin, SMA, NSE, neurofilament, S-100 protein, HMB-45, CD34, factor Ⅷ, EMA, AE1/AE3 and AFP were negatively stained. The tumor was diagnosed as an undifferentiated embryonal sarcoma (malignant mesenchymoma).

Table 1 The immunohistochemical findings

Antibody	Expression
Vimentin	4+
AACT	1+
CD68	2+
Lysozyme	1+
α-sarcomeric actin	-
Myoglobin	-
SMA	-
NSE	-
Factor Ⅷ	-
EMA	-
AE1/AE3	-
AFP	-
HMB-45	-
S-100	-
CD34	-

DISCUSSION

UESL has a predilection for children and young adults in the first 2 decades of their life. To our knowledge, few cases have been reported in patients over the age of 40 years[3]. The overall clinical outcome is poor with a long-term disease-free survival rate of less than 30% in all series. In 2 other series, the outcome was better[4]. In the first series of 9 patients, 4 were alive with no evidence of disease, whereas in the second series, 3 out of 4 patients were alive with no evidence of disease[4]. In all series, the long-term survival was achieved only in those whose tumors could be resected completely.

According to the literature, the principal pathological features of UESL in children include an expansive intrahepatic growth with massive necrosis, hemorrhage, and occasional gelatinous appearance[1,2]. Laboratory evaluations including alfa-fetoprotein showed no specific abnormality. Radiologic characteristics of USL are characterized by enhanced peripheral rim, some solid portions at the periphery or adjacent to the septa, and discrepancy in internal architecture between computed tomography (CT) and ultrasound scan[5].

UESL is histologically surrounded by a fibrous

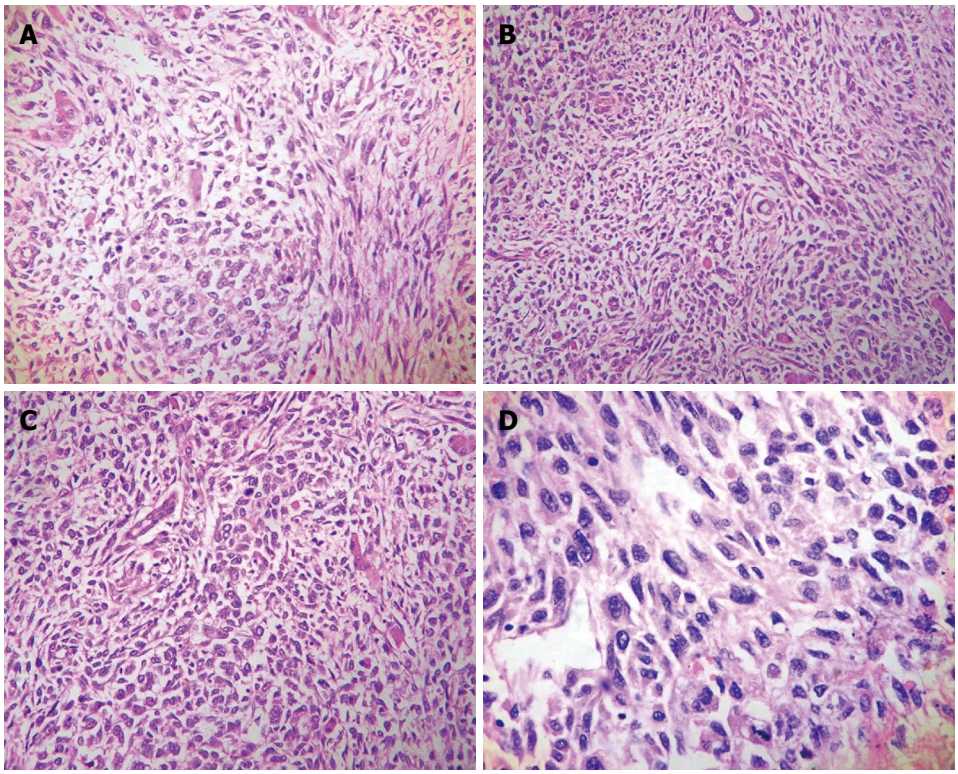

Figure 2 UESL. A: Atypical spindle cells mixed with stellated cells (HE, x 200); B: Normal bile ducts in the tumor (HE, x 200); C: Normal hepatic cells in the tumor (HE, x 200); D: Giant cells containing eosinophilic hyaline globules in the cytoplasm (HE, x 400).

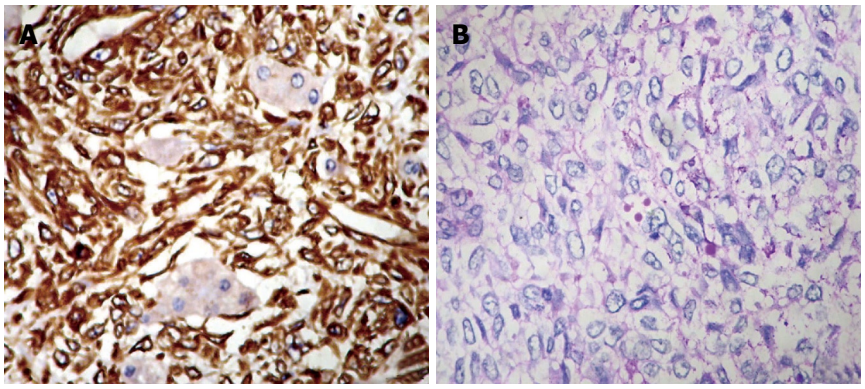

Figure 3 Immunohistochemical expression of UESL. A: Strong reactivity for vimentin in spindle and polygonal or round cells (streptavidin-biotin method, x 200); B: Tumor cells containing eosinophilic hyaline globules with positive PAS.

pseudocapsule with a slightly direct invasion of the adjacent hepatic parenchyma. The cellular component is composed of medium-large spindle or satellite cells with marked nuclear pleomorphism. At the periphery, trapped hepatocytes and abnormal bile duct cells are apparent[6]. In addition, there are prominent eosinophilic, PAS-positive, and diastase-resistant globules in the cytoplasm of tumor cells[1,7]. Generally, immunohistochemistry using various cell-specific markers reveals that UESL is highly positive for histiocytic markers such as AACT and CD68, and vimentin is commonly positive. A few case reports demonstrate that tumor cells are reactive to cytokeratin, S-100 protein, and NSE[1,7,8-11]. In our case, both vimentin and histiocytic markers were positive, whereas cytokeratins, S-100 protein, and NSE were negative. In some cases, the smooth muscle differentiation was confirmed by the positivity of desmin and alpha-SMA. However, our case did not exhibit any myogenic features. The differential diagnosis of UESL in old-aged adults includes malignant fibrous histiocytoma (MFH), leiomyosarcoma, liposarcoma, and angiomyolipoma (AML). MFH is characterized by fibroblastic, histiocytic, and pleomorphic tumor cells forming a storiform pattern. In the present study, the tumor was basically composed of primitive mesenchymal cells with a divergent differentiation toward fibroblastic, histiocytic lines. Although these cellular compositions are quite similar to those of MFH[7,12], it is possible to distinguish UESL from MFH on the basis of tissue pattern[13]. In our case, the diagnosis of leiomyosarcoma was inappropriate, because the tumor did not show any variable size of the foci and any fascicular pattern of the brightly eosinophilic spindle cells in hematoxylin, and SMA was negative. Lipoblastic differentiation has been suggested by some investigators on the basis of oil red O staining, and cells resembling lipoblasts seen at light microscopy[14]. However, no multivacuolated lipoblasts were observed in our case under light microscope, and the tumor cells were negative for S-100 protein. AML is characteristically composed of a varying heterogeneous mixture of 3 tissue components: blood vessels, smooth muscle cells, and adipose cells. However, the proportions

of the 3 tissue components vary markedly from case to case and from area to area within the same tumor[15]. AML with spindle cells and pleomorphic features may be misdiagnosed as various sarcomas, including MFH, leiomyosarcoma, dedifferentiated liposarcoma, and undifferentiated sarcoma[16-20]. Recent studies indicate that HMB-45 is a promising marker for AML and can facilitate differentiation of this tumor from other lesions[20-22]. In our case, the tumor cells were negative for HMB-45. Whenever pathologists encounter spindle cell tumors in the liver, immunostaining with HMB-45 should be performed to distinguish AML from other neoplasms.

The prognosis of UESL is poor. Even after complete resection of the tumor, few UESL patients can achieve a long-term, disease-free survival. Our patient died 8 mo after the tumor resection. Since 1990, long-term survivors after multiagent chemotherapy have been reported and their outcome appears to have improved substantially over the last decades[23,24]. The chance of cure depends on radical resection and vigorous multiple approaches including chemotherapy[23,24]. We hold that multiagent chemotherapy after resection can significantly improve the survival and the identified pathologic changes may offer important clues to the pathogenesis and development of UESL and other pediatric mesenchymal tumors.

REFERENCES

1 **Stocker JT**, Ishak KG. Undifferentiated (embryonal) sarcoma of the liver: report of 31 cases. *Cancer* 1978; **42**: 336-348
2 **Lack EE**, Schloo BL, Azumi N, Travis WD, Grier HE, Kozakewich HP. Undifferentiated (embryonal) sarcoma of the liver. Clinical and pathologic study of 16 cases with emphasis on immunohistochemical features. *Am J Surg Pathol* 1991; **15**: 1-16
3 **Psatha EA**, Semelka RC, Fordham L, Firat Z, Woosley JT. Undifferentiated (embryonal) sarcoma of the liver (USL): MRI findings including dynamic gadolinium enhancement. *Magn Reson Imaging* 2004; **22**: 897-900
4 **Leuschner I**, Schmidt D, Harms D. Undifferentiated sarcoma of the liver in childhood: morphology, flow cytometry, and literature review. *Hum Pathol* 1990; **21**: 68-76
5 **Moon WK**, Kim WS, Kim IO, Yeon KM, Yu IK, Choi BI, Han MC. Undifferentiated embryonal sarcoma of the liver: US and CT findings. *Pediatr Radiol* 1994; **24**: 500-503
6 **Walker NI**, Horn MJ, Strong RW, Lynch SV, Cohen J, Ong TH, Harris OD. Undifferentiated (embryonal) sarcoma of the liver. Pathologic findings and long-term survival after complete surgical resection. *Cancer* 1992; **69**: 52-59
7 **Aoyama C**, Hachitanda Y, Sato JK, Said JW, Shimada H. Undifferentiated (embryonal) sarcoma of the liver. A tumor of uncertain histogenesis showing divergent differentiation. *Am J Surg Pathol* 1991; **15**: 615-624
8 **Miettinen M**, Kahlos T. Undifferentiated (embryonal) sarcoma of the liver. Epithelial features as shown by immunohistochemical analysis and electron microscopic examination. *Cancer* 1989; **64**: 2096-2103
9 **Leuschner I**, Schmidt D, Harms D. Undifferentiated sarcoma of the liver in childhood: morphology, flow cytometry, and literature review. *Hum Pathol* 1990; **21**: 68-76
10 **Parham DM**, Kelly DR, Donnelly WH, Douglass EC. Immunohistochemical and ultrastructural spectrum of hepatic sarcomas of childhood: evidence for a common histogenesis. *Mod Pathol* 1991; **4**: 648-653
11 **Weiss SW**, Enzinger FM. Malignant fibrous histiocytoma: an analysis of 200 cases. *Cancer* 1978; **41**: 2250-2266
12 **Keating S**, Taylor GP. Undifferentiated (embryonal) sarcoma of the liver: ultrastructural and immunohistochemical similarities with malignant fibrous histiocytoma. *Hum Pathol* 1985; **16**: 693-699
13 **Yamamoto I**, Oshiro Y, Fukuda T, Tsuneyoshi M. Pleomorphic leiomyosarcoma of the soft parts: a reassessment by histology and immunohistochemistry of pleomorphic soft tissue sarcomas. *Oncol Rep* 1999; **6**: 533-537
14 **Cozzutto C**, De Bernardi B, Comelli A, Soave F. Malignant mesenchymoma of the liver in children: a clinicopathologic and ultrastructural study. *Hum Pathol* 1981; **12**: 481-485
15 **Nonomura A**, Mizukami Y, Kadoya M, Matsui O, Shimizu K, Izumi R. Angiomyolipoma of the liver: Its clinical and pathological diversity. *J Hepatobiliary Pancr Surg* 1996; **3**: 122-132
16 **Kimura N**, Kubota M, Nagura H. A hepatic tumor associated with bilateral renal angiomyolipomas: a variant of angiomyolipoma? *Pathol Int* 1994; **44**: 540-547
17 **Nonomura A**, Mizukami Y, Muraoka K, Yajima M, Oda K. Angiomyolipoma of the liver with pleomorphic histological features. *Histopathology* 1994; **24**: 279-281
18 **Nonomura A**, Mizukami Y, Shimizu K, Kadoya M, Matsui O. Angiomyolipoma mimicking true lipoma of the liver: report of two cases. *Pathol Int* 1996; **46**: 221-227
19 **Salisbury JR**, Portmann BC. Oncocytic liver cell adenoma. *Histopathology* 1987; **11**: 533-539
20 **Tsui WM**, Yuen AK, Ma KF, Tse CC. Hepatic angiomyolipomas with a deceptive trabecular pattern and HMB-45 reactivity. *Histopathology* 1992; **21**: 569-573
21 **Pea M**, Bonetti F, Zamboni G, Martignoni G, Riva M, Colombari R, Mombello A, Bonzanini M, Scarpa A, Ghimenton C. Melanocyte-marker-HMB-45 is regularly expressed in angiomyolipoma of the kidney. *Pathology* 1991; **23**: 185-188
22 **Weeks DA**, Malott RL, Arnesen M, Zuppan C, Aitken D, Mierau G. Hepatic angiomyolipoma with striated granules and positivity with melanoma--specific antibody (HMB-45): a report of two cases. *Ultrastruct Pathol* 1991; **15**: 563-571
23 **O'Sullivan MJ**, Swanson PE, Knoll J, Taboada EM, Dehner LP. Undifferentiated embryonal sarcoma with unusual features arising within mesenchymal hamartoma of the liver: report of a case and review of the literature. *Pediatr Dev Pathol* 2001; **4**: 482-489
24 **Walker NI**, Horn MJ, Strong RW, Lynch SV, Cohen J, Ong TH, Harris OD. Undifferentiated (embryonal) sarcoma of the liver. Pathologic findings and long-term survival after complete surgical resection. *Cancer* 1992; **69**: 52-59

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Differentiation of Behcet's disease from inflammatory bowel diseases: Anti-saccharomyces cerevisiae antibody and anti-neutrophilic cytoplasmic antibody

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Abstract

The differential diagnosis of Behcet's disease (BD) from inflammatory bowel disease (IBD) is sometimes difficult and challenging. Hereby, we suggested the utility of anti-saccharomyces cerevisiae antibody (ASCA) and anti-neutrophilic cytoplasmic antibody (p-ANCA) in the differential diagnosis of BD from IBD.

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Key words: Anti-neutrophilic cytoplasmic antibody; Anti-saccharomyces cerevisiae antibody; Behcet's disease

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TO THE EDITOR

The clinical diagnosis of Behcet's disease (BD) may pose considerable difficulties. Since the disease is multisystemic and does not have any pathognomonic symptom or laboratory findings, the diagnosis is based on a group of clinical features (oral, genital, skin or ocluar lesions)[1,2]. Gastrointestinal manifestation is relatively common in Japanese patients with BD (50%-60%), and the rate is significantly lower in Turkish and American population (5% and 8%, respectively)[3,4]. Similarly, accurate diagnosis of inflammatory bowel diseases (IBD) is very important. Also, because the treatment strategies in Crohn's disease (CD) and ulcerative colitis (UC) differ, especially when surgery is required, much effort has been expended over the years to distinguish some cases. Non-invasive tests are expected to display a crucial role in the differential diagnosis. The presence of a relatively specific laboratory marker can substantially facilitate the diagnosis of BD, and possibly support a diagnosis before all disease manifestations have occurred. Serum was obtained from 18 patients with BD whose diagnosis was fulfilled with the criteria of Behcet's Disease International Study Group[5]. Both anti-saccharomyces cerevisiae antibody (ASCA) and anti-neutrophilic cytoplasmic antibody (p-ANCA) were measured with indirect immunofluorescence assay. None of the patients was ASCA or p-ANCA positive.

ASCA is more frequently found in CD patients (50%-80%) compared to patients with UC (2%-14%)[6]. ANCA is present in the sera of 60%-80% of the patients with UC and approximately 10%-30% of patients with CD[6]. Since none of the patients in this study was ASCA or p-ANCA positive, we might conclude that both serologic tests may aid in the differential diagnosis of IBD and BD.

REFERENCES

1 **Shimizu T**, Ehrlich GE, Inaba G, Hayashi K. Behcet disease (Behcet syndrome). *Semin Arthritis Rheum* 1979; **8**: 223-260
2 **Rhee SH**, Kim YB, Lee ES. Comparison of Behcet's disease and recurrent aphthous ulcer according to characteristics of gastrointestinal symptoms. *J Korean Med Sci* 2005; **20**: 971-976
3 **Yurdakul S**, Tuzuner N, Yurdakul I, Hamuryudan V, Yazici H. Gastrointestinal involvement in Behcet's syndrome: a controlled study. *Ann Rheum Dis* 1996; **55**: 208-210
4 **Balabanova M**, Calamia KT, Perniciaro C, O'Duffy JD. A study of the cutaneous manifestations of Behcet's disease in patients from the United States. *J Am Acad Dermatol* 1999; **41**: 540-545
5 **Criteria for diagnosis of Behcet's disease**. International Study Group for Behcet's Disease. *Lancet* 1990; **335**: 1078-1080
6 **Papp M**, Norman GL, Altorjay I, Lakatos PL. Utility of serological markers in inflammatory bowel diseases: gadget or magic? *World J Gastroenterol* 2007; **13**: 2028-2036

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

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Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

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April 3-5, November 27-29
Laparoscopic Digestive Surgery

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May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

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]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

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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

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Contents

Contents

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Volume 14 **Number** 48 **December** 28, 2008

Contents

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EDITORIAL

Spectrum of non-inflammatory bowel disease and non-infectious colitis

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Abstract

A variety of inflammatory diseases of the colon, which can be differentiated from inflammatory bowel disease (IBD) and infectious colitis by their clinical, endoscopic and histological characteristics, are reported as non-IBD and non-infectious colitis. These diseases include microscopic colitis, ischemic colitis, segmental colitis associated with diverticula, radiation colitis, diversion colitis, eosinophilic colitis and Behcet's colitis. The etiopathogenesis of most of these diseases remains obscure and the epidemiological data are rather limited. These conditions are often troublesome for the patient and are associated with diagnostic difficulties for the physician. In many cases the treatment is empirical and there is a need for future research using randomized controlled trials.

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Key words: Diversion colitis; Ischemic colitis; Microscopic colitis; Radiation colitis; Segmental colitis

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This issue of *World Journal of Gastroenterology* contains a number of articles focusing on diagnosis and management of non-inflammatory bowel disease (IBD) and non-infectious colitis. This term includes a variety of inflammatory diseases of the colon, which can be differentiated from IBD and infectious colitis by their clinical, endoscopic and histological characteristics[1,2]. These diseases include microscopic colitides (collagenous and lymphocytic colitis), ischemic colitis, segmental colitis associated with diverticula (SCAD), radiation colitis, diversion colitis, eosinophilic colitis and Behcet's colitis. The etiopathogenesis of most of these diseases remains obscure. Clinical presentations include chronic, watery diarrhoea, abdominal pain and intermittent rectal bleeding. Constitutional symptoms are typically absent and laboratory data are often non-specific. Colonoscopic evaluation and mucosal biopsy are essential in establishing these diagnoses and to exclude IBD and infectious colitis. Prognosis and responses to treatment are variable. In general these conditions are often troublesome for both the patient and the physician. Most of these diseases are uncommon; therefore, epidemiologic data and data from controlled trials are not readily available.

Experts for these diseases were invited to write clinical guidelines for the diagnosis and management of the most common and more important of these diseases, although the scarcity of original data for the recently characterized forms of colitis make this task rather difficult.

Ischemic colitis is the most common form of gastrointestinal ischemia and accounts for 1 in 1000 hospitalizations. However, due to its mild and transient nature the incidence of IC is believed to be underestimated[3]. Although frequent in the elderly, younger patients may also be affected. The first two articles[4,5] deal with the diagnosis and management of ischemic colitis and the diagnostic approach of chronic GI ischemia.

Segmental colitis (or diverticular colitis) has been defined as the chronic mucosal inflammation associated with diverticular disease. This condition, which is usually called segmental colitis associated with diverticulosis (SCAD), is mainly characterized by the involvement of the sigmoid colon with sparing of the rectum and proximal colon. SCAD often mimics IBD at endoscopic and histological examination[6]. Freeman[7] has recently reviewed the clinical, pathogenetic and therapeutic features of this disease.

Collagenous colitis and lymphocytic colitis are the two major conditions that are characterized by chronic

watery diarrhoea, without endoscopic or radiological lesions, but with histological abnormalities and are therefore considered as "microscopic colitis". Recent data suggests that the incidence of microscopic colitis is slightly less than the incidence of chronic idiopathic inflammatory bowel diseases (IBD)[8,9]. In their review Tysk *et al*[10] provide the current concepts on the diagnosis and management of microscopic colitis.

Radiation colitis has been known for years as an insidious and progressive iatrogenic disease that frequently develops 6 months to 5 years after regional radiotherapy for malignancy. Although improvements have been made in radiotherapy delivery, the incidence of radiation colitis is increasing. Kountouras *et al*[11] present an extensive review on the recent advances in the management and prophylaxis of radiation colitis.

The articles cited in this review of non-IBD and non-infectious colitis hopefully serve to remind us that there are a variety of inflammatory diseases of the colon. The articles aid in early diagnosis of these diseases and provide us with current therapeutic options, as well as future prospects.

Other diseases that are rather rare and not included in these articles are diversion colitis, eosinophilic colitis and Behcet's colitis.

Diversion colitis is a non-specific colonic inflammation following surgical diversion of the fecal stream away from the colorectal mucosa. Such surgery may be necessary in cases of colon cancer, trauma or inflammatory diseases. Diversion colitis is characterized histopathologically by a chronic lymphoplasmacytic inflammatory infiltrate, and the existence of lymphoid follicular hyperplasia is considered to be a hallmark feature[12]. The development of diversion colitis is attributed to a lack of short chain fatty acids (SCFA), normally produced from the breakdown of complex carbohydrates by resident bacteria. SCFA are the preferred energy substrate for colonocytes and are necessary for normal metabolism. Although most patients are asymptomatic, common symptoms are rectal bleeding, tenesmus and mucous discharge. It is observed in up to 91% of adults following diversion and it is usually mild or moderate but rarely severe (only in 4% of cases). The restoration of fecal continuity is the treatment of choice and is curative. However, prolonged diversion causes involution and atrophy of the segment leading to a poor functional outcome. Other possible treatment options are SCFA enemas (or 5-ASA enemas)[13,14].

Eosinophilic colitis is an etiologically obscure and rare colonic inflammation which can be associated with involvement of other sections of the gastrointestinal tract from esophagus to rectum in a diffuse or segmentary manner. An infiltrate of eosinophilic granulocytes is found to varying degrees in all wall layers. Eosinophilic gastroenteritis may involve any part of the gastrointestinal tract, however colonic involvement is usually confined to the right colon. The common clinical symptoms are acute colicky pain, diarrhoea, rectal bleeding and weight loss. A history of food intolerance or allergy is present in most of the patients and peripheral eosinophilia is present in 80% of cases. Colonoscopy is usually inconclusive but histology reveals an inflammatory infiltrate by eosinophils in the mucosal and submucosal layers. Treatment includes initially dietary manipulation and avoidance of specific foods, but in refractory cases, corticosteroids, immunosuppressants and sodium cromoglycate are effective although the published data on treatment of eosinophilic colitis are rather limited[15,16].

Behcet's disease is a chronic inflammatory disease characterized by systemic manifestations such as recurrent oral and genital ulcerations, ocular and cutaneous lesions, arthritis and vascular disease. Gastrointestinal involvement is rare; its frequency has been reported to be 3%-25%, with geographical differences[17]. Cases of Behcet's disease cluster along the ancient Silk Road, which extends from eastern Asia to the Mediterranean basin. In cases with ileocolonic involvement, it is often difficult to distinguish Behcet's disease from other inflammatory bowel diseases. Intestinal Behcet's disease commonly accompanies ulcerative lesions in the small and large bowel. The diagnosis of intestinal Behcet's disease, therefore, often depends on clinical manifestations of systemic Behcet's disease and intestinal ulcerative lesions. Treatment options include corticosteroids, azathioprine, or cyclosporine thalidomide and infliximab[18,19].

In conclusion, there is a wide variety of rarer causes of colitis included in the term non-IBD non-infectious colitis. The etiopathogenesis of most of these diseases remains obscure and the epidemiological data are rather limited. In many cases the treatment is empirical and there is a need for future research using randomized controlled trials.

REFERENCES

1 **Sanderson IR**. Unusual colitides. *Baillieres Clin Gastroenterol* 1994; **8**: 181-196
2 **Nielsen OH**, Vainer B, Rask-Madsen J. Non-IBD and noninfectious colitis. *Nat Clin Pract Gastroenterol Hepatol* 2008; **5**: 28-39
3 Brandt LJ, Boley SJ. AGA technical review on intestinal ischemia. American Gastrointestinal Association. *Gastroenterology* 2000; **118**: 954-968
4 **Kolkman JJ**, Bargeman M, Huisman AB, Geelkerken RH. Diagnosis and management of splanchnic ischemia. *World J Gastroenterol* 2008; **14**: 7309-7320
5 **Theodoropoulou A**, Koutroubakis IE. Ischemic colitis: Clinical practice in diagnosis and treatment. *World J Gastroenterol* 2008; **14**: 7302-7308
6 **Peppercorn MA**. The overlap of inflammatory bowel disease and diverticular disease. *J Clin Gastroenterol* 2004; **38**: S8-S10
7 **Freeman HJ**. Segmental colitis associated with diverticulosis syndrome. *World J Gastroenterol* 2008; **14**: 6442-6443
8 **Pardi DS**, Loftus EV Jr, Smyrk TC, Kammer PP, Tremaine WJ, Schleck CD, Harmsen WS, Zinsmeister AR, Melton LJ 3rd, Sandborn WJ. The epidemiology of microscopic colitis: a population based study in Olmsted County, Minnesota. *Gut* 2007; **56**: 504-508
9 **Williams JJ**, Kaplan GG, Makhija S, Urbanski SJ, Dupre M, Panaccione R, Beck PL. Microscopic colitis-defining incidence rates and risk factors: a population-based study. *Clin Gastroenterol Hepatol* 2008; **6**: 35-40

10 **Tysk C**, Bohr J, Nyhlin N, Wickbom A, Eriksson S. Diagnosis and management of microscopic colitis. *World J Gastroenterol* 2008; **14**: 7280-7288
11 **Kountouras J**, Zavos C. Recent advances in the management of radiation colitis. *World J Gastroenterol* 2008; **14**: 7289-7301
12 **Edwards CM**, George B, Warren B. Diversion colitis--new light through old windows. *Histopathology* 1999; **34**: 1-5
13 **Cook SI**, Sellin JH. Review article: short chain fatty acids in health and disease. *Aliment Pharmacol Ther* 1998; **12**: 499-507
14 **Giardiello FM**, Lazenby AJ. The atypical colitides. *Gastroenterol Clin North Am* 1999; **28**: 479-490, x
15 **Gonsalves N**. Food allergies and eosinophilic gastrointestinal illness. *Gastroenterol Clin North Am* 2007; **36**: 75-91, vi
16 **Rothenberg ME**. Eosinophilic gastrointestinal disorders (EGID). *J Allergy Clin Immunol* 2004; **113**: 11-28; quiz 29
17 **Yurdakul S**, Tuzuner N, Yurdakul I, Hamuryudan V, Yazici H. Gastrointestinal involvement in Behcet's syndrome: a controlled study. *Ann Rheum Dis* 1996; **55**: 208-210
18 **Gul A**. Standard and novel therapeutic approaches to Behcet's disease. *Drugs* 2007; **67**: 2013-2022
19 **Naganuma M**, Sakuraba A, Hisamatsu T, Ochiai H, Hasegawa H, Ogata H, Iwao Y, Hibi T. Efficacy of infliximab for induction and maintenance of remission in intestinal Behcet's disease. *Inflamm Bowel Dis* 2008; **14**: 1259-1264

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TOPIC HIGHLIGHT

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Diagnosis and management of microscopic colitis

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Abstract

Microscopic colitis, comprising collagenous and lymphocytic colitis, is characterized clinically by chronic watery diarrhea, and a macroscopically normal colonic mucosa where diagnostic histopathological features are seen on microscopic examination. The annual incidence of each disorder is 4-6/100 000 inhabitants, with a peak incidence in 60-70-year-old individuals and a noticeable female predominance for collagenous colitis. The etiology is unknown. Chronic diarrhea, abdominal pain, weight loss, fatigue and fecal incontinence are common symptoms, which impair the health-related quality of life of the patient. There is an association with other autoimmune disorders such as celiac disease, diabetes mellitus, thyroid disorders and arthritis. Budesonide is the best-documented short-term treatment, but the optimal long-term strategy needs further study. The long-term prognosis is good and the risk of complications including colonic cancer is low.

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INTRODUCTION

Chronic diarrhea, reported in 4%-5% of individuals in Western populations, is a common cause for consulting a physician in general practice or in internal medicine, and for referral to a gastroenterologist[1]. Microscopic colitis (MC), previously regarded as rare, and certainly overlooked, has now emerged as a common cause of chronic diarrhea especially in elderly women. The condition is characterized clinically by chronic watery diarrhea, and a macroscopically normal or almost normal colonic mucosa, where microscopic examination of mucosal biopsies reveals characteristic histopathological changes[2]. MC comprises the two entities collagenous colitis (CC) and lymphocytic colitis (LC), which have indistinguishable clinical presentations but are separated by histopathological characteristics. This review will highlight epidemiology, clinical features, diagnosis and management of MC.

EPIDEMIOLOGY

CC and LC, first described in 1976[3] and in 1989[4], respectively, have mostly been reported from European or North American centers, but the disease is found worldwide[5-10]. Currently, epidemiological data have been reported from seven different regions (Table 1)[5,6,11-17]. Long-term epidemiological data from Sweden and US since the 1980s show a rising incidence, which seems to have levelled off during the last study periods in the Swedish study. Whether the increasing incidence figures are an artefact, reflecting an increased awareness and improved diagnosis of the condition, or in fact represents a true rise is at present unknown. MC may be diagnosed in 10%-20% of cases investigated for chronic

Table 1 Annual incidence/100 000 inhabitants in population-based epidemiological studies of CC and LC[5,6,11-17]

Region and study period	CC	LC
Örebro, Sweden 1984-1988	0.8	
Örebro, Sweden 1989-1993	2.7	
Örebro, Sweden 1993-1995	3.7	3.1
Örebro, Sweden 1996-1998	6.1	5.7
Örebro, Sweden 1999-2004	5.2	5.5
Terassa, Spain 1993-1997	2.3	3.7
Iceland 1995-1999	5.2	4.0
Olmsted County, USA 1985-1989	0.3	0.5
Olmsted County, USA 1990-1993	1.6	1.0
Olmsted County, USA 1994-1997	3.9	6.4
Olmsted County, USA 1997-2001	6.2	12.9
Lothian, UK 1998-2003	0.8	
Tayside, UK 1999-2004	1.1	0.6
Calgary, Canada 2002-2004	4.6	5.4

watery diarrhea[5].

CC mainly affects middle-aged women with a peak incidence around 65 years of age, and the female:male ratio is about 7:1 (Figure 1)[6,18]. However, the disease can occur in all ages, including children[19]. In LC, the peak incidence is in the same age group as CC, but the female predominance is less pronounced with a female:male ratio of 2-3:1 (Figure 1)[20].

CLINICAL PRESENTATION

The clinical symptoms of CC and LC are similar and the diseases cannot be differentiated on clinical grounds. Both disorders cause chronic or recurrent non-bloody, watery diarrhea, often associated with nocturnal diarrhea, diffuse abdominal pain, and weight loss, which may be substantial[18,20,21]. Although some patients may suffer from severe diarrhea, serious dehydration is rare. Fatigue, nausea and fecal incontinence are other associated symptoms and the disease may significantly impair quality of life in the affected patient[22,23].

The onset of disease can be sudden and mimic infectious diarrhea[18,20]. The clinical course is often chronic relapsing and benign. Severe complications are rare, although there are reports of colonic perforation in CC[24-26]. No increased risk of colorectal cancer has been reported in CC[27]. A few cases with concomitant lymphoproliferative disorders and CC have been presented but further studies are required to assess if there is an increased risk[28].

Some patients may have mild symptoms that may be misinterpreted as irritable bowel syndrome[29]. Morphological findings of LC have been reported even in constipated or asymptomatic patients[30]. The natural history of the condition in these patients is unknown.

Patients with MC often have concomitant autoimmune diseases[18,20,21]. The most common are thyroid disorders, celiac disease, diabetes mellitus and rheumatoid arthritis. The occurrence of such associations, reported in up to 40%-50% of patients in some cases, is variable depending on the study, and differences between LC and CC with respect to

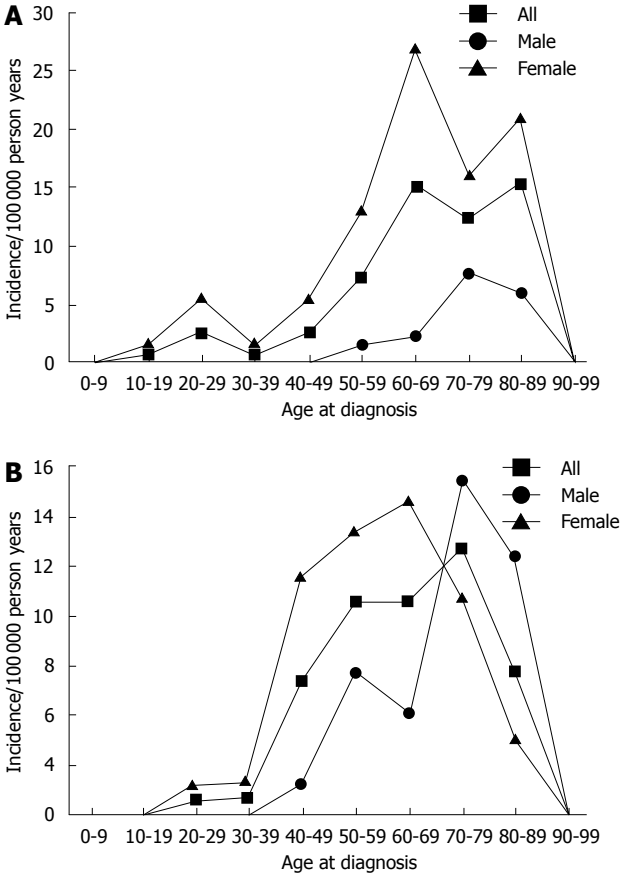

Figure 1 Age- and sex-specific incidence of CC (A) and LC (B). Reprinted with permission from *Gut* 2004; 53: 346-350[5].

associated conditions have been described[18,20,21,31]. Bile acid malabsorption can often co-exist with MC and lead to worsening of symptoms[32]. An interchange between ulcerative colitis or Crohn's disease and MC has been reported occasionally[33,34]. Whether this merely is a chance association of two fairly common disorders occurring in the same individual, or results from a common genetic predisposition or shared immunological pathways remains unknown.

ETIOLOGY AND PATHOGENESIS OF MUCOSAL INFLAMMATION

The cause of MC is multifactorial and largely unknown. CC and LC are presently considered to represent specific mucosal responses in predisposed individuals to various noxious luminal agents. As CC and LC have many clinical similarities and share histopathological features, except for the subepithelial collagen layer found in CC, it has been discussed whether LC and CC are in fact the same disease seen in different stages of development. Conversion of LC to CC or *vice versa* has been reported. However, conversion is seen infrequently and this fact, together with the observed difference in sex ratio, makes it more likely to consider CC and LC as two separate but related entities.

Data on the mucosal inflammation in MC are limited. In the epithelium, mainly CD8+ T lymphocytes are

found that carry the α/β form of the T-cell receptor, and in the lamina propria there are mainly CD4+ T lymphocytes[35]. By means of segmental colorectal perfusion, increased luminal levels of eosinophilic cationic protein (ECP), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been found in CC[36-38]. By immunohistochemistry, others have verified increased mucosal levels of VEGF that are not affected following therapy with budesonide[39]. A study of cytokines in MC found a TH1 mucosal cytokine profile with interferon γ, tumor necrosis factor (TNF)α and interleukin-15 as the predominantly up-regulated cytokines[40]. Using Ussing chamber technology, transcellular and paracellular mucosal permeability has been found to be increased in patients with CC[41,42]. The excess subepithelial collagen in CC may be caused by an imbalance of collagen turnover. An increased collagen synthesis is supported by the finding of an increase in the number or the activity of myofibroblasts[43]. Among degrading enzymes, matrix-metalloproteinases (MMPs) have a central role that is regulated by tissue endogenous inhibitors of metalloproteinases (TIMPs)[44]. Impaired collagen degradation in CC is supported by the finding of restricted MMP-1 RNA expression and increased TIMP expression[45].

GENETICS

A familial occurrence of MC has been reported, but the role of genetic factors still remains largely unknown[46-49]. Human leukocyte antigen (HLA) studies have shown an association between MC and HLA-DQ2 or DQ1/3, and recently an association has reported between MC and HLA-DR3-DQ2 haplotype and with TNF2 allele carriage, irrespective of the presence of concomitant celiac disease[50,51]. Variants of the MMP-9 gene have been reported to be associated with CC[52]. No association with NOD2/CARD15 polymorphisms and susceptibility to CC has been found[53].

LUMINAL FACTORS

The mucosal inflammation with an increased number of intraepithelial T lymphocytes has suggested that MC may be caused by an immunological response to a luminal agent in predisposed individuals. This theory is supported by the observation that diversion of the fecal stream by an ileostomy normalizes or reduces the characteristic histopathological changes in CC[54]. After closure of the ileostomy, recurrence of symptoms and histopathological changes occur.

Drug-induced MC

There are several reports on drug-induced MC and a strong likelihood of association has been found with acarbose, aspirin, Cyclo3 Fort, non-steroidal anti-inflammatory drugs, lansoprazole, ranitidine, sertraline and ticlopidine[55]. Assessment of concomitant drug use in patients with MC is therefore important to identify and consider withdrawal of drugs that might cause or worsen the condition.

Infection

An infectious cause has been suspected, especially in patients with a sudden onset of disease. An association with MC and *Campylobacter jejuni, Yersinia enterocolitica* or *Clostridium difficile* has been reported occasionally[56-59]. LC shares many features with "Brainerd diarrhea", which refers to outbreaks of acute watery diarrhea with long duration, first reported among 122 residents of Brainerd, Minnesota, USA[60]. Colonic biopsies of these patients show epithelial lymphocytosis similar to LC, but no crypt distortion or epithelial destruction[61]. Investigations of several outbreaks of Brainerd diarrhea have established an incubation period of 10-30 d and median duration of illness of 16 mo[62]. Although an infectious agent is thought to be the cause of Brainerd diarrhea, no microorganism has yet been identified. Furthermore, a seasonal pattern of onset of LC[20,63] may support an infectious cause. However, in most cases of MC with a sudden onset, stool cultures remain negative.

Bile acids

Bile acid malabsorption can coexist with MC, which leads to worsening of symptoms. Concurrent bile acid malabsorption was found in 27%-44% of patients with CC and in 9%-60% of patients with LC[32,64,65]. These observations are the rationale for recommendations on bile acid binding treatment in MC. The treatment is especially effective in patients with concomitant bile acid malabsorption, but improvement has also been shown in patients without bile acid malabsorption.

Autoimmunity

The association with other autoimmune diseases such as thyroid disease, celiac disease, diabetes mellitus or arthritis has suggested an autoimmune process. However, no specific autoantibody or marker has been identified.

Nitric oxide (NO)

Colonic NO production is greatly increased in active MC caused by upregulation of inducible nitric oxide synthase (iNOS) in the colonic epithelium[66-69]. A major transcriptional inducer of iNOS gene expression is the transcription factor nuclear factor-κB (NF-κB). In active CC, colonic mucosal NF-κB has been found to be activated in epithelial cells but not in lamina propria macrophages, in contrast to ulcerative colitis[70]. The levels of NO are correlated to clinical and histological disease activity[67]. NO has been suggested to be involved in the pathophysiology of diarrhea in CC, as infusion in the colon of N^G-monomethyl-L-arginine, an inhibitor of NOS, reduced colonic net secretion by 70% and the addition of L-arginine, a precursor of NO synthesis, increased colonic net secretion by 50%[68]. Further

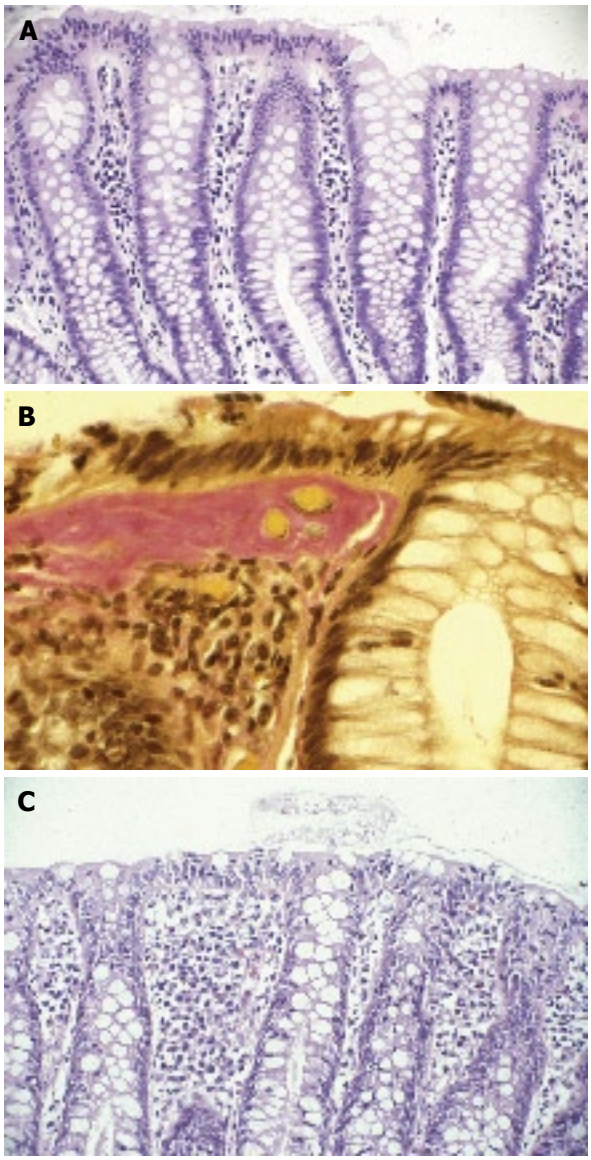

Figure 2 Biopsy from colon. A: normal colonic mucosa (H&E stain); B: typical findings of CC-increased subepithelial collagen layer, inflammation of lamina propria and epithelial cell damage with intraepithelial lymphocytes (Van Gieson's stain); C: typical findings of LC-epithelial cell damage with intraepithelial lymphocytes and inflammation in the lamina propria (H&E stain).

support for NO being involved in the pathogenesis of CC comes from therapeutic studies. Treatment with budesonide, in contrast to placebo, has resulted in a significant reduction of iNOS mRNA that is correlated with clinical and histopathological improvement[71].

Secretory or osmotic diarrhea

The exact mechanism of diarrhea in MC has not been clarified fully. In CC, diarrhea has been regarded as secretory and caused by reduced net absorption of Na^+ and Cl^- ions caused by epithelial cell lesions, and the thickened collagenous layer as a co-factor that causes a diffusion barrier, and by additional active Cl^- secretion[72]. Fasting, on the other hand, seems to reduce diarrhea, which indicates an osmotic component in some patients as well[73].

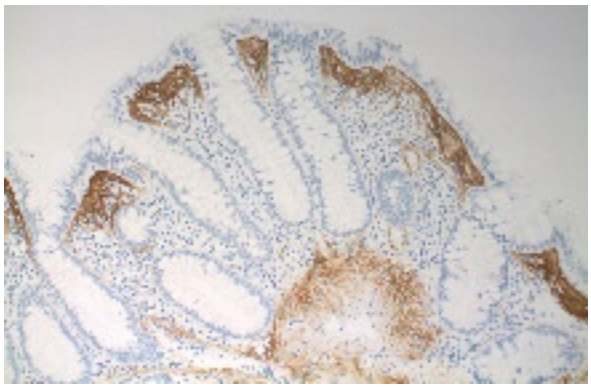

Figure 3 Tenascin immunostaining in CC.

DIAGNOSIS

Diagnosis of MC relies solely on typical microscopic changes seen in colonic mucosal biopsies[74]. In CC, a thickening of the subepithelial collagen layer is seen together with a chronic mononuclear inflammation in the lamina propria, and epithelial cell damage, with an occasionally increased number of intraepithelial lymphocytes (Figure 2). The thickened subepithelial collagen layer in CC is $\geqslant$ 10 μm in well-orientated sections, in contrast to a normal basal membrane of $<$ 3 μm. The thickening of the collagen layer may be variable and is most prominent in the ascending or transverse colon, and may be absent in biopsies from the sigmoid colon or rectum, which emphasizes the importance of obtaining biopsies from the proximal colon when diagnosing CC[75]. Generally, the histopathological changes are restricted to the large bowel, but a thickened collagen layer has infrequently been found in the stomach, duodenum or terminal ileum. In addition to conventional histological staining, the use of tenascin immunostaining has been suggested in uncertain cases of CC (Figure 3)[43,76].

The diagnostic features of LC (Figure 2) are an increased number of intraepithelial lymphocytes ($\geqslant$ 20/100 surface epithelial cells), in conjunction with surface epithelial cell damage and infiltration of lymphocytes and plasma cells into the lamina propria, but the collagen layer is normal, in contrast to CC[74]. In uncertain cases, immunostaining of CD3+ T lymphocytes facilitates the assessment of intraepithelial lymphocyte count (Figure 4).

Barium enema and colonoscopy are usually normal, although subtle mucosal changes can be seen such as edema, erythema and abnormal vascular pattern[18,20]. Tears of colonic mucosa have occasionally been seen during colonoscopy, which might be a sign of increased risk of colonic perforation during the procedure[26,77-79]. In the future, the use of confocal laser microscopy may enable *in vivo* diagnosis of MC[80-82].

Laboratory tests are non-diagnostic and only non-specific abnormalities such as moderately elevated C-reactive protein, erythrocyte sedimentation rate, or mild anemia are found. Stool tests reveal no pathological microorganisms, but fecal calprotectin can be slightly elevated[83].

Table 2 Data from four randomized, placebo-controlled trials of oral budesonide in CC and LC

Author year	Number of cases	Dosage	Clinical response budesonide *vs* placebo	Histological response budesonide *vs* placebo	Adverse events
Collagenous colitis					
Baert *et al*[91] 2002	28	9 mg/d Budenofalk 8 wk	Improvement: 8/14 *vs* 3/14 ($P = 0.05$)	Reduction of lamina propria inflammation in 9/13 *vs* 4/12 ($P < 0.001$) No difference in collagen layer	Mild No difference between treatment groups
Miehlke *et al*[93] 2002	45	9 mg/d Entocort 6 wk	Remission: 15/23 *vs* 0/22 ($P < 0.0001$)	Improvement in 17/23 *vs* 5/22 ($P < 0.01$) No difference in collagen layer	Mild 38% *vs* 12% $P = 0.052$
Bonderup *et al*[92] 2003	20	9 mg/d Entocort 8 wk	Response: 10/10 *vs* 2/10 ($P < 0.001$)	Reduction of overall inflammation ($P < 0.01$) and of collagen layer in sigmoid colon ($P < 0.02$)	None
Lymphocytic colitis					
Miehlke *et al*[95] 2007	41	9 mg/d Budenofalk 6 wk	Remission: 18/21 *vs* 8/20 ($P = 0.004$)	Response in 11/15 *vs* 4/12 ($P = 0.04$)	Mild No difference between treatment groups

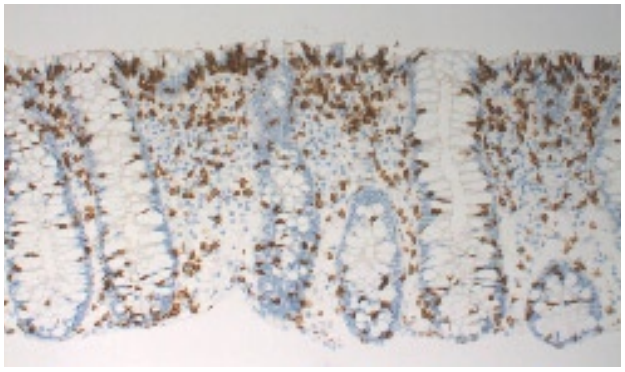

Figure 4 Immunostaining of CD3+ T lymphocytes in LC.

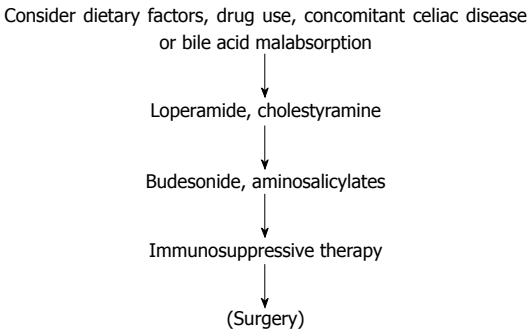

Figure 5 Treatment algorithm for MC.

ATYPICAL MC

In addition to CC and LC, other rare subtypes of MC have been described including MC with giant cells[84,85], paucicellular LC[86], cryptal LC[87], pseudomembranous CC[88], MC with granulomatous inflammation[89], and MC not otherwise specified[74]. The clinical features of these conditions are similar to those of classical MC, but histopathological appearance differs. Further studies are required to address the relationship and clinical significance of these atypical forms of MC[90].

THERAPY AND PROGNOSIS

A careful assessment of concomitant drug use and dietary factors such as excess use of caffeine, alcohol and dairy products that might worsen the condition is important. Concomitant bile acid malabsorption or celiac disease should be considered. In the patient with mild symptoms, loperamide or cholestyramine are recommended as the first step of treatment (Figure 5).

Budesonide is the best-documented treatment and significantly improves the clinical symptoms and the patient's quality of life. Three short-term, randomized controlled trials in CC have consistently shown that budesonide 9 mg daily for 6-8 wk is superior to placebo (Table 2)[91-93]. About 80% of patients responded to budesonide and had a decrease in the number of loose stools after 2-4 wk of therapy. In a Cochrane meta-analysis, the pooled odds ratio for clinical response with budesonide compared to placebo was 12.32 (95% CI 5.53-27.46), and the number needed to treat was two patients[94]. In a placebo-controlled trial including 41 patients, budesonide treatment was effective also in LC[95]. After 6 wk treatment, 18 of 21 patients (86%; 95% CI 65%-96%) in the budesonide group achieved a clinical response compared to eight of 20 patients (40%; 95% CI 22%-61%) in the placebo group, which yielded an odds ratio of 9.00 (95% CI 1.98-40.93; $P = 0.004$)[96]. The number needed to treat to achieve a clinical response with budesonide was three patients.

The relapse rate is high after cessation of successful short-term budesonide therapy in CC and 61%-80% of treated patients will have a recurrence of symptoms[91-93]. In clinical practice, tapering doses of budesonide to 3-6 mg/d have been used as maintenance therapy and may well control clinical symptoms. There is now evidence for such a strategy in CC, and two studies have proven maintenance therapy with budesonide 6 mg/d for 6 mo is well-tolerated and superior to placebo[97,98]. A total of 80 patients, who had responded to open-label budesonide, were randomized to budesonide 6 mg/d or placebo for 6 mo. Clinical response was maintained in 33/40 (83%) patients who received budesonide compared to 11/40 (28%) patients who

received placebo ($P = 0.0002$). Pooled odds ratio was 8.40 (95% CI, 2.73-25.81) with a number needed to treat of two patients for maintenance of clinical response with budesonide. Histological response was seen in 48% of patients who received budesonide compared to 15% of patients who received placebo ($P = 0.002$)[94]. However, 6 mo maintenance therapy did not alter the subsequent course, as the relapse risk after withdrawal of 24 wk maintenance treatment was similar to that observed after 6 wk induction therapy, and the median time to relapse was equal in the two groups (39 d *versus* 38 d)[97].

Other oral corticosteroids, such as prednisolone, are associated with more frequent side-effects, and the efficacy seems inferior to budesonide, although no formal comparative studies are available[99].

Bismuth subsalicylate has been shown to be effective in a small placebo-controlled study including nine patients with CC and five with LC[100]. This drug is not available in a number of countries because of concerns regarding drug toxicity.

Sulfasalazine or mesalazine have been extensively used in MC but not strictly evaluated in randomized placebo-controlled trials. In a recent trial, 64 patients with MC were randomized to mesalazine 2.4 g/d or mesalazine 2.4 g/d + cholestyramine 4 g/d for 6 mo. A high remission rate was seen in both treatment arms, and 85% of patients with LC and 91% of those with CC were in remission at study end. Combined therapy was superior in CC and induced an earlier clinical response in both diseases[101]. The benefit of mesalazine with or without cholestyramine needs to be confirmed in a placebo-controlled trial.

Antibiotics such as metronidazole or erythromycin have been used but not in a controlled fashion. Probiotic treatment shows uncertain results and need further evaluation[102]. *Boswelia serrata* extract has been tried in a placebo-controlled trial showing a non-significant trend in favor of active treatment[103].

In patients with unresponsive or steroid-resistant disease, immunosuppressive therapy may be considered, although the evidence is limited. An open study with azathioprine gave partial or complete remission in eight of nine patients with MC[104]. The efficacy of methotrexate has been assessed in a retrospective study[105]. Out of 19 patients with CC, a good response, generally seen within 2-3 wk of treatment, was seen in 16 and a partial response in two patients. The dose of methotrexate ranged from 5-25 mg/wk (median 7.5-10 mg/wk).

Surgical therapy may be considered for patients with severe unresponsive MC. Both split ileostomy and subtotal colectomy have been performed and reported as successful[54,106]. The indications for surgical therapy today are limited, considering the improvement of medical therapy.

The long-term prognosis of MC is generally good. In a follow-up study of CC, 63% of the patients had a lasting remission after 3.5 years, and in another cohort study, all 25 patients were improved 47 mo after diagnosis, and only 29% of them required ongoing medication[107,108]. A benign course was reported in 27 cases with LC, with resolution of diarrhea and normalization of histology in > 80% of patients within 38 mo[109]. Others have reported that 63% of patients with LC had a single attack, with a median duration from onset of symptoms to remission of 6 mo[20].

CONCLUSION

MC is a fairly common cause of chronic diarrhea, especially in elderly women, and may considerably impair the patient's quality of life. The correct diagnosis depends on the awareness of the condition by the clinician (referring the patient with chronic diarrhea to colonoscopy and not to barium enema), by the endoscopist (obtaining mucosal biopsies although the colonic mucosa is endoscopically normal) and by the pathologist (recognizing the histopathological features of MC). Treatment with budesonide is effective in the short term and improves the patient's symptoms and quality of life, but the optimal long-term therapy needs further study. The long-term prognosis is good and the risk of complications including colonic cancer is low.

REFERENCES

1 **Thomas PD**, Forbes A, Green J, Howdle P, Long R, Playford R, Sheridan M, Stevens R, Valori R, Walters J, Addison GM, Hill P, Brydon G. Guidelines for the investigation of chronic diarrhoea, 2nd edition. *Gut* 2003; **52** Suppl 5: v1-v15

2 **Pardi DS**. Microscopic colitis: an update. *Inflamm Bowel Dis* 2004; **10**: 860-870

3 **Lindström CG**. 'Collagenous colitis' with watery diarrhoea--a new entity? *Pathol Eur* 1976; **11**: 87-89

4 **Lazenby AJ**, Yardley JH, Giardiello FM, Jessurun J, Bayless TM. Lymphocytic ("microscopic") colitis: a comparative histopathologic study with particular reference to collagenous colitis. *Hum Pathol* 1989; **20**: 18-28

5 **Olesen M**, Eriksson S, Bohr J, Järnerot G, Tysk C. Microscopic colitis: a common diarrhoeal disease. An epidemiological study in Orebro, Sweden, 1993-1998. *Gut* 2004; **53**: 346-350

6 **Pardi DS**, Loftus EV Jr, Smyrk TC, Kammer PP, Tremaine WJ, Schleck CD, Harmsen WS, Zinsmeister AR, Melton LJ 3rd, Sandborn WJ. The epidemiology of microscopic colitis: a population based study in Olmsted County, Minnesota. *Gut* 2007; **56**: 504-508

7 **Rubio-Tapia A**, Martínez-Salgado J, García-Leiva J, Martínez-Benítez B, Uribe M. Microscopic colitides: a single center experience in Mexico. *Int J Colorectal Dis* 2007; **22**: 1031-1036

8 **Fekih M**, Ben Hriz F, Sassi A, Matri S, Filali A, Boubaker J. [Microscopic colitis. A 20 cases series] *Tunis Med* 2006; **84**: 403-406

9 **Tagkalidis P**, Bhathal P, Gibson P. Microscopic colitis. *J Gastroenterol Hepatol* 2002; **17**: 236-248

10 **Garg PK**, Singh J, Dhali GK, Mathur M, Sharma MP. Microscopic colitis is a cause of large bowel diarrhea in Northern India. *J Clin Gastroenterol* 1996; **22**: 11-15

11 **Agnarsdottir M**, Gunnlaugsson O, Orvar KB, Cariglia N, Birgisson S, Bjornsson S, Thorgeirsson T, Jonasson JG. Collagenous and lymphocytic colitis in Iceland. *Dig Dis Sci* 2002; **47**: 1122-1128

12 **Bohr J**, Tysk C, Eriksson S, Järnerot G. Collagenous colitis in Orebro, Sweden, an epidemiological study 1984-1993. *Gut* 1995; **37**: 394-397

13 **Fernández-Bañares F**, Salas A, Forné M, Esteve M, Espinós J, Viver JM. Incidence of collagenous and lymphocytic colitis: a 5-year population-based study. *Am J Gastroenterol* 1999; **94**: 418-423

14 **Heron T**, Walsh S, Mowat A. Microscopic colitis in Tayside: clinical features, associations, and behaviour. *Gut* 2005; **54** suppl 2: A84

15 **Rajan J**, Noble C, Anderson C, Satsangi J, Lessels A, Arnott I. The epidemiology and clinical features of collagenous colitis in Lothian. *Gut* 2005; **54** suppl 2: A99

16 **Wickbom A**, Nyhlin N, Eriksson S, Bohr J, Tysk C. Collagenous colitis and lymphocytic colitis in Örebro, Sweden 1999-2004; a continuous epidemiological study. *Gut* 2006; **55** suppl V : A111

17 **Williams JJ**, Kaplan GG, Makhija S, Urbanski SJ, Dupre M, Panaccione R, Beck PL. Microscopic colitis-defining incidence rates and risk factors: a population-based study. *Clin Gastroenterol Hepatol* 2008; **6**: 35-40

18 **Bohr J**, Tysk C, Eriksson S, Abrahamsson H, Järnerot G. Collagenous colitis: a retrospective study of clinical presentation and treatment in 163 patients. *Gut* 1996; **39**: 846-851

19 **Benchimol EI**, Kirsch R, Viero S, Griffiths AM. Collagenous colitis and eosinophilic gastritis in a 4-year old girl: a case report and review of the literature. *Acta Paediatr* 2007; **96**: 1365-1367

20 **Olesen M**, Eriksson S, Bohr J, Järnerot G, Tysk C. Lymphocytic colitis: a retrospective clinical study of 199 Swedish patients. *Gut* 2004; **53**: 536-541

21 **Pardi DS**, Ramnath VR, Loftus EV Jr, Tremaine WJ, Sandborn WJ. Lymphocytic colitis: clinical features, treatment, and outcomes. *Am J Gastroenterol* 2002; **97**: 2829-2833

22 **Madisch A**, Heymer P, Voss C, Wigginghaus B, Bästlein E, Bayerdörffer E, Meier E, Schimming W, Bethke B, Stolte M, Miehlke S. Oral budesonide therapy improves quality of life in patients with collagenous colitis. *Int J Colorectal Dis* 2005; **20**: 312-316

23 **Hjortswang H**, Tysk C, Bohr J, Benoni C, Kilander A, Vigren L, Larsson L, Taha Y, Ström M. Health-related quality of life is impaired in patients with collagenous colitis. *Gut* 2005; **54** Suppl VII: A183

24 **Allende DS**, Taylor SL, Bronner MP. Colonic perforation as a complication of collagenous colitis in a series of 12 patients. *Am J Gastroenterol* 2008; **103**: 2598-2604

25 **Bohr J**, Larsson LG, Eriksson S, Järnerot G, Tysk C. Colonic perforation in collagenous colitis: an unusual complication. *Eur J Gastroenterol Hepatol* 2005; **17**: 121-124

26 **Sherman A**, Ackert JJ, Rajapaksa R, West AB, Oweity T. Fractured colon: an endoscopically distinctive lesion associated with colonic perforation following colonoscopy in patients with collagenous colitis. *J Clin Gastroenterol* 2004; **38**: 341-345

27 **Chan JL**, Tersmette AC, Offerhaus GJ, Gruber SB, Bayless TM, Giardiello FM. Cancer risk in collagenous colitis. *Inflamm Bowel Dis* 1999; **5**: 40-43

28 **Freeman HJ**. Lymphoproliferative disorders in collagenous colitis. *Inflamm Bowel Dis* 2005; **11**: 781-782

29 **Limsui D**, Pardi DS, Camilleri M, Loftus EV Jr, Kammer PP, Tremaine WJ, Sandborn WJ. Symptomatic overlap between irritable bowel syndrome and microscopic colitis. *Inflamm Bowel Dis* 2007; **13**: 175-181

30 **Barta Z**, Mekkel G, Csípo I, Tóth L, Szakáll S, Szabó GG, Bakó G, Szegedi G, Zeher M. Microscopic colitis: a retrospective study of clinical presentation in 53 patients. *World J Gastroenterol* 2005; **11**: 1351-1355

31 **Koskela RM**, Niemelä SE, Karttunen TJ, Lehtola JK. Clinical characteristics of collagenous and lymphocytic colitis. *Scand J Gastroenterol* 2004; **39**: 837-845

32 **Ung KA**, Gillberg R, Kilander A, Abrahamsson H. Role of bile acids and bile acid binding agents in patients with collagenous colitis. *Gut* 2000; **46**: 170-175

33 **Aqel B**, Bishop M, Krishna M, Cangemi J. Collagenous colitis evolving into ulcerative colitis: a case report and review of the literature. *Dig Dis Sci* 2003; **48**: 2323-2327

34 **Pokorny CS**, Kneale KL, Henderson CJ. Progression of collagenous colitis to ulcerative colitis. *J Clin Gastroenterol* 2001; **32**: 435-438

35 **Mosnier JF**, Larvol L, Barge J, Dubois S, De La Bigne G, Hénin D, Cerf M. Lymphocytic and collagenous colitis: an immunohistochemical study. *Am J Gastroenterol* 1996; **91**: 709-713

36 **Taha Y**, Carlson M, Thorn M, Loof L, Raab Y. Evidence of local eosinophil activation and altered mucosal permeability in collagenous colitis. *Dig Dis Sci* 2001; **46**: 888-897

37 **Taha Y**, Raab Y, Larsson A, Carlson M, Lööf L, Gerdin B, Thörn M. Mucosal secretion and expression of basic fibroblast growth factor in patients with collagenous colitis. *Am J Gastroenterol* 2003; **98**: 2011-2017

38 **Taha Y**, Raab Y, Larsson A, Carlson M, Lööf L, Gerdin B, Thörn M. Vascular endothelial growth factor (VEGF)--a possible mediator of inflammation and mucosal permeability in patients with collagenous colitis. *Dig Dis Sci* 2004; **49**: 109-115

39 **Griga T**, Tromm A, Schmiegel W, Pfisterer O, Müller KM, Brasch F. Collagenous colitis: implications for the role of vascular endothelial growth factor in repair mechanisms. *Eur J Gastroenterol Hepatol* 2004; **16**: 397-402

40 **Tagkalidis PP**, Gibson PR, Bhathal PS. Microscopic colitis demonstrates a T helper cell type 1 mucosal cytokine profile. *J Clin Pathol* 2007; **60**: 382-387

41 **Münch A**, Söderholm JD, Wallon C, Ost A, Olaison G, Ström M. Dynamics of mucosal permeability and inflammation in collagenous colitis before, during, and after loop ileostomy. *Gut* 2005; **54**: 1126-1128

42 **Münch A**, Söderholm JD, Öst A, Ström M. Increased transmucosal uptake of E. coli in collagenous colitis is not reversed by budesonide. *Gut* 2007; **56** Suppl III: A72

43 **Salas A**, Fernández-Bañares F, Casalots J, González C, Tarroch X, Forcada P, González G. Subepithelial myofibroblasts and tenascin expression in microscopic colitis. *Histopathology* 2003; **43**: 48-54

44 **Medina C**, Radomski MW. Role of matrix metalloproteinases in intestinal inflammation. *J Pharmacol Exp Ther* 2006; **318**: 933-938

45 **Günther U**, Schuppan D, Bauer M, Matthes H, Stallmach A, Schmitt-Gräff A, Riecken EO, Herbst H. Fibrogenesis and fibrolysis in collagenous colitis. Patterns of procollagen types I and IV, matrix-metalloproteinase-1 and -13, and TIMP-1 gene expression. *Am J Pathol* 1999; **155**: 493-503

46 **Freeman HJ**. Familial occurrence of lymphocytic colitis. *Can J Gastroenterol* 2001; **15**: 757-760

47 **Järnerot G**, Hertervig E, Grännö C, Thorhallsson E, Eriksson S, Tysk C, Hansson I, Björknäs H, Bohr J, Olesen M, Willén R, Kagevi I, Danielsson A. Familial occurrence of microscopic colitis: a report on five families. *Scand J Gastroenterol* 2001; **36**: 959-962

48 **Abdo AA**, Zetler PJ, Halparin LS. Familial microscopic colitis. *Can J Gastroenterol* 2001; **15**: 341-343

49 **van Tilburg AJ**, Lam HG, Seldenrijk CA, Stel HV, Blok P, Dekker W, Meuwissen SG. Familial occurrence of collagenous colitis. A report of two families. *J Clin Gastroenterol* 1990; **12**: 279-285

50 **Fine KD**, Do K, Schulte K, Ogunji F, Guerra R, Osowski L, McCormack J. High prevalence of celiac sprue-like HLA-DQ genes and enteropathy in patients with the microscopic colitis syndrome. *Am J Gastroenterol* 2000; **95**: 1974-1982

51 **Koskela RM**, Karttunen TJ, Niemelä SE, Lehtola JK, Ilonen J, Karttunen RA. Human leucocyte antigen and TNFalpha polymorphism association in microscopic colitis. *Eur J Gastroenterol Hepatol* 2008; **20**: 276-282

52 **Madisch A**, Miehlke S, Schreiber S, Bethke B, Stolte M, Hellmig S. Matrix metalloproteinase-9 gene polymorphism is associated with collagenous colitis. *Gut* 2006; **55** SupplV:

A113
53 **Madisch A**, Hellmig S, Schreiber S, Bethke B, Stolte M, Miehlke S. NOD2/CARD15 gene polymorphisms are not associated with collagenous colitis. *Int J Colorectal Dis* 2007; **22**: 425-428
54 **Järnerot G**, Tysk C, Bohr J, Eriksson S. Collagenous colitis and fecal stream diversion. *Gastroenterology* 1995; **109**: 449-455
55 **Beaugerie L**, Pardi DS. Review article: drug-induced microscopic colitis - proposal for a scoring system and review of the literature. *Aliment Pharmacol Ther* 2005; **22**: 277-284
56 **Erim T**, Alazmi WM, O'Loughlin CJ, Barkin JS. Collagenous colitis associated with Clostridium difficile: a cause effect? *Dig Dis Sci* 2003; **48**: 1374-1375
57 **Perk G**, Ackerman Z, Cohen P, Eliakim R. Lymphocytic colitis: a clue to an infectious trigger. *Scand J Gastroenterol* 1999; **34**: 110-112
58 **Bohr J**, Nordfelth R, Järnerot G, Tysk C. Yersinia species in collagenous colitis: a serologic study. *Scand J Gastroenterol* 2002; **37**: 711-714
59 **Mäkinen M**, Niemelä S, Lehtola J, Karttunen TJ. Collagenous colitis and Yersinia enterocolitica infection. *Dig Dis Sci* 1998; **43**: 1341-1346
60 **Osterholm MT**, MacDonald KL, White KE, Wells JG, Spika JS, Potter ME, Forfang JC, Sorenson RM, Milloy PT, Blake PA. An outbreak of a newly recognized chronic diarrhea syndrome associated with raw milk consumption. *JAMA* 1986; **256**: 484-490
61 **Bryant DA**, Mintz ED, Puhr ND, Griffin PM, Petras RE. Colonic epithelial lymphocytosis associated with an epidemic of chronic diarrhea. *Am J Surg Pathol* 1996; **20**: 1102-1109
62 **Mintz E**. A riddle wrapped in a mystery inside an enigma: Brainerd diarrhoea turns 20. *Lancet* 2003; **362**: 2037-2038
63 **LaSala PR**, Chodosh AB, Vecchio JA, Schned LM, Blaszyk H. Seasonal pattern of onset in lymphocytic colitis. *J Clin Gastroenterol* 2005; **39**: 891-893
64 **Fernandez-Bañares F**, Esteve M, Salas A, Forné TM, Espinos JC, Martín-Comin J, Viver JM. Bile acid malabsorption in microscopic colitis and in previously unexplained functional chronic diarrhea. *Dig Dis Sci* 2001; **46**: 2231-2238
65 **Ung KA**, Kilander A, Willén R, Abrahamsson H. Role of bile acids in lymphocytic colitis. *Hepatogastroenterology* 2002; **49**: 432-437
66 **Lundberg JO**, Herulf M, Olesen M, Bohr J, Tysk C, Wiklund NP, Morcos E, Hellström PM, Weitzberg E, Järnerot G. Increased nitric oxide production in collagenous and lymphocytic colitis. *Eur J Clin Invest* 1997; **27**: 869-871
67 **Olesen M**, Middelveld R, Bohr J, Tysk C, Lundberg JO, Eriksson S, Alving K, Järnerot G. Luminal nitric oxide and epithelial expression of inducible and endothelial nitric oxide synthase in collagenous and lymphocytic colitis. *Scand J Gastroenterol* 2003; **38**: 66-72
68 **Perner A**, Andresen L, Normark M, Fischer-Hansen B, Sørensen S, Eugen-Olsen J, Rask-Madsen J. Expression of nitric oxide synthases and effects of L-arginine and L-NMMA on nitric oxide production and fluid transport in collagenous colitis. *Gut* 2001; **49**: 387-394
69 **Perner A**, Nordgaard I, Matzen P, Rask-Madsen J. Colonic production of nitric oxide gas in ulcerative colitis, collagenous colitis and uninflamed bowel. *Scand J Gastroenterol* 2002; **37**: 183-188
70 **Andresen L**, Jørgensen VL, Perner A, Hansen A, Eugen-Olsen J, Rask-Madsen J. Activation of nuclear factor kappaB in colonic mucosa from patients with collagenous and ulcerative colitis. *Gut* 2005; **54**: 503-509
71 **Bonderup OK**, Hansen JB, Madsen P, Vestergaard V, Fallingborg J, Teglbjaerg PS. Budesonide treatment and expression of inducible nitric oxide synthase mRNA in colonic mucosa in collagenous colitis. *Eur J Gastroenterol Hepatol* 2006; **18**: 1095-1099
72 **Bürgel N**, Bojarski C, Mankertz J, Zeitz M, Fromm M, Schulzke JD. Mechanisms of diarrhea in collagenous colitis. *Gastroenterology* 2002; **123**: 433-443
73 **Bohr J**, Järnerot G, Tysk C, Jones I, Eriksson S. Effect of fasting on diarrhoea in collagenous colitis. *Digestion* 2002; **65**: 30-34
74 **Warren BF**, Edwards CM, Travis SP. 'Microscopic colitis': classification and terminology. *Histopathology* 2002; **40**: 374-376
75 **Tanaka M**, Mazzoleni G, Riddell RH. Distribution of collagenous colitis: utility of flexible sigmoidoscopy. *Gut* 1992; **33**: 65-70
76 **Müller S**, Neureiter D, Stolte M, Verbeke C, Heuschmann P, Kirchner T, Aigner T. Tenascin: a sensitive and specific diagnostic marker of minimal collagenous colitis. *Virchows Arch* 2001; **438**: 435-441
77 **Cruz-Correa M**, Milligan F, Giardiello FM, Bayless TM, Torbenson M, Yardley JH, Jackson FW, Wilson Jackson F. Collagenous colitis with mucosal tears on endoscopic insufflation: a unique presentation. *Gut* 2002; **51**: 600
78 **Wickbom A**, Lindqvist M, Bohr J, Ung KA, Bergman J, Eriksson S, Tysk C. Colonic mucosal tears in collagenous colitis. *Scand J Gastroenterol* 2006; **41**: 726-729
79 **Smith RR**, Ragput A. Mucosal tears on endoscopic insufflation resulting in perforation: an interesting presentation of collagenous colitis. *J Am Coll Surg* 2007; **205**: 725
80 **Kiesslich R**, Hoffman A, Goetz M, Biesterfeld S, Vieth M, Galle PR, Neurath MF. In vivo diagnosis of collagenous colitis by confocal endomicroscopy. *Gut* 2006; **55**: 591-592
81 **Meining A**, Schwendy S, Becker V, Schmid RM, Prinz C. In vivo histopathology of lymphocytic colitis. *Gastrointest Endosc* 2007; **66**: 398-399, discussion 400
82 **Zambelli A**, Villanacci V, Buscarini E, Bassotti G, Albarello L. Collagenous colitis: a case series with confocal laser microscopy and histology correlation. *Endoscopy* 2008; **40**: 606-608
83 **Wildt S**, Nordgaard-Lassen I, Bendtsen F, Rumessen JJ. Metabolic and inflammatory faecal markers in collagenous colitis. *Eur J Gastroenterol Hepatol* 2007; **19**: 567-574
84 **Libbrecht L**, Croes R, Ectors N, Staels F, Geboes K. Microscopic colitis with giant cells. *Histopathology* 2002; **40**: 335-338
85 **Sandmeier D**, Bouzourene H. Microscopic colitis with giant cells: a rare new histopathologic subtype? *Int J Surg Pathol* 2004; **12**: 45-48
86 **Goldstein NS**, Bhanot P. Paucicellular and asymptomatic lymphocytic colitis: expanding the clinicopathologic spectrum of lymphocytic colitis. *Am J Clin Pathol* 2004; **122**: 405-411
87 **Rubio CA**, Lindholm J. Cryptal lymphocytic coloproctitis: a new phenotype of lymphocytic colitis? *J Clin Pathol* 2002; **55**: 138-140
88 **Yuan S**, Reyes V, Bronner MP. Pseudomembranous collagenous colitis. *Am J Surg Pathol* 2003; **27**: 1375-1379
89 **Saurine TJ**, Brewer JM, Eckstein RP. Microscopic colitis with granulomatous inflammation. *Histopathology* 2004; **45**: 82-86
90 **Chang F**, Deere H, Vu C. Atypical forms of microscopic colitis: morphological features and review of the literature. *Adv Anat Pathol* 2005; **12**: 203-211
91 **Baert F**, Schmit A, D'Haens G, Dedeurwaerdere F, Louis E, Cabooter M, De Vos M, Fontaine F, Naegels S, Schurmans P, Stals H, Geboes K, Rutgeerts P. Budesonide in collagenous colitis: a double-blind placebo-controlled trial with histologic follow-up. *Gastroenterology* 2002; **122**: 20-25
92 **Bonderup OK**, Hansen JB, Birket-Smith L, Vestergaard V, Teglbjaerg PS, Fallingborg J. Budesonide treatment of collagenous colitis: a randomised, double blind, placebo controlled trial with morphometric analysis. *Gut* 2003; **52**: 248-251
93 **Miehlke S**, Heymer P, Bethke B, Bästlein E, Meier E,

Bartram HP, Wilhelms G, Lehn N, Dorta G, DeLarive J, Tromm A, Bayerdörffer E, Stolte M. Budesonide treatment for collagenous colitis: a randomized, double-blind, placebo-controlled, multicenter trial. *Gastroenterology* 2002; **123**: 978-984

94 **Chande N**, McDonald JW, Macdonald JK. Interventions for treating collagenous colitis. *Cochrane Database Syst Rev* 2008; CD003575

95 **Miehlke S**, Madisch A, Karimi D, Wonschik S, Beckmann R, Kuhlisch E, Morgner A, Müller R, Greinwald R, Baretton G, Seitz G, Stolte M. Budesonide for treatment of lymphocytic colitis - a randomized, double-blind, placebo-controlled trial. *Gut* 2007; **56** Suppl III: A156

96 **Chande N**, McDonald JW, Macdonald JK. Interventions for treating lymphocytic colitis. *Cochrane Database Syst Rev* 2008; CD006096

97 **Bonderup OK**, Hansen JB, Teglbjoerg PS, Christensen LA, Fallingborg JF. Long-term budesonide treatment of collagenous colitis: a randomised, double-blind, placebo-controlled trial. *Gut* 2009; **58:** 68-72. Epub 2008 Jul 31

98 **Miehlke S**, Madisch A, Bethke B, Morgner A, Kuhlisch E, Henker C, Vogel G, Andersen M, Meier E, Baretton G, Stolte M. Oral budesonide for maintenance treatment of collagenous colitis: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 2008; **135**: 1510-1516

99 **Munck LK**, Kjeldsen J, Philipsen E, Fischer Hansen B. Incomplete remission with short-term prednisolone treatment in collagenous colitis: a randomized study. *Scand J Gastroenterol* 2003; **38**: 606-610

100 **Fine KD**, Ogunji F, Lee E, Lafon G, Tanzi M. Randomized, double blind, placebo-controlled trial of bismuth subsalicylate for microscopic colitis. *Gastroenterology* 1999; **116**: A880

101 **Calabrese C**, Fabbri A, Areni A, Zahlane D, Scialpi C, Di Febo G. Mesalazine with or without cholestyramine in the treatment of microscopic colitis: randomized controlled trial. *J Gastroenterol Hepatol* 2007; **22**: 809-814

102 **Wildt S**, Munck LK, Vinter-Jensen L, Hanse BF, Nordgaard-Lassen I, Christensen S, Avnstroem S, Rasmussen SN, Rumessen JJ. Probiotic treatment of collagenous colitis: a randomized, double-blind, placebo-controlled trial with Lactobacillus acidophilus and Bifidobacterium animalis subsp. Lactis. *Inflamm Bowel Dis* 2006; **12**: 395-401

103 **Madisch A**, Miehlke S, Eichele O, Mrwa J, Bethke B, Kuhlisch E, Bästlein E, Wilhelms G, Morgner A, Wigginghaus B, Stolte M. Boswellia serrata extract for the treatment of collagenous colitis. A double-blind, randomized, placebo-controlled, multicenter trial. *Int J Colorectal Dis* 2007; **22**: 1445-1451

104 **Pardi DS**, Loftus EV Jr, Tremaine WJ, Sandborn WJ. Treatment of refractory microscopic colitis with azathioprine and 6-mercaptopurine. *Gastroenterology* 2001; **120**: 1483-1484

105 **Riddell J**, Hillman L, Chiragakis L, Clarke A. Collagenous colitis: oral low-dose methotrexate for patients with difficult symptoms: long-term outcomes. *J Gastroenterol Hepatol* 2007; **22**: 1589-1593

106 **Varghese L**, Galandiuk S, Tremaine WJ, Burgart LJ. Lymphocytic colitis treated with proctocolectomy and ileal J-pouch-anal anastomosis: report of a case. *Dis Colon Rectum* 2002; **45**: 123-126

107 **Goff JS**, Barnett JL, Pelke T, Appelman HD. Collagenous colitis: histopathology and clinical course. *Am J Gastroenterol* 1997; **92**: 57-60

108 **Bonner GF**, Petras RE, Cheong DM, Grewal ID, Breno S, Ruderman WB. Short- and long-term follow-up of treatment for lymphocytic and collagenous colitis. *Inflamm Bowel Dis* 2000; **6**: 85-91

109 **Mullhaupt B**, Güller U, Anabitarte M, Güller R, Fried M. Lymphocytic colitis: clinical presentation and long term course. *Gut* 1998; **43**: 629-633

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TOPIC HIGHLIGHT

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Recent advances in the management of radiation colitis

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Abstract

Radiation colitis, an insidious, progressive disease of increasing frequency, develops 6 mo to 5 years after regional radiotherapy for malignancy, owing to the deleterious effects of the latter on the colon and the small intestine. When dealing with radiation colitis and its complications, the most conservative modality should be employed because the areas of intestinal injury do not tend to heal. Acute radiation colitis is mostly self-limited, and usually, only supportive management is required. Chronic radiation colitis, a poorly predictable progressive disease, is considered as a precancerous lesion; radiation-associated malignancy has a tendency to be diagnosed at an advanced stage and to bear a dismal prognosis. Therefore, management of chronic radiation colitis remains a major challenge owing to the progressive evolution of the disease, including development of fibrosis, endarteritis, edema, fragility, perforation, partial obstruction, and cancer. Patients are commonly managed conservatively. Surgical intervention is difficult to perform because of the extension of fibrosis and alterations in the gut and mesentery, and should be reserved for intestinal obstruction, perforation, fistulas, and severe bleeding. Owing to the difficulty in managing the complications of acute and chronic radiation colitis, particular attention should be focused onto the prevention strategies. Uncovering the fibrosis mechanisms and the molecular events underlying radiation bowel disease could lead to the introduction of new therapeutic and/or preventive approaches. A variety of novel, mostly experimental, agents have been used mainly as a prophylaxis, and improvements have been made in radiotherapy delivery, including techniques to reduce the amount of exposed intestine in the radiation field, as a critical strategy for prevention.

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INTRODUCTION

Radiation colitis is an insidious, progressive disease of increasing frequency. It is usually iatrogenic and unavoidable and frequently develops 6 mo to 5 years after regional radiotherapy for malignancy[1,2]. About half of all patients with malignancies undergo irradiation as part of their therapy. Considerable morbidity and mortality accompany radiation treatment because of the deleterious effects on adjacent normal tissues, mainly the colon and the small intestine. The type and extent of injury, depending on the dose of the radiation and the radiation sensitivity of the gut and the duration, is highly variable, ranging from 3 mo to 30 years[1,3]. Serious consequences may develop after years of gestation, and the disease, its treatment, and the disability produced are formidable. Apart from acute radiation colitis, manifestations of chronic radiation injury include proctitis, hemorrhages, fistulas, abscesses with signs of sepsis, perforations, strictures, and even cancer. Therefore, novel means to increase resistance of the intestine to radiation damage and effective therapeutic strategies are needed to prevent and manage this disease.

MANAGEMENT OF COLITIS CAUSED BY IRRADIATION

In general, prior to start, each treatment should be

individualized, and any predisposing factor should be identified during its course in order to early recognize and treat complications. Once complications have arisen, it is best to deal with the irradiated tissue by the most conservative modality, because the areas of intestinal injury do not tend to heal. This may require early diversion or resection as conservative therapy, because fistulas and bleeding will become recurrent and intractable. The effectiveness of non-surgical approaches remains far from desirable, and bleeding recurrence represents a major drawback that leads to a need for consecutive therapeutic sessions and combination of techniques[4]. If diversion fails to control bleeding, resection is necessary, even if it involves an abdominoperineal resection.

From another general viewpoint, there is a similarity in the activation of mucosal cytokines between inflammatory bowel disease (IBD) and radiation proctosigmoiditis. Indeed, as in the case of IBD patients, the mucosal levels of interleukin (IL)-2, -6, and -8 are significantly higher in both diseased and normal segments of colon in patients with radiation proctitis, compared with normal controls. In addition, IL-1β levels are significantly higher in diseased segments, compared with endoscopically normal-appearing segments in radiation proctitis. Tumor necrosis factor-alpha (TNF-α) levels are also significantly elevated in irradiated mice compared with non-irradiated controls[5]. These data may partially explain the beneficial effects of similar systemic and topical drugs including mesalamine compounds and steroids when used in radiation-induced proctosigmoiditis[6].

ACUTE RADIATION COLITIS (TABLE 1)

Empirical-experimental management

The majority of acute radiation colitis is self-limited, and only supportive management is required[7]. It must be emphasized, however, that acute radiation syndrome with a threshold dose of 8 Gy in man, represents a lethal clinical-pathological unit, enteritis and proctocolitis necro-hemorrhagica, with unknown causal therapy. In this respect, the detection of phospho-Elk-1, a protein acting as a transcription factor activating specific genes, might be considered as a suitable and very sensitive marker of acute radiation-induced injury of large and small intestine[8]. Whether Elk-1 inhibitors, such as the compound A (CpdA) or the protective agent U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene], the effect of which probably results from the IL-1β mRNA reduction *via* the inhibition of ERK pathway, can be used in the management of this syndrome remains to be investigated[9,10].

Inflammatory cell infiltration of the colon is observed at an early stage of radiation-induced colitis. The migration of inflammatory cells from the circulation requires interactions between cell adhesion molecules on the vascular endothelium and molecules on the surface of leukocytes. Specifically, circulating leukocytes are recruited to sites of inflammation by a well-regulated and coordinated process that largely occurs in postcapillary venules. Adhesion molecules are expressed on the surface of endothelial cells, and leukocytes are involved in an orderly sequence of cell-cell interactions that include leukocyte adherence to vascular endothelium and the subsequent transendothelial migration into the inflamed tissue. Finally, reactive oxygen metabolites produced by activated leukocytes can induce damage to various cellular components, including structural and regulatory proteins, carbohydrates, lipids, DNA and RNA. In this respect, upregulation of intercellular adhesion molecule (ICAM)-1 and the accumulation of inflammatory myeloperoxidase-positive cells have been observed during acute radiation colitis prior to an overt radiation-induced ulcer, thereby playing important roles in the development of radiation-induced colonic ulcer[11]. Moreover, there is direct *in vivo* evidence that antioxidant mechanisms of the intestinal mucosa are not mobilized during the acute tissue radiation response; four days after exposure, during the inflammatory phase, superoxide dismutases (SOD) and catalase are decreased and glutathione peroxidases and metallothioneins are induced. Dexamethasone treatment modulates only glutathione peroxidase expression and does not influence either metallothionein or SOD expression. These experimental data indicate that during the radiation-induced acute inflammatory response, an imbalance of the antioxidant network of intestinal mucosa occurs[12].

In view of the aforementioned data, modulation of the leukocyte recruitment and activation pathway seems to be a potential therapeutic strategy against acute radiation colitis. Further supporting this consideration, experimental studies have demonstrated that leukocyte rolling is mediated by P-selectin and that firm leukocyte adhesion is supported by lymphocyte function antigen-1 in radiation-induced colitis. P-selectin-dependent leukocyte rolling is a precondition for subsequent leukocyte adhesion in radiation-induced intestinal damage. Therefore, targeting P-selectin and/or lymphocyte function antigen-1 might protect against pathologic inflammation in the colon induced by radiotherapy[13]. Moreover, Cu/Zn-SOD1 supplementation in an

Table 1 Management of acute radiation colitis

Management of acute radiation colitis
Supportive management
Anti-diarrheal medications and by reducing fat and lactose intake; In intractable cases hospitalization is required for parenteral feeding and elementary diet
Elk-1 inhibitors
Compound A (CpdA)
U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene]
Modulation of leukocyte recruitment and activation pathway
Targeting P-selectin and/or lymphocyte function antigen-1
Cu/Zn-SOD1 supplementation
Synthetic somatostatin analog octreotide
Other measures
Antiemetics
Steroid-containing suppositories
Recombinant granulocyte colony-stimulating factor in neutropenia
Epidermal growth factor

experimental model of radiation-induced intestinal inflammation has also been shown to decrease oxidative stress and adhesion molecule upregulation in response to abdominal irradiation. Specifically, a significant increase in the flux of rolling leukocytes and number of firmly adherent leukocytes in intestinal venules is observed after irradiation. Although administration of SOD1 has no effect on leukocyte rolling, it decreases leukocyte adhesion to intestinal venules significantly and in a dose-dependent way. Treatment with SOD1, at doses that reduce leukocyte recruitment, abrogates the increase in hydroperoxides in intestinal tissue and ICAM-1 upregulation in intestinal endothelial cells. The inflammatory score, but not a combined histology damage score, is also significantly reduced by SOD1[14].

Diarrhea associated with acute radiation colitis frequently resolves with anti-diarrheal medications and by reducing fat and lactose intake. The diarrhea rarely requires discontinuation of treatment unless chemotherapy is given concurrently with radiation[15]. Intractable diarrhea during the combined treatment may require hospital admission for administration of parenteral feeding. Elementary diet may also be introduced as an alternative to parenteral nutrition[16].

Patients refractory to anti-diarrheal medications may benefit from administration of the synthetic somatostatin analog octreotide[7]. Specifically, it has been shown that subcutaneous octreotide administration (150 μg t.i.d.) for 5 d is apparently an effective, well-tolerated treatment modality for concurrent chemoradiotherapy-induced diarrhea refractory to loperamide[17]. Octreotide appears to be more effective than conventional therapy with diphenoxylate and atropine in controlling acute radiation-induced diarrhea and eliminating the need for radiotherapy interruptions[18].

Apart from anti-diarrheal medications, other measures of general management of acute radiation enteropathy include administration of antiemetics. Steroid-containing suppositories may be helpful in the treatment of patients with anorectal inflammation[7]. Severe neutropenia from chemotherapy might require growth factors, such as recombinant granulocyte colony-stimulating factor (G-CSF, filgrastim) or granulocyte-macrophage colony-stimulating factor (GM-CSF, sargramostim) to shorten the period of neutropenia, and avoid excessively delayed therapy from the bone marrow depression[19]. G-CSF is a cytokine known to activate neutrophils *in vivo* and GM-CSF mediates its effects on the neutrophil lineage through its effects on phagocytic accessory cells and its synergy with G-CSF[20,21].

Epidermal growth factor, an endogenous peptide, trophic to the gastrointestinal tract, significantly decreases the acute clinical manifestations of experimental radiation enteritis[22]. Therefore, it may be effective in human acute radiation colitis[23].

CHRONIC RADIATION COLITIS (TABLE 2)

Empirical-experimental management

Chronic radiation colitis is recognized as a frequent and clinically important sequel of abdominal and pelvic irradiation treatment for malignant disease. Since radiotherapy is now being used more than ever before in the therapy of solid organ neoplasms of the abdomen and the pelvis, the incidence of radiation colitis is likely to increase in the future[24-26]. Importantly, it is a precancerous lesion: Radiation-associated rectal cancer originates from dysplasia due to radiation colitis and has a tendency to be diagnosed at an advanced stage and to bear a dismal prognosis[27,28]. Therefore, management of chronic radiation colitis remains a major challenge owing to the progressive evolution of the disease that includes development of fibrosis, endarteritis, edema, fragility, perforation, partial obstruction, and even cancer. Patients with this condition are commonly managed conservatively. Because the obstruction is only partial, decompression is easily achieved by nasogastric suction and parenteral support. The patient is then often discharged on a liquid-to-soft diet. However, this therapeutic regimen does nothing for the underlying pathology. Although total parenteral nutrition corrects denutrition and facilitates deferred surgery in some patients, severe radiation enteritis remains a poorly predictable progressive disease with numerous relapses[29]. The problem, sooner or later, will return with the patient further depleted by the chronic radiation colitis. In a recent meta-analysis assessing the incidence and significance of malnutrition and examining the efficacy of therapeutic nutritional interventions used to manage gastrointestinal side effects in patients undergoing pelvic radiotherapy, it has been shown that there is no evidence favoring the use of nutritional interventions to prevent or manage bowel symptoms attributable to radiotherapy[30]. Regarding the underlying

Table 2 Management of chronic radiation colitis (IL; TNF-α)

Management of chronic radiation colitis
Empirical-experimental management
Total parenteral nutrition
Anti-IL-6R
Cyclooxygenase-2 inhibitors
Rho kinase inhibitors
Small molecular inhibitors of TNF-α
Targeting cadherin-catenin complex pathways
Recombinant human IL-11
Low-residue diet combined with bismuth subsalicylate or opiate drugs, such as loperamide or diphenoxylate (for mild diarrhea)
Aminosalicylates
Prostaglandin-inhibiting compounds
Oral steroids (for severe cases)
Probiotics (Lactobacillus bulgaricus)
Antioxidants
Colestyramine Balsalazide (in radiation-induced proctosigmoiditis)
Peroxisome proliferation-activated receptor activators
Sucralfate enemas
Short-chain fatty acids
Hyperbaric oxygen
Control of bleeding (by endoscopic cauterization using a heater, BICAP probe, Nd: YAG or argon laser)
Surgery (indicated in intestinal obstruction, perforation, fistulas, and severe bleeding)

pathology, vascular damage consisting of fibrin thrombi, fibrinoid necrosis and subintimal thickening of the arterioles leads to persistent local ischemia, which results in diffuse fibrosis of the lamina propria and submucosa. The diffuse fibrosis, in turn, accelerates vascular damage and further worsens local ischemia, forming a vicious cycle, finally leading to ulceration of the bowel wall and serious complications including massive gastrointestinal hemorrhages and perforations[31]. Therefore, surgical intervention appears to be appropriate when the diagnosis of chronic radiation colitis is confirmed[32].

Nevertheless, chronic changes in cytokine levels after abdominal irradiation in rodents have recently been documented[33]. Structural injury of the bowel wall and mesentery were scored and correlated with the levels of TNF-α, IL-6, transforming growth factor (TGF)-β1, -β2, -β3 and interferon (IFN)-γ mRNA in large and small bowel of mice 18-25 wk after whole abdominal irradiation with 12.5 and 13.5 Gy. Abdominal irradiation seems to induce considerable bowel damage associated with increased levels of all cytokines compared with sham-irradiated (0 Gy) mice. These experimental data demonstrate long-term cytokine expression changes in the bowel wall after irradiation that parallel the responses noticed in other tissues prone to radiation-induced fibrosis, such as cutaneous and pulmonary tissues, thereby having implications for the prediction, treatment and/or prevention of chronic radiation colitis. For instance, chronic IL-6 elevations, even prior to the start of irradiation, may predict patients at risk of radiation fibrotic bowel damage in the same way that IL-6 baseline elevations have been shown to identify patients with an increased risk of radiation pneumonitis and pulmonary fibrosis following thoracic irradiation[33]. Since studies in animal models of IBD have shown that various antibodies to pro-inflammatory cytokines and their receptors, such as IL-6 receptor (IL-6R) or TNF, appear to suppress chronic intestinal inflammation by inducing T-cell apoptosis[34], it is reasonable to assume that such antibodies (anti-IL-6R) might also be used to manage radiation colitis. In addition, reduction in cytokine expression with cyclooxygenase (COX)-2 inhibitors and small molecular inhibitors of TNF-α may reduce the frequency and severity of long-term bowel damage. There is some evidence that the COX-2 pathway is implicated in radiation-induced gut injury[31,35,36]. COX-2 and nuclear factor κB (NF-κB) expression have been associated with histopathological changes in the human colon and rectum following abdominal radiotherapy[31].

Besides, in radiation colitis involving aberrant glands, cellular proliferation increases and spotted oncogene p53 expression is noticed. Therefore, radiation colitis and aberrant glands with p53 overexpression might predict malignant potential of this condition[37].

Three typical phases of radiation proctitis are defined on histological grounds (acute damage, and early and late regenerative phases), essentially correlating with the time interval between radiotherapy and surgery. Such characteristics are mirrored by alterations in cadherin-catenin expression and localization in rectal crypts; morphology at both cellular and glandular levels in the large bowel is dependent to an extent on cell-cell adhesion mediated by cadherin-catenin complexes. In this regard, P-cadherin is highly expressed in the acute radiation damage and early regenerative phases, with a decreased level of expression during late regeneration. E-cadherin and associated catenins are translocated from the membrane to the cytoplasm in degenerating crypts, with return to normal membranous expression in regenerating crypts. Therefore, radiation-induced proctitis represents an *in vivo* model of mucosal damage and regeneration, thereby providing a valid model to study events during epithelial injury and repair: altered cadherin and associated catenins expressions appear to be predictive indicators closely associated with these processes[38]. On the other hand, because the E-cadherin-catenin complex plays a critical role in the maintenance of normal tissue architecture, mutation of any of its components is believed to result in loss of cell-cell adhesion, thereby contributing to neoplasia development. In this respect, adenomatous polyposis coli (APC) gene abnormalities, found to be the "gate-keeping" event for the initiation of colorectal neoplasia, may lead to a disruption of normal cell-cell adhesion through altered association with catenins and the cell adhesion molecule E-cadherin that binds catenins[39]. Translocation of the β-catenin protein, a key downstream effector of the Wnt signal transduction pathway, is frequently found in colorectal cancer. This protein is also observed in the cytoplasm and/or nucleus of non-neoplastic irradiated colonocytes. Nuclear translocation of β-catenin correlates with loss of APC and gain of cyclin D1 expression, suggesting the activation of the Wnt pathway during radiation-induced colorectal carcinogenesis. Because the translocation of β-catenin is found in irradiated-colonic mucosa as well as in colon cancer, the disruption of the β-catenin expression may be one of the early events in radiation-induced colonic oncogenesis[40]. Based on these data, interventions on cadherin-catenin complex pathways may also be used against chronic radiation colitis and radiation-induced colonic carcinogenesis. Finally, in a novel mouse model of radiation-induced colitis, a combination of high-dose γ-irradiation and lack of major histocompatibility complex (MHC) class Ⅱ expression on cells of hematopoietic origin results in the development of radiation colitis. Therefore, protection and/or inhibition from radiation-induced colitis seems to require MHC class Ⅱ antigen expression by cells of hematopoietic origin[41]. In this regard, administration of the recombinant pleiotropic human cytokine IL-11, which stimulates bone marrow stem cells to proliferate, has been shown to decrease intestinal mucosal injury produced by radiation in animals, thereby providing a potential therapeutic regimen for the treatment and/or prevention of chronic radiation colitis[42].

Diarrhea, with or without abdominal cramps, is the most common symptom of chronic radiation colitis[26]. The etiology of chronic radiation-induced diarrhea may be attributable to accelerated small and large

bowel transit, bacterial overgrowth, increased intestinal permeability, malabsorption of bile salts, lactose, fat and carbohydrate, and pancreatic insufficiency, all of which can exist with or without small bowel or large bowel strictures[43,44]. In case of colonic strictures, spurious diarrhea can occur. Moreover, the above mentioned microvascular changes in the bowel wall lead also to mucosal atrophy and a non-specific chronic inflammatory cell infiltrate, which has resulted in a mistaken diagnosis of celiac sprue[45]. However, in most cases, the pathophysiology of the diarrhea is uncertain. While changes in intestinal absorption and motility, unrelated to bacterial overgrowth, have been implicated in the etiology of diarrhea, there has been no comprehensive evaluation of gastrointestinal function in chronic radiation colitis. Perhaps partly as a result of this, present approaches to treatment have often been empirical. A low-residue diet (i.e. a low-fiber diet poor in foods that increase bowel activity) combined with bismuth subsalicylate or opiate drugs, such as loperamide or diphenoxylate, might be sufficient for mild diarrhea[1]; loperamide-N-oxide slows small intestinal transit, increases bile acid absorption, and is effective in the treatment of diarrhea associated with chronic radiation colitis[26]. Other antidiarrheal agents can be administered, including aminosalicylates and prostaglandin (PG)-inhibiting compounds[46,47]. In severe cases of radiation colitis, oral steroids have been tried with limited success[48]. Randomized controlled trials are not available, and all treatment regimens are based on evidence from small pilot studies, including the administration of sulfasalazine[49], glutathione (GSH)[50], and antioxidants[51]. Furthermore, antibiotics are indicated if there is small bowel bacterial overgrowth syndrome[52,53]. Preliminary results suggest that probiotics may also be useful for treatment of radiation bowel disease, although no robust data exist[54]. Other studies suggested that colestyramine, an agent that binds bile acids in the colonic lumen, might be effective in preventing radiation-induced diarrhea if administered in dosages of 4 g t.i.d. during radiation therapy[55]. In the presence of low serum magnesium levels, intravenous administration of magnesium sulfate, together with low residue diet and antidiarrheals, may also ameliorate the diarrhea[56].

Anti-diarrheal and bulk-forming agents have a role in the management of rectal urgency, frequency, and fecal incontinence, which might be induced by radiation damage of the myenteric plexus of the rectum and internal anal sphincter[57]. Sulfasalazine, 5-aminosalicylic acid (5-ASA) preparations and corticosteroid enemas have minimal or no effects on rectal tenesmus or bleeding[48].

However, recent pilot studies indicate that balsalazide, a new 5-ASA drug that yields a high concentration of active drug to the distal colon, is able to prevent or reduce symptoms of radiation-induced proctosigmoiditis[58]. In addition, irradiation-induced inflammatory response could be modulated pharmacologically based on the anti-inflammatory properties of 5-ASA, which is a peroxisome proliferation-activated receptor (PPAR) activator. PPAR agonists are now emerging as therapeutic drugs for various inflammatory diseases characterized by impaired PPAR expression: Irradiation drastically reduces mRNA and protein levels of PPAR-α and -γ. Specifically, 5-ASA treatment normalizes both PPAR-α and PPAR-γ during the post-irradiation period (after 7 and 3 d, respectively). By promoting PPAR expression and its nuclear translocation, 5-ASA interferes with the NF-κB pathway, both reducing irradiation-induced NF-κB p65 translocation/activation and increasing the expression of NF-κB inhibitor (IκB) mRNA and protein. Therefore, 5-ASA prevents irradiation-induced inflammatory processes as well as expression of TNF-α, monocyte chemotactic protein-1, inducible nitric-oxide synthase, and macrophage infiltration. In addition, 5-ASA restores the IFN-γ/signal transducer and activator of transcription (STAT)-1 and STAT-3 concentrations that were impaired at 3 and 7 d post-irradiation and are correlated with suppressor of cytokine signaling-3 repression. Collectively, these data suggest that PPAR agonists might be effective in the prevention of inflammatory processes and immune responses during and after pelvic radiotherapy[59].

Fecal incontinence appears to be a late complication that causes symptoms years after radiation treatment. The specific mechanisms that cause incontinence are changes in anal resting tone, squeeze pressure, and rectal volume or rectal compliance. Other aspects associated with incontinence include further disorders such as proctitis, colitis, and other disturbances involving the lower digestive tract. The therapeutic options mainly comprise management of associated aspects, such as proctitis or diarrhea; surgical intervention should be the absolute exception[60].

It has been reported that sucralfate treatment has a protective effect against experimental radiation colitis. Sucralfate enemas prior to radiation lead to reduction in: (a) the number of apoptotic colonic crypt cells; (b) the number of caspase-3 positive cells; (c) oncogene p53 accumulation and p21 expression; and (d) proapoptotic Bax/anti-apoptotic Bcl-2 ratio in rats. Therefore, the protective effects of sucralfate against radiation colitis might be partially due to the suppression of radiation-induced apoptosis in the colon and the protection of the colonic epithelial stem cell region[61]. Sucralfate administration may be also effective in human radiation proctocolitis[62]. In addition, when compared with oral sulfasalazine plus rectal prednisolone enemas, sucralfate enemas give a better clinical response in human proctosigmoiditis, are better tolerated, and, because of the lower cost, they might be the preferred short-term regimen[48]. Moreover, topical sucralfate induces a lasting remission in the majority of patients with moderate to severe rectal hemorrhage due to radiation proctosigmoiditis[63].

Clinically, short-chain fatty acids (SCFAs) have been proposed as possible therapeutic agents in several conditions including radiation proctitis. Although some promising effects have been observed in uncontrolled studies, a specific therapeutic role for SCFAs remains to be defined[64].

Hyperbaric oxygen

Hyperbaric oxygen application appears to be a very effective means of treatment of chronic radiation colitis and non-healing wounds in the involved anorectal region[65]. Hyperbaric oxygen therapy can be considered as a treatment option after failure of standard treatments in patients with severe radiation proctopathy[66]. The rationale for hyperbaric oxygen is the creation of an oxygen gradient in hypoxic tissue that stimulates the creation of new blood vessels. Neoangiogenesis improves the blood supply and reduces the ischemia and necrosis responsible for severe complications. In a retrospective study of patients with severe radiation colitis refractory to medical management, hyperbaric oxygen therapy provided clinical relief and can thus prove to be a useful alternative to conventional treatment in patients with chronic radiation-induced necrosis of the digestive tract[67]. Moreover, in a systematic review of the literature on the application of hyperbaric oxygen prevention and treatment of delayed radiation injuries, all but seven of the 74 publications analyzed reported positive results when hyperbaric oxygen was delivered as treatment for or prevention of delayed radiation injury. These results are particularly impressive in the context of alternative interventions[68]. Hyperbaric oxygen may also be helpful in management of bleeding due to chronic radiation colitis in patients not controlled with conservative measures such as formalin and laser therapy[69,70]. Hyperbaric oxygen treatment and infusion of PG E1 abolishes completely tarry stools and hematuria, and reverses the endoscopic findings of radiation colitis and cystitis[71].

Control of bleeding

Rectal bleeding due to radiation colitis usually results from telangiectasias. It is frequently minor, but blood transfusions may be required. Endoscopic cauterization using a heater, BICAP probe, Nd:YAG or argon laser can reduce bleeding.

Argon plasma coagulation therapy appears to be a simple, safe, and effective technique in the management of hemorrhagic radiation-induced proctosigmoiditis and is now generally accepted as the treatment of choice followed by local application of formalin if this fails[31,72,73]. Argon plasma coagulation, a non-contact thermal coagulation technique that reduces rectal bleeding in 80%-90% of cases, is applied endoscopically, with a probe passing through the endoscope that delivers a field of argon gas to the mucosal surface, where it is ionized by a high-voltage filament resulting in superficial mucosal heating and coagulation of friable blood vessels. Topical formalin therapy depends on direct application of a 4% concentration of the chemical soaked in gauze to the hemorrhagic areas under direct vision using a rigid sigmoidoscope. Thrombosis of the neovasculature and coagulation necrosis of the superficial mucosa ensues, with a complete response rate of 78%. While topical formalin appears to be slightly less effective than argon plasma coagulation therapy, formalin application alone or a combination of the two treatments has been advocated for severe cases of hemorrhagic radiation proctitis. Although formalin installation may be effective in controlling refractory bleeding due to radiation-induced proctitis, the procedure is not risk-free and may induce major complications such as acute colitis[74]. Preliminary results of a randomized study of the two therapeutic interventions, however, show equivalent efficacy but an absence of effect of either treatment on anorectal dysfunction.

Another approach to treat hemorrhagic radiation proctitis involves use of low-dose thalidomide, a potent inhibitor of (neo)angiogenesis, following a case report with successful outcome[75]. In addition, hormone therapy consisting of an estrogen-progesterone combination might provide a promising new additional symptomatic therapy for bleeding radiation colitis[76].

Rectal strictures should be managed initially non-operatively with a low-fiber diet, stool softeners, mineral oil enemas, and analgesics. Manual or endoscopic dilations of rectal strictures might be required. Short strictures with minimal angulation can be dilated by transendoscopic balloons or other dilators, albeit with considerable risk of perforation. Long, tortuous strictures should be managed operatively[77].

Surgery

About one third of patients with chronic radiation enteritis will need to be operated during follow-up. Surgical intervention is indicated in intestinal obstruction, perforation, fistulas, and severe bleeding. Surgery should be performed by an experienced team familiar with the treatment of radiation colitis. It is difficult to perform surgery for chronic radiation colitis because of the diffuse process of fibrosis and alterations in the gut and mesentery. The risk of anastomotic leak is high if the anastomosis is performed using irradiated tissue[78]. The risk can be lowered if at least one limb of the anastomosis did not receive prior radiotherapy[79]. It is difficult to distinguish between the normal tissue area and the irradiated area of the gut by gross evaluation during operation even when the fresh tissue is sent for frozen section. The accuracy in localizing injured intestine may be improved by intraoperative endoscopic evaluation, which can detect radiation-induced mucosal injury[80].

Resection of the affected intestine is significantly better than an enteric bypass procedure in overall outcome. However, extensive surgical resection of the diseased bowel may lead to short bowel syndrome and increase the need for total parenteral nutrition. Moreover, because of the progressive evolution of the fibrosis, the patient may require additional surgery. Surgical bypass of the damaged bowel is associated with a blind loop syndrome, and the patient may be still at risk of perforation, bleeding, abscess, and fistulae due to the persistence of the affected bowel. Bypass procedures should be performed when resection is not possible or as a temporary management before resection at a later date. Limited resection of the diseased intestine is the goal, but if the lesion is too diffuse, a bypass procedure

might be attempted.

Management of a pelvic fistula (e.g. vaginal or bladder fistula) is also complex and requires fecal diversion before the corrective surgery. Patients with fistulae frequently present with additional challenges such as electrolyte imbalance, malnutrition and infections. Many surgical techniques have been described to repair fistulae, but corrective surgery is best done when the patient is medically stable and enough time has elapsed after the surgical diversion. This permits the healing and decreased inflammation of the affected tissues[81,82].

In cases with severe fibrotic strictures, surgical intervention with establishment of a primary anastomosis may be required[83]. Strictureplasty may be an effective and safe tool to conserve intestinal length in certain highly selected patients with chronic radiation colitis and small-bowel strictures, namely those with limited intestinal reserve where strictures are located within long segments of diseased bowel which, if resected or bypassed, would have significant nutritional or metabolic consequences. Strictureplasty is not indicated for the treatment of perforation, hemorrhage, fistula, or short segments of disease in patients with adequate intestinal reserve[84].

Surgical complications of chronic radiation colitis such as intestinal obstruction, enterocutaneous fistula, intestinal stenosis, intestinal bleeding, severe proctocolitis and intestinal perforation should be managed operatively[85].

It is important to note that vigorous preoperative and postoperative nutritional support and evaluation are vital because of the poor healing qualities of the irradiated gut.

If conservative measures and local intervention to control bleeding prove unsuccessful, resection or ligation of the affected area(s) is preferred over a bypass procedure because the latter will allow the hemorrhage to continue and may lead to a higher mortality rate[86]. A promising surgical approach is small bowel transplantation, which may be considered in the pediatric population with radiation colitis.

PREVENTION OF RADIATION COLITIS

Based on the above data, it appears that the management of radiation bowel damage can be difficult and problematic; chronic radiation colitis is complex and rarely curable. Recent advances in the approaches to its prevention or amelioration are therefore particularly encouraging. Research to uncover the mechanisms of fibrosis and the molecular events underlying radiation bowel disease could lead to the development of new therapeutic and/or preventive approaches and provide the basis for predicting the risk of bowel damage and oncogenesis using levels and expressions of the mentioned cytokine IL-6, oncogene p53 or cadherin-catenin complexes, and for amelioration of bowel damage through inhibition, for example, of the COX-2 and Rho/Rho kinase pathways[33,87]. In this regard, the COX-2 inhibitor Rofecoxib® has been shown to suppress cytokine expression and to reduce acute bowel damage in rodents following abdominal irradiation[36]. In addition, piroxicam (a nonsteroidal anti-inflammatory agent) significantly decreases the incidence of colonic neoplasia in general and also delays the endoscopic appearance of colonic neoplasia in rats after pelvic irradiation[88]. Moreover, specific inhibition of Rho kinase is a promising approach for the amelioration of radiation fibrotic gut damage, as reported by a study investigating molecular pathways involved in the maintenance of fibrosis of the bowel wall of late radiation colitis patients[87]. Alterations in expressions of genes coding for Rho proteins was first established by molecular profile analysis of ileal biopsies. Primary cultures of gut smooth muscle cells derived from the ileal biopsies are associated with retention of fibrogenic differentiation *in vitro* and exhibit a typical cytoskeletal network, a high constitutive connective tissue growth factor level, increased collagen secretory capacity and altered expression of genes coding for the Rho family. Rho kinase blockade induces a simultaneous reduction in the number of actin stress fibers, α-smooth muscle actin and heat shock protein (Hsp) 27 levels. It also reduces connective tissue growth factor levels, the latter probably through NF-κB inhibition, leading to decreased expression of the type 1 collagen gene[87]. These observations show the involvement of the Rho/Rho kinase pathway in radiation fibrosis and intestinal smooth muscle cell fibrogenic differentiation, suggesting the potential role of Rho kinase inhibitors in ameliorating the radiation bowel damage.

Additional biomarkers potentially playing a role in the prediction, reduction or prevention of radiation colitis include genetic alterations of the cellular radiation response genes, such as the ataxia telangiectasia gene, and micronutrients, such as selenium and zinc. Genetic variants of the ataxia telangiectasia gene have been correlated with the risk of rectal hemorrhage associated with chronic radiation proctitis among prostate cancer patients who received the full brachytherapy prescription dose to defined volumes of the rectum[89]. The ataxia telangiectasia sequence alterations lead to an approximately sevenfold increase in mild to moderate (Radiation Therapy Oncology Group grades 1 and 2) radiation proctitis among patients who had received the full prescription dose to either low (< 0.7 mL) or moderate (0.7-1.4 mL) volumes of their rectum. Patients contemplating this increasingly popular radiation treatment modality for early prostate cancer should not only be better informed about the risks of bowel complications, but could also have their radiation dose prescriptions individualized based on genetic profiling.

Dietary supplementation of selenium and zinc may be useful in reducing anorectal sequelae after pelvic radiotherapy; an indirect relationship between baseline plasma levels of these micronutrients and abnormalities in anorectal function one year after radiotherapy for prostate cancer has been suggested. Notably, the heavy metal zinc induces Hsps, also known as stress proteins and molecular chaperones, which play a central role in protecting cellular homeostatic processes from environmental and physiologic insults by preserving the

structure of normal proteins and repairing or removing damaged ones. Lowering Hsps in cancer tissues can amplify the effectiveness of chemo- or radiotherapy[90].

Importantly, improvements in the delivery of radiotherapy, including techniques to reduce the amount of exposed intestine in the radiation field, also represent a critical strategy for prevention. The ideal radiation toxicity preventive therapy must have high efficacy, low toxicity, low cost, and not afford cancer protection. Unfortunately, the currently available therapy often does not fulfill all of these objectives and there is a need to identify patients who may truly benefit from preventive therapies. Specifically, the radiation therapy technique plays an essential role in reducing the rate of complications; particular attention should be paid to optimizing radiotherapy technique and dose prescriptions. The use of only anterior and posterior fields for pelvic radiation should be avoided, if possible, because of the high dose and large volume of intestine irradiated. A higher operative mortality was reported in trials using this technique preoperatively for rectal cancers[91,92]. The toxicity of radiation is directly related to the volume of small bowel being irradiated[93]. In many patients, therapy in the prone position with a special "belly" board allows the protrusion of the small intestine out of the radiation field[94,95]. Patients should be instructed to maintain a full bladder during the radiation session, which mechanically displaces the intestine out of the pelvis[96].

Modern radiation treatment techniques, such as three-dimensional treatment planning, also optimize the treatment technique by developing more accurate dose distributions. Notably, three-dimensional conformal radiotherapy techniques, including intensity-modulated radiotherapy, may not reduce late intestinal toxicity because margins around the cancer may not be able to be safely reduced and because of the prescription of higher radiation doses[97,98]. Brachytherapy, alone or as a supplement to external beam radiotherapy, is now increasingly being utilized to decrease normal tissue toxicity, without compromising treatment efficacy, in the management of prostate carcinoma[99,100]. Brachytherapy is a kind of radiotherapy whereby the source of radiation is located either within the malignant tissue (interstitial brachytherapy) or within a cavity in its immediate vicinity (intracavitary brachytherapy), rather than at a distance (typically 100 cm) from the center of the neoplasm target, as it is the case with external beam radiotherapy. Brachytherapy exploits the physical characteristics inherent with this modality of radiotherapy, whereby the high radiation dose is limited to the neoplasm target, while the surrounding normal tissues are spared from radiation by the rapid dose reduction (with the square of the distance). Brachytherapy alone, in the therapy of low-risk prostate carcinoma, is well tolerated, even in patients with a history of IBD[101].

Another related treatment, such as intensity-modulated radiotherapy (IMRT), uses sophisticated planning techniques to avoid critical structures. IMRT uses multiple segments of beams to shape the dose distribution to a desired result.

Operation, as a major risk factor, leads to the prolapse of the small intestine into the pelvis, exposing it to a full dose of radiation. Postoperative bowel adhesions also increase the volume of gut irradiated compared with normal intestine, usually mobile and able to move out of the radiation field. With gut adhesions, the intestine is trapped and is more likely to receive a high dose of radiation. If radiation therapy is anticipated after surgery, every attempt should be made at the time of surgery to displace the bowel outside of the radiation field[102]. One simple technique is the surgical placement of a polyglycolic, biodegradable mesh that moves the intestine out of the pelvis[103,104]. The procedure has negligible morbidity and it does not increase the operating time significantly. It also does not require a second operation to remove the mesh because it is absorbed 3 to 4 mo postoperatively. MRI can be used post-operatively to verify the position of the mesh, the small bowel, and its disappearance. Placement of a mesh during surgery allows a higher dose of radiation to be given postoperatively when indicated, thereby decreasing by 50% the volume of the small bowel exposed to the radiation[105,106]. Other techniques such as pelvic reconstruction, omentoplasty, and transposition of the large bowel also reduce the volume of gut at risk for radiotherapy up to 60%[106-109].

Amifostine (WR-2721) is an amino-thiol with well-established radioprotective effects. Recent studies have documented its effectiveness in protection of the salivary glands in patients receiving radiotherapy for head-and-neck cancer[110]. It has also been investigated for the prevention of chronic radiation colitis. According to preclinical studies, amifostine protects both the small and large intestine[111]. Specifically, it is converted intracellularly to an active metabolite, WR-1065, which in turn binds to free radicals and protects the cell from radiation damage[112]. In a randomized study, the late effects of radiation were significantly reduced in the group receiving parenterally administered amifostine. However, the median follow-up was quite short (24 mo), and longer follow-up is necessary to confirm the benefits of this medication because the incidence of late complications increases with time[113]. There is also evidence suggesting that intrarectal application of amifostine directly onto the rectum may reduce the risk of proctitis in patients undergoing radiotherapy for prostate cancer[114]; its intrarectal application is feasible and well tolerated. Systemic absorption of amifostine and its metabolites is negligible, and close monitoring of patients is not required after rectal administration[115]. Systemic administration of amifostine, used concurrently with radiotherapy in advanced rectal cancer, has been reported to reduce acute and late pelvic radiation toxicity[113]. Other investigators[116], however, were not able to demonstrate any protection afforded by amifostine.

Since cytotoxic effects of ionizing radiation on gastrointestinal epithelium may be related to oxidative stress, a number of agents have been used as a prophylaxis

treatment. Eicosanoids and free radicals release have been implicated in the pathogenesis. Selenium and/or vitamin E pretreatments are shown to improve post-irradiation disturbances in pro-oxidant-antioxidant balance, such as increased intestinal lipid peroxide and decreased GSH levels, increased intestinal SOD and GSH peroxidase activities and decreased glutathione transferase activity. This amelioration has been confirmed by histopathological findings[117]. In another study, the early side effects of radiation were suggested to be prevented by vitamin A supplementation[118].

PGs have been investigated as potential radioprotectors. PGE2 and the PG analogs enprostil and misoprostol (Cytotec®) display radiation protection in animal studies[119-122]. Misoprostol suppositories also reduced symptoms of acute radiation colitis in patients undergoing radiation therapy for prostate cancer[123]. With respect to the mechanism of action, PGE2 has pro-proliferative and anti-apoptotic effects on epithelial cells in gastrointestinal injury. PGE2 decreases radiation-induced apoptosis and increases crypt survival[124].

Experimental data indicate that in control animals, glucagon-like peptide-2 (GLP-2) induces an increase in intestinal mucosal mass, along with an increase in villus height and crypt depth. GLP-2 administration before and after irradiation completely prevents the acute radiation-induced mucosal ulcerations and strikingly reduces the late radiation damage. Microscopic observations show an improved organization of the intestinal wall and an efficient wound healing process, especially in the smooth muscle layers. This therapeutic effect is mediated through an increased mucosal mass before tissue injury and the stimulation of still unknown mechanisms of tissue response to radiation damage. Although these preliminary results still need to be confirmed, GLP-2 might be a way to limit patient discomfort during radiotherapy and reduce the risk of consequential late effects[125].

Irradiated intestine consistently exhibits increased immunoreactivity of transforming growth factor (TGF)-β1. It has been demonstrated that mucosal barrier breakdown is closely associated with increased TGF-β immunoreactivity in subsequent radiation enteropathy. The highly significant correlation between TGF-β expression levels and alterations in late-responding tissue compartments also suggest a role for TGF-β in primary radiation colitis. A recent preclinical study showed a role for possible anti-TGF-β1 interventions to reduce delayed radiation fibrosis and enteropathy[126].

Preliminary studies also suggest that IFN-γ may be effective in the treatment of patients with radiation-induced cutaneous fibrosis. IFN-γ should be considered in Phase Ⅰ-Ⅱ studies to assess its toxicity and efficacy in the treatment of patients with radiation colitis[127].

Many special diets and nutrients, such as the mentioned fiber, elemental diets, SCFAs and amino acids like glutamine, may reduce small-bowel radiation toxicity. Specifically, probiotics (*Lactobacillus bulgaricus* strain isolated from yogurt) added as substrates can be given by an oral or enteral route to patients who undergo radiotherapy to prevent radiation-induced colitis and related malnutrition[128].

Glutamine and arginine support the mucosal barrier in several ways. In experimental studies, a 7-d glutamine- or arginine-enriched diet administered both pre- and post-irradiation showed that they have protective effects on gut mucosa in the post-irradiation state. However, pre- and post-irradiation administration together do not provide superior protection compared to post-irradiation administration alone[129].

Administration of insulin-like growth factor (IGF)-I immediately following abdominal irradiation increases small-intestinal mass and improves indicators of mucosal integrity, suggesting acceleration of small-intestinal mucosal recovery from radiation injury[130]. More recently, growth hormone and IGF-I have been demonstrated to protect intestinal cells from radiation-induced apoptosis both *in vitro*, by inhibiting apoptosis of the cells and preserving the mucosal integrity[131], and *in vivo*[132], utilizing IGF- I transgenic mice.

Pancreatic enzymes can exacerbate acute intestinal radiation toxicity, and suppressing pancreatic secretion with synthetic somatostatin receptor analogs, such as octreotide, can reduce both early and delayed radiation colitis[133]. Of note, in an experimental model, irradiation significantly increased intestinal and pancreatic myeloperoxidase activities and intestinal malondialdehyde levels of intestinal tissues, and octreotide treatment improved this elevation. The histopathologic evaluation of the mucosal structure was also preserved in the octreotide-treated group. Inflammation of pancreatic tissue was also confirmed with histopathological examinations. Moreover, irradiation seems to induce NF-κB overexpression, and octreotide treatment decreases the end organ damage and inflammation of the small intestine. Thus, octreotide appears to have beneficial effects on intestinal and pancreatic damage in abdominal irradiation through the inflammatory process[134].

Despite the aforementioned promising agents used in acute and chronic radiation colitis, further understanding of the pathophysiological mechanisms involved in the pathogenesis of acute and chronic irradiation colitis and the interaction of the molecular events controlling mainly apoptosis and fibrosis may assist in the development and establishment of new therapeutic approaches.

REFERENCES

1 **Nielsen OH**, Vainer B, Rask-Madsen J. Non-IBD and noninfectious colitis. *Nat Clin Pract Gastroenterol Hepatol* 2008; **5**: 28-39

2 **Berthrong M**, Fajardo LF. Radiation injury in surgical pathology. Part II. Alimentary tract. *Am J Surg Pathol* 1981; **5**: 153-178

3 **Berthrong M**. Pathologic changes secondary to radiation. *World J Surg* 1986; **10**: 155-170

4 **Cotti G**, Seid V, Araujo S, Souza AH Jr, Kiss DR, Habr-Gama A. Conservative therapies for hemorrhagic radiation proctitis: a review. *Rev Hosp Clin Fac Med Sao Paulo* 2003; **58**: 284-292

5 **Skwarchuk MW**, Travis EL. Changes in histology and

fibrogenic cytokines in irradiated colorectum of two murine strains. *Int J Radiat Oncol Biol Phys* 1998; **42**: 169-178
6 **Indaram AV**, Visvalingam V, Locke M, Bank S. Mucosal cytokine production in radiation-induced proctosigmoiditis compared with inflammatory bowel disease. *Am J Gastroenterol* 2000; **95**: 1221-1225
7 **Cho LC**, Antoine JE. Radiation Injury to the Gastrointestinal Tract. In: Feldman M, Friedman LS, Sleisenger MH, eds. Sleisenger & Fordtran's Gastrointestinal and Liver Disease. 8th ed. Philadelphia: WB Saunders, 2006: 813-826
8 **Driak D**, Osterreicher J, Rehakova Z, Vilasova Z, Vavrova J. Expression of phospho-Elk-1 in rat gut after the whole body gamma irradiation. *Physiol Res* 2008; **57**: 753-759
9 **Yemelyanov A**, Czwornog J, Gera L, Joshi S, Chatterton RT Jr, Budunova I. Novel steroid receptor phyto-modulator compound a inhibits growth and survival of prostate cancer cells. *Cancer Res* 2008; **68**: 4763-4773
10 **Wang ZQ**, Chen XC, Yang GY, Zhou LF. U0126 prevents ERK pathway phosphorylation and interleukin-1beta mRNA production after cerebral ischemia. *Chin Med Sci J* 2004; **19**: 270-275
11 **Ikeda Y**, Ito M, Matsuu M, Shichijo K, Fukuda E, Nakayama T, Nakashima M, Naito S, Sekine I. Expression of ICAM-1 and acute inflammatory cell infiltration in the early phase of radiation colitis in rats. *J Radiat Res* (Tokyo) 2000; **41**: 279-291
12 **Haton C**, Francois A, Vandamme M, Wysocki J, Griffiths NM, Benderitter M. Imbalance of the antioxidant network of mouse small intestinal mucosa after radiation exposure. *Radiat Res* 2007; **167**: 445-453
13 **Mihaescu A**, Thornberg C, Mattsson S, Wang Y, Jeppsson B, Thorlacius H. Critical role of P-selectin and lymphocyte function antigen-1 in radiation-induced leukocyte-endothelial cell interactions in the colon. *Dis Colon Rectum* 2007; **50**: 2194-2202
14 **Molla M**, Gironella M, Salas A, Closa D, Biete A, Gimeno M, Coronel P, Pique JM, Panes J. Protective effect of superoxide dismutase in radiation-induced intestinal inflammation. *Int J Radiat Oncol Biol Phys* 2005; **61**: 1159-1166
15 **Classen J**, Belka C, Paulsen F, Budach W, Hoffmann W, Bamberg M. Radiation-induced gastrointestinal toxicity. Pathophysiology, approaches to treatment and prophylaxis. *Strahlenther Onkol* 1998; **174** Suppl 3: 82-84
16 **Goschke H**, Buess H, Gyr K, Leutenegger A, Ott S, Stalder GA, Tholen H, Fahrlander H. [Elementary diet as an alternative to parenteral feeding in severe gastrointestinal diseases] *Schweiz Med Wochenschr* 1977; **107**: 43-49
17 **Topkan E**, Karaoglu A. Octreotide in the management of chemoradiotherapy-induced diarrhea refractory to loperamide in patients with rectal carcinoma. *Oncology* 2006; **71**: 354-360
18 **Yavuz MN**, Yavuz AA, Aydin F, Can G, Kavgaci H. The efficacy of octreotide in the therapy of acute radiation-induced diarrhea: a randomized controlled study. *Int J Radiat Oncol Biol Phys* 2002; **54**: 195-202
19 **Lyman GH**. A novel approach to maintain planned dose chemotherapy on time: a decision-making tool to improve patient care. *Eur J Cancer* 2000; **36** Suppl 1: S15-S21
20 **Bermudez LE**, Petrofsky M, Stevens P. Treatment with recombinant granulocyte colony-stimulating factor (Filgrastin) stimulates neutrophils and tissue macrophages and induces an effective non-specific response against Mycobacterium avium in mice. *Immunology* 1998; **94**: 297-303
21 **Glaspy JA**. Hematopoietic management in oncology practice. Part 1. Myeloid growth factors. *Oncology* (Williston Park) 2003; **17**: 1593-1603
22 **McKenna KJ**, Ligato S, Kauffman GL Jr, Abt AB, Stryker JA, Conter RL. Epidermal growth factor enhances intestinal mitotic activity and DNA content after acute abdominal radiation. *Surgery* 1994; **115**: 626-632
23 **Villareal DT**, Morley JE. Trophic factors in aging. Should older people receive hormonal replacement therapy? *Drugs Aging* 1994; **4**: 492-509
24 **Toomey DP**, Cahill RA, Geraghty J, Thirion P. Radiation enteropathy. *Ir Med J* 2006; **99**: 215-217
25 **Watanabe H**, Suda T. [Precancerous lesions of the colon and rectum] *Gan To Kagaku Ryoho* 1984; **11**: 1-9
26 **Yeoh EK**, Horowitz M, Russo A, Muecke T, Robb T, Chatterton BE. Gastrointestinal function in chronic radiation enteritis--effects of loperamide-N-oxide. *Gut* 1993; **34**: 476-482
27 **Tamai O**, Nozato E, Miyazato H, Isa T, Hiroyasu S, Shiraishi M, Kusano T, Muto Y, Higashi M. Radiation-associated rectal cancer: report of four cases. *Dig Surg* 1999; **16**: 238-243
28 **Narui K**, Ike H, Fujii S, Nojiri K, Tatsumi K, Yamagishi S, Saito S, Kunisaki C, Imada T, Nozawa A, Ohki S, Ota M, Ichikawa Y, Shimada H. [A case of radiation-induced rectal cancer] *Nippon Shokakibyo Gakkai Zasshi* 2006; **103**: 551-557
29 **Silvain C**, Besson I, Ingrand P, Beau P, Fort E, Matuchansky C, Carretier M, Morichau-Beauchant M. Long-term outcome of severe radiation enteritis treated by total parenteral nutrition. *Dig Dis Sci* 1992; **37**: 1065-1071
30 **McGough C**, Baldwin C, Frost G, Andreyev HJ. Role of nutritional intervention in patients treated with radiotherapy for pelvic malignancy. *Br J Cancer* 2004; **90**: 2278-2287
31 **Yeoh E**. Radiotherapy: long-term effects on gastrointestinal function. *Curr Opin Support Palliat Care* 2008; **2**: 40-44
32 **O'Brien PH**, Jenrette JM 3rd, Garvin AJ. Radiation enteritis. *Am Surg* 1987; **53**: 501-504
33 **Okunieff P**, Cornelison T, Mester M, Liu W, Ding I, Chen Y, Zhang H, Williams JP, Finkelstein J. Mechanism and modification of gastrointestinal soft tissue response to radiation: role of growth factors. *Int J Radiat Oncol Biol Phys* 2005; **62**: 273-278
34 **Kountouras J**, Kouklakis G, Zavos C, Chatzopoulos D, Moschos J, Molyvas E, Zavos N. Apoptosis, inflammatory bowel disease and carcinogenesis: overview of international and Greek experiences. *Can J Gastroenterol* 2003; **17**: 249-258
35 **Yeoh AS**, Bowen JM, Gibson RJ, Keefe DM. Nuclear factor kappaB (NFkappaB) and cyclooxygenase-2 (Cox-2) expression in the irradiated colorectum is associated with subsequent histopathological changes. *Int J Radiat Oncol Biol Phys* 2005; **63**: 1295-1303
36 **Keskek M**, Gocmen E, Kilic M, Gencturk S, Can B, Cengiz M, Okten RM, Koc M. Increased expression of cyclooxygenase-2 (COX-2) in radiation-induced small bowel injury in rats. *J Surg Res* 2006; **135**: 76-84
37 **Minami K**, Matsuzaki S, Hayashi N, Mokarim A, Ito M, Sekine I. Immunohistochemical study of p53 overexpression in radiation-induced colon cancers. *J Radiat Res* (Tokyo) 1998; **39**: 1-10
38 **Hardy RG**, Brown RM, Miller SJ, Tselepis C, Morton DG, Jankowski JA, Sanders DS. Transient P-cadherin expression in radiation proctitis; a model of mucosal injury and repair. *J Pathol* 2002; **197**: 194-200
39 **Kountouras J**, Zavos C, Chatzopoulos D, Katsinelos P. New aspects of Helicobacter pylori infection involvement in gastric oncogenesis. *J Surg Res* 2008; **146**: 149-158
40 **Nakashima M**, Meirmanov S, Matsufuji R, Hayashida M, Fukuda E, Naito S, Matsuu M, Shichijo K, Kondo H, Ito M, Yamashita S, Sekine I. Altered expression of beta-catenin during radiation-induced colonic carcinogenesis. *Pathol Res Pract* 2002; **198**: 717-724
41 **Marguerat S**, MacDonald HR, Kraehenbuhl JP, van Meerwijk JP. Protection from radiation-induced colitis requires MHC class II antigen expression by cells of hemopoietic origin. *J Immunol* 1999; **163**: 4033-4040
42 **Keith JC Jr**, Albert L, Sonis ST, Pfeiffer CJ, Schaub RG. IL-11, a pleiotropic cytokine: exciting new effects of IL-11 on gastrointestinal mucosal biology. *Stem Cells* 1994; **12** Suppl 1: 79-89; discussion 89-90
43 **Andreyev J**. Gastrointestinal complications of pelvic radiotherapy: are they of any importance? *Gut* 2005; **54**: 1051-1054

44 **Skinn AC**, Vergnolle N, Cellars L, Sherman PM, MacNaughton WK. Combined challenge of mice with Citrobacter rodentium and ionizing radiation promotes bacterial translocation. *Int J Radiat Biol* 2007; **83**: 375-382

45 **Jazwinski A**, Palazzo J, Kastenberg D. Capsule endoscopy diagnosis of radiation enteritis in a patient previously considered to have celiac sprue. *Endoscopy* 2007; **39** Suppl 1: E66

46 **Baughan CA**, Canney PA, Buchanan RB, Pickering RM. A randomized trial to assess the efficacy of 5-aminosalicylic acid for the prevention of radiation enteritis. *Clin Oncol* (R Coll Radiol) 1993; **5**: 19-24

47 **Vane JR**. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971; **231**: 232-235

48 **Kochhar R**, Patel F, Dhar A, Sharma SC, Ayyagari S, Aggarwal R, Goenka MK, Gupta BD, Mehta SK. Radiation-induced proctosigmoiditis. Prospective, randomized, double-blind controlled trial of oral sulfasalazine plus rectal steroids versus rectal sucralfate. *Dig Dis Sci* 1991; **36**: 103-107

49 **Goldstein F**, Khoury J, Thornton JJ. Treatment of chronic radiation enteritis and colitis with salicylazosulfapyridine and systemic corticosteroids. A pilot study. *Am J Gastroenterol* 1976; **65**: 201-208

50 **De Maria D**, Falchi AM, Venturino P. Adjuvant radiotherapy of the pelvis with or without reduced glutathione: a randomized trial in patients operated on for endometrial cancer. *Tumori* 1992; **78**: 374-376

51 **Kennedy M**, Bruninga K, Mutlu EA, Losurdo J, Choudhary S, Keshavarzian A. Successful and sustained treatment of chronic radiation proctitis with antioxidant vitamins E and C. *Am J Gastroenterol* 2001; **96**: 1080-1084

52 **Meyers JS**, Ehrenpreis ED, Craig RM. Small Intestinal Bacterial Overgrowth Syndrome. *Curr Treat Options Gastroenterol* 2001; **4**: 7-14

53 **Attar A**, Flourie B, Rambaud JC, Franchisseur C, Ruszniewski P, Bouhnik Y. Antibiotic efficacy in small intestinal bacterial overgrowth-related chronic diarrhea: a crossover, randomized trial. *Gastroenterology* 1999; **117**: 794-797

54 **Floch MH**, Madsen KK, Jenkins DJ, Guandalini S, Katz JA, Onderdonk A, Walker WA, Fedorak RN, Camilleri M. Recommendations for probiotic use. *J Clin Gastroenterol* 2006; **40**: 275-278

55 **Heusinkveld RS**, Manning MR, Aristizabal SA. Control of radiation-induced diarrhea with cholestyramine. *Int J Radiat Oncol Biol Phys* 1978; **4**: 687-690

56 **Cohen L**, Kitzes R. Early radiation-induced proctosigmoiditis responds to magnesium therapy. *Magnesium* 1985; **4**: 16-19

57 **Varma JS**, Smith AN, Busuttil A. Function of the anal sphincters after chronic radiation injury. *Gut* 1986; **27**: 528-533

58 **Jahraus CD**, Bettenhausen D, Malik U, Sellitti M, St Clair WH. Prevention of acute radiation-induced proctosigmoiditis by balsalazide: a randomized, double-blind, placebo controlled trial in prostate cancer patients. *Int J Radiat Oncol Biol Phys* 2005; **63**: 1483-1487

59 **Linard C**, Gremy O, Benderitter M. Reduction of peroxisome proliferation-activated receptor gamma expression by gamma-irradiation as a mechanism contributing to inflammatory response in rat colon: modulation by the 5-aminosalicylic acid agonist. *J Pharmacol Exp Ther* 2008; **324**: 911-920

60 **Petersen S**, Jongen J, Petersen C, Sailer M. Radiation-induced sequelae affecting the continence organ: incidence, pathogenesis, and treatment. *Dis Colon Rectum* 2007; **50**: 1466-1474

61 **Matsuu-Matsuyama M**, Shichijo K, Okaichi K, Ishii K, Wen CY, Fukuda E, Nakayama T, Nakashima M, Okumura Y, Sekine I. Sucralfate protects intestinal epithelial cells from radiation-induced apoptosis in rats. *J Radiat Res* (Tokyo) 2006; **47**: 1-8

62 **Pienkowski P**, Fioramonti J, Skalli F, Frexinos J. [Effects of corticoids, 5-aminosalycilic acid and sucralfate on the potential difference of the rectum in inflammatory colitis in man] *Gastroenterol Clin Biol* 1989; **13**: 202-207

63 **Kochhar R**, Sriram PV, Sharma SC, Goel RC, Patel F. Natural history of late radiation proctosigmoiditis treated with topical sucralfate suspension. *Dig Dis Sci* 1999; **44**: 973-978

64 **Cook SI**, Sellin JH. Review article: short chain fatty acids in health and disease. *Aliment Pharmacol Ther* 1998; **12**: 499-507

65 **Bem J**, Bem S, Singh A. Use of hyperbaric oxygen chamber in the management of radiation-related complications of the anorectal region: report of two cases and review of the literature. *Dis Colon Rectum* 2000; **43**: 1435-1438

66 **Nakabayashi M**, Beard C, Kelly SM, Carr-Locke DL, Oh WK. Treatment of a radiation-induced rectal ulcer with hyperbaric oxygen therapy in a man with prostate cancer. *Urol Oncol* 2006; **24**: 503-508

67 **Gouello JP**, Bouachour G, Person B, Ronceray J, Cellier P, Alquier P. [The role of hyperbaric oxygen therapy in radiation-induced digestive disorders. 36 cases] *Presse Med* 1999; **28**: 1053-1057

68 **Feldmeier JJ**, Hampson NB. A systematic review of the literature reporting the application of hyperbaric oxygen prevention and treatment of delayed radiation injuries: an evidence based approach. *Undersea Hyperb Med* 2002; **29**: 4-30

69 **Zimmermann FB**, Feldmann HJ. Radiation proctitis. Clinical and pathological manifestations, therapy and prophylaxis of acute and late injurious effects of radiation on the rectal mucosa. *Strahlenther Onkol* 1998; **174** Suppl 3: 85-89

70 **Feldmeier JJ**, Heimbach RD, Davolt DA, Court WS, Stegmann BJ, Sheffield PJ. Hyperbaric oxygen an adjunctive treatment for delayed radiation injuries of the abdomen and pelvis. *Undersea Hyperb Med* 1996; **23**: 205-213

71 **Miura M**, Sasagawa I, Kubota Y, Iijima Y, Sawamura T, Nakada T. Effective hyperbaric oxygenation with prostaglandin E1 for radiation cystitis and colitis after pelvic radiotherapy. *Int Urol Nephrol* 1996; **28**: 643-647

72 **Silva RA**, Correia AJ, Dias LM, Viana HL, Viana RL. Argon plasma coagulation therapy for hemorrhagic radiation proctosigmoiditis. *Gastrointest Endosc* 1999; **50**: 221-224

73 **de Parades V**, Bauer P, Marteau P, Chauveinc L, Bouillet T, Atienza P. [Nonsurgical treatment of chronic radiation-induced hemorrhagic proctitis] *Presse Med* 2008; **37**: 1113-1120

74 **Pikarsky AJ**, Belin B, Efron J, Weiss EG, Nogueras JJ, Wexner SD. Complications following formalin installation in the treatment of radiation induced proctitis. *Int J Colorectal Dis* 2000; **15**: 96-99

75 **Craanen ME**, van Triest B, Verheijen RH, Mulder CJ. Thalidomide in refractory haemorrhagic radiation induced proctitis. *Gut* 2006; **55**: 1371-1372

76 **Wurzer H**, Schafhalter-Zoppoth I, Brandstatter G, Stranzl H. Hormonal therapy in chronic radiation colitis. *Am J Gastroenterol* 1998; **93**: 2536-2538

77 **Qadeer MA**, Vargo JJ. Approaches to the prevention and management of radiation colitis. *Curr Gastroenterol Rep* 2008; **10**: 507-513

78 **Girvent M**, Carlson GL, Anderson I, Shaffer J, Irving M, Scott NA. Intestinal failure after surgery for complicated radiation enteritis. *Ann R Coll Surg Engl* 2000; **82**: 198-201

79 **Galland RB**, Spencer J. Surgical management of radiation enteritis. *Surgery* 1986; **99**: 133-139

80 **Kuroki F**, Iida M, Matsui T, Matsumoto T, Fujishima M, Yao T. Intraoperative endoscopy for small intestinal damage in radiation enteritis. *Gastrointest Endosc* 1992; **38**: 196-197

81 **Frileux P**, Berger A, Zinzindohoue F, Cugnenc PH, Parc R. [Rectovaginal fistulas in adults] *Ann Chir* 1994; **48**: 412-420

82 **Mann WJ**. Surgical management of radiation enteropathy. *Surg Clin North Am* 1991; **71**: 977-990

83 **Yoshimura K**, Hirata I, Maemura K, Sugi K, Tahara T. Radiation enteritis: a rare complication of the transverse colon in uterine cancer. *Intern Med* 2000; **39**: 1060-1063

84 **Dietz DW**, Remzi FH, Fazio VW. Strictureplasty for obstructing small-bowel lesions in diffuse radiation enteritis--successful outcome in five patients. *Dis Colon Rectum* 2001; **44**: 1772-1777

85 **Li N**, Zhu WM, Ren JA, Li YX, Zhao YZ, Jiang ZW, Li YS, Li JS. [Surgical management of chronic radiation enteritis] *Zhonghua Waike Zazhi* 2006; **44**: 23-26

86 **Libotte F**, Autier P, Delmelle M, Gozy M, Pector JC, Van Houtte P, Gerard A. Survival of patients with radiation enteritis of the small and the large intestine. *Acta Chir Belg* 1995; **95**: 190-194

87 **Bourgier C**, Haydont V, Milliat F, Francois A, Holler V, Lasser P, Bourhis J, Mathe D, Vozenin-Brotons MC. Inhibition of Rho kinase modulates radiation induced fibrogenic phenotype in intestinal smooth muscle cells through alteration of the cytoskeleton and connective tissue growth factor expression. *Gut* 2005; **54**: 336-343

88 **Northway MG**, Scobey MW, Cassidy KT, Geisinger KR. Piroxicam decreases postirradiation colonic neoplasia in the rat. *Cancer* 1990; **66**: 2300-2305

89 **Cesaretti JA**, Stock RG, Atencio DP, Peters SA, Peters CA, Burri RJ, Stone NN, Rosenstein BS. A genetically determined dose-volume histogram predicts for rectal bleeding among patients treated with prostate brachytherapy. *Int J Radiat Oncol Biol Phys* 2007; **68**: 1410-1416

90 **Tytell M**, Hooper PL. Heat shock proteins: new keys to the development of cytoprotective therapies. *Expert Opin Ther Targets* 2001; **5**: 267-287

91 **Preoperative short-term radiation therapy in operable rectal carcinoma.** A prospective randomized trial. Stockholm Rectal Cancer Study Group. *Cancer* 1990; **66**: 49-55

92 **Goldberg PA**, Nicholls RJ, Porter NH, Love S, Grimsey JE. Long-term results of a randomised trial of short-course low-dose adjuvant pre-operative radiotherapy for rectal cancer: reduction in local treatment failure. *Eur J Cancer* 1994; **30A**: 1602-1606

93 **Letschert JG**, Lebesque JV, de Boer RW, Hart AA, Bartelink H. Dose-volume correlation in radiation-related late small-bowel complications: a clinical study. *Radiother Oncol* 1990; **18**: 307-320

94 **Caspers RJ**, Hop WC. Irradiation of true pelvis for bladder and prostatic carcinoma in supine, prone or Trendelenburg position. *Int J Radiat Oncol Biol Phys* 1983; **9**: 589-593

95 **Shanahan TG**, Mehta MP, Bertelrud KL, Buchler DA, Frank LE, Gehring MA, Kubsad SS, Utrie PC, Kinsella TJ. Minimization of small bowel volume within treatment fields utilizing customized "belly boards". *Int J Radiat Oncol Biol Phys* 1990; **19**: 469-476

96 **Green N**. The avoidance of small intestine injury in gynecologic cancer. *Int J Radiat Oncol Biol Phys* 1983; **9**: 1385-1390

97 **Yeoh EE**, Holloway RH, Fraser RJ, Botten RJ, Di Matteo AC, Butters J, Weerasinghe S, Abeysinghe P. Hypofractionated versus conventionally fractionated radiation therapy for prostate carcinoma: updated results of a phase III randomized trial. *Int J Radiat Oncol Biol Phys* 2006; **66**: 1072-1083

98 **Su AW**, Jani AB. Chronic genitourinary and gastrointestinal toxicity of prostate cancer patients undergoing pelvic radiotherapy with intensity-modulated versus 4-field technique. *Am J Clin Oncol* 2007; **30**: 215-219

99 **Chen AB**, D'Amico AV, Neville BA, Earle CC. Patient and treatment factors associated with complications after prostate brachytherapy. *J Clin Oncol* 2006; **24**: 5298-5304

100 **Lee WR**, Bae K, Lawton C, Gillin M, Morton G, Firat S, Baikadi M, Kuettel M, Greven K, Sandler H. Late toxicity and biochemical recurrence after external-beam radiotherapy combined with permanent-source prostate brachytherapy: analysis of Radiation Therapy Oncology Group study 0019. *Cancer* 2007; **109**: 1506-1512

101 **Peters CA**, Cesaretti JA, Stone NN, Stock RG. Low-dose rate prostate brachytherapy is well tolerated in patients with a history of inflammatory bowel disease. *Int J Radiat Oncol Biol Phys* 2006; **66**: 424-429

102 **Waddell BE**, Rodriguez-Bigas MA, Lee RJ, Weber TK, Petrelli NJ. Prevention of chronic radiation enteritis. *J Am Coll Surg* 1999; **189**: 611-624

103 **Meric F**, Hirschl RB, Mahboubi S, Womer RB, Goldwein J, Ross AJ 3rd, Schnaufer L. Prevention of radiation enteritis in children, using a pelvic mesh sling. *J Pediatr Surg* 1994; **29**: 917-921

104 **Rodier JF**, Janser JC, Rodier D, Dauplat J, Kauffmann P, Le Bouedec G, Giraud B, Lorimier G. Prevention of radiation enteritis by an absorbable polyglycolic acid mesh sling. A 60-case multicentric study. *Cancer* 1991; **68**: 2545-2549

105 **Dasmahapatra KS**, Swaminathan AP. The use of a biodegradable mesh to prevent radiation-associated small-bowel injury. *Arch Surg* 1991; **126**: 366-369

106 **Logmans A**, van Lent M, van Geel AN, Olofsen-Van Acht M, Koper PC, Wiggers T, Trimbos JB. The pedicled omentoplasty, a simple and effective surgical technique to acquire a safe pelvic radiation field; theoretical and practical aspects. *Radiother Oncol* 1994; **33**: 269-271

107 **Logmans A**, Trimbos JB, van Lent M. The omentoplasty: a neglected ally in gynecologic surgery. *Eur J Obstet Gynecol Reprod Biol* 1995; **58**: 167-171

108 **Smedh K**, Moran BJ, Heald RJ. Fixed rectal cancer at laparatomy: a simple operation to protect the small bowel from radiation enteritis. *Eur J Surg* 1997; **163**: 547-548

109 **Chen JS**, ChangChien CR, Wang JY, Fan HA. Pelvic peritoneal reconstruction to prevent radiation enteritis in rectal carcinoma. *Dis Colon Rectum* 1992; **35**: 897-901

110 **Brizel DM**, Wasserman TH, Henke M, Strnad V, Rudat V, Monnier A, Eschwege F, Zhang J, Russell L, Oster W, Sauer R. Phase III randomized trial of amifostine as a radioprotector in head and neck cancer. *J Clin Oncol* 2000; **18**: 3339-3345

111 **Ito H**, Meistrich ML, Barkley HT Jr, Thames HD Jr, Milas L. Protection of acute and late radiation damage of the gastrointestinal tract by WR-2721. *Int J Radiat Oncol Biol Phys* 1986; **12**: 211-219

112 **Dorr RT**. Radioprotectants: pharmacology and clinical applications of amifostine. *Semin Radiat Oncol* 1998; **8**: 10-13

113 **Liu T**, Liu Y, He S, Zhang Z, Kligerman MM. Use of radiation with or without WR-2721 in advanced rectal cancer. *Cancer* 1992; **69**: 2820-2825

114 **Ben-Josef E**, Han S, Tobi M, Vargas BJ, Stamos B, Kelly L, Biggar S, Kaplan I. Intrarectal application of amifostine for the prevention of radiation-induced rectal injury. *Semin Radiat Oncol* 2002; **12**: 81-85

115 **Ben-Josef E**, Han S, Tobi M, Shaw LM, Bonner HS, Vargas BJ, Prokop S, Stamos B, Kelly L, Biggar S, Kaplan I. A pilot study of topical intrarectal application of amifostine for prevention of late radiation rectal injury. *Int J Radiat Oncol Biol Phys* 2002; **53**: 1160-1164

116 **Montana GS**, Anscher MS, Mansbach CM 2nd, Daly N, Delannes M, Carke-Pearson D, Gaydica EF. Topical application of WR-2721 to prevent radiation-induced proctosigmoiditis. A phase I/II trial. *Cancer* 1992; **69**: 2826-2830

117 **Mutlu-Turkoglu U**, Erbil Y, Oztezcan S, Olgac V, Toker G, Uysal M. The effect of selenium and/or vitamin E treatments on radiation-induced intestinal injury in rats. *Life Sci* 2000; **66**: 1905-1913

118 **Beyzadeoglu M**, Balkan M, Demiriz M, Tibet H, Dirican B, Oner K, Pak Y. Protective effect of vitamin A on acute radiation injury in the small intestine. *Radiat Med* 1997; **15**: 1-5

119 **Hanson WR**, Thomas C. 16, 16-dimethyl prostaglandin E2 increases survival of murine intestinal stem cells when given before photon radiation. *Radiat Res* 1983; **96**: 393-398

120 **Tomas-de la Vega JE**, Banner BF, Hubbard M, Boston DL, Thomas CW, Straus AK, Roseman DL. Cytoprotective effect

of prostaglandin E2 in irradiated rat ileum. *Surg Gynecol Obstet* 1984; **158**: 39-45
121 **Keelan M**, Walker K, Cheeseman CI, Thomson AB. Two weeks of oral synthetic E2 prostaglandin (Enprostil) improves the intestinal morphological but not the absorptive response in the rat to abdominal irradiation. *Digestion* 1992; **53**: 101-107
122 **Delaney JP**, Bonsack ME, Felemovicius I. Misoprostol in the intestinal lumen protects against radiation injury of the mucosa of the small bowel. *Radiat Res* 1994; **137**: 405-409
123 **Khan AM**, Birk JW, Anderson JC, Georgsson M, Park TL, Smith CJ, Comer GM. A prospective randomized placebo-controlled double-blinded pilot study of misoprostol rectal suppositories in the prevention of acute and chronic radiation proctitis symptoms in prostate cancer patients. *Am J Gastroenterol* 2000; **95**: 1961-1966
124 **Stenson WF**. Prostaglandins and epithelial response to injury. *Curr Opin Gastroenterol* 2007; **23**: 107-110
125 **Torres S**, Thim L, Milliat F, Vozenin-Brotons MC, Olsen UB, Ahnfelt-Ronne I, Bourhis J, Benderitter M, Francois A. Glucagon-like peptide-2 improves both acute and late experimental radiation enteritis in the rat. *Int J Radiat Oncol Biol Phys* 2007; **69**: 1563-1571
126 **Zheng H**, Wang J, Koteliansky VE, Gotwals PJ, Hauer-Jensen M. Recombinant soluble transforming growth factor beta type II receptor ameliorates radiation enteropathy in mice. *Gastroenterology* 2000; **119**: 1286-1296
127 **Nguyen NP**, Antoine JE, Dutta S, Karlsson U, Sallah S. Current concepts in radiation enteritis and implications for future clinical trials. *Cancer* 2002; **95**: 1151-1163
128 **Demirer S**, Aydintug S, Aslim B, Kepenekci I, Sengul N, Evirgen O, Gerceker D, Andrieu MN, Ulusoy C, Karahuseyinoglu S. Effects of probiotics on radiation-induced intestinal injury in rats. *Nutrition* 2006; **22**: 179-186
129 **Ersin S**, Tuncyurek P, Esassolak M, Alkanat M, Buke C, Yilmaz M, Telefoncu A, Kose T. The prophylactic and therapeutic effects of glutamine- and arginine-enriched diets on radiation-induced enteritis in rats. *J Surg Res* 2000; **89**: 121-125
130 **Howarth GS**, Fraser R, Frisby CL, Schirmer MB, Yeoh EK. Effects of insulin-like growth factor-I administration on radiation enteritis in rats. *Scand J Gastroenterol* 1997; **32**: 1118-1124
131 **Mylonas PG**, Matsouka PT, Papandoniou EV, Vagianos C, Kalfarentzos F, Alexandrides TK. Growth hormone and insulin-like growth factor I protect intestinal cells from radiation induced apoptosis. *Mol Cell Endocrinol* 2000; **160**: 115-122
132 **Wilkins HR**, Ohneda K, Keku TO, D'Ercole AJ, Fuller CR, Williams KL, Lund PK. Reduction of spontaneous and irradiation-induced apoptosis in small intestine of IGF-I transgenic mice. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G457-G464
133 **Wang J**, Zheng H, Hauer-Jensen M. Influence of Short-Term Octreotide Administration on Chronic Tissue Injury, Transforming Growth Factor beta (TGF-beta) Overexpression, and Collagen Accumulation in Irradiated Rat Intestine. *J Pharmacol Exp Ther* 2001; **297**: 35-42
134 **Olgac V**, Erbil Y, Barbaros U, Oztezcan S, Giris M, Kaya H, Bilge H, Guler S, Toker G. The efficacy of octreotide in pancreatic and intestinal changes: radiation-induced enteritis in animals. *Dig Dis Sci* 2006; **51**: 227-232

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TOPIC HIGHLIGHT

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Ischemic colitis: Clinical practice in diagnosis and treatment

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Abstract

Ischemic colitis is the most common form of ischemic injury of the gastrointestinal tract and can present either as an occlusive or a non-occlusive form. It accounts for 1 in 1000 hospitalizations but its incidence is underestimated because it often has a mild and transient nature. The etiology of ischemic colitis is multifactorial and the clinical presentation variable. The diagnosis is based on a combination of clinical suspicion, radiographic, endoscopic and histological findings. Therapy and outcome depends on the severity of the disease. Most cases of the non-gangrenous form are transient and resolve spontaneously without complications. On the other hand, high morbidity and mortality and urgent operative intervention are the hallmarks of gangrenous ischemic colitis.

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Key words: Colon ischemia; Intestinal blood flow; Ischemic colitis; Thrombosis

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INTRODUCTION

Ischemic colitis (IC), first described by Boley *et al*, is the most common form of ischemic injury to the gastrointestinal tract representing more than half of the cases with gastrointestinal ischemia[1,2]. The incidence of IC is underestimated because it often has a mild and transient nature. Moreover, many cases are misdiagnosed as suffering from other diseases such as inflammatory bowel disease or infectious colitis.

An acute, self-limited compromise in intestinal blood flow which is inadequate for meeting the metabolic demands of a region of the colon is the underlying pathophysiology[3]. Colonic blood flow may be compromised by changes in the systemic circulation or by anatomic or functional changes in the local mesenteric vasculature. The original insult precipitating the ischemic event often cannot be established, but frequently occurs in the elderly patient with diffuse disease in small segmental vessels and various co-morbidities. Approximately 90% of cases of colonic ischemia occur in patients over 60 years of age although younger patients may also be affected[4].

IC presents either as an occlusive or a non-occlusive form. In most cases no specific occlusive lesion is recognized on angiography, and patients are referred to as suffering from non-occlusive colon ischemia.

The aim of this review is to transfer the current knowledge on diagnosis and management of ischemic colitis into daily clinical practice.

RISK FACTORS

A plethora of conditions may predispose to IC: Mesenteric artery emboli, thrombosis, or trauma may lead to occlusive vascular disease and impaired colonic perfusion[5]. Hypo-perfusion states due to congestive heart failure, transient hypotension in the perioperative period or strenuous physical activities and shock due to a variety of causes such as hypovolemia or sepsis can result in IC[3]. Mechanical colonic obstruction due to tumors, adhesions, volvuli, hernias, diverticulitis or prolapse may also infrequently cause IC[3]. There is a long list of medications that predispose to colon ischemia. Major classes of pharmacologic agents known to be associated with IC include the following[6]: antibiotics, appetite suppressants (phentermine), chemotherapeutic agents (vinca alkaloids and taxanes), constipation inducing medications, decongestants (pseudoephedrine), cardiac glucosides, diuretics, ergot alkaloids, hormonal therapies, statins, illicit drugs,

immunosuppressive agents, laxatives, nonsteroidal anti-inflammatory drugs, psychotropic medications, serotonin agonists/antagonists and vasopressors. Iatrogenic causes may result in IC. Ischemic colitis follows aortic reconstruction with an incidence of 2% to 3% and is higher after abdominal aortic aneurysm repair[7,8]. IC may be a complication of coronary artery bypass surgery or a rare complication of colonic surgery or colonoscopy[3].

A state of increased coagulability, although not extensively investigated, has been raised as a significant factor in the pathogenesis of IC. Some cases of IC have been reported to be associated with genetic defects such as deficiencies of protein C, protein S, and antithrombin Ⅲ[9-11], factor V Leiden (FVL) mutation[12,13], and prothrombin 20210G/A mutation[14], as well as acquired factors such as antiphospholipid antibodies[15]. Protein Z deficiency has also been reported in IC patients[16]. A thrombophilic tendency in the majority of patients was shown in a study of comprehensive thrombophilic screening in colon ischemia[17]. The most significant associations were found with the antiphospholipid antibodies and the FVL mutation[17]. These results were confirmed by another recent study in which thrombophilic disorders were found in 28% of patients studied[18].

IC might also spontaneously appear in apparently healthy individuals. In these cases no clear cause for the ischemia is identified. This idiopathic or "spontaneous" form is generally thought to be related to localized non-occlusive ischemia of the bowel[5]. In younger patients a predisposing cause is more easily recognized. Vasculitides, estrogens, cocaine and methamphetamine use, psychotropic drugs, sickle cell disease, long-distance running and heritable disorders of coagulation should be considered[19-24]. In a recent study[25], the frequency of the 506 Q allele of the factor V (FV) 506 RQ (Leiden) mutation and the mutant 4G allele of plasminogen activator inhibitor (PAI) polymorphism were found to be significantly higher in young patients with IC compared with healthy controls.

PATHOPHYSIOLOGY

The colon is predisposed to ischemia by its relatively low blood flow and its less developed microvasculature plexus compared with the small bowel. Two major arteries supply most of the blood to the colon: the superior mesenteric artery (which supplies the ascending and transverse colon) and the inferior mesenteric artery (IMA) (which supplies the descending and sigmoid colon). The internal iliac arteries supply the rectum.

The colon is protected from ischemia by a collateral blood supply *via* a system of arcades connecting the two major arteries. The anatomy is highly variable, however, and certain areas are more vulnerable in some people[26]. The splenic flexure and sigmoid colon are regions where two circulations meet each other (so-called watershed areas), have more limited collateral networks and therefore ischemic damage is more common in these areas. The marginal artery of Drummond is one of the collateral vessels supplying the splenic flexure; 5% of the population has a diminished or absent marginal artery of Drummond[27]. These patients are at particular risk of ischemia. The right colon may be vulnerable in systemic low-flow states, as the marginal artery of Drummond is poorly developed here in 50% of the population[28]. The vasa recta are smaller and less developed in the right colon compared to the left colon. Collateral flow between the IMA and the internal iliac arteries occurs *via* the superior and middle/inferior rectal (hemorrhoidal) vessels. Ischemic damage of the rectum is rare because of its dual blood supply from the mesenteric and iliac arteries.

Classification

Clinically, ischemic colitis may be classified into gangrenous and non-gangrenous forms. The latter can also be subdivided into transient and chronic forms.

According to the classification of Brandt and Boley the following types are suggested[29]: (1) Reversible ischemic colonopathy; (2) Transient IC; (3) Chronic ulcerative IC; (4) Ischemic colonic stricture; (5) Colonic gangrene; and (6) Fulminant universal.

The non-gangrenous form accounts for 80%-85% of cases[26]. The disease is transient, and reversible in about 50% of cases. Chronic forms, presenting either as chronic segmental colitis or strictures, occur in 20%-25% and 10%-15% of cases, respectively[26,29]. Predictive factors of the chronic form are older age, longer elapsed time from the onset of illness to the termination of subjective symptoms, and a prolonged period until normalization of the white blood cell count or the erythrocyte sedimentation rate[5]. Gangrene occurs in about 15% of patients and requires laparotomy as soon as possible[26]. Fulminant pancolitis is rare, occurring in only 1% of cases[26].

A worse prognosis has been reported in elderly patients. There are conflicting results with regard to the relationship between the medical history of patients and the severity of IC. High blood pressure, history of cancer, diabetes mellitus, aortic surgery, peripheral vascular disease and involvement of the right side of the colon have been suggested by some authors to be predisposing factors for a worse evolution of the disease[30-32]. In the study by Anon *et al*[33], factors predicting poor prognosis in ischemic colitis were the absence of hematochezia, tachycardia and peritonism, anemia, hyponatremia and colonic stenosis.

Any part of the colon may be affected but the left colon is the predominant location in approximately 75% of patients[5]. Splenic flexure is involved in approximately one-quarter of patients[5] and isolated right colon ischemia (IRCI) in about 10% of cases[29]. In a recent biopsy-proven study, IRCI accounted for 26% of cases[34]. Its clinical presentation was found to be different in patients who presented more commonly with abdominal pain without bloody diarrhea. IRCI has been reported to be associated with hemodialysis and chronic renal failure and in patients with shock. It is associated with severe colitis and patients have a worse outcome than those with colon ischemia involving other regions, including

a five-fold need for surgery and a two-fold increase in mortality[34]. Patients on hemodialysis who develop IRCI have a particularly unfavorable outcome[34]. Insufficient collateralization and blood flow to the right side of the colon is believed to be the reason for the poor prognosis in these patients. Alternatively, it is possible that the presence of an acute superior mesenteric artery occlusion and thus its outcome reflects that of acute mesenteric ischemia.

Clinical presentation

The clinical presentation varies, depending on the severity and extent of the disease. None of the symptoms and signs is specific. Most patients present with a sudden onset of crampy abdominal pain, diarrhea and an urge to defecate. The pain is mild, located over the affected bowel, usually to the left side of the lower abdomen and hypogastrium, followed by mild rectal bleeding within 24 h. The blood may be bright red or maroon, frequently mixed with the stools. Rectal bleeding is usually minimal. Significant hematochezia accompanied with hemodynamic instability or the need for blood transfusion suggests a different diagnosis. The presence of an associated ileus may be manifested by anorexia, nausea and vomiting.

Clinical examination of the abdomen reveals mild to moderate tenderness over the affected area of the colon. Rectal examination shows heme-positive stools. Fever is unusual while the white cell count is generally raised. In cases of severe ischemia with transmural infarction and necrosis, marked tenderness with peritoneal signs may be present on physical examination accompanied by metabolic acidosis and septic shock.

DIAGNOSIS

Given that the presentation of colon ischemia is not specific and is highly variable, diagnosis and management is clinically challenging. Diagnosis requires a high index of clinical suspicion. The chronology of symptoms and the clinical situations upon which these symptoms appear must be taken into account.

Special attention must be paid to the presence of conditions that predispose to the disease, such as strenuous physical activity, dehydration, illicit drugs, thrombophilic tendency, aortic surgery or cardiac bypass, vasculitis, major cardiovascular episode accompanied by hypotension or an obstructing lesion of the colon.

The presence of diarrhea, abdominal pain and tenderness as well as mild lower gastrointestinal bleeding, even in the absence of any risk factor, should prompt consideration of IC as a cause. Early and repeated clinical evaluation in addition to radiological and endoscopic assessment is necessary to avoid complications. Common clinical conditions should be excluded. The differential diagnosis includes infectious colitis, inflammatory bowel disease, pseudomembranous colitis, diverticulitis and colon carcinoma. Severe forms may be difficult to distinguish from acute mesenteric ischemia.

All patients with clinical suspicion of IC should have stool cultures for *Salmonella, Shigella, Campylobacter* and *Escherichia coli* O157:H7[35]. The latter organism has been implicated in causing colonic ischemia. Infection with parasites or viruses such as cytomegalovirus should also be excluded.

Laboratory tests

Various laboratory markers of ischemia have been investigated such as: lactate, LDH, CPK, amylase levels, leucocytes, alkaline phosphatase, inorganic phosphate, intestinal fatty acid binding protein and alfa-glutathione S-transferase[36]. These markers have been studied mainly in acute bowel ischemia, and none has been found to be sufficiently specific to diagnose IC. They are uncommon in mild ischemia and only increase with advanced and severe ischemic damage, late in the course of the disease.

Imaging techniques

Plain abdominal radiography can reveal nonspecific findings such as thumbprinting, air-filled loops, colonic aperistalsis, mural thickening and exhausted bowel in up to 21% of patients[3]. It is a useful examination for excluding colon infarction[37]. When intra-abdominal air secondary to perforation, air within the bowel wall, or air in the portal vein, is demonstrated by plain radiography, an emergency exploratory laparotomy is indicated.

Barium enema may suggest colon ischemia in up to 75% of patients with thumbprinting being the most common finding. Ulcers, ridges, edema, eccentric mural deformity, succulation and strictures may also be seen. Findings are non specific[38]. Barium enema should be avoided in cases where there is a suspicion of gangrene or perforation. Barium enema also makes the later use of angiography or endoscopy more difficult because of residual contrast agent.

Computed tomography (CT) is often used as the initial diagnostic test when assessing patients with nonspecific abdominal pain. It may suggest the diagnosis and location, exclude other serious medical conditions, narrow the differential diagnosis possibilities and illustrate the complications. Although intrinsic colonic abnormalities cannot be used to diagnose or predict the development of infarction[39].

In non-transmural IC, the initial bowel wall thickening, thumbprinting, and pericolonic stranding, with or without peritoneal fluid, can be seen on CT images. In these cases, CT usually demonstrates the double halo or target sign. After reperfusion of the ischemic bowel wall, the sign may be produced by edema in the submucosa and appear as low attenuation or by hemorrhage and appear as high attenuation. If there is total vascular occlusion without reperfusion (infarction), the colonic wall remains thin and unenhancing, associated with dilatation of the lumen. In these cases, CT may demonstrate a thrombus in the corresponding mesenteric vessel. If ischemia is transmural, strictures may form. Occasionally, a toxic megacolon develops. Pneumatosis and/or gas in the mesenteric veins are ominous signs when associated with bowel wall thickening and are due to bowel infarction.

Pneumatosis coli or pneumatosis intestinalis can be diagnosed by demonstrating air bubbles in the colonic or intestinal wall. The gas bubbles are arranged in a linear fashion and are best visualized with the window settings for bone or lung[40].

Mesenteric angiography usually has no role in the evaluation and management of IC because at the time of symptom onset, colon blood flow has returned to normal. Damage from hypoperfusion is often at the arteriolar level, whereas mesenteric vessels and arcades are patent. There are two exceptions where angiography may have some utility: when acute mesenteric ischemia is considered and cannot be clearly distinguished from IC by clinical presentation, or when there is isolated involvement of the right side of the colon, suggesting superior mesenteric artery occlusion.

Sonography is a sensitive technique for the early detection of changes in the colon wall resulting from ischemia, and it can suggest this cause in the appropriate clinical setting. Location and length of the involved colonic segment, colon wall thickening, bowel wall stratification and abnormal echogenicity of the pericolic fat and peritoneal fluid are some of the findings on sonography[41].

Color Doppler sonography may be useful in the differentiation between inflammatory and ischemic bowel wall thickening[42]. Sonography may provide data for identifying patients who will develop necrosis. In one study, altered pericolic fat or the absence of improvement in sonographic follow-up studies were factors associated with transmural necrosis[41]. Nevertheless, overlying bowel gas, operator-dependent quality and poor sensitivity for low flow vessel disease limit its use.

Scintigraphy has recently been used in the diagnosis of ischemic colitis. In-111 or Tc-99m-labeled leukocyte scintigraphy has been studied and has demonstrated successful imaging of bowel infarction while Tc-99m(V) DMSA was recently found to have no role in the detection and diagnosis of IC[43-45].

Colonoscopy

In recent years, colonoscopy has replaced barium enema as the most common diagnostic method and the gold standard for confirmation of IC. It is more sensitive and allows visualization of colonic mucosa and histological analysis of biopsies. However, with the exception of colonic gangrene, neither endoscopic nor histological findings are specific[29] and highly depend on the duration and severity of ischemic injury. Diagnosis requires early colonoscopy (< 48 h). Serial studies in connection with the clinical setting are necessary to establish the diagnosis.

Ischemic tissue damage to the colon is thought to be a result of both local hypoperfusion during the ischemic period and reperfusion injury when blood flow returns. When the ischemic period is brief, reperfusion may be significant and accounts for most of the histologic and endoscopic damage present in IC[2]. Reperfusion injury may be associated with the release of oxygen free radicals which cause lipid peroxidation within cell membranes, resulting in cell lysis and tissue damage.

When the ischemic period is of long duration, hypoperfusion deprives the involved bowel of oxygen and nutrients, leading to hypoxia and direct cell death[2]; damage progresses from the lumen outwards to the serosa (from the mucosa and submucosa to deeper layers).

In the early stages only the mucosa and the submucosa are involved. Hemorrhagic nodules may be seen at colonoscopy and represent bleeding into the submucosa. These findings parallel the "thumbprints" or "pseudotumors" found on barium studies[5]. The purple submucosal hemorrhages usually dissipate within 48 h or are followed by ulceration. Hence, the initial diagnostic study should be performed soon after the onset of symptoms. Focal areas of pale and edematous mucosa interspersed with areas of petechial hemorrhage or superficial ulceration may also be seen in mild cases[2,46]. Later, segmental erythema with or without ulcerations and bleeding may be observed. A single longitudinal ulcerated or inflamed colon strip represents the characteristic single-stripe sign[47]. In more severe ischemia when transmural infarction of the bowel wall occurs, the mucosa appears gray-green or black over a significant area. Pseudopolyps and pseudomembranes may also co-exist[5]. In chronic stages, weeks or months later, stricture, mucosal atrophy and granularity or a mucosal pattern suggestive of "segmental ulcerative colitis" may occur[2].

Histologic changes in IC include edema, distorted crypts, mucosal and submucosal hemorrhage, inflammatory infiltration in the lamina propria, granulation tissue, intravascular platelet thrombi and necrosis. In the phase of stricture, inflammation is minimal and fibrosis predominates[5].

Endoscopic findings which distinguish between IC and inflammatory bowel disease are the segmental distribution, rectum sparing and rapid resolution on serial examinations[3]. Special care should be taken during colonoscopy to avoid overinflation which can lead to the risk of perforation. Distention of the bowel with room air may cause a further reduction in intestinal perfusion. Using carbon dioxide as the insufflating agent which is rapidly absorbed, and has the benefit of vasodilation and direct improvement in colonic perfusion, may minimize these risks[26]. When signs of peritonitis are present, endoscopy should be avoided. When endoscopy reveals findings of gangrene, colonoscopy should be stopped and laparotomy performed as soon as possible.

Total colonoscopy when it is considered safe, is preferred because 30% of IC cases occur proximal to the left flexure[3]. Given the high morbidity and mortality of IRCI and the vague presenting symptoms, early diagnosis and aggressive management is critical.

TREATMENT

Treatment depends on acuteness and severity of presentation. Most cases of IC are transient and resolve

spontaneously. Such patients do not require specific therapy. Very mild cases can be managed on an outpatient basis with liquid diet, close observation and antibiotics. Patients with more severe symptoms must be hospitalized. In the absence of colonic gangrene or perforation, general measures of supportive care are recommended. Patients should be placed on bowel rest and given intravenous fluids to resuscitate extracellular volume and reduce intestinal oxygen requirements. Parenteral nutrition should be considered for patients who need prolonged bowel rest and have major medical contraindications to surgery[26]. Cardiac function and oxygenation should be optimized. Swan-Ganz catheterization may assist in guiding fluid status and cardiac function in hemodynamically unstable patients. Vasopressors or any medications which are associated with colon ischemia should be withdrawn if possible. Oral cathartics and bowel preparations should not be given because they can, in some cases, precipitate colonic perforation or toxic dilatation of the colon. Likewise, the use of systemic corticosteroids may potentiate ischemic damage and predispose to colonic perforation. Local corticosteroids may have a role in the treatment of patients with chronic IC although no published experience supports their use. A nasogastric tube should be placed if ileus is present. Decompression of a distended colon by use of a rectal tube may be useful. Empiric broad-spectrum antibiotics are given to cover aerobic and anaerobic bacteria and minimize bacterial translocation and sepsis which has been shown to occur with the loss of mucosal integrity[48]. The use of antibiotics is based on several experimental studies which showed a reduction in severity and extent of bowel damage when antibiotics were given before or during an ischemic event[2,49]. Antibiotics have resulted in prolonged survival after intestinal ischemia in rats[49]. Although there is a lack of substantial evidence in humans, this practice is justified because of the difficulty in predicting who will progress to gangrenous colitis. In experimental studies[5], substances such as papaverine, isoproterenol, bradykinin, histamine, serotonin, adenosine, vasoactive intestinal polypeptide and glucagon have been found to dilate colonic vasculature and improve local colonic blood flow and tissue oxygenation.

Frequent clinical follow up of the abdomen, careful monitoring of vital signs and serial radiographic and colonoscopic examinations are needed. Clinical suspicion of colonic infarction justifying an emergency laparotomy may arise if there are signs of clinical deterioration despite conservative therapy, such as sepsis, persistent fever and leukocytosis, peritoneal irritation, protracted pain, diarrhea or bleeding, protein-losing colopathy for more than 14 d, free intra-abdominal air, or endoscopically-proved extensive gangrene[26].

About 20% of patients with acute IC will require surgery with an associated mortality rate of up to 60%[31]. At laparotomy, the diagnosis is confirmed and all affected bowel resected. It is important to ensure normal surgical margins. The external appearance of the bowel may be normal during laparotomy since the serosa may be unaffected, despite extensive mucosal damage. The extent of resection should be guided by the distribution of disease seen on preoperative studies. Some authors have reported on intraoperative techniques such as Doppler ultrasonography, intraoperative colonoscopy, evaluation of the antimesenteric serosal surface by hand-held photoplethysmography, pulse oximetry or transcolonic oxygen saturation and intravenous fluorescein for assessment of colonic viability[49,50]. In general, the resected segment should be examined in the operating room for mucosal injury. If needed, additional colon should be removed. Questionably viable areas of colon are generally resected. A colectomy is followed by colostomy or ileostomy. Patients with left-sided IC undergo resection with a proximal stoma and a distal mucous fistula or Hartman pouch. Primary anastomosis is unusual. Rarely, an ileocolostomy may be performed in patients with right-sided IC and viable ileum and transverse colon. In a series by Longo *et al*[51], the stoma was closed in 75% of patients with IC who underwent segmental resection *vs* only a third of those with total colonic involvement.

Fortunately, in the majority of patients, signs and symptoms of the disease resolve within 24 to 48 h and complete clinical, radiographic and endoscopic resolution occurs within 2 wk. In these circumstances no further therapy is indicated. In severe but reversible injury, when segmental ulcerative colitis exists, the colon may take 1 to 6 mo to heal[29]. Asymptomatic patients should have frequent follow-up examinations to document healing or the development of strictures or persistent colitis. In such cases, the patient may have persistent diarrhea, rectal bleeding or repeated episodes of sepsis, which may lead to perforation. Chronic ischemia may respond to topical steroid preparations in addition to general conservative measures. Resection of the affected segment is curative and subsequent development of further ischemic disease is rare. Asymptomatic strictures should be observed, since some may return to normal within 12 to 24 mo with no specific therapy[31]. When a stricture produces symptoms of obstruction, segmental resection is adequate while endoscopic dilation has been proposed as an alternative to surgery[48].

CONCLUSION

The etiology of ischemic colitis is multifactorial and the clinical presentation variable. The diagnosis is based on a combination of clinical suspicion, endoscopic and histological findings. Therapy and outcome depend on the severity of the disease. Most cases of the non-gangrenous form are transient and resolve spontaneously without complications. High morbidity and mortality and urgent operative intervention are the hallmarks of gangrenous ischemic colitis.

REFERENCES

1 **Boley SJ**, Schwartz S, Lash J, Sternhill V. Reversible vascular occlusion of the colon. *Surg Gynecol Obstet* 1963; **116**: 53-60
2 **Greenwald DA**, Brandt LJ. Colonic ischemia. *J Clin*

Gastroenterol 1998; **27**: 122-128
3 **Green BT**, Tendler DA. Ischemic colitis: a clinical review. *South Med J* 2005; **98**: 217-222
4 **Binns JC**, Isaacson P. Age-related changes in the colonic blood supply: their relevance to ischaemic colitis. *Gut* 1978; **19**: 384-390
5 **Gandhi SK**, Hanson MM, Vernava AM, Kaminski DL, Longo WE. Ischemic colitis. *Dis Colon Rectum* 1996; **39**: 88-100
6 **Hass DJ**, Kozuch P, Brandt LJ. Pharmacologically mediated colon ischemia. *Am J Gastroenterol* 2007; **102**: 1765-1780
7 **Steele SR**. Ischemic colitis complicating major vascular surgery. *Surg Clin North Am* 2007; **87**: 1099-1114, ix
8 **Champagne BJ**, Lee EC, Valerian B, Mulhotra N, Mehta M. Incidence of colonic ischemia after repair of ruptured abdominal aortic aneurysm with endograft. *J Am Coll Surg* 2007; **204**: 597-602
9 **Blanc P**, Bories P, Donadio D, Parelon G, Rouanet C, Paleirac G, Michel H. [Ischemic colitis and recurrent venous thrombosis caused by familial protein S deficiency] *Gastroenterol Clin Biol* 1989; **13**: 945
10 **Verger P**, Blanc C, Feydy P, Boey S. [Ischemic colitis caused by protein S deficiency] *Presse Med* 1996; **25**: 1350
11 **Knot EA**, ten Cate JW, Bruin T, Iburg AH, Tytgat GN. Antithrombin III metabolism in two colitis patients with acquired antithrombin III deficiency. *Gastroenterology* 1985; **89**: 421-425
12 **Ludwig D**, Stahl M, David-Walek T, Bruning A, Siemens A, Zwaan M, Schmucker G, Stange EF. Ischemic colitis, pulmonary embolism, and right atrial thrombosis in a patient with inherited resistance to activated protein C. *Dig Dis Sci* 1998; **43**: 1362-1367
13 **Yee NS**, Guerry D 4th, Lichtenstein GR. Ischemic colitis associated with factor V Leiden mutation. *Ann Intern Med* 2000; **132**: 595-596
14 **Balian A**, Veyradier A, Naveau S, Wolf M, Montembault S, Giraud V, Borotto E, Henry C, Meyer D, Chaput JC. Prothrombin 20210G/A mutation in two patients with mesenteric ischemia. *Dig Dis Sci* 1999; **44**: 1910-1913
15 **Cervera R**, Espinosa G, Cordero A, Oltra MR, Unzurrunzaga A, Rossinol T, Plaza J, Bucciarelli S, Ramos-Casals M, Ingelmo M, Asherson RA, Font J. Intestinal involvement secondary to the antiphospholipid syndrome (APS): clinical and immunologic characteristics of 97 patients: comparison of classic and catastrophic APS. *Semin Arthritis Rheum* 2007; **36**: 287-296
16 **Koutroubakis IE**, Theodoropoulou A, Sfiridaki A, Kouroumalis EA. Low plasma protein Z levels in patients with ischemic colitis. *Dig Dis Sci* 2003; **48**: 1673-1676
17 **Koutroubakis IE**, Sfiridaki A, Theodoropoulou A, Kouroumalis EA. Role of acquired and hereditary thrombotic risk factors in colon ischemia of ambulatory patients. *Gastroenterology* 2001; **121**: 561-565
18 **Midian-Singh R**, Polen A, Durishin C, Crock RD, Whittier FC, Fahmy N. Ischemic colitis revisited: a prospective study identifying hypercoagulability as a risk factor. *South Med J* 2004; **97**: 120-123
19 **Lee JR**, Paik CN, Kim JD, Chung WC, Lee KM, Yang JM. Ischemic colitis associated with intestinal vasculitis: histological proof in systemic lupus erythematosus. *World J Gastroenterol* 2008; **14**: 3591-3593
20 **Boutros HH**, Pautler S, Chakrabarti S. Cocaine-induced ischemic colitis with small-vessel thrombosis of colon and gallbladder. *J Clin Gastroenterol* 1997; **24**: 49-53
21 **Dowd J**, Bailey D, Moussa K, Nair S, Doyle R, Culpepper-Morgan JA. Ischemic colitis associated with pseudoephedrine: four cases. *Am J Gastroenterol* 1999; **94**: 2430-2434
22 **Charles JA**, Pullicino PM, Stoopack PM, Shroff Y. Ischemic colitis associated with naratriptan and oral contraceptive use. *Headache* 2005; **45**: 386-389
23 **Zervoudis S**, Grammatopoulos T, Iatrakis G, Katsoras G, Tsionis C, Diakakis I, Calpaktsoglou C, Zafiriou S. Ischemic colitis in postmenopausal women taking hormone replacement therapy. *Gynecol Endocrinol* 2008; **24**: 257-260
24 **Sreenarasimhaiah J**. Diagnosis and management of ischemic colitis. *Curr Gastroenterol Rep* 2005; **7**: 421-426
25 **Theodoropoulou A**, Sfiridaki A, Oustamanolakis P, Vardas E, Livadiotaki A, Boumpaki A, Paspatis G, Koutroubakis IE. Genetic risk factors in young patients with ischemic colitis. *Clin Gastroenterol Hepatol* 2008; **6**: 907-911
26 **Baixauli J**, Kiran RP, Delaney CP. Investigation and management of ischemic colitis. *Cleve Clin J Med* 2003; **70**: 920-921, 925-926, 928-930 passim
27 **Griffiths JD**. Surgical anatomy of the blood supply of the distal colon. *Ann R Coll Surg Engl* 1956; **19**: 241-256
28 **Sonneland J**, Anson BJ, Beaton LE. Surgical anatomy of the arterial supply to the colon from the superior mesenteric artery based upon a study of 600 specimens. *Surg Gynecol Obstet* 1958; **106**: 385-398
29 **Brandt LJ**, Boley SJ. Colonic ischemia. *Surg Clin North Am* 1992; **72**: 203-229
30 **Barouk J**, Gournay J, Bernard P, Masliah C, Le Neel JC, Galmiche JP. [Ischemic colitic in the elderly: predictive factors of gangrenous outcome] *Gastroenterol Clin Biol* 1999; **23**: 470-474
31 **Longo WE**, Ballantyne GH, Gusberg RJ. Ischemic colitis: patterns and prognosis. *Dis Colon Rectum* 1992; **35**: 726-730
32 **Medina C**, Vilaseca J, Videla S, Fabra R, Armengol-Miro JR, Malagelada JR. Outcome of patients with ischemic colitis: review of fifty-three cases. *Dis Colon Rectum* 2004; **47**: 180-184
33 **Anon R, Bosca MM**, Sanchiz V, Tosca J, Almela P, Amoros C, Benages A. Factors predicting poor prognosis in ischemic colitis. *World J Gastroenterol* 2006; **12**: 4875-4878
34 **Flobert C**, Cellier C, Berger A, Ngo A, Cuillerier E, Landi B, Marteau P, Cugnenc PH, Barbier JP. Right colonic involvement is associated with severe forms of ischemic colitis and occurs frequently in patients with chronic renal failure requiring hemodialysis. *Am J Gastroenterol* 2000; **95**: 195-198
35 **Su C**, Brandt LJ, Sigal SH, Alt E, Steinberg JJ, Patterson K, Tarr PI. The immunohistological diagnosis of E. coli O157: H7 colitis: possible association with colonic ischemia. *Am J Gastroenterol* 1998; **93**: 1055-1059
36 **Kurland B**, Brandt LJ, Delany HM. Diagnostic tests for intestinal ischemia. *Surg Clin North Am* 1992; **72**: 85-105
37 **Scholz FJ**. Ischemic bowel disease. *Radiol Clin North Am* 1993; **31**: 1197-1218
38 **Iida M**, Matsui T, Fuchigami T, Iwashita A, Yao T, Fujishima M. Ischemic colitis: serial changes in double-contrast barium enema examination. *Radiology* 1986; **159**: 337-341
39 **Balthazar EJ**, Yen BC, Gordon RB. Ischemic colitis: CT evaluation of 54 cases. *Radiology* 1999; **211**: 381-388
40 **Thoeni RF**, Cello JP. CT imaging of colitis. *Radiology* 2006; **240**: 623-638
41 **Teefey SA**, Roarke MC, Brink JA, Middleton WD, Balfe DM, Thyssen EP, Hildebolt CF. Bowel wall thickening: differentiation of inflammation from ischemia with color Doppler and duplex US. *Radiology* 1996; **198**: 547-551
42 **Ripolles T**, Simo L, Martinez-Perez MJ, Pastor MR, Igual A, Lopez A. Sonographic findings in ischemic colitis in 58 patients. *AJR Am J Roentgenol* 2005; **184**: 777-785
43 **Moallem AG**, Gerard PS, Japanwalla M. Positive In-111 WBC scan in a patient with ischemic ileocolitis and negative colonoscopies. *Clin Nucl Med* 1995; **20**: 483-485
44 **Hyun H**, Pai E, Blend MJ. Ischemic colitis: Tc-99m HMPAO leukocyte scintigraphy and correlative imaging. *Clin Nucl Med* 1998; **23**: 165-167
45 **Stathaki MI**, Koutroubakis IE, Koukouraki SI, Kouroumalis EA, Karkavitsas NS. Is there a role for Tc-99m (V) DMSA scintigraphy in ischemic colitis? *World J Gastroenterol* 2008;

14: 5432-5435

46 **Habu Y**, Tahashi Y, Kiyota K, Matsumura K, Hirota M, Inokuchi H, Kawai K. Reevaluation of clinical features of ischemic colitis. Analysis of 68 consecutive cases diagnosed by early colonoscopy. *Scand J Gastroenterol* 1996; **31**: 881-886

47 **Zuckerman GR**, Prakash C, Merriman RB, Sawhney MS, DeSchryver-Kecskemeti K, Clouse RE. The colon single-stripe sign and its relationship to ischemic colitis. *Am J Gastroenterol* 2003; **98**: 2018-2022

48 **Brandt LJ**, Boley SJ. AGA technical review on intestinal ischemia. American Gastrointestinal Association. *Gastroenterology* 2000; **118**: 954-968

49 **Maupin GE**, Rimar SD, Villalba M. Ischemic colitis following abdominal aortic reconstruction for ruptured aneurysm. A 10-year experience. *Am Surg* 1989; **55**: 378-380

50 **Bergman RT**, Gloviczki P, Welch TJ, Naessens JM, Bower TC, Hallett JW Jr, Pairolero PC, Cherry KJ Jr. The role of intravenous fluorescein in the detection of colon ischemia during aortic reconstruction. *Ann Vasc Surg* 1992; **6**: 74-79

51 **Longo WE**, Ward D, Vernava AM 3rd, Kaminski DL. Outcome of patients with total colonic ischemia. *Dis Colon Rectum* 1997; **40**: 1448-1454

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TOPIC HIGHLIGHT

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Diagnosis and management of splanchnic ischemia

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Abstract

Splanchnic or gastrointestinal ischemia is rare and randomized studies are absent. This review focuses on new developments in clinical presentation, diagnostic approaches, and treatments. Splanchnic ischemia can be caused by occlusions of arteries or veins and by physiological vasoconstriction during low-flow states. The prevalence of significant splanchnic arterial stenoses is high, but it remains mostly asymptomatic due to abundant collateral circulation. This is known as chronic splanchnic disease (CSD). Chronic splanchnic syndrome (CSS) occurs when ischemic symptoms develop. Ischemic symptoms are characterized by postprandial pain, fear of eating and weight loss. CSS is diagnosed by a test for actual ischemia. Recently, gastro-intestinal tonometry has been validated as a diagnostic test to detect splanchnic ischemia and to guide treatment. In single-vessel CSD, the complication rate is very low, but some patients have ischemic complaints, and can be treated successfully. In multi-vessel stenoses, the complication rate is considerable, while most have CSS and treatment should be strongly considered. CT and MR-based angiographic reconstruction techniques have emerged as alternatives for digital subtraction angiography for imaging of splanchnic vessels. Duplex ultrasound is still the first choice for screening purposes. The strengths and weaknesses of each modality will be discussed. CSS may be treated by minimally invasive endoscopic treatment of the celiac axis compression syndrome, endovascular antegrade stenting, or laparotomy-assisted retrograde endovascular recanalization and stenting. The treatment plan is highly individualized and is mainly based on precise vessel anatomy, body weight, co-morbidity and severity of ischemia.

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Key words: Splanchnic ischemia; Mesenteric ischemia; Tonometry; Blood flow; Chronic splanchnic syndrome; Chronic splanchnic disease; Chronic mesenteric ischemia; Celiac artery compression syndrome; Ischemic colitis

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INTRODUCTION

In this review we will cover the current insights in splanchnic or gastrointestinal ischemia. This disorder is still rarely seen in daily practice, and randomized controlled trials are absent, therefore the view of this paper is highly personal and partly authority-based in its conclusions. The spectrum of ischemic bowel disease is broad, ranging from transient left-sided ischemic colitis (with a good prognosis) to full blown intestinal infarction, with a high death rate. We will focus on new developments in clinical presentation, diagnostic approaches, and treatment options. Splanchnic ischemia can develop during low-flow states in patients with patent vessels, and in subjects with varying degree of splanchnic artery stenoses or splanchnic venous thrombosis. The prevalence of significant splanchnic arterial stenoses, or chronic splanchnic disease (CSD) is high, ranging from 30% to 50%[1,2]. Chronic splanchnic syndrome (CSS) occurs when ischemic symptoms develop. The most characteristic ischemic symptoms consist of postprandial pain, with resultant fear of eating and weight loss. When epigastric bruit is included, these are the so-called classical triad of CSS. In most patients

with CSS, this triad is incomplete. The true incidence of CSS is currently unclear, but is rare compared to CSD due to abundant collateral circulation.

Two important developments occurred in the last decade. Firstly, validation of the gastric exercise tonometry, which is currently the only clinically available and validated diagnostic test to ascertain the presence of splanchnic ischemia[3,4]. Using an ischemia-specific test it should be possible (1) to identify patients with symptomatic vessel stenoses, or CSS, which can be treated, and (2) to make this diagnosis in time and thus prevent the disaster of acute intestinal infarction. Secondly, the increasing evidence that one vessel CSD may cause splanchnic ischemia resulting in one vessel CSS, and can be successfully treated with appropriate selection procedures[5]. An important difference in presentation, treatment and outcome has been shown to exist between single and multi-vessel disease[6]. In the latter group, the clinical presentation is often less typical, with diarrhea, unexplained gastric ulcers, or dyspepsia-like symptoms. These insights stem mainly from our work with tonometry.

An entirely different entity consists of patients suffering from splanchnic ischemia without splanchnic stenoses; the so-called non-occlusive mesenteric ischemia (NOMI). It can be seen as a consequence of physiological adaptation mechanisms during low-flow states were blood is dispersed from the gastrointestinal region to more vital organs[7]. This situation is very common in intensive care and operative units, but can also be seen in outpatients. Treatment consists of aggressive fluid resuscitation and medication. However, bowel infarction can still occur.

In the last decade a change in imaging of the splanchnic vessels occurred. Duplex ultrasound, although operator dependent and suitable for 80% of patients, is still the first choice. Visceral angiography has increasingly been replaced by CT and MR-based angiographic reconstruction techniques. The clinically important advantages and disadvantages of these techniques will be discussed. Whichever technique is used, it leaves the clinician with only anatomical information. To decide whether a given stenosis has caused the symptoms, information on actual ischemia is required. This information can be obtained using tonometry, which has a proven accuracy of 80%-90%. Other tests including, serological iFABP, endothelial progenitor cell measurement, or MR angiography (MRA)-based saturation measurements, may serve that purpose in the near future.

Treatment options have changed considerably over the last decade. Apart from the classical transabdominal vascular reconstructive surgery techniques, minimally invasive endoscopic treatment of the celiac axis compression syndrome, endovascular antegrade stenting, or laparotomy-assisted retrograde endovascular recanalization and stenting have broadened our therapeutic "armory" considerably. The main patient characteristics to guide therapy choice, which include anatomical considerations, as well as body weight, co-morbidity and severity of ischemia, will be discussed.

EPIDEMIOLOGY

The prevalence of CSD is not insignificant, and rises with increasing age. In a 30-year-old angiographic study of 713 patients, 5% of the splanchnic arteries were occluded and in 70% of these occlusions the IMA was involved[8]. In a retrospective study including 980 patients with a mean age of 68 years who underwent angiography for various indications, 8% had significant stenoses of at least one splanchnic artery[9]. In a screening study with duplex ultrasonography in 553 healthy elderly subjects with a mean age of 84 years, stenoses in the celiac artery (CA) or superior mesenteric artery (SMA) were found in 18%[10]. In patients with atherosclerotic disorders of aorta, iliac and femoral vessels the incidence ranged from 25% to 40%[11,12].

A minority of patients with CSD will develop CSS or acute splanchnic syndrome (ASS). A follow-up of the study in elderly subjects in whom duplex had shown CSD, revealed no CSD-related mortality after 6 years of follow-up[13]. This risk is increased in subjects with 2 and 3 vessel CSD. In the study by Thomas *et al* 4.5% of patients with three-vessel CSD developed CSS and another 1.5% died of ASS after a follow-up of an average of 2.6 years[9]. In the Detroit experience, of the 23 patients with severe acute intestinal ischemia studied between 1963 and 2000, 12 (52%) patients had undetected CSS symptoms well before presentation[14].

ANATOMY AND (PATHO)-PHYSIOLOGY

Anatomy

Three major arteries supply blood to the stomach, small intestine and colon. The first branch, the celiac artery (CA) supplies the stomach, proximal duodenum, liver and spleen. The second, the superior mesenteric artery (SMA) supplies the distal duodenum, small intestine and proximal colon. The third branch supplies the distal colon and the rectum. There is an abundance of collateral vessels to protect the gastrointestinal tract from ischemia. Branches of these arteries enter the serosa of the gut on the mesenteric side and form a vascular plexus around the gut. After penetration of the bowel wall, a dense submucosal plexus is formed. From this plexus, arterioles penetrate the muscularis mucosa to the superficial mucosal layers. At the mucosal tip they branch into an intense capillary network of capillaries and venules. Each villus has a single, central arteriole. This arteriole travels to the tip of the villus, then splits into a network of capillaries, which form a central venule at the base of the villus. This is why countercurrent exchange can take place[15]. The tip of the villus is quite susceptible to ischemia[16].

Blood flow

Under normal conditions, approximately 20% of the cardiac output goes through the splanchnic vessels. This splanchnic blood flow doubles after a meal to approximately 2000 mL/min. Blood draining from the bowel enters the splanchnic veins and finally drains into

the portal vein. The liver, therefore, receives its blood supply from two sources: venous blood from the portal vein and arterial blood from the hepatic artery, which branches from the CA in 75% and from the SMA in remaining 25%. This dual blood supply renders the liver relatively protected against ischemia. When the blood flow to the bowel decreases below a certain level (the critical O2 delivery level), the cells will switch to anaerobic glycolysis, resulting in lactate production[17]. In the gastrointestinal system this occurs when blood flow is reduced below 50% of the basal rate[18,19]. In most cases of splanchnic ischemia, the arterial lactate levels will remain normal despite increased lactate production by the gut. The reason for this discrepancy is the large lactate metabolizing capacity of the liver. Thus, systemic lactic acidosis is a late phenomenon in these patients, indicating severe transmural ischemia and probably liver involvement as well.

The regulation of the splanchnic blood flow involves both vasoconstrictive and vasodilating substances. The main vasoconstricting substances are the catecholamines and endothelin, especially endothelin-1[20,21]. The main splanchnic vasodilators are nitric oxide (NO) and prostaglandins. It is assumed that part of the gastrointestinal toxicity of NSAIDs can be attributed to vasoconstriction because of reduced mucosal prosta-glandin concentration[22].

During low-flow states, splanchnic vasoconstriction is an early and profound phenomenon[23], which may lead to blood flow reduction below the 50% threshold. Splanchnic ischemia develops well before systemic hemodynamic instability arises[24]. This splanchnic vasoconstriction may be triggered by shock states, including hemorrhage, sepsis, dehydration or cardiac failure, from vasoactive medications, nicotine and cocaine abuse, or even in strenuous exercise[19,25-27] or severe psychological stress[28]. This combination of ischemia despite normal vessel anatomy, has given rise to the term non-occlusive mesenteric ischemia (NOMI).

Ischemic and reperfusion damage

After the onset of ischemia, an ischemic and reperfusion phase can be distinguished. When this ischemia lasts for less than 6-8 h all processes are reversible; thereafter, transmural gangrene may be the result of completely interrupted blood flow. The ischemic phase starts when the energy-containing ATP is depleted due to lack of oxygen, leading to disruptions of the tight junctions. Also, membrane-bound enzymatic pumps stop functioning, leading to inflow of luminal electrolytes and water into epithelial cells resulting in cell death. Both effects lead to reduced intestinal epithelial barrier function, with bacteria and other luminal contents entering the blood stream[29]. At the same time, the mucosal enzyme xanthine dehydrogenase is converted to xanthine oxidase (XO), which at this stage is harmless. These early effects of the ischemic phase alone are localized and can remain clinically undetected for many hours. The reperfusion phase starts when oxygen-enriched blood re-enters the ischemic tissue. This reperfusion may begin after partial dissolution of an embolus or thrombus or after revascularization. This oxygen is transformed into reactive oxygen species (ROS) by the abundantly present XO. ROS are toxic to proteins and DNA[30], and diffuse into tissues, leading to intensification and spreading of the damaged area. This so-called ischemia-reperfusion cascade initiates an inflammatory and thrombotic response in the submucosal layer of the villus. In the future, antagonists of leukocyte vessel wall adhesions, an early event in the ischemia-reperfusion cascade, could attenuate these inflammatory and thrombotic responses[31]. Successful restoration of splanchnic blood flow, containing toxic products, from the ischemic area into the systemic circulation might trigger multiple organ failure.

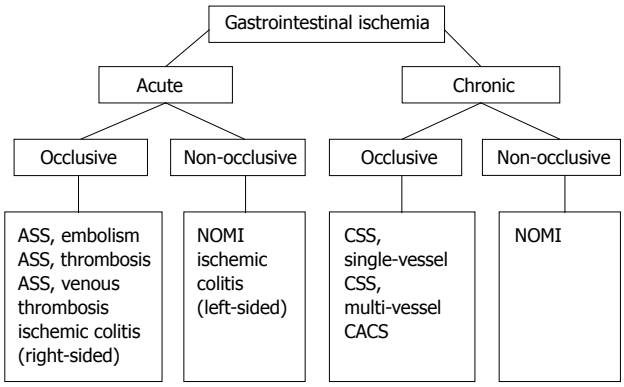

Figure 1 Classification of splanchnic, or gastrointestinal ischemia. ASS: Acute splanchnic syndrome; CSS: Chronic splanchnic syndrome; CACS, Celiac artery compression syndrome; NOMI: Non-occlusive mesenteric ischemia.

CLINICAL PRESENTATION

The naming of gastrointestinal ischemic syndromes is often confusing and requires a brief introduction. We will divide the different syndromes based on duration of complaints and vessel abnormalities (Figure 1). Acute splanchnic syndrome (ASS; synonym: acute mesenteric ischemia, acute bowel infarction) is characterized by a sudden onset of abdominal pain due to interrupted splanchnic circulation. It consists of occlusive disorders: acute splanchnic emboli, venous thromboses and end-stage arterial thrombotic occlusions, and the non-occlusive disorder, NOMI. Chronic splanchnic syndrome (CSS) is defined by a combination of chronic splanchnic disease (CSD) with ischemic symptoms. Celiac artery compression syndrome (CACS) is defined by the combination of eccentric celiac artery compression by the arcuate ligament of the diaphragm and chronic abdominal symptoms caused by ischemia. NOMI may be diagnosed with chronic or remittent splanchnic hypoperfusion, for example in heart failure[32] or in dialysis patients[33]. Finally, ischemic colitis is a separate entity and will be discussed separately.

Acute splanchnic syndrome

Acute splanchnic ischemia can result from arterial thrombosis, acute embolism, venous thrombosis or non-occlusive ischemia[34-39]. Most often the superior mesenteric

artery is involved. Clinically, it is recognized by an acute onset of abdominal pain, which might be accompanied by nausea, vomiting and hypotension. On physical examination and laboratory testing there are usually minimal abnormalities at first[40]. If left untreated, the pain often disappears. Without restoration of blood flow and depending on the collateral circulation, a full blown peritonitis follows within hours or days, with translocation of bacteria and SIRS, or systemic inflammatory response syndrome, and multiple organ failure. Mortality is high, ranging from 32%-80% depending on the etiology. In the last four decades a reduced mortality rate was observed for venous thrombosis and arterial embolism (now 32% and 51%), while the mortality of NOMI and arterial thrombosis remained unchanged at 73% and 77%[39]. Therefore, the most important factors for improvement of survival should be a high index suspicion, a proper diagnosis of CSS before ASS develops and an immediate restoration of blood flow.

Chronic splanchnic syndrome

Chronic splanchnic syndrome (CSS), a synonym for chronic mesenteric ischemia, gastrointestinal ischemia or intestinal angina, is a relatively rare disorder and may be under-diagnosed. After institution of a multidisciplinary approach team for the evaluation of insufficiently explained abdominal pain in the Medisch Spectrum Twente Hospital, the recognition of CSS increased from seven to 23 persons per million per year[41]. The major symptoms of CSS are outlined in Table 1. The most characteristic is postprandial pain, starting 15-30 min after a meal, and persisting for 1-3 h. Patients often report fear of eating, and take smaller meals, with less fat and proteins. Weight loss, the second characteristic finding in CSS, is almost always caused by reduced intake due to this fear of eating and not to malabsorption. Diarrhea, unexplained gastric ulcers or even gastroparesis can also be presenting symptoms.

This multidisciplinary team, with nationwide referrals, also found a differentiation between single- and multi-vessel disease[6]. Although the clinical presentation is quite similar, the course and outcome justifies separate discussion of both groups[42].

Single-vessel disease

The etiology of isolated stenoses of the splanchnic arteries, most often the celiac artery, is caused in most cases by splanchnic arteriosclerosis or external compression by the crux of the diaphragm[5]. Due to the presence of abundant collateral vessels, it was generally assumed that a single stenotic lesion rarely, if ever, causes complaints[43]. In 1972, Szilagyi *et al*[44] reviewed the entire literature on CA compression syndrome and found no proof of any abnormality of intestinal structure or function that could be attributed to this compression, nor proof that treatment had more than a placebo effect. However, several papers were published with good results for CA decompression operations[45-47]. These opinions were challenged recently by our group. We have shown that by using tonometry as a functional test, we could successfully distinguish patients that benefited from surgery from those who did not, and that the disappearance of symptoms after successful revascularization was associated with normalization of this functional test[5]. The prognosis of single-vessel CSS seems rather benign. In 50 patients with an isolated stenosis, no mortality and low post-operative morbidity were seen on follow-up, which contrasts sharply with multi-vessel CSS[6]. No difference in short-term outcome between patients with CACS or atherosclerotic lesions was observed. In our view, single-vessel CSS can be diagnosed when (1) there is a significant stenosis on duplex ultrasound or angiography (> 70%), (2) the clinical history fits CSS and (3) the functional test (gastric exercise tonometry) indicates splanchnic ischemia.

Table 1 Clinical picture in 107 CSS patients[6]

Patient characteristics in 107 CSS patients	
Age	Mean 55 years, range 18-85
Male:Female	26%:74%
Duration of symptoms	Mean 18 mo, range 3-192
Reported weight loss	78%
BMI	Mean 20.8 kg/m^2, range 12.0-33.2
BMI < 20 kg/m^2	35%
Weight loss (kg/mo)	Mean 1.3 kg/mo, range 0-8
Pain after meal	86%
Pain after exercise	43%
Abdominal buit	24%
Classical abdominal angina	22%
Cardiovascular history	40.20%
Nicotine use	45.80%

Multi-vessel disease

When 2 or 3 of the main splanchnic arteries have significant stenoses the ratio of CSS versus CSD increases to almost 90% in patients referred to our unit (unpublished data). Although the clinical presentation is in essence not very different from patients with single vessel disease, (postprandial pain and weight loss as the main symptoms) sometimes quite atypical presentations can be seen. Even experienced clinicians can miss an adapted lifestyle that masks a case of slowly progressive CSS. In multi-vessel CSS the clinical presentation can mimic simple dyspepsia with bloating and fullness, gastroparesis, unexplained diarrhea or simply lack of energy. When the disease progresses, the pain may be provoked by small triggers such as a simple drink, or even during rest. Abdominal vascular resting pain, persisting abdominal pain not related to a meal, are important prognostic indicators, and often indicate imminent or ongoing bowel infarction, or ASS. It should be remembered that the time to develop irreversible ischemic changes is about 6-8 h in end-stage 2- or 3-vessel CSS.

Ischemic colitis

There are two types of ischemic colitis: left-sided and right-sided ischemic colitis. Left-sided ischemic colitis usually presents with abrupt onset abdominal pain, followed by bloody diarrhea, which may persist for days

to weeks. It is often associated with low-flow states[48], coagulation disorders[49], cardiac abnormalities or after abdominal aortal surgery. Low-flow states may be induced by arrhythmias or cardiac dysfunction, drugs, dehydration or (aortic) surgery. Isolated right-sided ischemic colitis usually presents with abdominal pain, but rarely with bloody diarrhea[50]. Right-sided ischemic colitis is usually associated with SMA stenosis or occlusion. It should therefore be considered as part of CSS or ASS.

The late stages of ischemic colitis can be a clinical challenge, with clinical presentation and endoscopic findings mimicking both Crohn's disease and ulcerative colitis. In most cases isolated ischemic colitis will be transient, although persistent colitis, stricture formation and even gangrenous colitis have been seen to develop[48].

NOMI

NOMI has already been mentioned as cause of ASS in 20%-30% of patients. Most NOMI patients however, never develop ASS, and this condition is quite common due to the early splanchnic vasoconstriction with reduced circulating blood volume of any cause. Moreover, the bowel has limited capacity to preserve aerobic metabolism. NOMI is very common and widely recognized in intensive care and peri-operative medicine, where it is referred to as intramucosal acidosis or mucosal ischemia[7]. Here, the clinical signs of NOMI may range from abdominal tenderness, nausea, diarrhea, ulceration, bleeding and full thickness ischemia, and may lead to bowel wall necrosis and even death. In critically ill patients, this process can easily lead to translocation of bacteria and endotoxins, causing SIRS and multi organ failure[51].

We also distinguished a group of patients with 'abdominal migraine' characterized by symptoms compatible with splanchnic ischemia, abnormal functional tests (tonometry) indicating ischemia, splanchnic angiography without relevant pathology, and good response to vasodilators[4,52].

DIAGNOSTIC METHODS

Duplex ultrasound

Duplex-ultrasound is widely used as screening tool for detection of splanchnic stenosis. In experienced hands the CA and the SMA can be visualized in 80%-90% of patients. Proper visualization can be difficult because of the location behind the, often air-filled, stomach. First, vessel anatomy is established using the B-mode. This is followed by assessment of blood-flow pattern and velocity. The arterial blood-flow in the splanchnic vessels varies during the cardiac cycle. The normal CA has a biphasic signal. Retrograde flow in the common hepatic artery may indicate proximal CA stenosis or occlusion. The SMA normally has a triphasic signal. A biphasic signal in the SMA is normal after a meal or when the right, or rarely the common hepatic artery, comes from the SMA as an anatomic variant; it may indicate a proximal stenosis if it occurs in the fasting state. Blood-flow measurement is performed using a measurement angle of less than 60 degrees. A significant stenosis is characterized by areas of high velocity jets (small streaks of very high, sometimes turbulent flow) within the artery, and overall increased flow velocity. The widely accepted cut-off values, published by Moneta in 1993, are: for the CA a Peak Systolic Velocity (PSV) and End Diastolic Velocity (EDV) of 200 m/s and 55 m/s; and for the SMA, PSV and EDV of 275 and 45 m/s, respectively[53]. Using these threshold values, the sensitivity and specificity for stenoses > 70% were 89% and 44% for the inspiration CA, 89% and 62% for the expiration CA, 100% and 61% for the inspiration SMA, and 80% and 42% for the expiration SMA. One criticism of the "Moneta criteria" has been that he used a cohort from the general population with atherosclerotic disease, who did not necessarily suffer from chronic abdominal symptoms. In daily practice, duplex ultrasound is performed under fasting conditions. Some studies suggested measurement after test meals, because patients with ischemia had suppressed augmentation of blood-flow following a meal compared to subjects with normal vessels[54-58]. Until recently, post-test meal splanchnic duplex made no headway in daily practice. Duplex-derived flow velocities after splanchnic artery bypass grafting may be affected by graft diameter or type of reconstruction and are not equal to the preoperative criteria[59].

MRA

By using contrast, so-called contrast enhanced (ce)-MRA it is possible to identify splanchnic artery stenoses and even collaterals, for example Riolan's artery[60-62]. Surprisingly, we could identify only two studies that compared digital subtraction angiography (DSA) with MRA in 19[63] and 23 patients[61] with good correlation. In our own experience, with 25 patients in whom DSA and MRA were performed within 2 mo, the MRA had a 95% sensitivity and 90% specificity, compared to DSA as the "gold standard". We observed a significant inter and intra observer variation in grading of stenoses in 45% of the cases[64]. Although widely used, it can therefore not be considered a gold standard investigation for assessment of vessel anatomy. A potential advantage of MRA is its ability to measure actual flow through the splanchnic and portal circulation. The arterial flow is harder to measure than in veins, because of the smaller caliber and the pulsatile character of arterial flow[56]. However, a consistent relationship between flow in the arteries and veins was observed[65,66]. Several studies with healthy volunteers and patients have shown augmentation of flow after a meal and significant differences between patients with vessel stenoses and healthy volunteers[54,58]. These results may be promising for future use in diagnosing CSS.

CT angiography

With the introduction of the multi-slice CT scan, CT angiography of the abdominal arteries has become possible. There are several studies showing that CT angiography is an accurate way to image the splanchnic arteries, veins, and collaterals[67-70]. Surprisingly, studies focusing on CSD and comparing this technique to the gold

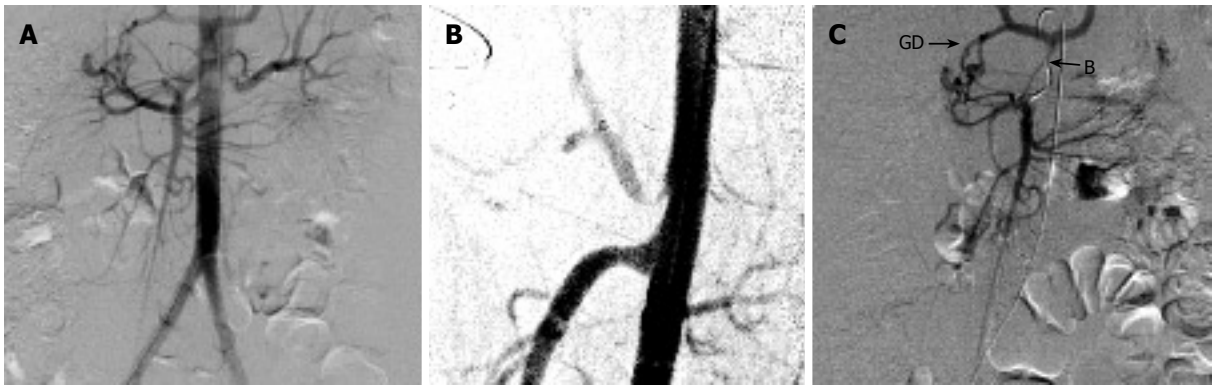

Figure 2 Collateral vessels: gastroduodenal (GD) artery and Buehlers artery (B). A: The gastroduodenal (GD) artery and Buehlers artery (B) are visible on non-selective aortography indicating stenosis of the origin of either the CA or SMA. The late filling of the CA points to a stenosis in its origin; B: Lateral aortography showing an asymmetrical stenosis of the CA; C: On selective cannulation of the SMA, both collaterals are more clearly visible.

standard (DSA) in a representative group of patients, are lacking to our knowledge. It is essential to use multi-slice scanners with slice thicknesses of 2 mm at most (preferably 1 mm) to allow accurate visualization of the arteries. With the state-of-the-art technology, CT-scanning in inspiration and expiration is possible and is a prerequisite when there is suspicion of celiac artery compression syndrome. The advantages of CT angiography are clear: in a patient with acute abdominal complaints it can show or exclude arterial and venous obstruction, bowel involvement (wall distention and thickening, presence or absence of contrast enhancement, pneumatics), as well as alternative diagnoses. Among these are perforations, pancreatitis, and abscesses. Similarly, in the setting of chronic unexplained postprandial pain, CT angiography may also show alternative diagnoses. In our experience, these include pancreatitis, pancreatic cancer, and retroperitoneal tumors. Adding the advantages of minimal invasiveness and lower costs, CT angiography can be a serious competition for conventional angiography[67,69,71,72].

Angiography

Intra-arterial digital subtraction angiography (DSA) of the splanchnic vessels can be used to perform endovascular therapeutic procedures in the same session, including infusion of papaverine and angioplasty or stenting of stenoses. The combination of high diagnostic accuracy and the possibility for intervention makes angiography the procedure of choice in patients suspected of symptomatic splanchnic stenoses, especially with imminent or ongoing infarction. In acute splanchnic infarction, angiography serves as guideline for endovascular or operative revascularization.

A state-of-the art visceral angiography involves three steps: (1) a non-selective anterior-posterior abdominal aortic angiography. When collaterals show in this stage, they indicate significant splanchnic artery stenosis and are considered pathological (Figure 2A and B); (2) a lateral aortography during maximal inspiration and expiration, for detection of external compression of the CA and/or, rarely, the SMA; and (3) selective angiography of all three splanchnic vessels to obtain a detailed view of the vascular anatomy, stenoses and anatomical variations[42]. Although CT and MR angiography are gaining ground as the principal investigative tools for splanchnic vessel anatomy, detailed angiographic information of anatomy, stenosis, collaterals and anatomical variations is, in our view, essential in the preparation of an optimal revascularization strategy.

Endoscopy

Although almost all patients with CSS underwent upper GI endoscopy during the work-up of their complaints, abnormalities were rarely noted. Reports on gastroparesis and gastric gangrene have been published, but are also rare. In our experience, 7% of patients with CSS presented with unexplained gastroduodenal ulcers (Helicobacter negative, no NSAID-use). In colonic ischemia endoscopy is the mainstay of diagnosis. The typical picture consists of superficial ulceration, mucosal friability, edema, and patchy areas of either bleached or cyanotic mucosa[73,74]. Colonic ischemia of longer duration may mimic ulcerative colitis or even Crohn's disease. Some papers have focused on endoluminal light spectroscopy oximetry. With this technique, mucosal hemoglobin oxygen saturation can be measured[75-80]. In a recent study it was shown that mucosal saturations are low in patients with chronic splanchnic ischemia, compared to healthy subjects. After successful treatment in these patients, mucosal oxygen saturations increased substantially[76]. There are a several potential limitations to this technique. Firstly, ischemia is patchy in nature, and can therefore be missed. Secondly, ischemia might be present in parts of the small bowel that are difficult to reach endoscopically. Thirdly, ischemia might only be present in a situation of increased oxygen demand (after a meal, or during exercise), especially earlier in the course of the disease.

Tonometry

Tonometry of the gastrointestinal tract has the unique potential to the detect ischemia, irrespective of flow or metabolism. Tonometry is based on a general physiological principle that during ischemia, anaerobic metabolism leads to increased production of acids, which are buffered locally by bicarbonate ions, leading to increased carbon dioxide tension (PCO_2) in the tissue. This relation between

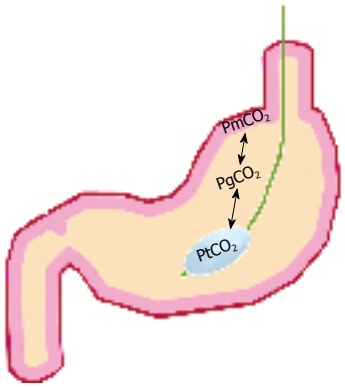

Figure 3 Tonometer balloon placed in the stomach nasogastrically. CO_2 diffuses rapidly over different membranes, therefore the tonometer PCO_2 ($PtCO_2$) will be in equilibrium with gastric luminal PCO_2 ($PgCO_2$) and mucosal PCO_2 ($PmCO_2$). The PCO_2 can be measured from the catheter either from injected saline using blood gas analyzers or by connection to a semi-automated Tonocap device. The underlying physiological principle is that ischemia is always associated with PCO_2 increase. Therefore, focal measurement of ischemia is possible for long periods *via* a minimally invasive technique.

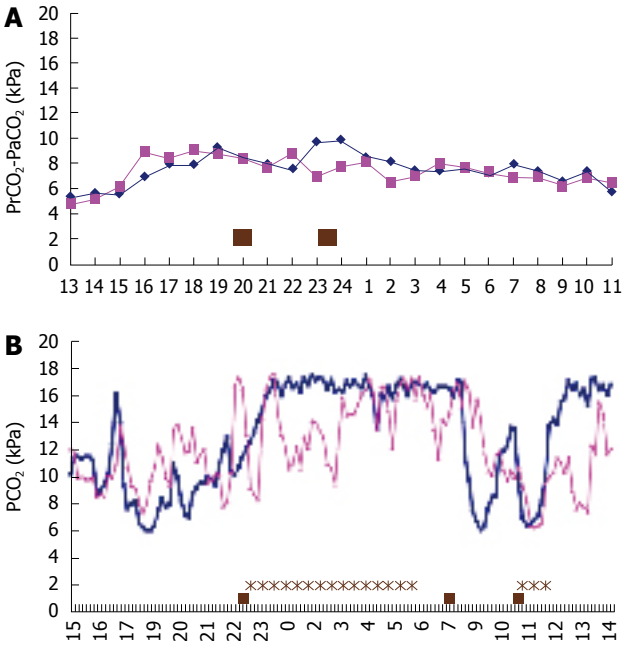

Figure 4 Imminent ASS and normal gastric and jejunal PCO_2 pattern. A: Normal 24 h PCO_2 pattern in the stomach (squares) and jejunum (diamonds) with variation in PCO_2, but no peaks above 11 kPa following meals; B: Imminent bowel infarction in a patient with severe 3 vessel CSS. After her evening meal she had pain for almost 6 h, and extreme ischemia with PCO_2 > 16 kPa for 7 h. She was treated with endovascular stent placement the day after this measurement, with immediate relief of complaints. She is still doing well, over 3 years later.

ischemia and increased PCO_2 has been observed in all ischemic models and animals studied. The most specific marker of ischemia is an increased difference between luminal and arterial CO2, the PCO_2 gradient, which is barely influenced by other systemic factors, including hyper- or hypoventilation. The luminal PCO2 can be measured conveniently using a nasogastric tonometry catheter and air tonometry (Figure 3). The unique property of tonometry in measuring ischemia *per se*[3] sets it apart from all other diagnostic methods. Indeed, when blood flow is gradually reduced, the PCO2 gradient remains normal until the blood flow decreases to < 50% of the basal flow and then increases sharply[18,19].

In patients with suspected chronic ischemia, gastric tonometry has been used initially using a test meal with variable, but overall disappointing, results[81-83]. The main methodological problems involved buffering effects by gastric acid and dilution effects of the ingested test meals[84]. We therefore developed a tonometric test involving 10 min of submaximal exercise, in order to provoke GI ischemia[19]. The diagnostic accuracy of the gastric exercise tonometry test (GET) was evaluated in a cohort of patients referred for suspected CSS. GET had a 78% sensitivity and 92% specificity for ischemia detection[4]. We have used GET to guide treatment in patients with single vessel stenoses. The main finding in this study was the tight relationship between normalization of GET and disappearance of symptoms after anatomically successful revascularization[5]. We also re-examined the potential use of a 24-h tonometry test, including test meals, with standardization of the test circumstances, including potent acid suppression and standard test meals[85]. In a pilot study in 33 patients referred for suspected CSS, the 24 h tonometry showed promising results, with a sensitivity of 76% and a specificity of 94%, comparable to those of exercise testing[86]. We are now analyzing a study comparing GET and 24-h tonometry. The preliminary data suggest that 24-h tonometry permits accurate measurement of postprandial and fasting PCO_2 levels; following meals, gastric and small bowel PCO_2 gradients may physiologically increase up to 10 kPa. During ischemia, gradients exceed 11 kPa (60 mmHg)[87]. Also, prolonged PCO2 increases for several hours (up to 7 h in one subject, Figure 4), especially in combination with abdominal pain during fasting, indicate imminent infarction, and thereby provide invaluable extra information.

Serological markers

Currently, there is no reliable marker to diagnose gastrointestinal ischemia. Studies have been performed using several markers, including (L and D)-lactate, LDH, D-dimer; however, none of these was proven to be sensitive or specific. In contrast, various animal models have successfully used markers like D-lactate and i-FABP (intestinal Fatty Acid Binding Protein) as an early marker of intestinal ischemia. This enzyme is present in the mature enterocytes of the small intestine, in the highest concentration at the villi[88,89], the region most susceptible to ischemia, and is released early after an ischemic insult. Therefore it seems a good candidate marker for early ischemia detection[90]. There are a few patient studies indicating its potential as marker for ASS[89,91], but also during pancreatitis[92] or inflammatory bowel disease[93]. We performed a pilot study comparing tonometric responses to a test meal and indeed found increased i-FABP in these subjects[94]. More studies are needed before the role of this and other promising plasma markers can be established.

WORK-UP AND TREATMENT

In the work-up of patients in whom splanchnic ischemia is suspected four questions should be addressed: (1) is the history compatible with splanchnic ischemia, (2) which of the three vessels are narrowed, to what degree and are pathological collaterals present, (3) is there (functional) evidence of actual ischemia, and (4) is the impairment of the splanchnic blood flow in the short-term threatening for the bowel.

Endovascular techniques have emerged allowing for stent placement in CA and SMA in most patients. The choice is between dilation or stent placement. Dilation alone has a low short-term success rate, and we currently use it only as diagnostic tool to distinguish between CSS and CSD. Although some vessels can be treated *via* the femoral artery; the sharp downward angulation (60 degrees) of the AMS and CA often necessitates brachial artery cannulation in many cases. In our center, both techniques are often combined, using the femoral catheter as a guide for the stent placed *via* the brachial catheter. Compared to operative revascularization, the main disadvantage of stent placement is the shorter long-term patency. The latter was shown in three recent studies, of which two compared endovascular and open repair. Atkins *et al* reported in a cohort of 80 patients with CSS primary patency at 1 year of 58% after endovascular and 90% after open repair and a primary assisted patency of 65% *vs* 96% respectively[95]. Bieble *et al* reported that in a cohort of 49 patients with CSS, 75% after endovascular and 89% after open repair were symptom free after 2 years[96]. The main difference in this study was the restenosis rate of 8% after open versus 25% after endovascular treated patients, with lower complication rates in the latter. Sarac *et al*[97] reported a primary, primary assisted and secondary patency of 65%, 97% and 99% in a cohort of 87 endovascular treated splanchnic arteries. The low short-term morbidity makes it an excellent choice in patients with limited life expectancy or those too weak or underweight for operative revascularization.

A variety of surgical techniques have been advocated for open repair of the splanchnic arteries, including reimplantation, transarterial and transaortic endarterectomy, antegrade and retrograde aortovisceral bypass using vein or arterial autograft bypasses and prosthetic bypass, with early success rates between 91% and 96% and late success rates between 80% and 90%[98]. The choice of technique is usually based on the preference and experience of the surgeon. However, the majority of centers with wide experience believe that antegrade autogenous revascularization techniques of both the CA and the SMA in selected cases offers the best long-term results. The disadvantage of major aortic surgery is the not inconsiderable burden for the patient. In general, we prefer antegrade two-vessel reconstruction for young patients with CSS and a body mass index above 19.5 kg/m^2, and endovascular or minimal invasive retrograde single or multi-vessel reconstructions for patients with relevant comorbidity or reduced life expectancy. Also endovascular repair could act as bridge to open repair after full recovery and weight gain in selected young patients with end stage CSS.

Acute splanchnic syndrome

The diagnosis of ASS begins with a high index of suspicion. Any patient with acute onset of abdominal pain that remains unexplained after proper investigation for two hours should be suspected to have ASS. An urgent investigation of vessel patency should be ordered. The choice is between an acute angiography and a CT scan. Simultaneously with the CT scan or DSA, the volume status should be aggressively restored to counterbalance the splanchnic vasoconstriction, which is almost always present in these patients. All necrotic bowel should be removed and blood flow restored as soon as possible. The latter will involve intravenous heparin in venous ASS, revascularization or embolectomy in arterial ASS, and in selected cases, intra-arterial vasodilation in ASS-NOMI[73]. In arterial ASS, the choice of revascularization, and whether it should be done before or after bowel resection, depends on local expertise. Bowel vitality can be hard to assess initially, therefore second- and third look operations to ascertain bowel vitality are often advised and seem prudent. It has been shown that aggressive treatment might be responsible for the modest improvement in outcome of ASS[39]. In cases of limited extent of severe bowel ischemia we advise immediate retrograde endovascular revascularization[99,100] and resection of the ischemic bowel to diminish the detrimental cascade of ischemia-reperfusion resulting in multi organ failure and high mortality.

Acute ischemic colitis

Isolated acute left-sided ischemia can usually be treated conservatively, as it is almost always non-occlusive in nature. Right-sided ischemic colitis should be considered as ASS and treated accordingly. Patients with left-sided ischemic colitis should be treated with intravenous fluid and bowel rest. Broad spectrum antibiotics are advised, which might reduce bowel damage[48,101], but there is no evidence to back this up. Left-sided ischemic colitis subsides in 2 wk[48] in most patients. About 20% of patients with acute ischemic colitis ultimately need surgery, either because the ischemic colitis persists or complications occur. A non-responsive left-sided ischemic colitis can manifest as ongoing sepsis refractory to medical treatment, persistent diarrhea, bleeding, or protein-losing enteropathy for more than 14 d. In some patients progressive peritonitis or gangrene of the colon develops, with a mortality rate of 30%-60%[48,102].

CHRONIC SPLANCHNIC SYNDROME

Single-vessel CSS and CACS

Patient selection is crucial in these patients. When single-vessel CSS is diagnosed by history, vessel anatomy and functional tests, treatment may help to relieve the symptoms. The prognosis quod vitam is good; therefore

treatment is aimed at relief of symptoms only. Some patients prefer conservative measures including small meals and proton pump inhibitors. When a revascularization is indicated, the choice of technique depends on vessel abnormalities as well as local experience.

In CACS patients primary stent placement is not an option because the repeated force by the diaphragm with each respiration will fracture the stent in the short-term. Different techniques are currently used to release the CA from compression by the diaphragm's crux. One potential complication is the development of reflux disease[5], which is related to the damage to the crux, which also plays an important role in the physiological anti-reflux barrier. Recently, we have performed the release by an endoscopic retroperitoneal technique with equally good results compared to the open approach in the short-term[103]. The problem of reflux seems to be reduced, although more studies with longer follow-up times are needed.

Multi-vessel CSS

Most patients with abdominal symptoms and multi-vessel stenoses have, in our experience, CSS. The risk of developing ASS is considerable[6], therefore the treatment goal is aimed at symptom relief as well as prevention of ASS. There are no prospective studies on the conservative treatment of CSS, but advice on diet and lifestyle have been described. Most patients have already changed their eating pattern, with smaller and more frequent meals that contain less fat and protein. It should be strongly advised to stop smoking because it causes strong splanchnic vasoconstriction. The use of proton pump inhibition is not evidence based, however, it makes sense as these drugs reduce the secretion of gastric acid, and thus gastric metabolic demand, while increasing the gastric blood flow[104]. Atherosclerosis is a generalized disorder; therefore the usual measures should be initiated including treatment of hypertension, hyperlipidemia, and diabetes.

Most multi-vessel CSS patients have an indication for revascularization. Whether patients with multi-vessel (asymptomatic) CSD should be treated to prevent ASS is uncertain. It may be considered in young patients in good health, but firm evidence is lacking. The choice of revascularization in multi-vessel CSS again depends on anatomy, experience, and comorbidity as discussed before.

NOMI

Chronic splanchnic ischemia due to NOMI comes in two different patient groups. The first group has severe underlying medical conditions, with reduced effective circulating volume and splanchnic vasoconstriction and ischemia. These include dialysis patients[33] and chronic heart failure[32] patients. Treatment is difficult because the treatment of their underlying disease requires reduction of intravascular volume, which may worsen their abdominal complaints. In our experience the use of nitrates, ketanserin and alpha-inhibitors may have a positive effect on the abdominal symptoms, whereas calcium channel blockers seemed to worsen these. A second group we encountered are patients with a clinical presentation similar to CSS, with normal microvasculature but functional tests indicating ischemia. As discussed earlier, in some of these we have observed vascular spasms during angiography, with onset of pain within minutes thereafter[4]. In a pilot study, we treated them with vasodilators, nitrates, ketanserin or nicorandil. Over 50% of patients show a reduction of abdominal pain of at least 50% on a visual analog scale, which is sustained for years in most cases[52]. Further studies are needed to assess the prevalence, precise mechanism and the best treatment options of this disorder.

Post-intervention care

After revascularization, severe reperfusion injury can occur. The exact risk factors for its development are unknown. In our experience it is more common in patients with serious and long-standing multi-vessel disease. The following pattern in reperfusion damage after revascularization is typical. The first 1-2 d after revascularization the patient has good clinical recovery. Most could start eating again without pain or special discomfort. After 2-3 d symptoms develop, in hours to days, consisting of nausea, abdominal pain, similar or even worse than before treatment, diarrhea and in extreme cases, protein-losing enteropathy with very low serum albumin levels. Massive ascites may develop during reperfusion.

It is crucial to distinguish reperfusion injury from vessel occlusion; therefore vessel patency has to be ascertained with CTA or DSA. This bowel reperfusion syndrome can persist for days to weeks. We therefore treat these patients with parenteral nutrition, intravenous fluids, and proton pump inhibitors. The end of the reperfusion syndrome is usually heralded by increased appetite, reduced pain and reduced diarrhea. Patients can restart oral intake, be taken off parenteral nutrition and generally have uneventful recovery within weeks thereafter. No long-term complications have been observed in these patients.

CONCLUSION

Splanchnic ischemia has developed into a broad spectrum of diseases. These are characterized by onset, vessel anatomy, and presence of ischemia. Each syndrome has different characteristics, outcome, and treatment options, therefore a state-of-the art vessel anatomy assessment and accurate functional test are crucial. Tonometry is the only validated test assessing the adequacy of the splanchnic blood-flow and consequently is crucial in proper patient selection. Treatment options, including noninvasive, minimal invasive and classical open vascular reconstructive techniques, are wide and require a multi-disciplinary team-approach for proper selection and follow-up.

REFERENCES

1 **Derrick JR**, Pollard HS, Moore RM. The pattern of

arteriosclerotic narrowing of the celiac and superior mesenteric arteries. *Ann Surg* 1959; **149**: 684-689
2 **Reiner L**, Jimenez FA, Rodriguez FL. Atherosclerosis in the mesenteric circulation. observations and correlations with aortic and coronary atherosclerosis. *Am Heart J* 1963; **66**: 200-209
3 **Kolkman JJ**, Otte JA, Groeneveld AB. Gastrointestinal luminal PCO2 tonometry: an update on physiology, methodology and clinical applications. *Br J Anaesth* 2000; **84**: 74-86
4 **Otte JA**, Geelkerken RH, Oostveen E, Mensink PB, Huisman AB, Kolkman JJ. Clinical impact of gastric exercise tonometry on diagnosis and management of chronic gastrointestinal ischemia. *Clin Gastroenterol Hepatol* 2005; **3**: 660-666
5 **Mensink PB**, van Petersen AS, Kolkman JJ, Otte JA, Huisman AB, Geelkerken RH. Gastric exercise tonometry: the key investigation in patients with suspected celiac artery compression syndrome. *J Vasc Surg* 2006; **44**: 277-281
6 **Mensink PB**, van Petersen AS, Geelkerken RH, Otte JA, Huisman AB, Kolkman JJ. Clinical significance of splanchnic artery stenosis. *Br J Surg* 2006; **93**: 1377-1382
7 **Kolkman JJ**, Mensink PB. Non-occlusive mesenteric ischaemia: a common disorder in gastroenterology and intensive care. *Best Pract Res Clin Gastroenterol* 2003; **17**: 457-473
8 **Bron KM**, Redman HC. Splanchnic artery stenosis and occlusion. Incidence; arteriographic and clinical manifestations. *Radiology* 1969; **92**: 323-328
9 **Thomas JH**, Blake K, Pierce GE, Hermreck AS, Seigel E. The clinical course of asymptomatic mesenteric arterial stenosis. *J Vasc Surg* 1998; **27**: 840-844
10 **Hansen KJ**, Wilson DB, Craven TE, Pearce JD, English WP, Edwards MS, Ayerdi J, Burke GL. Mesenteric artery disease in the elderly. *J Vasc Surg* 2004; **40**: 45-52
11 **Valentine RJ**, Martin JD, Myers SI, Rossi MB, Clagett GP. Asymptomatic celiac and superior mesenteric artery stenoses are more prevalent among patients with unsuspected renal artery stenoses. *J Vasc Surg* 1991; **14**: 195-199
12 **Glockner JF**. Incidental findings on renal MR angiography. *AJR Am J Roentgenol* 2007; **189**: 693-700
13 **Wilson DB**, Mostafavi K, Craven TE, Ayerdi J, Edwards MS, Hansen KJ. Clinical course of mesenteric artery stenosis in elderly americans. *Arch Intern Med* 2006; **166**: 2095-2100
14 **Cho JS**, Carr JA, Jacobsen G, Shepard AD, Nypaver TJ, Reddy DJ. Long-term outcome after mesenteric artery reconstruction: a 37-year experience. *J Vasc Surg* 2002; **35**: 453-460
15 **Lundgren O**, Haglund U. The pathophysiology of the intestinal countercurrent exchanger. *Life Sci* 1978; **23**: 1411-1422
16 **Haglund U**, Hulten L, Ahren C, Lundgren O. Mucosal lesions in the human small intestine in shock. *Gut* 1975; **16**: 979-984
17 **Schlichtig R**, Bowles SA. Distinguishing between aerobic and anaerobic appearance of dissolved CO2 in intestine during low flow. *J Appl Physiol* 1994; **76**: 2443-2451
18 **Knichwitz G**, Rotker J, Mollhoff T, Richter KD, Brussel T. Continuous intramucosal PCO2 measurement allows the early detection of intestinal malperfusion. *Crit Care Med* 1998; **26**: 1550-1557
19 **Otte JA**, Oostveen E, Geelkerken RH, Groeneveld AB, Kolkman JJ. Exercise induces gastric ischemia in healthy volunteers: a tonometry study. *J Appl Physiol* 2001; **91**: 866-871
20 **Burgener D**, Laesser M, Treggiari-Venzi M, Oi Y, Jolliet P, Strasser S, Hadengue A, Aneman A. Endothelin-1 blockade corrects mesenteric hypoperfusion in a porcine low cardiac output model. *Crit Care Med* 2001; **29**: 1615-1620
21 **Kawano S**, Tsuji S. Role of mucosal blood flow: a conceptional review in gastric mucosal injury and protection. *J Gastroenterol Hepatol* 2000; **15** Suppl: D1-D6
22 **Kawano S**, Tsuji S, Sato N, Kamada T. NSAIDS and the microcirculation of the stomach. *Gastroenterol Clin North Am* 1996; **25**: 299-315
23 **Toung T**, Reilly PM, Fuh KC, Ferris R, Bulkley GB. Mesenteric vasoconstriction in response to hemorrhagic shock. *Shock* 2000; **13**: 267-273
24 **Hamilton-Davies C**, Mythen MG, Salmon JB, Jacobson D, Shukla A, Webb AR. Comparison of commonly used clinical indicators of hypovolaemia with gastrointestinal tonometry. *Intensive Care Med* 1997; **23**: 276-281
25 **Heer M**, Repond F, Hany A, Sulser H, Kehl O, Jager K. Acute ischaemic colitis in a female long distance runner. *Gut* 1987; **28**: 896-899
26 **Moses FM**. The effect of exercise on the gastrointestinal tract. *Sports Med* 1990; **9**: 159-172
27 **Nielsen HB**, Svendsen LB, Jensen TH, Secher NH. Exercise-induced gastric mucosal acidosis. *Med Sci Sports Exerc* 1995; **27**: 1003-1006
28 **Veenstra RP**, Geelkerken RH, Verhorst PM, Huisman AB, Kolkman JJ. Acute stress-related gastrointestinal ischemia. *Digestion* 2007; **75**: 205-207
29 **Wattanasirichaigoon S**, Menconi MJ, Delude RL, Fink MP. Effect of mesenteric ischemia and reperfusion or hemorrhagic shock on intestinal mucosal permeability and ATP content in rats. *Shock* 1999; **12**: 127-133
30 **Nielsen VG**, Tan S, Baird MS, McCammon AT, Parks DA. Gastric intramucosal pH and multiple organ injury: impact of ischemia-reperfusion and xanthine oxidase. *Crit Care Med* 1996; **24**: 1339-1344
31 **Beuk RJ**, Tangelder GJ, Maassen RL, Quaedackers JS, Heineman E, Oude Egbrink MG. Leucocyte and platelet adhesion in different layers of the small bowel during experimental total warm ischaemia and reperfusion. *Br J Surg* 2008; **95**: 1294-1304
32 **Krack A**, Richartz BM, Gastmann A, Greim K, Lotze U, Anker SD, Figulla HR. Studies on intragastric PCO2 at rest and during exercise as a marker of intestinal perfusion in patients with chronic heart failure. *Eur J Heart Fail* 2004; **6**: 403-407
33 **Diebel L**, Kozol R, Wilson RF, Mahajan S, Abu-Hamdan D, Thomas D. Gastric intramucosal acidosis in patients with chronic kidney failure. *Surgery* 1993; **113**: 520-526
34 **Rogers DM**, Thompson JE, Garrett WV, Talkington CM, Patman RD. Mesenteric vascular problems. A 26-year experience. *Ann Surg* 1982; **195**: 554-565
35 **Sitges-Serra A**, Mas X, Roqueta F, Figueras J, Sanz F. Mesenteric infarction: an analysis of 83 patients with prognostic studies in 44 cases undergoing a massive small-bowel resection. *Br J Surg* 1988; **75**: 544-548
36 **Kaleya RN**, Sammartano RJ, Boley SJ. Aggressive approach to acute mesenteric ischemia. *Surg Clin North Am* 1992; **72**: 157-182
37 **Bergan JJ**, Dean RH, Conn J Jr, Yao JS. Revascularization in treatment of mesenteric infarction. *Ann Surg* 1975; **182**: 430-438
38 **Jrvinen O**, Laurikka J, Salenius JP, Tarkka M. Acute intestinal ischaemia. A review of 214 cases. *Ann Chir Gynaecol* 1994; **83**: 22-25
39 **Schoots IG**, Koffeman GI, Legemate DA, Levi M, van Gulik TM. Systematic review of survival after acute mesenteric ischaemia according to disease aetiology. *Br J Surg* 2004; **91**: 17-27
40 **Hunter GC**, Guernsey JM. Mesenteric ischemia. *Med Clin North Am* 1988; **72**: 1091-1115
41 **Otte J**, Geelkerken R, Huisman A, Kolkman JJ. Assessment of the incidence of chronic gastrointestinal ischemia after institution of a multidisciplinary working group. *Gastroenterology* 1999; **116**: A915
42 **Kolkman JJ**, Mensink PB, van Petersen AS, Huisman AB, Geelkerken RH. Clinical approach to chronic gastrointestinal ischaemia: from ‚intestinal angina' to the spectrum of chronic splanchnic disease. *Scand J Gastroenterol Suppl* 2004; 9-16

43 **Brandt LJ**, Boley SJ. AGA technical review on intestinal ischemia. American Gastrointestinal Association. *Gastroenterology* 2000; **118**: 954-968

44 **Szilagyi DE**, Rian RL, Elliott JP, Smith RF. The cardiac artery compression syndrome: does it exist? *Surgery* 1972; **72**: 849-863

45 **Kernohan RM**, Barros D'Sa AA, Cranley B, Johnston HM. Further evidence supporting the existence of the celiac artery compression syndrome. *Arch Surg* 1985; **120**: 1072-1076

46 **Reilly LM**, Ammar AD, Stoney RJ, Ehrenfeld WK. Late results following operative repair for celiac artery compression syndrome. *J Vasc Surg* 1985; **2**: 79-91

47 **Loffeld RJ**, Overtoom HA, Rauwerda JA. The celiac axis compression syndrome. Report of 5 cases. *Digestion* 1995; **56**: 534-537

48 **Brandt LJ**, Boley SJ. Colonic ischemia. *Surg Clin North Am* 1992; **72**: 203-229

49 **Koutroubakis IE**, Sfiridaki A, Theodoropoulou A, Kouroumalis EA. Role of acquired and hereditary thrombotic risk factors in colon ischemia of ambulatory patients. *Gastroenterology* 2001; **121**: 561-565

50 **Sotiriadis J**, Brandt LJ, Behin DS, Southern WN. Ischemic colitis has a worse prognosis when isolated to the right side of the colon. *Am J Gastroenterol* 2007; **102**: 2247-2252

51 **Mythen MG**, Purdy G, Mackie IJ, McNally T, Webb AR, Machin SJ. Postoperative multiple organ dysfunction syndrome associated with gut mucosal hypoperfusion, increased neutrophil degranulation and C1-esterase inhibitor depletion. *Br J Anaesth* 1993; **71**: 858-863

52 **Kolkman JJ**, Mensink PBF, Huisman AB, Kuipers E, Geelkerken RH. Gastric ischemic pain with normal mesenteric vessels: A new disease entity? Report on diagnosis, treatment and outcome in 14 patients. *Gastroenterology* 2004; **126**: A254

53 **Moneta GL**, Lee RW, Yeager RA, Taylor LM Jr, Porter JM. Mesenteric duplex scanning: a blinded prospective study. *J Vasc Surg* 1993; **17**: 79-84; discussion 85-86

54 **Burkart DJ**, Johnson CD, Reading CC, Ehman RL. MR measurements of mesenteric venous flow: prospective evaluation in healthy volunteers and patients with suspected chronic mesenteric ischemia. *Radiology* 1995; **194**: 801-806

55 **Szinnai C**, Mottet C, Gutzwiller JP, Drewe J, Beglinger C, Sieber CC. Role of gender upon basal and postprandial systemic and splanchnic haemodynamics in humans. *Scand J Gastroenterol* 2001; **36**: 540-544

56 **Lycklama a Nijeholt GJ**, Burggraaf K, Wasser MN, Schultze Kool LJ, Schoemaker RC, Cohen AF, de Roos A. Variability of splanchnic blood flow measurements using MR velocity mapping under fasting and post-prandial conditions--comparison with echo-Doppler. *J Hepatol* 1997; **26**: 298-304

57 **Hoost U**, Kelbaek H, Rasmusen H, Court-Payen M, Christensen NJ, Pedersen-Bjergaard U, Lorenzen T. Haemodynamic effects of eating: the role of meal composition. *Clin Sci* (Lond) 1996; **90**: 269-276

58 **Tsukuda T**, Ito K, Koike S, Sasaki K, Shimizu A, Fujita T, Miyazaki M, Kanazawa H, Jo C, Matsunaga N. Pre- and postprandial alterations of portal venous flow: evaluation with single breath-hold three-dimensional half-Fourier fast spin-echo MR imaging and a selective inversion recovery tagging pulse. *J Magn Reson Imaging* 2005; **22**: 527-533

59 **Liem T**K, Segall JA, Wei W, Landry GJ, Taylor LM, Moneta GL. Duplex scan characteristics of bypass grafts to mesenteric arteries. *J Vasc Surg* 2007; **45**: 922-927; discussion 927-928

60 **Billaud Y**, Beuf O, Desjeux G, Valette PJ, Pilleul F. 3D contrast-enhanced MR angiography of the abdominal aorta and its distal branches: Interobserver agreement of radiologists in a routine examination. *Acad Radiol* 2005; **12**: 155-163

61 **Holland GA**, Dougherty L, Carpenter JP, Golden MA, Gilfeather M, Slossman F, Schnall MD, Axel L. Breath-hold ultrafast three-dimensional gadolinium-enhanced MR angiography of the aorta and the renal and other visceral abdominal arteries. *AJR Am J Roentgenol* 1996; **166**: 971-981

62 **Meaney JF**, Prince MR, Nostrant TT, Stanley JC. Gadolinium-enhanced MR angiography of visceral arteries in patients with suspected chronic mesenteric ischemia. *J Magn Reson Imaging* 1997; **7**: 171-176

63 **Prince MR**, Narasimham DL, Stanley JC, Chenevert TL, Williams DM, Marx MV, Cho KJ. Breath-hold gadolinium-enhanced MR angiography of the abdominal aorta and its major branches. *Radiology* 1995; **197**: 785-792

64 **Mensink PBF**, Kolkman JJ, Geelkerken RH, van Petersen AS, Rozeboom AR, Huisman AB. Comparison of magnetic resonance angiography and conventional angiography of the mesenteric arteries in patients suspected of chronic mesenteric ischemia. *Gastroenterology* 2004; **126**: A96

65 **Burkart DJ**, Johnson CD, Ehman RL. Correlation of arterial and venous blood flow in the mesenteric system based on MR findings. 1993 ARRS Executive Council Award. *AJR Am J Roentgenol* 1993; **161**: 1279-1282

66 **Applegate GR**, Thaete FL, Meyers SP, Davis PL, Talagala SL, Recht M, Wozney P, Kanal E. Blood flow in the portal vein: velocity quantitation with phase-contrast MR angiography. *Radiology* 1993; **187**: 253-256

67 **Iannaccone R**, Laghi A, Passariello R. Multislice CT angiography of mesenteric vessels. *Abdom Imaging* 2004; **29**: 146-152

68 **Horton KM**, Fishman EK. Multidetector CT angiography in the diagnosis of mesenteric ischemia. *Radiol Clin North Am* 2007; **45**: 275-288

69 **Savastano S**, Teso S, Corra S, Fantozzi O, Miotto D. Multislice CT angiography of the celiac and superior mesenteric arteries: comparison with arteriographic findings. *Radiol Med* 2002; **103**: 456-463

70 **Ilica AT**, Kocaoglu M, Bilici A, Ors F, Bukte Y, Senol A, Ucoz T, Somuncu I. Median arcuate ligament syndrome: multidetector computed tomography findings. *J Comput Assist Tomogr* 2007; **31**: 728-731

71 **Stueckle CA**, Haegele KF, Jendreck M, Zipser MC, Kirchner J, Kickuth R, Liermann D. Multislice computed tomography angiography of the abdominal arteries: comparison between computed tomography angiography and digital subtraction angiography findings in 52 cases. *Australas Radiol* 2004; **48**: 142-147

72 **Laghi A**, Catalano C, Iannaccone R, Paolantonio P, Panebianco V, Sansoni I, Trenna S, Passariello R. [Multislice spiral CT angiography in the evaluation of the anatomy of splanchnic vessels: preliminary experience] *Radiol Med* 2001; **102**: 127-131

73 **Kozuch PL**, Brandt LJ. Review article: diagnosis and management of mesenteric ischaemia with an emphasis on pharmacotherapy. *Aliment Pharmacol Ther* 2005; **21**: 201-215

74 **Habu Y**, Tahashi Y, Kiyota K, Matsumura K, Hirota M, Inokuchi H, Kawai K. Reevaluation of clinical features of ischemic colitis. Analysis of 68 consecutive cases diagnosed by early colonoscopy. *Scand J Gastroenterol* 1996; **31**: 881-886

75 **Larson MV**, Ahlquist DA, Karlstrom L, Sarr MG. Intraluminal measurement of enteric mucosal perfusion: relationship to superior mesenteric artery flow during basal and postprandial states in the dog. *Surgery* 1994; **115**: 118-126

76 **Friedland S**, Benaron D, Coogan S, Sze DY, Soetikno R. Diagnosis of chronic mesenteric ischemia by visible light spectroscopy during endoscopy. *Gastrointest Endosc* 2007; **65**: 294-300

77 **Tollefson DF**, Wright DJ, Reddy DJ, Kintanar EB. Intraoperative determination of intestinal viability by pulse oximetry. *Ann Vasc Surg* 1995; **9**: 357-360

78 **Avino AJ**, Oldenburg WA, Gloviczki P, Miller VM, Burgart LJ, Atkinson EJ. Inferior mesenteric venous sampling to detect colonic ischemia: a comparison with laser Doppler flowmetry and photoplethysmography. *J Vasc Surg* 1995; **22**: 271-277; discussion 278-279

79 **MacDonald PH**, Dinda PK, Beck IT, Mercer CD. The use of oximetry in determining intestinal blood flow. *Surg Gynecol Obstet* 1993; **176**: 451-458

80 **Vahl AC**, van Rij GL, Visser JJ, Nauta SH, Vink GQ, Scheffer GJ, de Lange-de Klerk ES, Uyterlinde A, Brom HL, Rauwerda JA. Endoluminal pulse oximetry in ischemic colon in a swine model. *J Am Coll Surg* 1995; **180**: 57-64

81 **Boley SJ**, Brandt LJ, Veith FJ, Kosches D, Sales C. A new provocative test for chronic mesenteric ischemia. *Am J Gastroenterol* 1991; **86**: 888-891

82 **Geelkerken RH**, Schultze Kool LJ, Hermans J, Zarza MT, van Bockel JH. Chronic splanchnic ischaemia: is tonometry a useful test? *Eur J Surg* 1997; **163**: 115-121

83 **Fiddian-Green RG**. Provocative test for chronic mesenteric ischemia. *Am J Gastroenterol* 1992; **87**: 543

84 **Kolkman JJ**, Groeneveld AB, Meuwissen SG. Effect of gastric feeding on intragastric P(CO2) tonometry in healthy volunteers. *J Crit Care* 1999; **14**: 34-38

85 **Mensink PB**, Geelkerken RH, Huisman AB, Kuipers EJ, Kolkman JJ. Effect of various test meals on gastric and jejunal carbon dioxide: A study in healthy subjects. *Scand J Gastroenterol* 2006; **41**: 1290-1298

86 **Mensink PB**, Geelkerken RH, Huisman AB, Kuipers EJ, Kolkman JJ. Twenty-four hour tonometry in patients suspected of chronic gastrointestinal ischemia. *Dig Dis Sci* 2008; **53**: 133-139

87 **Veenstra R**, Mensink P, Huisman A, Geelkerken B, Kolkman J. The value of jejunal exercise tonometry for the diagnosis of chronic gastrointestinal ischemia. Comparison of jejunal with gastric exercise tonometry in 100 patients suspected of GI ischemia. *Gastroenterology* 2007; **132**: A369

88 **Ockner RK**, Manning JA. Fatty acid-binding protein in small intestine. Identification, isolation, and evidence for its role in cellular fatty acid transport. *J Clin Invest* 1974; **54**: 326-338

89 **Lieberman JM**, Sacchettini J, Marks C, Marks WH. Human intestinal fatty acid binding protein: report of an assay with studies in normal volunteers and intestinal ischemia. *Surgery* 1997; **121**: 335-342

90 **Pelsers MM**, Namiot Z, Kisielewski W, Namiot A, Januszkiewicz M, Hermens WT, Glatz JF. Intestinal-type and liver-type fatty acid-binding protein in the intestine. Tissue distribution and clinical utility. *Clin Biochem* 2003; **36**: 529-535

91 **Gollin G**, Zieg PM, Cohn SM, Lieberman JM, Marks WH. Intestinal mucosal injury in critically ill surgical patients: preliminary observations. *Am Surg* 1999; **65**: 19-21

92 **Rahman SH**, Ammori BJ, Holmfield J, Larvin M, McMahon MJ. Intestinal hypoperfusion contributes to gut barrier failure in severe acute pancreatitis. *J Gastrointest Surg* 2003; **7**: 26-35; discussion 35-36

93 **Wiercinska-Drapalo A**, Jaroszewicz J, Siwak E, Pogorzelska J, Prokopowicz D. Intestinal fatty acid binding protein (I-FABP) as a possible biomarker of ileitis in patients with ulcerative colitis. *Regul Pept* 2008; **147**: 25-28

94 **Hol L**, Mensink PB, Borghuis-Koertshuis N, Geelkerken R, Huisman AB, Doelman CJ, Kusters JG, Kuipers EJ, Kolkman JJ. Transient postprandial ischemia, detected with tonometry, is associated with increased I-FABP in patients with chronic GI-ischemia ("abdominal angina"). *Gastroenterology* 2005; **128**: A656

95 **Atkins MD**, Kwolek CJ, LaMuraglia GM, Brewster DC, Chung TK, Cambria RP. Surgical revascularization versus endovascular therapy for chronic mesenteric ischemia: a comparative experience. *J Vasc Surg* 2007; **45**: 1162-1171

96 **Biebl M**, Oldenburg WA, Paz-Fumagalli R, McKinney JM, Hakaim AG. Surgical and interventional visceral revascularization for the treatment of chronic mesenteric ischemia--when to prefer which? *World J Surg* 2007; **31**: 562-568

97 **Sarac TP**, Altinel O, Kashyap V, Bena J, Lyden S, Sruvastava S, Eagleton M, Clair D. Endovascular treatment of stenotic and occluded visceral arteries for chronic mesenteric ischemia. *J Vasc Surg* 2008; **47**: 485-491

98 **van Bockel JH**, Geelkerken RH, Wasser MN. Chronic splanchnic ischaemia. *Best Pract Res Clin Gastroenterol* 2001; **15**: 99-119

99 **Moyes LH**, McCarter DH, Vass DG, Orr DJ. Intraoperative retrograde mesenteric angioplasty for acute occlusive mesenteric ischaemia: a case series. *Eur J Vasc Endovasc Surg* 2008; **36**: 203-206

100 **Wyers MC**, Powell RJ, Nolan BW, Cronenwett JL. Retrograde mesenteric stenting during laparotomy for acute occlusive mesenteric ischemia. *J Vasc Surg* 2007; **45**: 269-275

101 **Cohn I Jr**, Floyd CE, Dresden CF, Bornside GH. Strangulation obstruction in germfree animals. *Ann Surg* 1962; **156**: 692-702

102 **Longo WE**, Ballantyne GH, Gusberg RJ. Ischemic colitis: patterns and prognosis. *Dis Colon Rectum* 1992; **35**: 726-730

103 **van Petersen AS**, Vriens BHR, Huisman AB, Kolkman JJ, Geelkerken RH. A new minimally invasive treatment for the celiac artery compression syndrome: retroperitoneal endoscopic release; experience in 46 patients. submitted

104 **Hinder RA**, Fimmel CJ, Rickards E, von Ritter C, Svensson LG, Blum AL. Stimulation of gastric acid secretion increases mucosal blood flow in immediate vicinity of parietal cells in baboons. *Dig Dis Sci* 1988; **33**: 545-551

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Scutellaria barbate extract induces apoptosis of hepatoma H22 cells *via* the mitochondrial pathway involving caspase-3

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Abstract

AIM: To study the growth inhibitory and apoptotic effects of *Scutellaria barbata* D.Don (*S. barbata*) and to determine the underlying mechanism of its antitumor activity in mouse liver cancer cell line H22.

METHODS: Proliferation of H22 cells was examined by MTT assay. Cellular morphology of PC-2 cells was observed under fluorescence microscope and transmission electron microscope (EM). Mitochondrial transmembrane potential was determined under laser scanning confocal microscope (LSCM) with rhodamine 123 staining. Flow cytometry was performed to analyze the cell cycle of H22 cells with propidium iodide staining. Protein level of cytochrome C and caspase-3 was measured by semi-quantitive RT-PCR and Western blot analysis. Activity of caspase-3 enzyme was measured by spectrofluorometry.

RESULTS: MTT assay showed that extracts from *S. barbata* (ESB) could inhibit the proliferation of H22 cells in a time-dependent manner. Among the various phases of cell cycle, the percentage of cells in S phase was significantly decreased, while the percentage of cells in G_1 phase was increased. Flow cytometry assay also showed that ESB had a positive effect on apoptosis. Typical apoptotic morphologies such as condensation and fragmentation of nuclei and blebbing membrane of apoptotic cells could be observed under transmission electron microscope and fluorescence microscope. To further investige the molecular mechanism behind ESB-induced apoptosis, ESB-treated cells rapidly lost their mitochondrial transmembrane potential, released mitochondrial cytochrome C into cytosol, and induced caspase-3 activity in a dose-dependent manner.

CONCLUSION: ESB can effectively inhibit the proliferation and induce apoptosis of H22 cells involving loss of mitochondrial transmembrane potential, release of cytochrome C, and activation of caspase-3.

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Key words: *Scutellaria barbate*; Hepatoma; Apoptosis; Mitochondrial transmembrane potential; Serum pharmacology

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INTRODUCTION

Scutellaria barbata D.Don (*S. barbata*) is a perennial herb, also known as Ban-Zhi-Lian (barbat sskullcap) in traditional Chinese medicine. It is mainly distributed in southern China and has been used as an antitumor agent for lung cancer, digestive system cancer, hepatoma,

breast cancer, and chorioepithelioma as well as an anti-inflammatory agent and a diuretic in China and Korea[1-9]. Extracts from *S. barbata* (ESB) have *in vitro* growth inhibitory effects on a number of human cancers including leukemia, colon cancer, hepatoma and skin cancer[4-10]. However, its antitumor mechanism still remains unclear.

It was reported that many Chinese herbs have anticancer properties and induce apoptosis[11]. Three apoptotic pathways have been addressed, including the mitochondrial pathway[12,13], death receptor pathway[14], and endoplasmic reticulum stress-mediated apoptosis pathway[15]. The mitochondrial pathway initiates apoptosis in most physiological and pathological situations. Permeabilization outside mitochondrial membrane plays the most important role in mitochondrial apoptosis. In the mitochondria-initiated pathway, mitochondria undergoing permeability transition release apoptogenic proteins such as cytochrome C or apoptosis-inducing factor from the mitochondrial intermembrane space into the cytosol[16]. Released cytochrome C can activate caspase-9, and activated caspase-9 in turn cleaves and activates executioner caspase-3. After caspase-3 activation, some specific substrates for caspase-3 such as poly (ADP-ribose) and polymerase (PARP) are cleaved, and eventually lead to apoptosis[17].

In this study, *S. barbata* extract showed anti-tumor activity *in vitro* and could inhibit the growth of mouse H22 hepatoma cells by inhibiting cell apoptosis and cytotoxic effects, demonstrating that the extract from *S. barbata* can strongly inhibit cell proliferation and induce apoptosis of H22 cells through the mitochondrial dysfunction pathway.

MATERIALS AND METHODS

Reagents and animals

New bovine serum (Gibco, USA), RPMI-1640 medium(Gibco, USA), propidium iodide (PI) (Sigma, USA), dimethyl sulfoxide (DMSO), ribonuclease (RNase A), rhodanmin123 (Rh123), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). Mouse monoclonal antibodies against caspase-3 and cytochrome C were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Apoptotic cell Hoechst 33258 detection kit was purchased from Nanjing Kai-ji Biotechnology Development Ltd (China), and fluorescence probes Rhodamine 123 was purchased from Sigma (USA). Male SD rats weighing 220-250 g were purchased from the Experiment Animal Center, Medical School of Xi'an Jiaotong University (China).

Preparation of S. barbata extract and drug containing serum

S. barbata crude extract (ESB) was purchased from Xi'an Zhongxin Biotechnology Development Ltd (China). One kilogram of *S. barbata* was extracted three times with water as previously described[18]. Final qualification was 10:1. More specifically, stems of SB were cut into small pieces, boiled in water for 2 h, put into a filtrate, and concentrated by spray drying until the specific density reached 1.15-1.18.

"Serum pharmacology" was used to study the *in vitro* pharmacological activity of herb medicine as previously described[19]. ESB-containing serum was prepared as previously described[18,20]. Twenty male SD rats were randomly divided into control group, high ESB dose group, medium ESB dose group, and low ESB dose group (n = 5). Rats in the high, medium and low ESB dose groups received intragastric ESB of 6, 3 and 1.5 g/d per kg of body weight. Rats in the control group received normal saline, twice a day for 3 d. Two hours after the last administration, blood was immediately obtained from the heart and kept at room temperature for 4 h. The serum was separated by centrifugation at 2400 r/min for 10 min, collected following twice of filtration with a 0.22 μm cellulose acetate membrane, calefied in 56℃ water for 30 min, and stored at -20℃ for use.

Cell lines and culture

Mouse H22 hepatoma cells, purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1×10^5 U/L penicillin and 100 mg/L streptomycin in an incubator containing a humidified atmosphere with 50 mL CO_2 at 37℃. The cells were subcultured until reaching logarithmic growth phase. The viability of H22 cells, stained with trypan blue, was above 97%.

Cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Sigma, USA). H22 cells were seeded at a concentration of 5×10^3 cells/well in a 96-well plate, and grown at 37℃ until adherence. At end of the treatment, 50 μg/10 μL of MTT was added and the cells were incubated for another 4 h. Two hundred μL of DMSO was added to each well after the supernatant was removed. After the plate was shaken for 10 min, cell viability was detected by measuring the absorbance at 490 nm wavelength using an enzyme-labeling instrument (EX-800 type) in quintuplicate.

Cell viability (%) = the absorbance of experimental group/the absorbance of blank control group × 100%.

Detection of morphological apoptosis

Staining of cells with uranyl acetate and lead citrate was performed to detect morphological changes. Briefly, adherent H22 cells were treated with ESB at a high dose for 48 h. The treated cells were digested with pancreatin and fixed in 3% glutaraldehyde precooled at 4℃ for 2 h. To make ultra-thin sections of copper, cells were washed with PBS, fixed in 1% osmic acid for an additional hour, dehydrated in acetone and embedded in epoxide resin. After stained with uranyl acetate and lead citrate, the

sections were examined under a Hitachi-800 transmission electron microscope as previously described[21].

Nuclear staining

H22 cells were harvested by centrifugation, washed with PBS and fixed in 1% glutaraldehyde for 1 h at room temperature. The fixed cells were washed with PBS, stained with 200 μmol/L Hoechst 33258 for 10 min. Changes in nuclei after stained with Hoechst 33258 were observed under a fluorescence microscope (Olympus, BX-60, Japan).

Cell cycle analysis

H22 cells were incubated at 5×10^5 cells/well in 6-well plates, treated with a homologous drug for 48 h. The detached and attached cells were harvested and fixed in 70% ice-cold ethanol at -20℃ overnight. After fixation, cells were washed with PBS, resuspended in 1 mL PBS containing 1 mg/mL RNase (Sigma) and 50 μg/mL PI (Sigma), and incubated at 37℃ for 30 min in the dark. Samples of 10 000 cells were then analyzed for DNA content by FACScan flow cytometry (Beckman, USA), and cell cycle phase distributions were analyzed with the CellQuest acquisition software(BD Biosciences).

Detection of mitochondrial membrane potential

Mitochondria transmembrane potential ($\Delta\psi m$) was detected under laser scanning confocal microscope(LSCM) with Rhodamine 123 (Rh123) staining as previously described[22]. About 1×10^6 cells were harvested by trypsinization, washed twice with PBS, and incubated with Rh123 at the final concentration of 1 μL/mL for 20 min at 37℃ in the dark, centrifuged at 1000 r/min for 5 min, washed twice with a medium, resuspended in the medium, cultured at 37℃ in an incubator containing 50 mL CO_2 for 60 min. Fluorescence intensity was determined at an excitation wavelength of 488 nm, emission wavelength of 530 nm under a laser scanning confocal microscope (Olympus, FluoView™ FV300, Japan). The fluorescence intensity of Rhodamine 123 in cells represents the mitochondrial membrane potential[23].

Western blot analysis

H22 cells (2.5×10^7) were collected by centrifugation at 2000 r/min for 10 min at 4℃, washed twice with cold PBS (pH 7.2), centrifuged at 2000 r/min for 10 min. Protein content was determined using a Bio-Rad protein assay reagent with bovine serum albumin as the standard. Total proteins (30 μg/lane) were separated by 15% SDS-PAGE gel electrophoresis, and transferred to a 0.45 μm PVDF membrane (Amersham Pharmacia Biotech). The blots were incubated with the desired primary antibody overnight at the following dilutions: caspase 3 (1:1000), cytochrome C (1:1500), and β-actin (1:1500). Primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Subsequently, the membrane was incubated with appropriate secondary antibodies for 1 h at room temperature. The immunoblots were analyzed by densitometry on a GelDoc 2000 system (Bio-Rad Laboratories Inc. USA) as previously described[17,24].

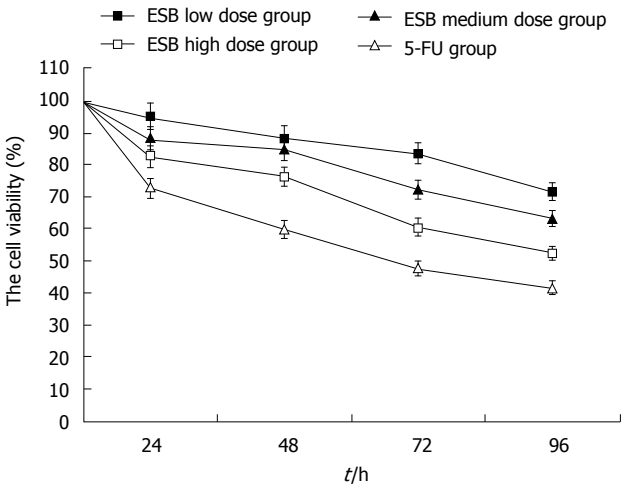

Figure 1 Inhibition of H22 cell proliferation by ESB. H22 cells were treated with different doses of ESB. The number of cells was determined at 0, 24, 48, 72, and 96 h, respectively. The viability of cells was detected by MTT assay. ANOVA analysis showed that the growth of H22 cells was inhibited by ESB in a dose- and time- dependent manner ($P < 0.05$).

Assay for caspase-3 activity

Caspase-3 activity was assayed using the caspase-3 activity assay kit (Nanjing Kai-ji, China) as previously described[25,26]. In brief, standard curve was plotted by detecting the absorbance of standard samples with terminal concentrations at each well, respectively. After 1 h incubation at room temperature, H22 cells were collected and lysed completely in a caspase assay buffer. The activity of caspase-3 was assayed in triplicate using a plate-reading luminometer (Turner Designs, Sunnyvale, CA) at the wavelength of 405 nm. Nkat was used to represent the activity measured[25].

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed with analysis of variance (ANOVA) using the statistical software SPSS 11.0. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of ESB on proliferation of H22 cells

H22 cells were treated with different doses of ESB. The growth rate of H22 cells was evaluated after 0, 24, 48, 72, and 96 h, respectively. The cell viability of H22 cells in different ESB treatment groups was significantly higher than that in 5-FU treatment group (Figure 1). High and medium dose ESB inhibited the proliferation of H22 cells ($P < 0.05$), while low dose ESB could not obviously inhibit the proliferation of H22 cells ($P > 0.05$). MTT assay showed that high and medium dose ESB inhibited the proliferation of H22 cells *in vitro* in a time-dependent manner.

Morphological observation of apoptosis of H22 cells induced by ESB

High resolution transmission electron microscopy showed that normal H22 cells were round and regular in shape

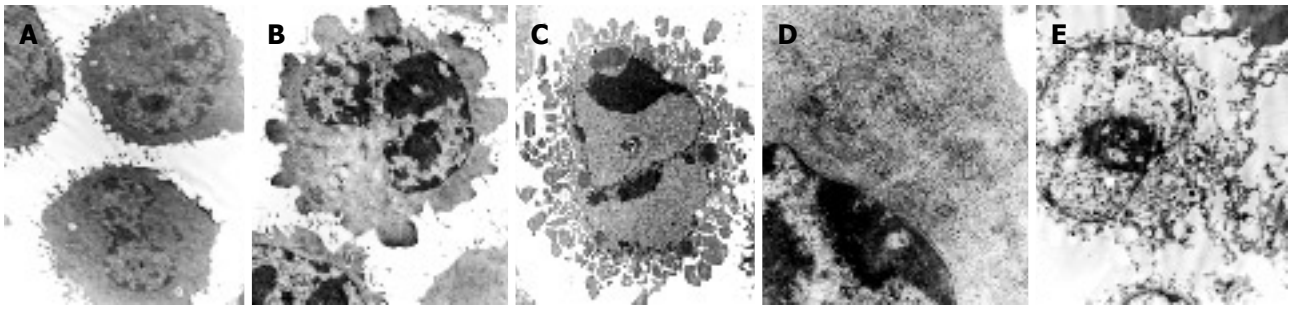

Figure 2 Morphological observation of H22 cells by EM after treatment. A: normal hepatoma H22 cells (5000 ×); B: karyopyknosis and chromatic agglutination in high ESB dose group (5000 ×); C: apoptotic body in high ESB dose group (5000 ×); D: chondriosome swelling in high ESB dose group (6000 ×); E: cellular swelling and necrosis in 5-FU group (5000 ×).

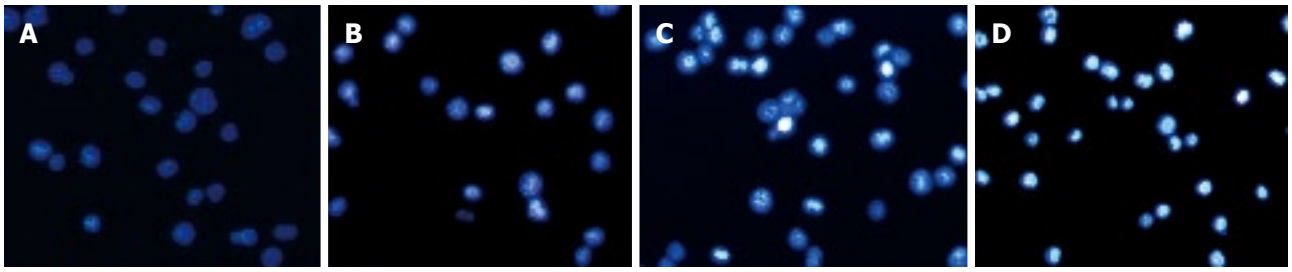

Figure 3 Cell apoptosis observed with Hoechst 33258 staining under a fluorescence microscope (× 200). After cells were treated with different doses of ESB for 48 h, Hoechst 33258 staining was used to observe apoptotic cells as described in MATERIALS AND METHODS. The number of apoptotic cells gradually increased in a dose-dependent manner with marked morphological changes found in cell apoptosis including condensation of chromatin and nuclear fragmentation. A: Control group; B: Low dose treated group; C: Medium dose treated group; D: High dose treated group.

with chromatin margination in few tumor cells (Figure 2A). After treatment with a high ESB dose for 48 h, a part of nuclear membrane domed outward with a sharp angle. The typical morphologies of apoptotic H22 cells such as chromatic agglutination and fragmentation of nuclei, chondriosome swelling, formation of apoptotic body, could be observed in high ESB dose group (Figure 2B-D), while in 5-FU group, cellular swelling and necrosis could be observed in many fields of vision.

Detection of apoptosis of H22 cells by Hoechst 33258 staining

After treatment with different doses of ESB for 48 h, H22 cells were stained with Hoechst 33258 and observed under a fluorescence microscope. The condensely stained chromatin of apoptotic cells was more bright than that of normal cells. The characteristics of apoptosis, such as nuclear shrinkage, DNA condensation and fragmentation, were found in ESB treatment group (Figure 3B-D), while no apoptosis occurred in blank control group (Figure 3A). The percentage of apoptotic cells in control group and low and high ESB dose groups was 3.36% ± 2.14%, 14.57% ± 4.28%, 43.15% ± 5.33%, 72.65% ± 6.52%, respectively. Furthermore, the number of apoptotic cells gradually increased in a dose-dependent manner.

Effect of ESB on cell cycle distribution by flow cytometry

The effects of ESB on cell cycles were analyzed by flow cytometry. The percentage of cells was significantly decreased at S phase and increased at G_1 phase in high ESB dose group.

Table 1 Effect of ESB on cell cycle and apoptosis of H22 cells by flow cytometry (mean ± SD)

Groups	*n*	G_0/G_1	S	G_2/ M	Apoptosis rate
Control	5	37.63 ± 2.12	32.73 ± 2.24	21.75 ± 1.52	0.51 ± 0.12
ESB low dose	5	39.35 ± 2.25	33.56 ± 3.12	21.20 ± 1.27	1.07 ± 0.15
ESB medium dose	5	45.91 ± 2.56[a]	30.65 ± 2.64	17.15 ± 1.34[a]	3.15 ± 0.27[a]
ESB high dose	5	56.05 ± 2.37[b]	21.33 ± 3.42[b]	12.30 ± 1.25[b]	7.83 ± 0.43[b]

Cell cycle distributions in control and ESB-treated cells were determined by PI staining and flow cytometric analysis. Results presented were representative of three independent experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

The sub-G1 population indicated apoptotic-associated chromatin degradation. The ratio of cell apoptosis in blank control group, and low, medium, high ESB dose groups was 0.51%, 1.07%, 3.15%, 7.83%, respectively. There was significantly difference between the 4 groups ($P < 0.05$). These results suggest that high ESB dose can induce cell cycle arrest at G_0/G_1 phase and apoptosis in H22 cells (Table 1).

Effect of ESB on mitochondrial membrane potential

Mitochondria play an essential role in apoptosis. To assess whether ESB affects the function of mitochondria, mitochondrial membrane potential was detected under a laser scanning confocal microscope with Rh123 staining. The fluorescence intensity of Rhodamine123 in H22 cells of blank control group was the strongest (Figure 4).

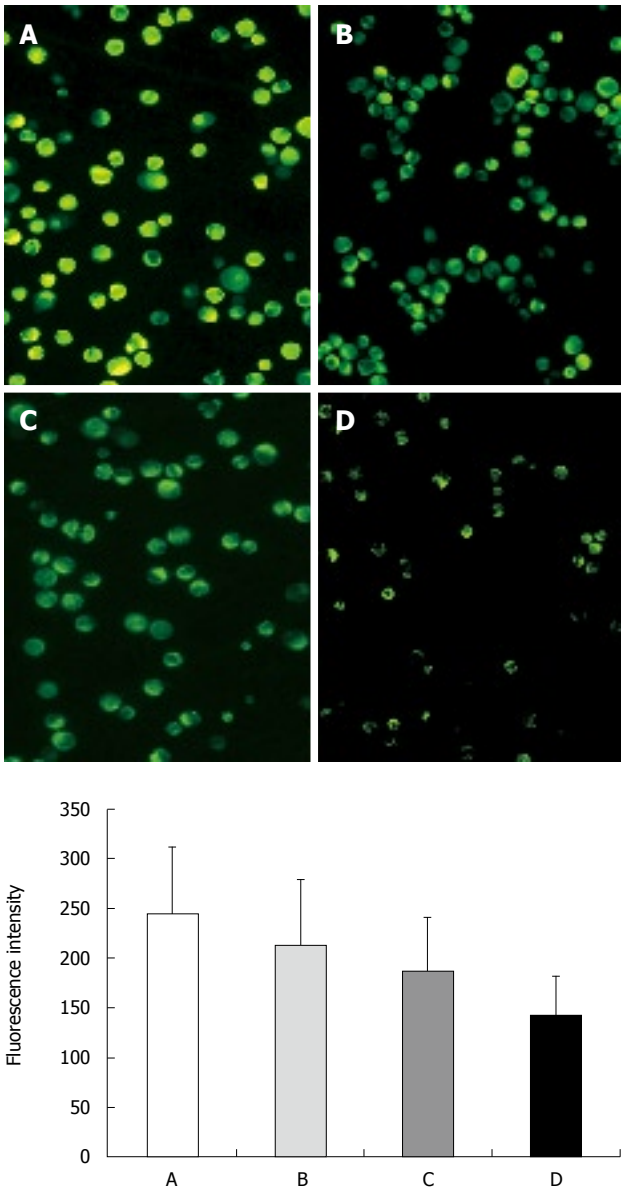

Figure 4 Effect of ESB on Δψm measured with laser scanning confocal microscope by staining with Rhodamine 123 (200 ×). A: Control group; B: Low ESB dose group; C: Medium ESB dose group; D: High ESB dose group. Fluorescence intensity (FI) indicates membrane potential of mitochondria in the cells. FI was decreased in a dose-dependent manner ($P < 0.05$, ANOVA analysis).

After treatment with different doses of ESB for 48 h, ANOVA analysis showed that the fluorescence intensity was decreased in a dose-dependent manner ($P < 0.05$).

Caspase-3 activity in ESB-induced apoptosis of H22 cells

Caspase-3, acting on downstream of the mitochondrial signaling pathway, is a major mediator of apoptosis. Dysfunction of mitochondria provoked us to detect the changes of caspase-3 activity in H22 cells following ESB treatment. The expression intensities of caspase-3 protein in the control and low-high ESB dose groups were 0.21 ± 0.02, 0.33 ± 0.04, 0.59 ± 0.03, and 0.85 ± 0.05, respectively (Figure 5). Western blot analysis revealed that there was a gradual increase in caspase-3 protein in low-high ESB dose groups ($P < 0.05$), indicating that

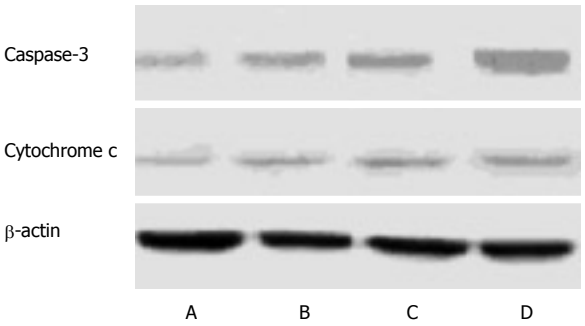

Figure 5 Protein level of caspase-3 and cytochrome C in H22 cells. A: Control group; B: Low ESB dose group; C: Medium ESB dose group; D: High ESB dose group. After treatment with different doses of ESB for 48 h, cellular proteins were detected by Western blot analysis.

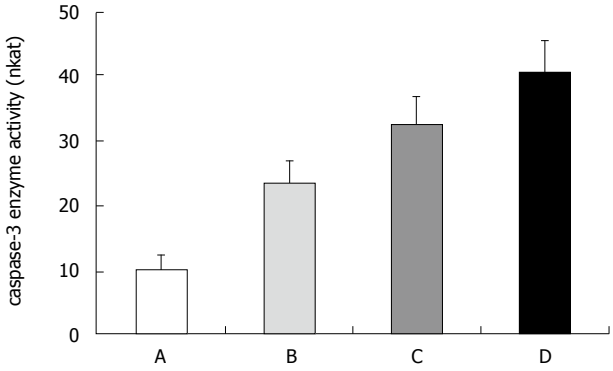

Figure 6 Effect of ESB on caspase-3 enzyme activity in H22 cells. A: Control group; B: Low ESB dose group; C: Medium ESB dose group; D: High ESB dose group. There was a dose dependent increase in activity of caspase-3 enzyme in ESB treated cells ($P < 0.05$). This assay was done triplicate, independently. Nkat was used to represent activity measured.

caspase-3 can be activated by ESB.

Caspase-3 activities were detected after treatment with different doses of ESB for 48 h, showing that caspase-3 activity was induced by ESB in a dose-dependent manner (Figure 6).

Release of cytochrome C from mitochondria in ESB-induced apoptosis

Cytochrome C release from mitochondria into cytosol is a critical step in the apoptotic cascade. The reduction of mitochondrial membrane potential may facilitate the release of cytochrome C, which will then activate the apoptotic pathway to trigger cell death. The protein level of cytochrome C in cytosol was measured in H22 cells treated with different doses of ESB by Western blot analysis with mouse monoclonal cytochrome C antibodies. As shown in Figure 5, the amount of cytosolic cytochrome C in the cytosolic fraction after ESB treatment was significantly increased in a dose-dependent manner ($P < 0.05$).

DISCUSSION

S. barbata, which has been traditionally used in treatment of inflammation, hepatitis, tumor and gynecological

diseases in China and Korea[1-9]. Studies have shown that *S. barbata* contains a large number of alkaloids and flavones, alkaloid, sterides, and polysaccharides[27,28]. However, the active site of chemical structure for antitumor activity has not been fully determined[29]. Recent studies indicate that *S. barbata* extract (ESB) is effective against hepatoma, lung and digestive system cancers, *et al*[4-10], and can be used in combination with other traditional Chinese medicines in treatment of other tumors.

In pharmacology study, crude Chinese drugs or their compounds are often added directly into the culture system of cells or organs *in vitro*[30]. However,, experimental results *in vitro* are often different from those *in vivo*. Serum pharmacology has been extensively used to study the effects and mechanisms of Chinese drugs *in vitro*[30]. It is believed that serum pharmacology is more scientific and beter for Chinese drugs than traditional pharmacology in which crude drugs are directly added into the culture system of cells or organs *in vitro*[19-20,30,31]. In this study, we investigated the effects of ESB on inducing apoptosis of H22 cells with serum pharmacology.

H22 cells were treated with different doses of ESB containing serum, and the growth rate of H22 cells was evaluated by MTT assay after 0, 24, 48, 72, and 96 h, respectively. High and medium ESB dose inhibited the proliferation of H22 cells, while low ESB dose could not obviously inhibit the proliferation of H22 cells. MTT assay showed high and medium ESB dose inhibited the proliferation of H22 cells *in vitro* in a time-dependent manner, which may provide useful information for development of anti-tumor drugs.

Morphological changes of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies[32]. These morphological changes were also observed in our study under transmission electron microscopy and fluorescence microscope after treatment with a high ESB dose for 48 h. Typical morphologies of apoptotic H22 cells, such as chromatic agglutination and fragmentation of nuclei, chondriosome swelling, formation of apoptotic body, were observed in ESB high ESB dose group (Figure 2B-D), but no apoptosis occurred in blank control group. Furthermore, fluorescence microscopy showed that the number of apoptotic cells gradually increased in a dose-dependent manner.

Blocking of cell cycle is one of the mechanisms of ESB by which the growth and proliferation of tumor cells are inhibited[33]. Flow cytometry showed that cell apoptosis was significantly decreased at S-phase, increased at G_1-phase, and reached its peak at subG_1-phase. The blocking of cell cycle may be one of the mechanisms of ESB by which the growth of H22 cells is inhibited and cell apoptosis is induced.

Mitochondria play a critical role in apoptosis induced by chemotherapeutic agents[12-14]. Many agents can induce, directly or indirectly, apoptosis by insult to the mitochondria[34,35]. Apoptosis could cause loss of $\Delta\psi m$ and release of cytochrome C into cytosol, and induce caspase-9-dependent activation of caspase-3[13]. In this study, the effect of ESB on $\Delta\psi m$ was examined using Rhodamine 123, a mitochondrial potential probe, showing that H22 cells lost $\Delta\psi m$ following ESB treatment. Forty-eight hours after ESB treatment, the cells exhibited significant alterations in $\Delta\psi m$, and the fluorescence intensity of disruption of $\Delta\psi m$ gradually decreased in a dose-dependent manner

One of the major apoptotic pathways is activated by the release of cytochrome C from mitochondria into cytosol[36], which is the hallmark of cells undergoing apoptosis. In this study, Western blotting analysis was performed to measure the protein level of cytochrome C in H22 cells after treatment with different doses of ESB. The amount of cytosolic cytochrome C in the cytosolic fraction after ESB treatment was increased in a dose-dependent manner (Figure 6).

Caspases are cystein proteases that play a key role in the execution phase of apoptosis[37]. Caspase-3, a member of the family of caspases, extensively studied as "the executor of apoptosis", plays a crucial role in cell death[38]. Apoptosis mediated by caspase-3 occurs in many cancer cells. In this study, Western blot analysis revealed that caspase-3 protein was gradually increased in the low-high ESB dose groups. At the same time, caspase-3 enzyme activity was increased in a dose-dependent manner. These results indicate that caspase-3 can be activated by ESB. ESB treatment resulted in loss of mitochondrial membrane potential, release of cytochrome C and caspase-3, demonstrating that ESB induces apoptosis and mitochondria are involved in apoptosis mediated by ESB.

In conclusion, ESB has antiproliferative activities against H22 cells by inducing apoptosis involving loss of $\Delta\psi m$, release of cytochrome C, and activation of caspase-3.

COMMENTS

Background

Medicinal plants have been used as traditional remedies for hundreds of years. Among them, *Scutellaria barbata D.Don* (*S. barbata*) has been traditionally used in treatment of hepatitis, inflammation, osteomyelitis and gynecological diseases in China. Studies indicate that extracts from *S. barbata* have growth inhibitory effects on a number of human cancers. Reports are available on the treatment of lung, breast and digestive system cancer, hepatoma, and chorioepithelioma with *S. barbata* extracts. However, the underlying mechanism of the antitumor activity of *S. barbata* extracts remains unclear.

Research frontiers

Studies have confirmed that many Chinese herbs have antitumor properties and induce apoptosis. In the process of signal transduction of cell apoptosis induced by drugs, mitochondria play a great role in promoting apoptosis signal and releasing caspase. Permeabilization of the outside mitochondrial membrane plays the most important role in mitochondrial apoptosis, during which loss of $\Delta\psi m$ and release of cytochrome C into cytosol, and caspase-9- dependent activation of caspase-3 occur sequentially.

Innovations and breakthroughs

There is no evidence that the mitochondrial pathway is involved in apoptosis induced by S. barbata. The present study was undertaken by culturing mouse liver cancer H22 cells treated with serum containing different concentrations of ESB. ESB containing serum induced apoptosis of H22 cells, and apoptosis was

involved in loss of mitochondrial transmembrane potential, release of cytochrome C, and activation of caspase-3.

Applications

This experimental study on the mechanism of the antitumor activity of S. barbata, may offer new evidence for S. barbata in the treatment of hepatoma in clinical practice.

Terminology

ESB is an extract from *Scutellaria barbata*; Δψm indicates mitochondrial transmembrane potential;1 nanokatal defined as the amount of enzyme required to increase the rate of reaction by 1 nmol/s under defined assay conditions.

Peer review

This study examined the anti-tumour effects of Scutellaria barbate. The authors used serum containing extract from *S. Barbata* (ESB) to determine its effect on proliferation of H22 hepatoma cells *in vitro*. ESB inhibited cell proliferation by inducing cell cycle arrest at Go/G1 phase and by increasing apoptosis with a reduction in mitochondrial membrane potential, release of cytochrome C and capase-3 activation. This work is novel and improves our understanding of the mechanisms of action of ESB.

REFERENCES

1 **Lee TK**, Lee DK, Kim DI, Lee YC, Chang YC, Kim CH. Inhibitory effects of Scutellaria barbata D. Don on human uterine leiomyomal smooth muscle cell proliferation through cell cycle analysis. *Int Immunopharmacol* 2004; **4**: 447-454

2 **Lin CC**, Shieh DE. The anti-inflammatory activity of Scutellaria rivularis extracts and its active components, baicalin, baicalein and wogonin. *Am J Chin Med* 1996; **24**: 31-36

3 **Lee TK**, Kim DI, Song YL, Lee YC, Kim HM, Kim CH. Differential inhibition of Scutellaria barbata D. Don (Lamiaceae) on HCG-promoted proliferation of cultured uterine leiomyomal and myometrial smooth muscle cells. *Immunopharmacol Immunotoxicol* 2004; **26**: 329-342

4 **Goh D**, Lee YH, Ong ES. Inhibitory effects of a chemically standardized extract from Scutellaria barbata in human colon cancer cell lines, LoVo. *J Agric Food Chem* 2005; **53**: 8197-8204

5 **Yin X**, Zhou J, Jie C, Xing D, Zhang Y. Anticancer activity and mechanism of Scutellaria barbata extract on human lung cancer cell line A549. *Life Sci* 2004; **75**: 2233-2244

6 **Cha YY**, Lee EO, Lee HJ, Park YD, Ko SG, Kim DH, Kim HM, Kang IC, Kim SH. Methylene chloride fraction of Scutellaria barbata induces apoptosis in human U937 leukemia cells via the mitochondrial signaling pathway. *Clin Chim Acta* 2004; **348**: 41-48

7 **Suh SJ**, Yoon JW, Lee TK, Jin UH, Kim SL, Kim MS, Kwon DY, Lee YC, Kim CH. Chemoprevention of Scutellaria bardata on human cancer cells and tumorigenesis in skin cancer. *Phytother Res* 2007; **21**: 135-141

8 **Dai ZJ**, Liu XX, Xue Q, Ji ZZ, Wang XJ, Kang HF, Guan HT, Ma XB, Ren HT. [Anti-proliferative and apoptosis-inducing activity of Scutellaria barbate containing serum on mouse's hepatoma H22 cells] *Zhongyaocai* 2008; **31**: 550-553

9 **Lin JM**, Liu Y, Luo RC. [Inhibition activity of Scutellariae barbata extracts against human hepatocellular carcinoma cells] *Nanfang Yikedadue Xuebao* 2006; **26**: 591-593

10 **Lee TK**, Cho HL, Kim DI, Lee YC, Kim CH. Scutellaria barbata D. Don induces c-fos gene expression in human uterine leiomyomal cells by activating beta2-adrenergic receptors. *Int J Gynecol Cancer* 2004; **14**: 526-531

11 **Yu ZH**, Wei PK, Xu L, Qin ZF, Shi J. Anticancer effect of jinlongshe granules on in situ-transplanted human MKN-45 gastric cancer in nude mice and xenografted sarcoma 180 in Kunming mice and its mechanism. *World J Gastroenterol* 2006; **12**: 2890-2894

12 **Mohamad N**, Gutierrez A, Nunez M, Cocca C, Martin G, Cricco G, Medina V, Rivera E, Bergoc R. Mitochondrial apoptotic pathways. *Biocell* 2005; **29**: 149-161

13 **Delivani P**, Martin SJ. Mitochondrial membrane remodeling in apoptosis: an inside story. *Cell Death Differ* 2006; **13**: 2007-2010

14 **Gupta S**. Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). *Int J Oncol* 2003; **22**: 15-20

15 **Bakhshi J**, Weinstein L, Poksay KS, Nishinaga B, Bredesen DE, Rao RV. Coupling endoplasmic reticulum stress to the cell death program in mouse melanoma cells: effect of curcumin. *Apoptosis* 2008; **13**: 904-914

16 **Hoye AT**, Davoren JE, Wipf P, Fink MP, Kagan VE. Targeting mitochondria. *Acc Chem Res* 2008; **41**: 87-97

17 **Li H**, Wang LJ, Qiu GF, Yu JQ, Liang SC, Hu XM. Apoptosis of Hela cells induced by extract from Cremanthodium humile. *Food Chem Toxicol* 2007; **45**: 2040-2046

18 **Zhang YH**, Liu JT, Wen BY, Xiao XH. In vitro inhibition of proliferation of vascular smooth muscle cells by serum of rats treated with Dahuang Zhechong pill. *J Ethnopharmacol* 2007; **112**: 375-379

19 **Miura D**, Miura Y, Yagasaki K. Effect of apple polyphenol extract on hepatoma proliferation and invasion in culture and on tumor growth, metastasis, and abnormal lipoprotein profiles in hepatoma-bearing rats. *Biosci Biotechnol Biochem* 2007; **71**: 2743-2750

20 **Nishida S**, Satoh H. Mechanisms for the vasodilations induced by Ginkgo biloba extract and its main constituent, bilobalide, in rat aorta. *Life Sci* 2003; **72**: 2659-2667

21 **Ma G**, Yang C, Qu Y, Wei H, Zhang T, Zhang N. The flavonoid component isorhamnetin in vitro inhibits proliferation and induces apoptosis in Eca-109 cells. *Chem Biol Interact* 2007; **167**: 153-160

22 **Zhao JX**, Guo FL, Bai DC, Wang XX. [Effects of fuzheng yiliu granules on apoptotic rate and mitochondrial membrane potential of hepatocellular carcinoma cell line H22 from mice] *Zhongxiyi Jiehe Xuebao* 2006; **4**: 271-274

23 **Blattner JR**, He L, Lemasters JJ. Screening assays for the mitochondrial permeability transition using a fluorescence multiwell plate reader. *Anal Biochem* 2001; **295**: 220-226

24 **Chen CJ**, Hsu MH, Huang LJ, Yamori T, Chung JG, Lee FY, Teng CM, Kuo SC. Anticancer mechanisms of YC-1 in human lung cancer cell line, NCI-H226. *Biochem Pharmacol* 2008; **75**: 360-368

25 **Feeney B**, Pop C, Swartz P, Mattos C, Clark AC. Role of loop bundle hydrogen bonds in the maturation and activity of (Pro)caspase-3. *Biochemistry* 2006; **45**: 13249-13263

26 **Peng B**, Chang Q, Wang L, Hu Q, Wang Y, Tang J, Liu X. Suppression of human ovarian SKOV-3 cancer cell growth by Duchesnea phenolic fraction is associated with cell cycle arrest and apoptosis. *Gynecol Oncol* 2008; **108**: 173-181

27 **Wang WS**, Zhou YW, Ye YH, Du N. [Studies on the flavonoids in herb from Scutellaria barbata] *Zhongguo Zhongyao Zazhi* 2004; **29**: 957-959

28 **Dai SJ**, Sun JY, Ren Y, Liu K, Shen L. Bioactive ent-clerodane diterpenoids from Scutellaria barbata. *Planta Med* 2007; **73**: 1217-1220

29 **Yu J**, Lei J, Yu H, Cai X, Zou G. Chemical composition and antimicrobial activity of the essential oil of Scutellaria barbata. *Phytochemistry* 2004; **65**: 881-884

30 **Bochu W**, Liancai Z, Qi C. Primary study on the application of Serum Pharmacology in Chinese traditional medicine. *Colloids Surf B Biointerfaces* 2005; **43**: 194-197

31 **Er HM**, Cheng EH, Radhakrishnan AK. Anti-proliferative and mutagenic activities of aqueous and methanol extracts of leaves from Pereskia bleo (Kunth) DC (Cactaceae). *J Ethnopharmacol* 2007; **113**: 448-456

32 **Rello S**, Stockert JC, Moreno V, Gamez A, Pacheco M, Juarranz A, Canete M, Villanueva A. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis* 2005; **10**: 201-208

33 **Sigounas G**, Hooker J, Anagnostou A, Steiner M. S-allylmercaptocysteine inhibits cell proliferation and reduces the viability of erythroleukemia, breast, and prostate cancer

cell lines. *Nutr Cancer* 1997; **27**: 186-191
34 **Fulda S**, Susin SA, Kroemer G, Debatin KM. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. *Cancer Res* 1998; **58**: 4453-4460
35 **Kim KC**, Kim JS, Son JK, Kim IG. Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells by the Ganoderma lucidum and Duchesnea chrysantha extracts. *Cancer Lett* 2007; **246**: 210-217
36 **Lalier L**, Cartron PF, Juin P, Nedelkina S, Manon S, Bechinger B, Vallette FM. Bax activation and mitochondrial insertion during apoptosis. *Apoptosis* 2007; **12**: 887-896
37 **Chen YC**, Shen SC, Lee WR, Hsu FL, Lin HY, Ko CH, Tseng SW. Emodin induces apoptosis in human promyeloleukemic HL-60 cells accompanied by activation of caspase 3 cascade but independent of reactive oxygen species production. *Biochem Pharmacol* 2002; **64**: 1713-1724
38 **Park SY**, Cho SJ, Kwon HC, Lee KR, Rhee DK, Pyo S. Caspase-independent cell death by allicin in human epithelial carcinoma cells: involvement of PKA. *Cancer Lett* 2005; **224**: 123-132

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MLH1 promoter germline-methylation in selected probands of Chinese hereditary non-polyposis colorectal cancer families

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Abstract

AIM: To detect the *MLH1* gene promoter germline-methylation in probands of Chinese hereditary non-polyposis colorectal cancer (HNPCC), and to evaluate the role of methylation in *MLH1* gene promoter and molecular genetics in screening for HNPCC.

METHODS: The promoter germline methylation of *MLH1* gene was detected by methylation-specific PCR (MSP) in 18 probands from unrelated HNPCC families with high microsatellite-instability (MSI-H) phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. At the same time, 6 kindreds were collected with microsatellite-stability (MSS) phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes as controls. The results of MSP were confirmed by clone sequencing. To ensure the reliability of the results, family H65 with nonsense germline mutation at c.2228C > A in *MSH2* gene was used as the negative control and the cell line sw48 was used as the known positive control along with water as the blank control. Immunochemical staining of *MLH1* protein was performed with Envision two-step method in those patients with aberrant methylation to judge whether the status of *MLH1* gene methylation affects the expression of MLH1 protein.

RESULTS: Five probands with *MLH1* gene promoter methylation were detected in 18 Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. Two of the five probands from families H10 and H29 displayed exhaustive-methylation, fulfilling the Japanese criteria (JC) and the Amsterdam criteria (AC), respectively. The other 3 probands presented part-methylation fulfilling the AC. Of the 13 probands with unmethylation phenotype, 8 fulfilled the JC and the Bethesda guidelines (BG), 5 fulfilled the AC. The rate of aberrant methylation in *MLH1* gene in the AC group (22.2%, 4/18) was higher than that in the JC/BG groups (5.6%, 1/18) in all HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. However, no proband with methylation in *MLH1* gene was found in the families with MSS phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. No expression of MLH1 protein was found in tumor tissues from two patients with exhaustive-methylation phenotype, whereas positive expression of MLH1 protein was observed in tumor tissues from patients with partial methylation phenotype (excluding family H42 without tumor tissue), indicating that exhaustive-methylation of *MLH1* gene can cause defective expression of MLH1 protein.

CONCLUSION: Methylation phenotype of *MLH1* gene is correlated with microsatellite phenotype of *MMR* genes, especially with MSI-H. Exhaustive-methylation of *MLH1* gene can silence the expression of MLH1 protein. *MLH1* promoter methylation analysis is a promising tool for molecular genetics screening for HNPCC.

Key words: Hereditary non-polyposis colorectal cancer; *MLH1*; Methylation; Germline; Methylation-specific PCR; Microsatellite phenotype

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INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is characterized by an autosomal dominant inheritance of early-onset microsatellite instability (MSI)-positive colorectal cancer and an increased risk of other cancers, including cancers of the endometrium, stomach, ovary, urinary tract, pancreas, and small bowel. HNPCC accounts for 5%-10% of all colorectal cancers and is caused by a mutation in one of the following DNA mismatch repair (MMR) genes: *MLH1, MSH2, MSH6, PMS1* and *PMS2*[1-3]. Germline mutations in *MLH1* and *MSH2* account for > 90% of all known MMR mutations in HNPCC[4], and germline mutations in *MSH6* account for 5%-10%, whereas mutations in other genes are rare[3,5]. MSI has been observed in approximately 13% of sporadic colorectal cancers (CRC) and in virtually all CRC arising in patients with HNPCC. Germline mutations in *MMR* genes, high-frequency microsatellite instability (MSI-H) and loss of MMR protein expression are the hallmarks of HNPCC. Epigenetic silencing is usually considered a kind of somatic phenomenon and somatic *MLH1* promoter hypermethylation is generally accepted in the tumorigenesis of sporadic tumours. However, little is known about the maintenance of epigenetic state in the germline[6] and abnormal *MLH1* gene promoter methylation in normal body cells is controversially discussed as a mechanism predisposing patients to HNPCC. Recently, aberrant methylation in MMR genes, *MLH1* or *MSH2*, has been supposed as a basic factor for cancer[7]. Promoter hypermethylation in *MLH1* gene of colorectal tumors correlates well with loss of MLH1 protein in sporadic MSI-positive cases[8,9]. This study was to investigate the *MLH1* gene germline epimutation by methylation-specific PCR (MSP) in 18 Chinese HNPCC kindreds with MSI-H but without germline mutations in *MSH2, MLH1,* or *MSH* gene, in order to identify HNPCC families and provide experimental information for HNPCC database.

MATERIALS AND METHODS

Materials

From January 1998 to October 2005, 24 Chinese HNPCC families fulfilling different clinical criteria were registered at the Department of Abdominal Surgery in Shanghai Cancer Hospital/Institution. Germline mutations in *MLH1*, *MSH2* and *MSH6* genes were excluded by DNA-PCR-based sequencing in the probands of all Chinese HNPCC families[10-12]. Of them, 18 unrelated HNPCC probands were selected for the study objects with the phenotype of MSI-H, and the remaining 6 were for the control group with the phenotype of microsatellite stability (MSS). Each participant was asked to give 10 microliters of peripheral blood and consented for access to archival tumor tissue. The characteristics of the selected cases are listed in Tables 1 and 2. To ensure the reliability of the results, family H65 with nonsense germline mutation at c.2228C > A in *MSH2* gene was used as the negative control and the cell line sw48 was used as the known positive control for the methylation in *MLH1* gene as well as water as the blank control. This study was proved by the Medical Ethical Committee of Cancer Hospital, Fudan University. The procedures of the study were in accordance with the international rules and regulations.

Table 1 Characteristics of 18 probands with MSI-H

Case	Gender	Age (yr)	Criteria	MSI	*MLH1/MSH2/MSH6* mutation study
H21	M	38	AC	MSI-H	NM
H22	M	46	AC	MSI-H	NM
H28	F	30	AC	MSI-H	NM
H29	F	37	AC	MSI-H	NM
H32	M	51	AC	MSI-H	NM
H42	M	65	AC	MSI-H	NM
H46	M	48	AC	MSI-H	NM
H57	F	47	AC Ⅱ	MSI-H	NM
H63	F	47	AC	MSI-H	NM
H10	M	41	JC	MSI-H	NM
H12	F	50	JC	MSI-H	NM
H41	M	46	JC	MSI-H	NM
H55	M	49	JC	MSI-H	NM
H7	M	38	BG	MSI-H	NM
H8	M	43	BG	MSI-H	NM
H30	M	48	BG	MSI-H	NM
H35	F	38	BG	MSI-H	NM
H51	F	27	BG	MSI-H	NM

AC: Amsterdam criteria; JC: Japanese criteria; BG: Bethesda guidelines; MSI-H: High microsatellite instability; NM: No mutation.

Table 2 Characteristics of 6 probands with MSS

Case	Gender	Age (yr)	Criteria	MSI	*MLH1/MSH2/MSH6* mutation study
H16	F	44	JC	MSS	NM
H20	F	54	BG	MSS	NM
H44	F	39	BG	MSS	NM
H48	M	28	BG	MSS	NM
H50	M	55	BG	MSS	NM
H54	M	43	BG	MSS	NM

JC: Japanese criteria; BG: Bethesda guidelines; MSS: Microsatellite stability; NM: No mutation.

DNA extraction

Genomic DNA from peripheral blood and the cell line sw48 was extracted with the QIAGEN (Hilden, Germany) DNA extraction kit following its manufacturer's introductions. Concentration of the genomic DNA was determined with an ultraviolet spectrophotometer (Beckman, DU640 type).

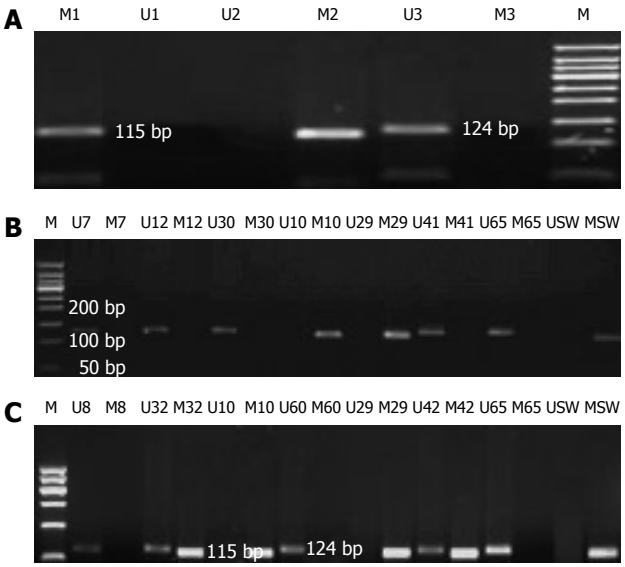

Figure 1 Results of *MLH1* MSP assay using primers that amplify methylated (M) or unmethylated (U) alleles (Lane M represents 100-bp DNA marker). A: MLH1 MSP assay in families H10, H65 and cell line SW48. M1, M2, U1and U2 indicate the methylated and unmethylated products of familiy H10, cell line SW48, and the methylaed band (115 bp); M3 and U3 indicate the methylated and unmethylated products in family H65; B: MLH1 MSP assay in families H7, H12, H30, H10, H29, H41, H65 and cell line SW48. U7, U12, U30, U41 and U65 indicate the unmethylated products of families H7, H12, H30, H41, and H65,respectively; M10, M29 and MSW indicate the methylated products of families H10, H29, and positive control SW48, respectively; C: MLH1 MSP assay in families H8, H32, H10, H60, H29, H42 H65 and cell line SW48. U8, U60 and U65 indicate the unmethylated products of families H8, H60 and H6, respectively; M10, M29 and MSW indicate the methylated products of families H10, H29, and positive control SW48, respectively; U32, M32 and U42, M42 are products of families H32 and H42, respectively.

PCR for methylation in MLH1

MSP exploits the effect of sodium bisulfite on DNA, which efficiently converts unmethylated cytosine to uracil with methylated cytosine unchanged. Consequently, after treatment, methylated and unmethylated alleles have different sequences that can be used to design allele-specific primers.

Genomic DNA was modified with sodium bisulfite as described previously[13,14]. The modified DNA was then subjected to MSP using primer pairs engineered to amplify either methylated or unmethylated DNA. Methylated and unmethylated primer pair sequences were also designed as previously described[15] and synthesized (Sangon, shanghai). PCR was carried out with HotstarTaq DNA polymerase (Cat. No. 203203): preheating at 94℃ for 10 min, followed by 40 cycles of denaturation at 94℃ for 45 s, annealing at 58℃ for 45 s and extension at 72℃ for 45 s, and a final elongation at 72℃ for 7 min. PCR products were subjected to 2% agarose gel electrophoresis. The products of exhaustive-methylation only indicated a methylated band of 124 bp and the unmethylated products only indicated an unmethylated band of 115 bp, while the partially methylated products indicated both of them. After observation of clear and expected bands, the products were purified using the QIAquick gel extraction kit (Qiagen) and sequenced on a 3700 DNA sequence system (ABI, USA) in order to check the correct bisulfite modification. Appropriate positive and negative reference samples were included. Each result of sequencing was analyzed by DNA Star 5.08 bioanalysis software.

Immunochemical staining for MLH1

A monoclonal antibody against MLH1 (Pharmingen, San Diego, CA, USA) was prepared at a 1:40 dilution and detected by the Envision two-step method to judge whether the status of methylation in *MLH1* gene would affect the expression of MLH1 protein. The expression of MLH1 was diminished in cancer tissues in the absence of detectable nuclear staining of neoplastic cells. Infiltrating lymphocytes and normal colonic crypt epithelium next to the tumor area served as internal positive controls.

RESULTS

Five probands with *MLH1* gene methylation were found in 18 unrelated Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. The rate of abnormal methylation in *MLH1* gene was approximately 27.8% (5/18). Among the 18 patients, 2 displayed exhaustively methylated phenotype and the other 3 presented partially-methylated phenotype. The exhaustive methylation accounted for 11.1% (2/18) in the HNPCC families with MSI-H but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. Perhaps, the changes might be much lower in all unselected HNPCC families. Among the 13 probands with unmethylation phenotype, 8 fulfilled the Japanese criteria (JC)/Bethesda guidelines (BG), 5 fulfilled the Amsterdam criteria (AC). All probands with partially-methylated phenotype fulfilled the AC, whereas probands of families H10 and H29 displaying exhaustively-methylated phenotype fulfilled the JC and AC, respectively. The rate of aberrant methylation in *MLH1* gene in the AC group (22.2%, 4/18) was higher than that in the JC/BG groups (5.6%, 1/18) in all HNPCC families with MSI-H phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. However, no proband with methylation in *MLH1* gene was found in HNPCC families with MSS phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. In our study, the expected size of bands was clear and specific. The study was repeated in triplicate to make sure all results credible (Figure 1A-C). Moreover, all exhaustively and partially methylated PCR products were purified and clone-sequenced in order to further substantiate the results of MSP (Figure 2). We believed that the methylation in *MLH1* gene might be related with microsatellite phenotype. No expression of MLH1 protein was observed in tumor tissues from two patients with exhaustively methylated phenotype, while positive expression of MLH1 protein was found in tumor tissues from patients with partially methylated phenotype (excluding family H42 without tumor tissue), suggesting that exhaustive-methylation in *MLH1* gene can cause defective expression of MLH1 protein and influence its function while the partial methylation in *MLH1* gene may have no impact on the expression of MLH1 protein.

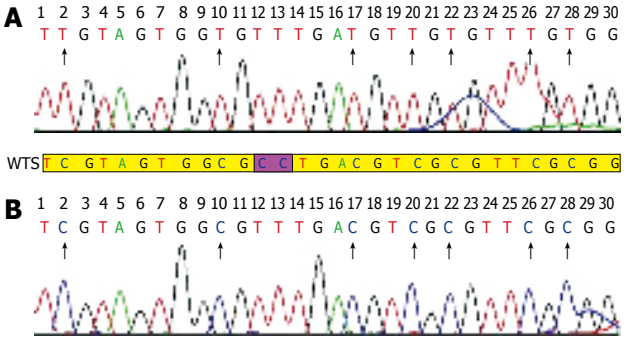

Figure 2 Methylation analysis of the promoter *MLH1* gene by clone sequencing. Arrow indicates CpG dinucleotide, WTS indicates the wild-type sequence of transcription start site. A: Unmethylated band presenting all unmethylated cytosines was converted to uracil after bisulfite modification; B: Methylated band presenting all unmethylated cytosines was unchanged after bisulfite modification.

DISCUSSION

HNPCC syndrome is the most common form of hereditary colorectal cancer. Predisposed individuals have a higher risk of developing cancer. The syndrome is primarily due to heterozygous germline mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2* genes. The resulting mismatch repair deficiency leads to MSI which is the hallmark of tumors arising within this syndrome, as well as a variable proportion of sporadic tumors. Diagnostic guidelines and criteria for molecular testing of suspected families have been proposed and continuously updated. However, not all families fulfilling these criteria show mutations in MMR genes and/or MSI implicating other unknown carcinogenic mechanisms and predisposition genes. This subset of tumors is the focus of current clinical and molecular research.

Germline mutations in the coding regions of *MSH2* and *MLH1* genes are known to be responsible for up to 45%-64% of all HNPCC families[16], and those of *MSH6* account for 10% of HNPCC kindreds[17]. We have previously detected germline mutations in the entire coding regions of *MSH2, MLH1* and *MSH6* genes in 24 probands meeting the AC, 15 probands fulfilling the JC and 19 probands meeting the BG by PCR-gene-sequencing with 20 germline mutations detected including two mutations occurring in a same patient and three novel mutations[10,11]. Subsequently, 3 new mutations are found by mRNA-based PCR sequencing[12]. It was speculated that the remaining probands without mutations in *MSH2*, *MLH1*, and *MSH6* genes might be associated with other aberrant types of genes. It was reported that DNA methylation associated with transcriptional silencing of *MLH1* is the underlying cause of MMR defects in most sporadic colorectal cancers with a MSI+ phenotype[9,18]. Moreover, reversal of methylation with 5-aza-deoxycytidine not only results in reexpression of MLH1 protein, but also restoration of the MMR capacity in MMR-deficient cell lines[9]. These findings further substantiate that the promoter methylation in *MLH1* gene is another deficient mechanism of *MLH1* gene.

Hypermethylation of CpG island in the promoter sequence has been proved to be an important mechanism of gene silencing and is particularly associated with transcriptional silencing of tumor suppressor genes in sporadic cancers[19,20]. Germline mutations might occur in individuals with a well-characterized genetic disease but lack an identifiable mutation in known disease genes[21]. It was recently reported that monoallelic promoter hypermethylation in *MLH1* gene is observed in peripheral blood from a number of patients with early-onset colorectal cancer[7,22-24]. The above results indicate that *MLH1* promoter-germline mutation might be related to HNPCC.

Our study demonstrated 5 probands with *MLH1* gene methylation (including 2 exhaustive-methylations which fulfill the JC and the AC, respectively, and 3 part-methylations fulfilling the AC) in 18 unrelated Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. The rate of aberrant methylation in *MLH1* gene (22.2%, 4/18) was higher in probands fulfilling the AC than that (5.6%, 1/18) in those meeting the JC and BG. Of the 13 probands with unmethylated phenotype, 8 fulfilled the JC and BG (61.5%, 8/13), 5 fulfilled the AC (38.5%, 5/13). However, no proband was detected with the aberrant methylation in *MLH1* gene in the 6 suspected HNPCC families with MSS phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. These findings illuminate that the promoter methylation in *MLH1* gene is likely another underlying cause of MMR defect in HNPCC fulfilling the AC. In order to ravel whether the aberrant methylation in *MLH1* gene influences the expression of MLH1 protein, immunostaining of MLH1 protein was carried out in 5 probands with *MLH1* aberrant methylation in our study. No expression of MLH1 protein was found in 2 probands with exhaustively methylated phenotype, whereas positive expression of MLH1 protein was observed in 2 probands with partially methylated phenotype (excluding family H42 without tumor tissue) suggesting that exhaustive methylation in *MLH1* gene can cause defective expression of MLH1 protein and influence its function while partial methylation of *MLH1* gene may have no impact on the expression of *MLH1* gene, revealing that methylation in *MLH1* gene may be related with the microsatellite phenotype and influence the expression of MLH1 protein and its function, which is consistent with the reported findings in other studies[8,9].

In neoplastic cells, stable allele-specific loss of transcription due to aberrant methylation in an unmutated promoter region can identify hypermethylation as a direct mechanism of tumor suppressor gene inactivation[25]. Moreover, the promoter methylation can be passed in somatic mitosis, which is reversible. Persons with hypermethylation in *MLH1* alleles of somatic cells can predispose to the development of cancer in patterns with hereditary nonpolyposis colorectal cancer. It was reported that epimutation can be transmitted from a mother to her son[26], which is consistent with transgenerational epigenetic inheritance.

In the present study, the rate of aberrant methyla-

tion in *MLH1* gene was only 27.8% (5/18) in selected HNPCC with MSI-H phenotype but without germline mutations in *MLH1*, *MSH2* and *MSH6* genes. Among the probands with aberrant methylation, the rate of methylation in those fulfilling the AC accounted for 80% (4/5), which was significantly higher than that [20% (1/5)] in those meeting the JC and BG. Methylation analysis of the *MLH1* promoter should be performed for all early-onset or multiple colorectal cancer patients with MSI-H tumors or loss of MLH1 protein expression due to unknown causes in HNPCC probands fulfilling the AC.

There is evidence that aberrant methylation in the promoter region of *MLH1* alleles is functionally equivalent to a pathogenic *MLH1* germline mutation and mimics the clinical phenotype of Lynch syndrome. 'Sporadic' HNPCC-patients need to be treated Lynch syndrome patients. Individuals carrying *MLH1* germline epimutations are at a high risk of developing colorectal and other tumors and should be considered carriers of germline mutations. Inheritance should be discarded in each case, until more conclusive data are obtained. *MLH1* promoter methylation analysis should be performed at least for the first degree relatives with positive methylation to exclude the inheritance of a familial epimutation[27]. Identification of hypermethylation as an epigenetic defect has important implications for surveillance recommendations, since these patients should be treated like Lynch syndrome patients.The heritability of methylation needs to be further investiged.

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COMMENTS

Background

Germline mutations in mismatched repair genes, such as MLH1, MSH2 and MSH6, lead to hereditary nonpolyposis colorectal cancer (HNPCC) and not all families fulfilling these criteria show mutations in mismatched repair genes. It is well known that MLH1 promoter methylation is related with sporadic colorectal cancer. However, MLH1 promoter germline-methylation in Chinese HNPCC patients has not yet been reported.

Research frontiers

Germline mutations in MMR genes, such as MSH2, MLH1 and MSH6 contribute to the early diagnosis of HNPCC and screening of HNPCC families. Few studies on MLH1 promoter germline-methylation are available.

Innovation and breakthroughs

Five patients with *MLH1* gene methylation were found in this study by methylation-specific PCR in 18 unrelated Chinese HNPCC probands with high microsatellite-instability phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* gene. The rate of abnormal methylation in *MLH1* gene was approximately 27.8% (5/18) and the rate (22.2%, 4/18) in probrands fulfilling the Amsterdam criteria, which was higher than that (5.6%, 1/18) in those meeting the Japanese criteria/Bethesda guidelines.

Applications

MLH1 promoter methylation analysis can be used for the microsatellite phenotype of mismatched repair genes and is a promising tool for molecular genetics screening of HNPCC.

Terminology

HNPCC is an abbreviation of hereditary nonpolyposis colorectal cancer; MSP is an abbreviation of methylation-specific PCR.

Peer review

In this study, MLH1 promoter germline-methylation was detected in 18 unrelated Chinese HNPCC probands with high microsatellite-instability phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* gene. The rate of aberrant methylation in probands meeting theAmsterdam criteria was higher than that in those fulfilling the Japanese criteria/Bethesda guidelines. However, the function of MLH1 promoter germline-methylation should be further studied with a large of samples.

REFERENCES

1 **Miyaki M**, Konishi M, Tanaka K, Kikuchi-Yanoshita R, Muraoka M, Yasuno M, Igari T, Koike M, Chiba M, Mori T. Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer. *Nat Genet* 1997; **17**: 271-272
2 **Muller A**, Fishel R. Mismatch repair and the hereditary non-polyposis colorectal cancer syndrome (HNPCC). *Cancer Invest* 2002; **20**: 102-109
3 **Peltomaki P**, Vasen HF. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. *Gastroenterology* 1997; **113**: 1146-1158
4 **Peltomaki P**. Deficient DNA mismatch repair: a common etiologic factor for colon cancer. *Hum Mol Genet* 2001; **10**: 735-740
5 **Liu B**, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, Watson P, Jass JR, Dunlop M, Wyllie A, Peltomaki P, de la Chapelle A, Hamilton SR, Vogelstein B, Kinzler KW. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nat Med* 1996; **2**: 169-174
6 **Reik W**, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001; **293**: 1089-1093
7 **Gazzoli I**, Loda M, Garber J, Syngal S, Kolodner RD. A hereditary nonpolyposis colorectal carcinoma case associated with hypermethylation of the MLH1 gene in normal tissue and loss of heterozygosity of the unmethylated allele in the resulting microsatellite instability-high tumor. *Cancer Res* 2002; **62**: 3925-3928
8 **Miyakura Y**, Sugano K, Konishi F, Ichikawa A, Maekawa M, Shitoh K, Igarashi S, Kotake K, Koyama Y, Nagai H. Extensive methylation of hMLH1 promoter region predominates in proximal colon cancer with microsatellite instability. *Gastroenterology* 2001; **121**: 1300-1309
9 **Herman JG**, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 1998; **95**: 6870-6875
10 **Cai Q**, Sun MH, Fu G, Ding CW, Mo SJ, Cai SJ, Ren SX, Min DL, Xu XL, Zhu WP, Zhang TM, Shi DR. [Mutation analysis of hMSH2 and hMLH1 genes in Chinese hereditary nonpolyposis colorectal cancer families] *Zhonghua Binglixue Zazhi* 2003; **32**: 323-328
11 **Yan SY**, Zhou XY, Du X, Zhang TM, Lu YM, Cai SJ, Xu XL, Yu BH, Zhou HH, Shi DR. Three novel missense germline mutations in different exons of MSH6 gene in Chinese hereditary non-polyposis colorectal cancer families. *World J Gastroenterol* 2007; **13**: 5021-5024
12 **Wang CF**, Zhou XY, Zhang TM, Sun MH, Xu Y, Shi DR. [The analysis for mRNA mutation of MLH1, MSH2 genes and the gene diagnosis for hereditary nonpolyposis colorectal cancer]

Zhonghua Yixue Yichuanxue Zazhi 2006; **23**: 32-36
13 **Herman JG**, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821-9826
14 **Grady WM**, Rajput A, Lutterbaugh JD, Markowitz SD. Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. *Cancer Res* 2001; **61**: 900-902
15 **Chung WB**, Hong SH, Kim JA, Sohn YK, Kim BW, Kim JW. Hypermethylation of tumor-related genes in genitourinary cancer cell lines. *J Korean Med Sci* 2001; **16**: 756-761
16 **Shin KH**, Shin JH, Kim JH, Park JG. Mutational analysis of promoters of mismatch repair genes hMSH2 and hMLH1 in hereditary nonpolyposis colorectal cancer and early onset colorectal cancer patients: identification of three novel germ-line mutations in promoter of the hMSH2 gene. *Cancer Res* 2002; **62**: 38-42
17 **Kariola R**, Raevaara TE, Lonnqvist KE, Nystrom-Lahti M. Functional analysis of MSH6 mutations linked to kindreds with putative hereditary non-polyposis colorectal cancer syndrome. *Hum Mol Genet* 2002; **11**: 1303-1310
18 **Cunningham JM**, Christensen ER, Tester DJ, Kim CY, Roche PC, Burgart LJ, Thibodeau SN. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res* 1998; **58**: 3455-3460
19 **Jones PA**, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999; **21**: 163-167
20 **Rountree MR**, Bachman KE, Herman JG, Baylin SB. DNA methylation, chromatin inheritance, and cancer. *Oncogene* 2001; **20**: 3156-3165
21 **Martin DI**, Ward R, Suter CM. Germline epimutation: A basis for epigenetic disease in humans. *Ann N Y Acad Sci* 2005; **1054**: 68-77
22 **Miyakura Y**, Sugano K, Akasu T, Yoshida T, Maekawa M, Saitoh S, Sasaki H, Nomizu T, Konishi F, Fujita S, Moriya Y, Nagai H. Extensive but hemiallelic methylation of the hMLH1 promoter region in early-onset sporadic colon cancers with microsatellite instability. *Clin Gastroenterol Hepatol* 2004; **2**: 147-156
23 **Suter CM**, Martin DI, Ward RL. Germline epimutation of MLH1 in individuals with multiple cancers. *Nat Genet* 2004; **36**: 497-501
24 **Hitchins M**, Williams R, Cheong K, Halani N, Lin VA, Packham D, Ku S, Buckle A, Hawkins N, Burn J, Gallinger S, Goldblatt J, Kirk J, Tomlinson I, Scott R, Spigelman A, Suter C, Martin D, Suthers G, Ward R. MLH1 germline epimutations as a factor in hereditary nonpolyposis colorectal cancer. *Gastroenterology* 2005; **129**: 1392-1399
25 **Myohanen SK**, Baylin SB, Herman JG. Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia. *Cancer Res* 1998; **58**: 591-593
26 **Hitchins MP**, Wong JJ, Suthers G, Suter CM, Martin DI, Hawkins NJ, Ward RL. Inheritance of a cancer-associated MLH1 germ-line epimutation. *N Engl J Med* 2007; **356**: 697-705
27 **Morak M**, Schackert HK, Rahner N, Betz B, Ebert M, Walldorf C, Royer-Pokora B, Schulmann K, von Knebel-Doeberitz M, Dietmaier W, Keller G, Kerker B, Leitner G, Holinski-Feder E. Further evidence for heritability of an epimutation in one of 12 cases with MLH1 promoter methylation in blood cells clinically displaying HNPCC. *Eur J Hum Genet* 2008; **16**: 804-811

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Metrically measuring liver biopsy: A chronic hepatitis B and C computer-aided morphologic description

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Abstract

AIM: To describe a quantitative analysis method for liver biopsy sections with a machine that we have named "Dioguardi Histological Metriser" which automatically measures the residual hepatocyte mass (including hepatocytes vacuolization), inflammation, fibrosis and the loss of liver tissue tectonics.

METHODS: We analysed digitized images of liver biopsy sections taken from 398 patients. The analysis with Dioguardi Histological Metriser was validated by comparison with semi-quantitative scoring system.

RESULTS: The method provides: (1) the metrical extension in two-dimensions (the plane) of the residual hepatocellular set, including the area of vacuoles pertinent to abnormal lipid accumulation; (2) the geometric measure of the inflammation basin, which distinguishes intra-basin space and extra-basin dispersed parenchymal leukocytes; (3) the magnitude of collagen islets, (which were considered truncated fractals and classified into three degrees of magnitude); and (4) the tectonic index that quantifies alterations (disorders) in the organization of liver tissue. Dioguardi Histological Metriser machine allows to work at a speed of 0.1 mm^2/s, scanning a whole section in 6-8 min.

CONCLUSION: The results are the first standardized metrical evaluation of the geometric properties of the parenchyma, inflammation, fibrosis, and alterations in liver tissue tectonics of the biopsy sections. The present study confirms that biopsies are still valuable, not only for diagnosing chronic hepatitis, but also for quantifying changes in the organization and order of liver tissue structure.

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Key words: Liver measurement; Image analysis; Liver lesion; Liver tectonics

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INTRODUCTION

The main purpose of this paper is to describe a rigorous method based on the fundamentals of measurement theory[1], which metrically defines the changes in magnitude of liver tissue prime basic structural elements that occurring during the course of chronic hepatitis B and C.

Each available score to evaluate hepatic lesions is characterized by some methodological inaccuracy[2-4]. In fact, transient elastography (Fibro-Scan)[5,6] is limited by the skill of the operator and because liver stiffness is not only dependent from fibrosis, and serological assays not directly involved in tissue evolution, but in patient diagnosis[7-11]. In addition to the inherent risks of excising a liver specimen[12], current morphometric analyses[13-17] are time-consuming, depend on subjective choices of the regions of interest, involve the interactive elimination of Glisson's capsule and staining artefacts, and use the International System (IS), which is unsuitable for measuring the irregular shapes found in histology[18-21].

The study concerning the status of the liver tissue affected by chronic viral hepatitis was suggested by three

main needs. The first was ethical because methodological accuracy and repeatability are essential. The second was clinical because many problems remain unsolved in hepatology, such as non-responders to therapy[3], and regression of cirrhosis[2]. The third need was economic, as the price of the metrical data supplied by the "Dioguardi Histological Metriser" analysis is relatively low, and the repeatable biopsy interpretation is obtained with a few minutes.

The lack of an appropriate geometry had prevented the real measurement of irregular liver structures, until Mandelbrot's fractal geometry[22] (also called the geometry of irregularity) offered a correct approach for obtaining reproducible and closer to reality metrical measurements of hepatocellular mass, inflammation, and fibrosis, and also provided a quantitative index for evaluating the organization of liver tissue tectonics. In order to apply these new measurements, we constructed a practical and fully-automated machine that we called the "Dioguardi Histological Metriser", which is capable of measuring 10 parameters to describe the status of the residual hepatocyte mass (including hepatocyte vacuolization), inflammation, fibrosis, and the loss of liver tissue tectonics in liver biopsy sections, at a speed of 0.1 mm^2/s. Hepatitis B and C virus infections do not usually affect the biliary system.

The study concerning the status of the liver tissue affected by chronic viral hepatitis B and C, was suggested by three main needs: (1) Ethical: methodological accuracy and reproducibility are essential; (2) Clinical: because many questions remain unsolved in hepatology, such as non-responders patients to therapy[3], and regression of cirrhosis[2]; (3) Economical: metrical data analysis supplied by the "Dioguardi Histological Metriser" is not expensive reproducible and is obtained within a few minutes.

MATERIALS AND METHODS

Case list

We studied 398 patients randomly collected (250 male) aged 52 ± 12 years with chronic hepatitis B or C, who were admitted to the hepatology departments of the Istituto Clinico Humanitas (ICH) IRCCS, Rozzano, and the University of Milan Department of Gastroenterology, Ospedale Maggiore IRCCS, Milan, Italy. The biopsies were performed in accordance with the guidelines of the Ethics Committees of ICH and Ospedale Maggiore IRCCS. All of the liver specimens were approximately 17 ± 12 mm^2.

The logarithmic curve of the ordered set according to the fibrosis data magnitude obtained from the 398 patients, can be interpreted as the trajectory (from α to ω)of the ideal dynamics of collagen deposition during the course of chronic hepatitis (Figure 1).

Histological methods

Three consecutive 2 μm thick sections were cut from formalin-fixed, paraffin-embedded biopsy specimens: the first was stained with hematoxylin and eosin for diagnostic purposes; the second was treated to identify inflammatory cells by using monoclonal antibodies raised against leukocyte common antigen (LCA: Dako, Milan, Italy) and a standardized immunoperoxidase method[23], and hepatocellular lipids vacuoles; and the third was stained with Sirius red to visualize fibrosis.

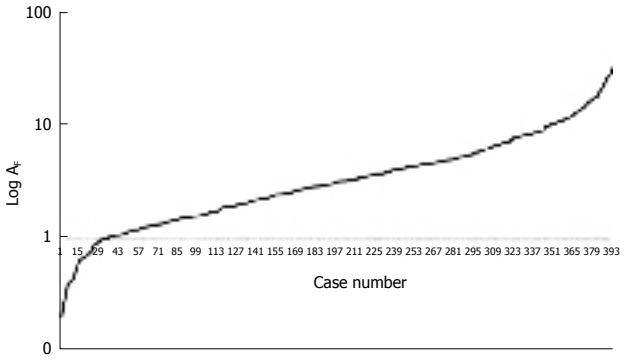

Figure 1 Markovian logarithmic curve obtained with the fibrosis content in each single biopsy section from 398 patients, ordered by increasing severity. A_F = area of fibrosis.

Semi-quantitative analyses

Expert hepatopathologists graded and staged the biopsy sections using the Knodell[24], Sheuer[25], Ishak[26], and METAVIR[27] semi-quantitative scoring systems.

Example of liver tissue geometric analysis

A specific example of the set of metrical parameters obtained by quantitatively evaluating liver residual parenchyma, inflammation, fibrosis, and disordered liver tissue tectonics is shown in Figure 2.

Methodology validations

Variations in the water bath temperatures used to distend the histological sections were tested at 41, 43, 45 and 47℃, (which accounted for 12% of the variations in fibrosis). Variations in paraffin section thickness were tested using five sequential thicknesses from 2-6 μm, (which accounted for 20% of the variations in fibrosis). Variations in staining times (tested using nine sequential sections stained with a freshly-made Sirius red solution for 15-135 min), (which accounted for 13% of the variations in fibrosis). Intra-sample variability in the tissue area covered with Sirius-red-stained collagen was assessed using three series of thirty 2 μm-thick sections obtained from three biopsies, two series of fifteen 4 μm-thick sections obtained from two further biopsies, and one series of ten 6 μm-thick sections obtained from a sixth biopsy. The results showed wide intra-sample variability, because of the highly irregular distribution of the collagen matrix. Also the loss of the thinner matrix components because of histological section processing might have play played a role in this result.

Statistical analysis

The results were analyzed using Statistica software (StatSoft Inc., Tulsa, OK, USA). Variability was evaluated using the coefficient of variation (CV) given by the formula CV = (SD/mean) × 100%. *P* values of less than 0.05 were considered statistically significant.

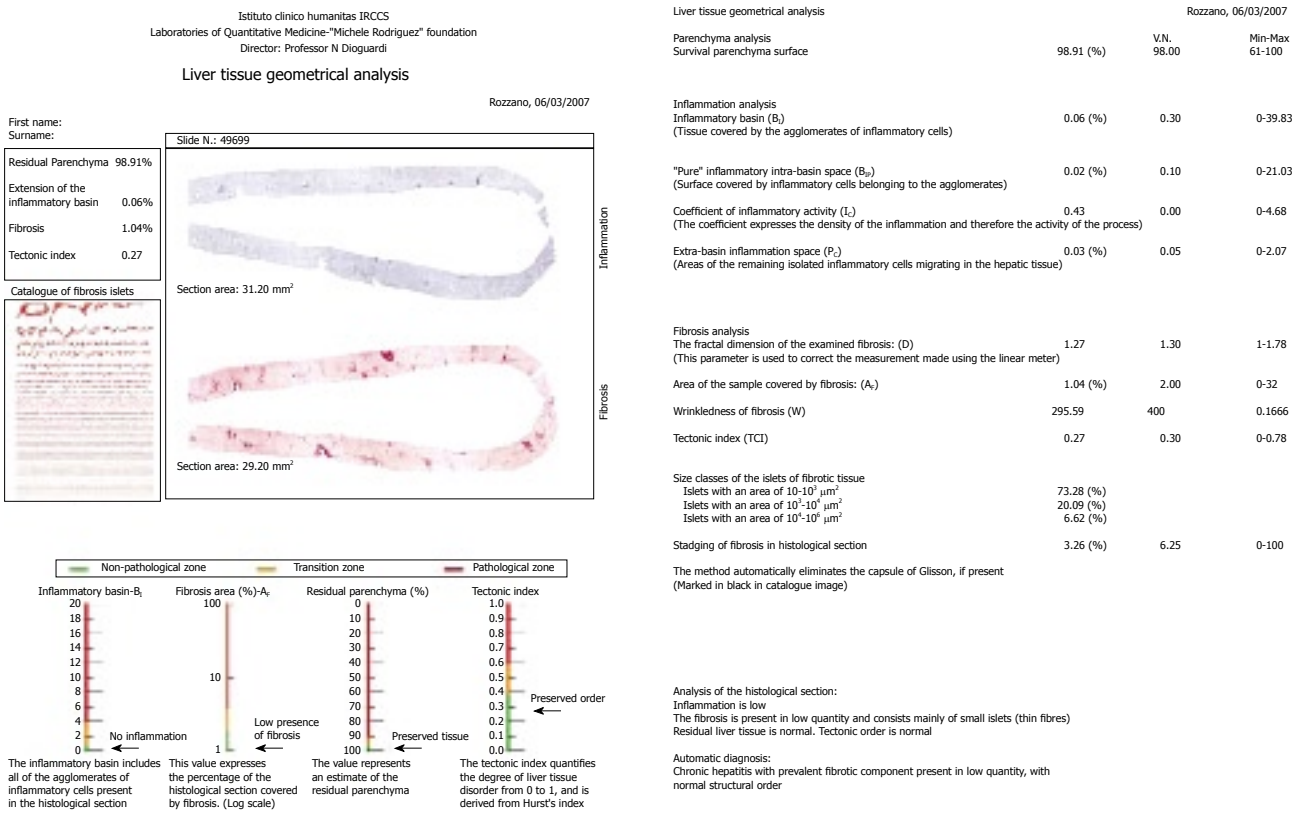

Figure 2 An example of LTGA. The machine completes the analysis by providing a common language description of the histological pattern and diagnosis.

The model and its pathological transformation

Our model for measuring the state of liver tissue is based on two canonical points: the choice of the prime structural elements of the tissue, and the most general kind of tissue organization[1].

Prime structural elements (determine property of the system) and are the most representative structural elements of an organ tissue insofar as they have the property of changing their shape and size over time, without losing their individuality.

Prime liver structural elements were considered: (1) the parenchyma (i.e. the *substantia jecuris*), which consists of the hepatocytic mass that, in this phase of the research, includes regenerative nodular hepatocytes; (2) the dispersed set of topical immunological cells; (3) the collagen scaffold that consists collagen fibers and included the portal spaces; and (4) the tectonic, defined by the Malpighi-Kiernan lobular organization of the liver. All of these elements were taken in their strictly structural form.

The most general kind of organization[1] of an organ tissue is the state of its prime structural elements defined by quantitative relationships. It determines tissue tectonics and the reference for every structural change in organ architecture which, in the case of the liver, is the lobular structure.

Pathological events occurring during the course of chronic viral hepatitis transform the shape and size of these prime physical structures, and consequently alter the most general organization of the liver system.

The main events altering the natural physical state of the prime structural elements and tectonics of liver tissue, are enlargement, reduction and "vacuolization". The pathological transformations determined by these events during the course of chronic viral hepatitis, can be interpreted as follows: (1) necrosis reduces lipid hepatocyte determines vacuolization increases the size of the parenchyma; (2) Increase in the number of dispersed cells generated by the topical general immune system determine inflammatory cell clusters; (3) Growth of septa that evolve into porto-portal or porto-central fibrotic bridges results in expansion of the collagen support network (which appears as Sirius-red-stained islets in a histological section). Taken together these individual transformations (in shape and size) generate a loss of the natural harmony in the inter-relationship of the prime elements. This loss of order is also measurable.

The Dioguardi Histological Metriser

We designed and built our own user-friendly Liver Tissue Geometric Analyser (LTGA or Dioguardi Histological Metriser; patent pending), which automatically ensures correct microscope focusing, metrically evaluates the image of an entire digitalized histological slide, and defines the areas covered by the residual parenchymal mass (including lipid vacuolization), inflammation and fibrosis; it also disregards any unfilled spaces (vascular and biliary cavities or sinusoidal spaces) and artifactual tissue-free spaces.

The Dioguardi Histological Metriser consists of two parts: a "client" (or dedicated microscope system) that captures and digitizes the images of the specifically stained histological section, and a central "server" that receives the images, automatically measures the parameters

Figure 3 Liver fibrosis. A: Prototypical examples of multifarious Sirius-red-stained collagen islets making-up the liver collagen network; B: the Metriser automatically selected and excluded Glisson's capsule (black islets) from the computation of fibrosis by means of an appropriate algorithm.

listed in Table 1, and sends the results back to the client.

In this study, the microscope system consisted of a Leica DMLA microscope (Leica, Milan, Italy) equipped with an X-Y translator table, a digital camera (QICam, QImaging, Surrey, Canada), and an Intel Pentium 4, 2.60 GHz computer. We used *ad hoc* built-in image analysis software that automatically filtered, selected, and marked the outlines of the images of interest using color thresholds based on the levels of red, green and blue. All of the measurements were made at 10 × objective magnification. The Dioguardi Histological Metriser automatically selected and excluded Glisson's capsule from the computation of fibrosis by means of an appropriate algorithm (Figure 3).

The minimum and maximum scalars obtained empirically on the basis of the Dioguardi Histological Metriser measurements of 398 biopsies are shown in Table 1.

Table 1 Quantitative parameters automatically obtained with the Metriser

Parameter	min	max
Residual hepatocellular set		
Residual hepatocellular set (%)	67.97	99.59
Inflammation		
Inflammatory cell cluster space (%)	0	8.71
Pure inflammatory cell cluster space (%)	0	3.67
Extra-basin inflammatory space (%)	0	1.14
Fibrosis		
Area of Sample covered by fibrosis (%)	0	32
Islets magnitude		
$10\text{-}10^3$ (%)	8.9	100
$10^3\text{-}10^4$ (%)	0	58.15
$> 10^4$ (%)	0	87.4
Wrinkledness	0	1666
Tectonic Index		
Liver tissue	0	0.78
Low or no disorder	0	0.4
Middle disorder	0.4	0.6
High disorder	0.6	1

Data are expressed as percentage of true liver surface. The value 0 is obtained when no cell clusters were recognized. Quantitative evaluation of steatosis. At this phase of the research this quantitative parameter is still obtained with an ad hoc software.

Measuring the pathological structures

We took as a reference for the following measurements the physical transformation average of the areas of the studied histological section.

Residual parenchymal mass: We consider the surviving hepatocellular set that remains after necrotic viral destruction, together with the nodular regenerated hepatocytes.

This surviving part of the hepatocellular set (residual mass) is expressed as a percentage of the reference area using the formula:

$H_S = 100\% - A_I - A_F$

Where H_S is the area of the residual hepatocellular set, A_I the sum of the area of the inflammation basin, and A_F the area covered by fibrosis. Vacuolization is due to the accumulation of lipids within hepatocytes (Figure 4). In this phase of the research we included lipid vacuoles in the residual parenchyma. The extension of these cytoplasmic enclaves was measured separately, to define the steatosis grade.

Inflammation basin: We called the inflammation basin (Figure 5A) the classic liver tissue pattern characterized by various sets of spatial immune-cell aggregates[23]. We consider three components. (1) Inflammatory cell clusters

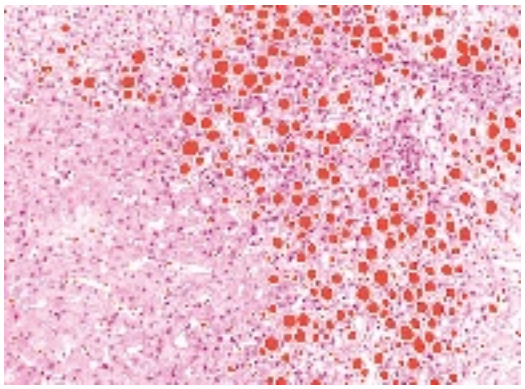

Figure 4 Computer-aided recognition of vacuolization, due to the accumulation of lipid vacuoles within hepatocytes. The image represents only a limited exemplificative area taken from the whole histological section (× 10).

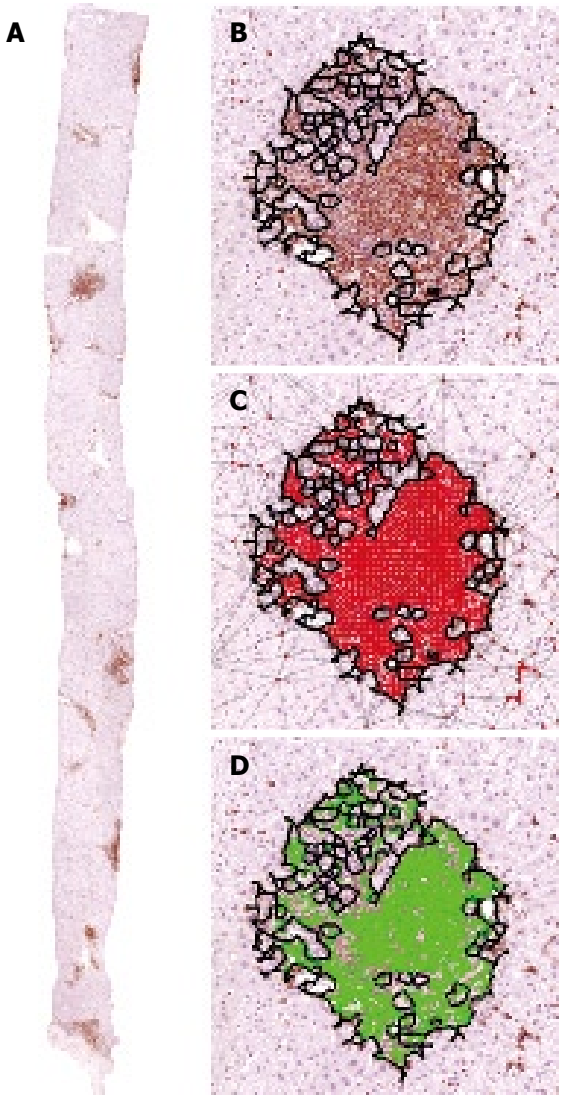

Figure 5 Liver inflammation (× 10). A: Morphological picture showing the inflammatory cell' clusters forming the inflammation basin; B: inflammatory cell cluster; C: discrimination of the cluster outline (black line) using the Delaunay' triangulation; D: pure intra-cluster inflammatory space covered by the inflammation cell bodies (green surface). The immunological staining was performed by treating the sections with monoclonal antibodies raised against LCA.

(Figure 5B). The boundaries of this dot-like pathological

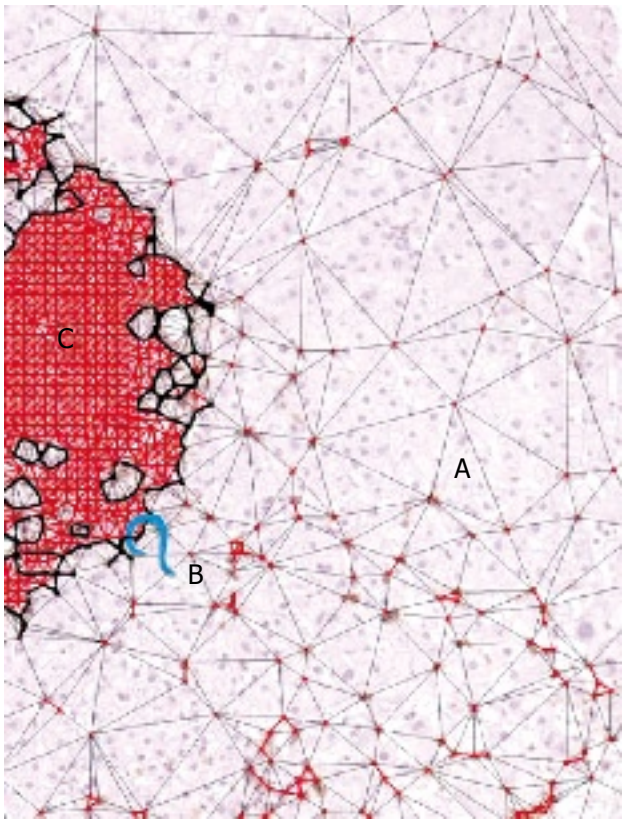

Figure 6 Liver inflammation (× 10). A: Extra-cluster inflammatory space, which is the sum of micro-areas covered by individual inflammatory cells represented by the nodes of the Delaunay' triangulation network; B: irregular outline (black line) in which lies the outermost cells distant each other no more than 20-μm; C: the irregular outline divided outermost cells from the inside resident cluster cells. The immunological staining was performed treating the sections with monoclonal antibodies raised against LCA.

structure are arbitrarily fixed using Delaunay's triangulation (Figure 5C), which defines their edges, as a continuous line, connecting the centers of the outermost cells (with a maximum distance of 20 μm)[23]. This line separates the intra-cluster inflammatory cells, from the immunologically evidenced parenchymal leukocytes throughout the tissue[23]. (2) Intra-cluster inflammatory space (Figure 5D), which is intra-cluster area covered by resident inflammatory cell bodies micro-areas[23]. (3) Extra-cluster inflammatory space, which is the sum of micro-areas covered by individual inflammatory cells that remain outside the clusters, within the liver tissue interstitium (Figure 6)[23].

Fibrosis: The fibrotic framework appears as a multifarious set of collagen islets (Figure 3). Three classes of collagen islets were arbitrarily identified on the basis of their area the first one included islets with an area of between 10 and 10^3 μm^2, the second are those with an area of between 10^3 and 10^4 μm^2, and the third are those with an area of > 10^4 μm^2[21] (Figure 7).

The wrinkledness of collagen islets is calculated using the formula:

$$W = \frac{P}{2\sqrt{\pi A}} - R$$

Where wrinkledness (W) is expressed as the ratio between the perimeter and area of an object[21], P is the frac-

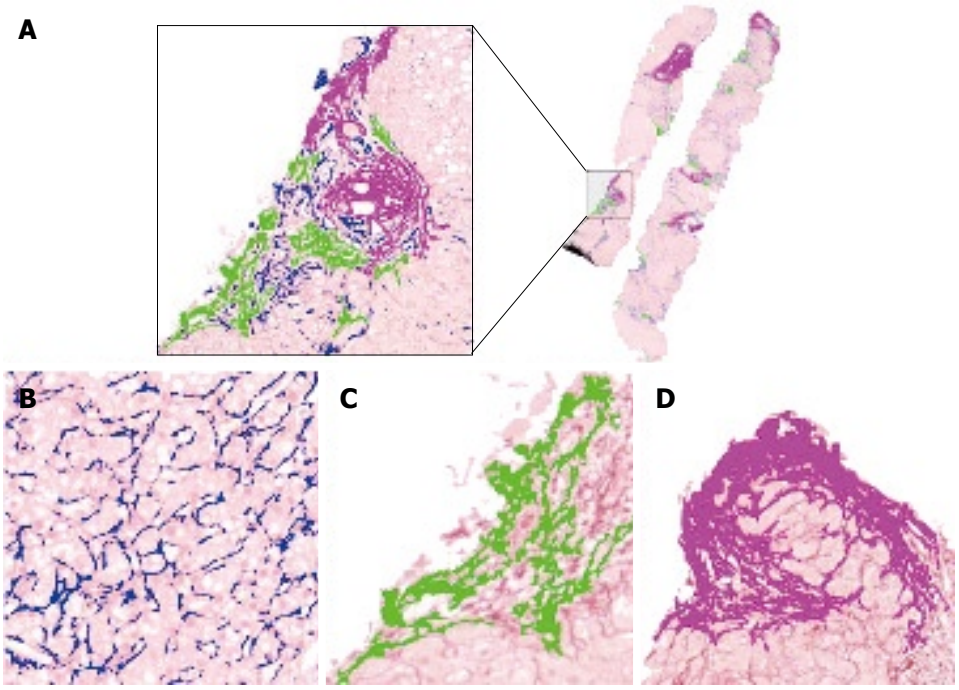

Figure 7 Liver collagen islets magnitude (× 10). A: The Histological Metriser Dioguardi distinguish and highlight with different colours three classes of Sirius-red stained collagen islets; B: islets with magnitudes arbitrarily fixed at 10-10^3 μm^2 (colored in blue); C: islets with magnitudes fixed at 10^3-10^4 μm^2 (colored in green); D: islets with magnitudes fixed at and > 10^4 μm^2 (coloured in pink).

tal-corrected perimeter of the collagen area, *A* the fractal-corrected collagen area, and *R* the roundness coefficient of the collagen islets[21].

Liver tectonics: Natural liver tectonics defines the organization of the intersecting elements of liver tissue. The tectonic order was quantitatively described by the Tectonic Index (*TCI*, which ranges from 0 to 1) obtained using the Euclidean and fractal dimensions and *TCI* was obtained from *H* using the conversion formula:

$TCI = 1 - H$

Where $H = D\gamma + 1 - D$, in which *D* is the fractal dimension and $D\gamma$ the Euclidean dimension.

TCI describes the loss of tissue organization or any deviation from natural order: a high *TCI* indicates a high degree of tissue disorder, and a low *TCI* indicates a low degree of tissue disorder. It can therefore be written:

$TCI = 1 - H = D - D\gamma$

Fractal dimension correction of the IS meter

The irregularity of collagen islets makes it impossible to measure them using IS linear units unless these units are corrected by means of fractal dimension[20,21]. This correction makes it possible to include details of shape that escape (or do not interact with) linear unit measurements at any given scale[20,21]. We derived the fractal dimension using the box-counting method[20,21,28,29]. Since the biological objects has been classified as "truncated fractals"[20,21,30] we used the fractal dimension to correct the reference units as a dilation factor[20,21].

Table 2 shows the differences between the uncorrected and fractal dimension-corrected IS measurements.

Table 2 Differences between the uncorrected and fractal-corrected IS measurements of fibrosis surface extension

n	Uncorrected fibrosis extension range (%)	Mean increase of fibrosis extension after IS meter correction (%)	min (%)	max (%)
193	0-3	25	0	65
156	3-10	10	5	18
49	10-40	4	2	6

n: Number of liver biopsy.

RESULTS

Dioguardi Histological Metriser resolution power

The Dioguardi Histological Metriser resolution was assessed by computing the surface area of liver fibrosis in 13 tissue sections, and repeating the measurements 10 times in order to define the instrument error. Two objects were considered distinct if, and only if, their values and 95% confidence intervals (twice the standard deviation of the experimental values) did not overlap. The Dioguardi Histological Metriser distinguished 68 different categories, as against the six of Ishak, the five of the METAVIR scoring system, and the four of Knodell's or Sheuer's methods. The mean distance between the data with no overlap was 0.786% (range: 0.056%-2.216%).

The selective power of the metrical quantifications defines the capacity of the method to distinguish small differences in magnitude.

Comparison of metrical and semi-quantitative data

A study a part, was performed to define differences of the metrical date concerning residual hepatocytic mass inflammation, fibrosis and liver tissue tectonics on the same patient. The digital images of 61 pairs of histological biopsies from patients with hepatitis C virus-dependent disease. The first measurement was made 4-15 years after of the interval (after the antiviral treatment). The aim was to study the date differences after a long and irregular time.

For each pair of biopsies, we studied: (1) the difference

Table 3 Residual parenchyma metrical and semi-quantitative case numbers

Δ metrical measure		Semi-quantitative evaluations				
		HAI	Scheuer	Ishak	METAVIR	Average
Increase	24	19	26	26	23	23.5
Stationarity	0	30	23	18	25	24.0
Decrease	37	12	12	17	13	13.5

Mean value of the four semi-quantitative scoring systems; Data refer to the difference between two biopsies taken at different times.

Table 4 Inflammatory basin metrical and semi-quantitative case numbers

Δ metrical measure		Semi-quantitative evaluations				
		HAI	Scheuer	Ishak	METAVIR	Average
Increase	35	16	16	23	7	15.5
Stationarity	1	25	22	19	36	25.5
Decrease	25	20	23	19	18	20.0

Mean value of the four semi-quantitative scoring systems; Data refer to the difference between two biopsies taken at different times.

Table 5 Fibrosis metrical and semi-quantitative case numbers

Δ metrical measure		Semi-quantitative evaluations				
		HAI	Scheuer	Ishak	METAVIR	Average
Increase	42	19	26	26	23	23.5
Stationarity	0	30	23	18	25	24.0
Decrease	19	12	12	17	13	13.5

Mean value of the four semi-quantitative scoring systems; Data refer to the difference between two biopsies taken at different times.

Table 6 Tectonic Index metrical and semi-quantitative case numbers

Δ of loss of the natural liver tissue order		Semi-quantitative evaluations				
		HAI	Scheuer	Ishak	METAVIR	Average
Increase	46	19	26	26	23	23.5
Stationarity	3	30	23	18	25	24.0
Decrease	12	12	12	17	13	13.5

Mean value of the four semi-quantitative scoring systems; Data refer to the difference between two biopsies taken at different times.

(Δ) between the scalar measurements performed with Dioguardi Histological Metriser; and (2) the changes of the semiquantitative evaluation with the four most widely used semi-quantitative methods (Knodell, Sheuer, Ishak, METAVIR).

Juxtaposing the quantitative variations in the metrical measurements with the time to the same parameters studied by means of the semi-quantitative methods in order to collate the results of the two scores, we found the following. (1) The metrical measurements of residual parenchyma gave, in comparison to semi-quantitative results, fewer indications of no change and more indications of decreases (Table 3). (2) The metrical measurements of inflammation gave more indications of increases, and fewer indications of no change than the semi-quantitative results (Table 4). (3) The metrical measurements of fibrosis gave more indications of increases and fewer indications of no change than the semi-quantitative results (Table 5). (4) The quantitative evaluations of the tectonic index of liver architecture gave more indications of increased disorder, and fewer indications of no change than the semiquantitative results (Table 6).

To compare the difference of the metrical scalars, with the ordinally numbered categories is not possible, because metrical measurements are *in continuum* (and thus additive with successive points separated by $\Delta = 0$), whereas semi-quantitative evaluations are discrete and not additive with intervals of $\Delta \neq 0$.

Differences between the metrical and semi-quantitative results

On the straight line of real numbers, we reported the value of the cases grouped by each category recognized with the current semi quantitative scoring systems.

The collate of the distribution of the data reported on the state shows the overlapping of the metrical measurements that correspond to patients classified in different semi quantitative categories (Knodell, Ishak, Sheuer, and METAVIR). This highlights the inadequacy of all four semi-quantitative methods in discriminating different states of liver fibrosis (Figure 8).

Classes of magnitude of collagen islets

Our method distinguished three classes of collagen islets with magnitudes arbitrarily fixed at 10-10^3 μm^2, 10^3-10^4 μm^2, and > 10^4 μm^2 (Figure 7). As the process of fibrosis is a progressive deposition of extracellular matrix that coalesces into islets that are subsequently thickened by matricial deposits, it can be speculated that thin islets indicate the initiation and persistence of inflammation.

Stad-ging and grading

Stad-ging indicates the part of the disease course already covered and the part that remains to be covered, before it reaches its end. It is established by placing the value of fibrosis (expressed as a scalar) on the ideal trajectory that indicates the phase of fibrosis, at the time of measurement (Figure 9)[21]. In brief, stad-ging indicates the tendency of the process to evolve in both senses from one state to another.

The staging and stad-ging of fibrosis are different insofar as the former indicates the current fibrotic state, and the latter indicates the phase of the process: i.e. the percentage of the course before collagen deposition reaches its maximum level of tolerance, which in our case, was empirically found to be 32% of fibrosis. The magnitude of inflammation defines the current status (grading) of the disease process.

DISCUSSION

The aim of this study was to test the means of rapidly

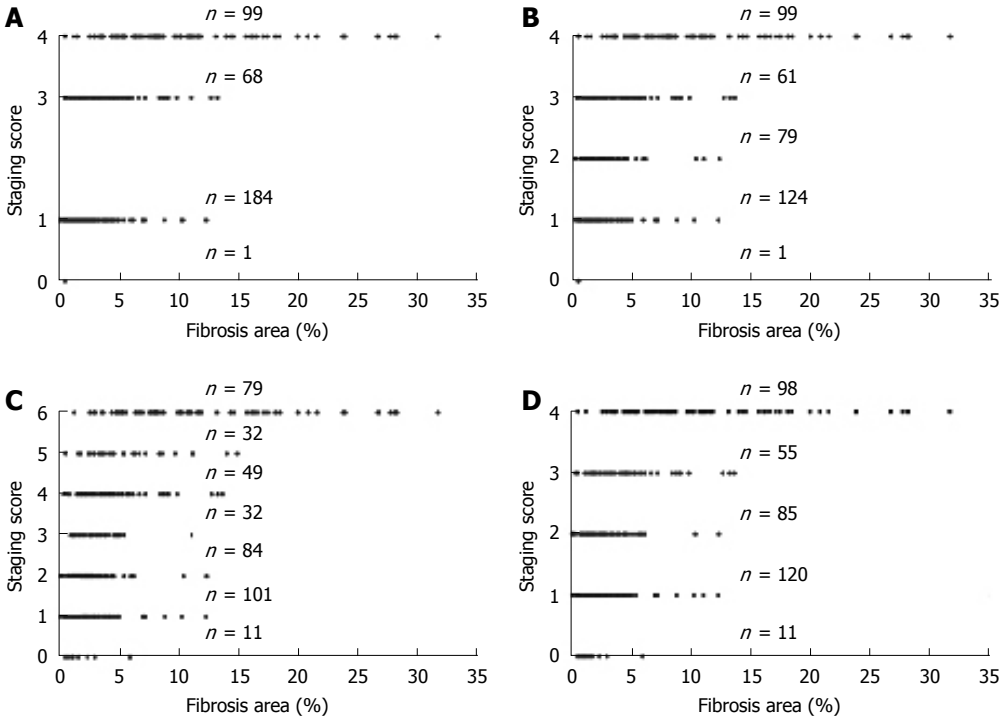

Figure 8 Comparison of the phase portraits obtained using the scalar values of fibrosis (%) calculated from each biopsy section, projected onto the state spaces. A: Knodell HAI; B: Sheuer; C: Ishak; and D: METAVIR categories (staging). All graphs highlight a considerable overlap of scalar data that corresponds to different categories. Forty-six cases for HAI, 34 for Sheuer, 10 for Ishak and 29 for METAVIR resulted uncertain to be classified in a unique category of severity.

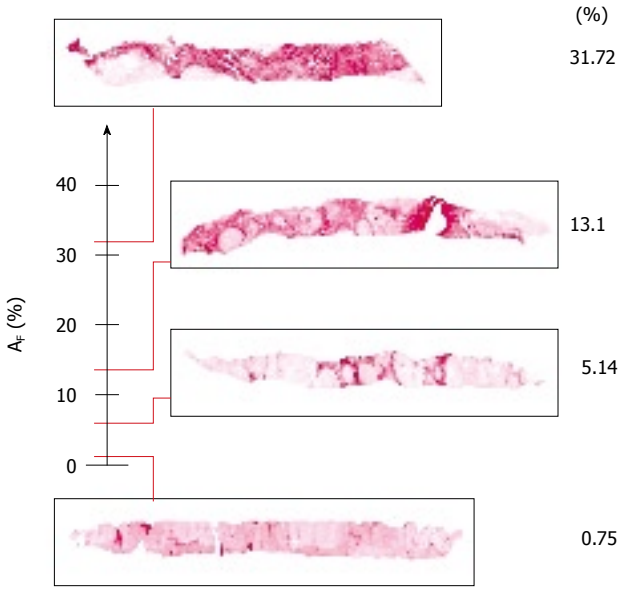

Figure 9 Stad-ging indicates the part of the disease course already covered and the part that remains to be covered before it reaches its end, and is established by placing the value of fibrosis (expressed as a scalar) on the ideal trajectory that indicates the phase of fibrosis at the time of measurement. A_F = fibrosis area (× 10).

making standardized and precise metrical measurements of pathological elements in a liver biopsy histological section using the rules of measurement theory.

The model used to make quantitative comparisons between the structural elements of the natural liver system and the same elements in liver tissue affected by chronic viral inflammation was constructed on the basis of a set of four observables, which were considered the prime structural elements of the system. Three of these (the hepatocyte sub-set, topical immune system and collagen support network) were taken as axioms for measuring the purely structural organized matter; the fourth (tissue tectonics) was taken as an axiom because it defines the harmony that orders the natural conformation of liver tissue. Recognizing these observables in their most strictly structural form makes it possible to define necrosis, the topical immune system, the collagen network and tissue tectonics in geometrical terms: (1) necrosis of the hepatocyte mass was considered a reduction, and steatosis an enlargement due to lipid deposition in the form of intercellular vacuoles; (2) inflammation was considered an enlargement of the topical immune cell system; (3) fibrosis an enlargement of natural liver collagen formed by the deposition of intercellular matrix; and (4) the transformations in liver tectonics as a reduction in the natural harmonic state of liver tissue that caused disorder in the organ's lobular structure.

This model allows the Dioguardi Histological Metriser to provide the following quantitative data: (1) the metrical extension of the residual hepatocellular set including the area of vacuoles pertinent to abnormal lipid accumulation; (2) the geometric measure of the inflammation basin (i.e. that part of the liver surface covered by inflammatory cell clusters), which distinguishes intra-basin space and extra-basin dispersed parenchymal leukocytes; (3) the magnitude of collagen islets, which were considered truncated or asymptotic planar fractals and classified into three degrees of magnitude; and (4) the TCI that quantifies alterations (disorders) in the organization of liver tissue.

To quantify these elements, the Dioguardi Histological Metriser uses three units of measurement. The first are the traditional IS units, which are used to measure the outlines of clusters and lipid vacuolization; the second are the traditional IS units corrected by the fractal dimension, which are used to measure irregular fractal collagen islets and the area of the residual hepatocellular set; and the third is the TCI, which is based on the inter-relationships between the Euclidean and fractal dimensions of liver tissue, and provides

Table 7 Side effects of liver biopsy sampling and other invasive techniques

Examination	Side effects		Ref.
	Serious complications (%)	Mortality (%)	
Liver biopsy	0.5	> 0.02	31
Oesophagogastroduodenoscopy		0.01	31
Colonoscopy	0.3	0.02	31-33
Flexible sigmoidoscopy	0.0001		32, 34
Endoscopic retrograde cholangiopancreatography	5-10	0.1-1	32, 35, 36
Percutaneous transhepatic cholangiography	3		32
Contrast agents	2-10		37-39

a quantitative estimate of the loss of natural tectonic order.

On the basis of our results, it is difficult not to consider biopsies a rich source of information regarding the course of chronic liver inflammation, despite their undeniable limitations[31]. Our perseverance in studying biopsies should be seen in the light of the risks[32-36] currently accepted in many fields of medical practice[37-39] (Table 7). Quantifying liver lesions in a biopsy sample raises many questions concerning the status and organization of natural and pathological liver systems that do not seem to be merely subsidiary matters to be dealt with within the confines of a specific investigation[1].

Why adopt a new measuring method in hepatology?

One fundamental question is whether hepatology researchers or practitioners need to adopt a metrical method of tissue analysis in order to confront the everyday problems they already solve in a less precise but what they still consider to be a satisfactory manner. Viral inflammation is a dynamic process influenced by a variety of factors that generate changes in the shapes (wrinkledness) of liver tissue lesions. However, studying the evolution of liver tissue status can use no more than one biopsy, which must therefore be examined in as much detail as possible, and this makes any attempt to improve precision crucial. It is clear that the status of the organ cannot be defined quantitatively without an appropriate technology that is capable of: (1) discriminating the most representative observables; (2) eliminating imprecise identifications and measurements; (3) using a suitable metrical unit for measuring the irregularly shaped elements of the tissue; (4) standardizing the measurement of previously unavailable histological elements; and (5) considering the smallest foci of inflammation and fibrotic islets that cannot be observed through an optical microscope.

The problem of the most representative observables was solved by the model, and that of the linear IS unit was solved by correcting it by the fractal dimension of the measured object[20,21]. The problem of standardizing the measurements was solved by constructing an innovative and completely automatic instrument that excludes human error, provides objective and reproducible results, and eliminates the need for the tedious work of light microscopic analysis (the automated analysis of an entire histological section takes place at a speed of 0.1 mm^2/s).

The problem of the representativeness of a biopsy fragment (which accounts for only 1/40 000-1/60 000 of the whole liver)[12] cannot be directly solved by our method which, however, can ensure objective and mathematical precision in measuring elements that may or may not be visible through a microscope.

At this point it has to be stressed that, although our metrical measures are rigorous and reproducible, their scalars provide definitions of magnitude and not interpretations, which remain the responsibility of pathologists and clinicians.

However, our machine can already describe a histological picture in verbal and repeatable terms, and thus provide a strictly morphological diagnosis. We can also say that we have begun to consider it in terms that make it more comparable with an intelligent collaborator than a sophisticated desk computer. Finally, our data come from machine-made metrical measurements of the pathological observables in a histological pattern, and are not hypotheses based on semi-quantitative methods that can only continue to generate new hypotheses.

This paper is closed with a dedication to Robert Rosen.

ACKNOWLEDGMENTS

The authors are grateful to Professor Massimo Colombo and Dr. Guido Ronchi for their kind concession to study the specimens of the archives of Department of Gastroenterology of Ospedale Maggiore IRCCS, Milan, Italy and Dr. Rosalind Roberts, Dr. Giuseppe Peverelli and Dr. Kevin Smart for their language revisions of the manuscript.

COMMENTS

Background

Liver biopsy can be considered the gold standard for grading, staging and stad-ging the chronic liver disease. In addition, it remains a primary source for acquiring new knowledge about liver pathology.

Innovations and breakthroughs

This study introduced a new kind of liver biopsy measurement that bases the tissue state description on scalars, not with subjective interpretations (i.e. hypothesis). Furthermore, the Dioguardi Histological Metriser can describe a histological picture in verbal and repeatable terms, and thus provide a strictly morphological diagnosis.

Applications

The method, with opportune software, based on the same principles can be used for investigating also non-viral or inflammatory liver disease of other organs.

Peer review

This paper reports an impressive method for automated biopsy scoring.

REFERENCES

1 **Rosen R**. Fundamentals of measurement and representation

of natural systems. Elsevier Science Ltd, 1978: 1-81
2 **Desmet VJ**, Roskams T. Cirrhosis reversal: a duel between dogma and myth. *J Hepatol* 2004; **40**: 860-867
3 **Goodman ZD**, Becker RL Jr, Pockros PJ, Afdhal NH. Progression of fibrosis in advanced chronic hepatitis C: evaluation by morphometric image analysis. *Hepatology* 2007; **45**: 886-894
4 **Fontana RJ**, Goodman ZD, Dienstag JL, Bonkovsky HL, Naishadham D, Sterling RK, Su GL, Ghosh M, Wright EC. Relationship of serum fibrosis markers with liver fibrosis stage and collagen content in patients with advanced chronic hepatitis C. *Hepatology* 2008; **47**: 789-798
5 **Sandrin L**, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, Beaugrand M, Palau R. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705-1713
6 **Ziol M**, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Ledinghen V, Marcellin P, Dhumeaux D, Trinchet JC, Beaugrand M. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48-54
7 **Poynard T**, Imbert-Bismut F, Munteanu M, Messous D, Myers RP, Thabut D, Ratziu V, Mercadier A, Benhamou Y, Hainque B. Overview of the diagnostic value of biochemical markers of liver fibrosis (FibroTest, HCV FibroSure) and necrosis (ActiTest) in patients with chronic hepatitis C. *Comp Hepatol* 2004; **3**: 8
8 **Rosenberg WM**, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, Hubscher S, Roskams T, Pinzani M, Arthur MJ. Serum markers detect the presence of liver fibrosis: a cohort study. *Gastroenterology* 2004; **127**: 1704-1713
9 **Patel K**, Gordon SC, Jacobson I, Hezode C, Oh E, Smith KM, Pawlotsky JM, McHutchison JG. Evaluation of a panel of non-invasive serum markers to differentiate mild from moderate-to-advanced liver fibrosis in chronic hepatitis C patients. *J Hepatol* 2004; **41**: 935-942
10 **Imbert-Bismut F**, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069-1075
11 **Afdhal NH**. Biopsy or biomarkers: is there a gold standard for diagnosis of liver fibrosis? *Clin Chem* 2004; **50**: 1299-1300
12 **Bravo AA**, Sheth SG, Chopra S. Liver biopsy. *N Engl J Med* 2001; **344**: 495-500
13 **Kage M**, Shimamatu K, Nakashima E, Kojiro M, Inoue O, Yano M. Long-term evolution of fibrosis from chronic hepatitis to cirrhosis in patients with hepatitis C: morphometric analysis of repeated biopsies. *Hepatology* 1997; **25**: 1028-1031
14 **Masseroli M**, Caballero T, O'Valle F, Del Moral RM, Perez-Milena A, Del Moral RG. Automatic quantification of liver fibrosis: design and validation of a new image analysis method: comparison with semi-quantitative indexes of fibrosis. *J Hepatol* 2000; **32**: 453-464
15 **Pilette C**, Rousselet MC, Bedossa P, Chappard D, Oberti F, Rifflet H, Maiga MY, Gallois Y, Cales P. Histopathological evaluation of liver fibrosis: quantitative image analysis vs semi-quantitative scores. Comparison with serum markers. *J Hepatol* 1998; **28**: 439-446
16 **Matalka II**, Al-Jarrah OM, Manasrah TM. Quantitative assessment of liver fibrosis: a novel automated image analysis method. *Liver Int* 2006; **26**: 1054-1064
17 **Wright M**, Thursz M, Pullen R, Thomas H, Goldin R. Quantitative versus morphological assessment of liver fibrosis: semi-quantitative scores are more robust than digital image fibrosis area estimation. *Liver Int* 2003; **23**: 28-34
18 **Friedenberg MA**, Miller L, Chung CY, Fleszler F, Banson FL, Thomas R, Swartz KP, Friedenberg FK. Simplified method of hepatic fibrosis quantification: design of a new morphometric analysis application. *Liver Int* 2005; **25**: 1156-1161
19 **Arima M**, Terao H, Kashima K, Arita T, Nasu M, Nishizono A. Regression of liver fibrosis in cases of chronic liver disease type C: quantitative evaluation by using computed image analysis. *Intern Med* 2004; **43**: 902-910
20 **Dioguardi N**, Franceschini B, Aletti G, Russo C, Grizzi F. Fractal dimension rectified meter for quantification of liver fibrosis and other irregular microscopic objects. *Anal Quant Cytol Histol* 2003; **25**: 312-320
21 **Dioguardi N**, Grizzi F, Franceschini B, Bossi P, Russo C. Liver fibrosis and tissue architectural change measurement using fractal-rectified metrics and Hurst's exponent. *World J Gastroenterol* 2006; **12**: 2187-2194
22 **Mandelbrot BB**. The fractal geometry of the nature. San Francisco: Freeman, 1982: 21-44
23 **Dioguardi N**, Franceschini B, Russo C, Grizzi F. Computer-aided morphometry of liver inflammation in needle biopsies. *World J Gastroenterol* 2005; **11**: 6995-7000
24 **Knodell RG**, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981; **1**: 431-435
25 **Scheuer PJ**. Classification of chronic viral hepatitis: a need for reassessment. *J Hepatol* 1991; **13**: 372-374
26 **Ishak K**, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**: 696-699
27 **Bedossa P**, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289-293
28 **Bassingthwaighte JB**, Liebovitch LS, West BJ. Fractal physiology. New York: Oxford University Press, 1994: 11-44
29 **Hastings HM**, Sugihara G. Fractals. A user's guide for the natural sciences. Oxford: Oxford Science Publications, 1993: 36-55
30 **Rigaut JP**, Schoevaert-Brossault D, Downs AM, Landini G. Asymptotic fractals in the context of grey-scale images. *J Microsc* 1998; **189**: 57-63
31 **Kumar P**, Clark M. Clinical medicine. WB Saunders, 1998: 296-297
32 **Tierney LM**, McPhee SJ, Papadakis MA. Current medical diagnosis & treatment. McGraw-Hill, 2002: 571-673
33 **Janes SE**, Cowan IA, Dijkstra B. A life threatening complication after colonoscopy. *BMJ* 2005; **330**: 889-890
34 **Levin TR**, Farraye FA, Schoen RE, Hoff G, Atkin W, Bond JH, Winawer S, Burt RW, Johnson DA, Kirk LM, Litin SC, Rex DK. Quality in the technical performance of screening flexible sigmoidoscopy: recommendations of an international multi-society task group. *Gut* 2005; **54**: 807-813
35 **Freeman ML**, Nelson DB, Sherman S, Haber GB, Herman ME, Dorsher PJ, Moore JP, Fennerty MB, Ryan ME, Shaw MJ, Lande JD, Pheley AM. Complications of endoscopic biliary sphincterotomy. *N Engl J Med* 1996; **335**: 909-918
36 **Baillie J**. ERCP training: for the few, not for all. *Gut* 1999; **45**: 9-10
37 **Kandzari DE**, Rebeiz AG, Wang A, Sketch MH Jr. Contrast nephropathy : an evidence-based approach to prevention. *Am J Cardiovasc Drugs* 2003; **3**: 395-405
38 **Sakai N**, Sendo T, Itoh Y, Hirakawa Y, Takeshita A, Oishi R. Delayed adverse reactions to iodinated radiographic contrast media after coronary angiography: a search for possible risk factors. *J Clin Pharm Ther* 2003; **28**: 505-512
39 **Panto PN**, Davies P. Delayed reactions to urographic contrast media. *Br J Radiol* 1986; **59**: 41-44

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CLINICAL RESEARCH

Disruption of colonic barrier function and induction of mediator release by strains of *Campylobacter jejuni* that invade epithelial cells

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Abstract

AIM: To study the mechanisms by which *Campylobacter jejuni* (*C. jejuni*) causes inflammation and diarrhea. In particular, direct interactions with intestinal epithelial cells and effects on barrier function are poorly understood.

METHODS: To model the initial pathogenic effects of *C. jejuni* on intestinal epithelium, polarized human colonic HCA-7 monolayers were grown on permeabilized filters and infected apically with clinical isolates of *C. jejuni*. Integrity of the monolayer was monitored by changes in monolayer resistance, release of lactate dehydrogenase, mannitol fluxes and electron microscopy. Invasion of HCA-7 cells was assessed by a modified gentamicin protection assay, translocation by counting colony forming units in the basal chamber, stimulation of mediator release by immunoassays and secretory responses in monolayers stimulated by bradykinin in an Ussing chamber.

RESULTS: All strains translocated across monolayers but only a minority invaded HCA-7 cells. Strains that invaded HCA-7 cells destroyed monolayer resistance over 6 h, accompanied by increased release of lactate dehydrogenase, a four-fold increase in permeability to [^{3}H] mannitol, and ultrastructural disruption of tight junctions, with rounding and lifting of cells off the filter membrane. Synthesis of interleukin (IL)-8 and prostaglandin E_2 was increased with strains that invaded the monolayer but not with those that did not.

CONCLUSION: These data demonstrate two distinct effects of *C. jejuni* on colonic epithelial cells and provide an informative model for further investigation of initial host cell responses to *C. jejuni*.

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Key words: *Campylobacter jejuni*; Cell invasion; Cell culture; Chloride secretion; Colonocyte; HCA-7 cells; Membrane permeability; Monolayer; Mucosal barrier; Ussing chamber

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INTRODUCTION

Campylobacters are small (1.5-6.0 μm long and 0.2-0.5 μm wide) Gram-negative spiral rods. *Campylobacter jejuni* (*C. jejuni*), a foodborne organism contracted from untreated water, milk and meat, especially chicken, is one of the most important causes of bacterial diarrhea worldwide[1-4]. The clinical spectrum ranges from non-inflammatory watery diarrhea to an acute entero-colitis with neutrophilic invasion of the mucosa and bloody diarrhea mimicking ulcerative colitis.

Much work has been conducted on laboratory strains such as NCT11168, which has been completely geno-

typed. This has allowed a number of virulence factors to be identified, including a number of flagellar proteins, which not only enable chemotaxis towards mucus and amino acids and epithelial cell invasion[5-7], but also facilitate secretion of non-flagellar virulence proteins[6], O-linked glycosylation, which is required for optimal flagella function[7], proteins secreted *via* flagella that result in epithelial cell invasion and apoptosis[8-10], a cytolethal distending toxin (CLDT)[11] with DNAse activity[12], associated with apoptosis[13] and secretion of interleukin (IL)-8 and other chemokines[14,15] and a lipo-oligosaccharide that resembles human neuronal gangliosides, which may pre-dispose to autoimmune phenomena such as Guillain-Barre syndrome[16].

Clinical isolates vary in the extent to which they express these virulence factors. *C. jejuni* display heterogeneity in its ability to invade cells of the intestinal epithelial layer[17-21]. Estimates of the proportion of clinical isolates that are characterized by toxin production range from 12% to 100%[22]. Cell death may occur by a variety of mechanisms, not all involving CLDT[23]. Release of chemokines such as IL-8 seems to occur by CLDT-dependent and independent mechanisms[14], and it is unclear how far inflammatory responses to *C. jejuni* infection, such as secretion of chemokines, correlate with reported differences in the ability of the bacteria to invade epithelial cells, or how much this is due to responses of cells in the lamina propria, such as macrophages. Similarly, the extent to which secretory responses by epithelial cells can maintain a secretory diarrhea, which is characteristic particularly of childhood infection, is also unclear. One possibility is that *C. jejuni* induces synthesis of prosecretory compounds such as prostaglandins directly in epithelial cells[24-27]. An earlier study that failed to show this might have been flawed because it used CaCo2 cells, which do not readily express cyclo-oxygenases (COXs) or synthesize prostaglandins[28].

In order to investigate the direct effects of *C. jejuni* on colonic epithelial function *in vitro*, we therefore exposed the well-differentiated colonocyte line HCA-7, clone 29 to a panel of primary clinical isolates. Our data suggest two distinct patterns of interaction between *C. jejuni* and colonic epithelium, with a minority of strains invading colonic epithelial cells, which causes barrier destruction and induces elaboration of potentially pro-inflammatory mediators.

MATERIALS AND METHODS

Bacterial strains

Nineteen consecutive *C. jejuni* clinical strains from community patients with acute bacterial enteritis were isolated and characterized by the Laboratory of Public Health, University Hospital, Nottingham, UK. Of these, three fresh clinical isolates (strains 2801055, 2102011 and 1702030*)* were used for detailed studies along with the laboratory strain 12189 (a kind gift from Dr. J Ketley), originally isolated from a patient with diarrhea and passaged several times in the laboratory[28]. Strains were grown microaerobically for 48 h on chocolate agar plates prior to inoculation into tissue culture flasks containing liquid medium (RPMI 1640 supplemented with 4% Isosensitest broth and 5% FCS) (Sigma, UK). A stock of the original isolates was aliquoted and frozen at -80℃. For study of bacterial-epithelial cell interactions, bacteria were resuspended in 4% Isosensitest broth with 5% FCS. After static microaerobic incubation for 24 h, bacteria were pelleted, washed and resuspended in PBS.

The bacteria were diluted in Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK) to different concentrations (10^5-10^8 bacteria/mL). Determination of the number of bacteria was standardized by optical density at a wavelength of 570 nm. Growth curves for the different strains and titration and culture of serial dilutions was used to establish the number of bacteria in culture. This was done by incubating the different strains grown on a chocolate agar plates in Isosensitest broth (5% FCS) overnight. After centrifugation (7000 r/min, 5 min) and washing in PBS, bacteria were resuspended in 1 mL PBS, and serial diluted. Each dilution was measured in a spectrophotometer at a wavelength of 540 nm. Each sample was then serially diluted in a 96-well plate and dilutions incubated on chocolate agar plates. After overnight growth, bacterial colonies were counted and plotted against absorbance. The results were used to establish bacterial numbers before incubating epithelial cells.

Cell culture

Tumor-derived colonic epithelial cells, HCA-7, colony 29 (a kind gift from Susan Kirkland) were cultured as described previously[29]. Briefly, cells were grown in DMEM with 10% FCS, glutamine (0.29 mg/mL), ampicillin (8 μg/mL), penicillin (40 μg/mL), streptomycin (368 μg/mL) and non-essential amino acids in an atmosphere of 5% CO_2 at 37℃. For studies of bacterial cell interactions, normal medium was replaced with antibiotic-free medium for at least 24 h. Tests for mycoplasma contamination were not performed. For electrophysiological studies, cells (10^5 cells/membrane) were seeded on Snapwell or Transwell filters (polycarbonate membrane, pore size 0.45 μm, surface area 1 cm^2; Costar UK Ltd) and formed confluent monolayers within 8-10 d, as assessed by an epithelial volt-ohmmeter (EVOM; World Precision Instruments, Stevenage, UK)[29].

Effect of C. jejuni on barrier function

Bacteria were grown and added to the apical side of monolayers grown on Transwell or Snapwell filters at a concentration of 10^4-10^8 bacteria/monolayer. Transepithelial resistance was measured with an EVOM at various timepoints up to 24 h after inoculation with different concentrations of bacteria. These data were supported by detailed studies of filter-grown monolayers voltage-clamped in Ussing chambers (World Precision Instruments) and by assessment of [^{3}H] mannitol flux[26].

Ussing chamber studies

Confluent HCA-7 cell monolayers were inoculated on the apical side with varying amounts of strains of *C. jejuni*. After different time periods, filters were

placed in an Ussing chamber and voltage-clamped by continuous application of a short circuit current (SCC)[30]. Resistance (Ω cm^2) was measured under basal conditions and the change in short circuit current (ΔSCC μA/cm^2) after basolateral stimulation by bradykinin (10^{-6} mol/L), and finally after similar stimulation by carbachol (10^{-4} mol/L).

Invasion assay

Invasion of epithelial cells was investigated using a gentamicin invasion assay[18]. Bacteria were grown in 4% Isosensitest broth and 5% FCS. After static microaerobic incubation for 24 h, bacteria were pelleted, washed and resuspended in PBS. Bacterial number was assessed spectrophotometrically and 10^6 organisms added to the cell monolayer. Infected monolayers were incubated for up to 6 h at 37℃, washed and covered with tissue culture medium containing gentamicin (100 μg/mL). After 90 min, the integrity of the monolayer was checked microscopically, then washed in PBS and flooded with 1 mL 10 mL/L Triton X-100 for 5 min, to release intracellular bacteria. Dilutions and viable counts were made of the bacteria within the lysed monolayer. Positive control was a *Yersinia enterocolitica* invasive strain 8081c, and the negative control was *Escherichia coli* non-invasive strain HB101. When it became clear that monolayer destruction occurred with some bacteria, we modified the assay to correct for the number of remaining cells per monolayer. We calculated the number of cells remaining per monolayer by using a hemocytometer, before lysing to obtain counts of intracellular protected bacteria.

Lactate dehydrogenase (LDH) release

HCA-7 cells were incubated with *C. jejuni* for 4 h. Media from the apical reservoir of bacterium-exposed and control monolayers were then collected and analyzed for spontaneous LDH using a colometric assay (Sigma)[31]. In addition, total intracellular LDH concentration from HCA-7 cells was determined by addition of 1 mL 0.1% Triton X-100 to wells of bacterium-exposed and control monolayers. After vigorous pipetting to ensure lysis of all cells, the homogenate was then collected from each well and was also assayed for LDH.

[^{3}H] mannitol flux

Mannitol flux studies were performed in an Ussing chamber[32]. Mannitol (final concentration 5 mmol/L) was added to both sides of the monolayer in Krebs solution. After equilibration of the epithelial monolayer, [^{3}H] mannitol (1 μCi/mL) was added to the apical side. Radioactivity was counted on samples from the basolateral compartment at 15-min intervals for 60 min. Basolateral chamber volume was maintained by replacing the sample aliquot with an equal volume of fresh Krebs buffer. Apical to basolateral flux, expressed as mol·h/cm^2, was calculated by relating the accumulation of tritium in the basolateral chamber compared to the apical.

Electron microscopy (EM)

Filters with HCA-7 cells inoculated with 10^7 bacteria for 6 h where fixed by immersion in 2.5% glutaraldehyde (in 0.1 mol/L cacodylate buffer, pH 7.4). Subsequent processing was performed as described previously[25].

Bacterial translocation

Translocation was measured by inoculating the apical side of monolayers with 10^7 bacteria/0.5 mL. Medium from the basolateral compartment was collected after 2, 4, 6 and 8 h inoculation, diluted 10-fold and cultured on agar plates[33,34]. After each collection, membranes were transferred to fresh wells to establish the number of bacteria translocating over each 2-h period. The number of bacteria was established by counting colony-forming units at each time interval to establish the total number of bacteria translocated. Colony forming units where counted after microaerophilic incubation for 24 h to assess the time course of bacterial translocation.

IL-8 measurement

Level of IL-8 in the basolateral supernatant after 6 h incubation with different strains of *C. jejuni* and of control monolayers was measured using a quantitative ELISA[14] (Amersham, UK). Samples were pipetted into wells coated with specific antibody for IL-8, followed by incubation with a biotinilated antibody reagent. After extensive washing, a streptavidin-horseradish peroxidase conjugate was added and developed with 3,3',5,5'-tetramethylbenzidine substrate. After terminating the reaction, the optical density was read at 450 nm. Detection level was 25-1000 pg/mL.

PGE2 measurement

Aliquots of supernatant were prepared in the same way as for IL-8 release. PGE2 levels were measured by ELISA (Amersham), based on competition between unlabeled PGE2 and a fixed quantity of peroxidase-labeled PGE2 for a limited amount of PGE2-specific antibody[35].

Statistical analysis

Analysis of variance was used in experiments where time or dose was varied, to investigate the influence of cellular invasive and non-invasive bacteria and the interaction with time. For simple comparisons, unpaired *t* tests were used. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using PRISM (Graphpad, San Diego, CA, USA) or Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA).

RESULTS

Transepithelial resistance

Fourteen of the 19 clinical strains tested, as well as the laboratory isolate 12189 had no effect on transepithelial resistance up to 24 h. In contrast, the other five fresh clinical isolates abrogated monolayer resistance entirely by 6 h. These differences did not correlate with rates of translocation. Detailed studies, with strain 2801055 which abrogated transepithelial resistance, showed that decrease in transepithelial resistance varied with bacterial load and was

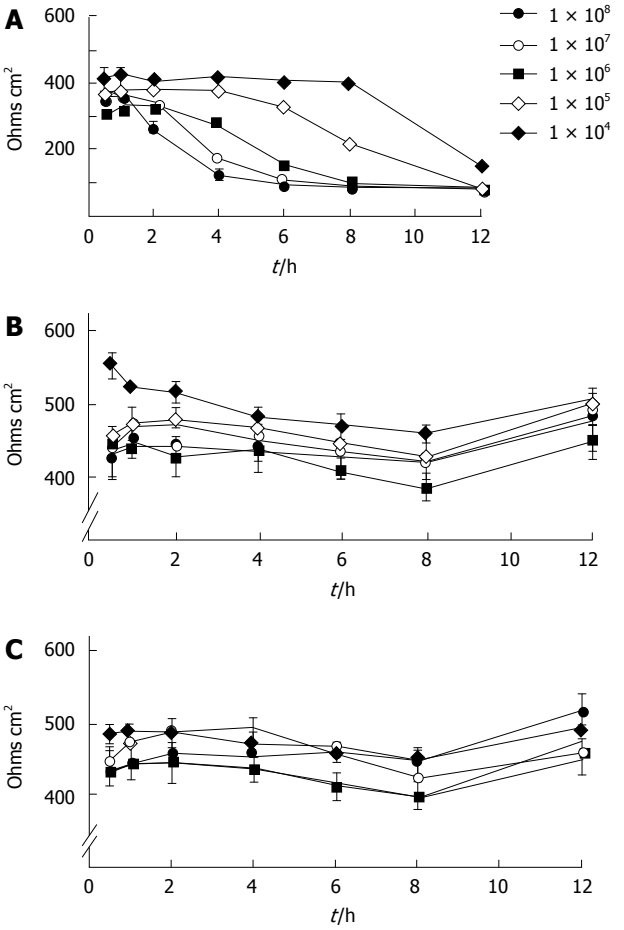

Figure 1 Time course and dose-response of changes in transepithelial resistance after inoculation of T84 cells with three different strains of C jejuni. A: Strain 2801055 reduced resistance to baseline over 6 h in a time- and dose-dependent manner [data are mean ± SE, n = 3 for each bacterial concentration; P < 0.05 compared with controls (uninfected monolayers)]. Baseline resistance across the filter membrane was 100 Ω.cm^2; B: Strain 12189 had no effect on resistance across the monolayer; C: Strain 2102011 had no effect on resistance across the monolayer.

time dependent, starting after 2 h of inoculation with 10^8 bacteria/0.5 mL (Figure 1). With strains 12189 or 2102011, no changes were seen at any time with any of the bacterial loads inoculated (Figure 1). Measurements in Ussing chambers mirrored those obtained with the EVOM with the different strain types. There was no significant difference in electrical resistance in monolayers infected with the strains 12189 and 2102011 at 4 and 8 h. However, strain 2801055 showed a time-dependent decrease in transepithelial resistance, with resistance falling to 71.3% and 17.7% of control at 4 and 8 h respectively (P < 0.05 *vs* control at 8 h).

Invasion of epithelial cells by C. jejuni

The standard gentamicin protection assay showed that 1.15% ± 0.05% of the positive control strain *Y. enterocolitica* invaded HCA-7 cells *vs* 0.0005% with the negative control strain *E. coli* HB101. Overall, *C. jejuni* showed levels of HCA-7 invasion that were intermediate between these values. Analysis of variance showed that the total number of *C. jejuni* per monolayer was higher for those that abrogated monolayer resistance compared to those that did not (P = 0.033), and that this increased with

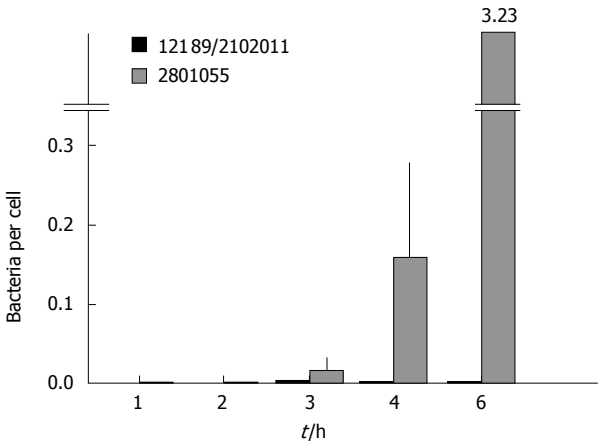

Figure 2 Invasion of monolayers of HCA-7 cells determined by a gentamicin protection assay, corrected for the number of cells remaining per monolayer. *C. jejuni* strain 2801055 invaded the cells of the monolayer in a time-dependent manner, while strains 12189 and 2102011 showed no cellular invasion. Data are mean ± SE, n = 4. Analysis of variance showed that the total number of *C. jejuni* per monolayer was higher for those strains that abrogated transepithelial resistance compared to those that did not (P = 0.033), and that this increased with time (P = 0.02).

time (P = 0.02). When the number of bacteria was related to the number of HCA7 cells remaining in the monolayer at the time of assessment, there was a substantial and highly significant difference between strains that destroyed and did not destroy the monolayer (Figure 2).

LDH release

LDH release from monolayers inoculated with the cell-invasive strain 2801055 was significantly increased after 24 h compared to inoculation with the non-invasive strains 12189 and 210211 (n = 4 per experiment, P < 0.05). LDH release with these strains did not differ from that seen with uninfected monolayers (Figure 3A).

Transepithelial mannitol flux

Cumulative flux data show that strains 12189 and 210211 did not change the flux of [^{3}H] mannitol, which indicated an intact paracellular resistance, whilst strain 2801055 increased the flux significantly (Figure 3B).

EM studies

In keeping with electrophysiological results, strains 12189 and 2102011 did not affect cellular morphology (Figure 4A and B). Monolayers infected with these strains showed close cell-to-cell contact, but bacteria were occasionally located in the pores of the filter membrane (Figure 4C). In contrast, strain 2801055 showed marked cellular and tight junction destruction at 6 h (Figure 4D). There was cell rounding and condensation of the plasma membrane (Figure 4E). In addition, cells were lifted off the filter membrane and multiple bacteria were seen between epithelial cells and the filter (Figure 4E and F).

Bacterial translocation

All strains tested translocated across the monolayer to become detectable after 2 h, with no obvious inter-strain differences.

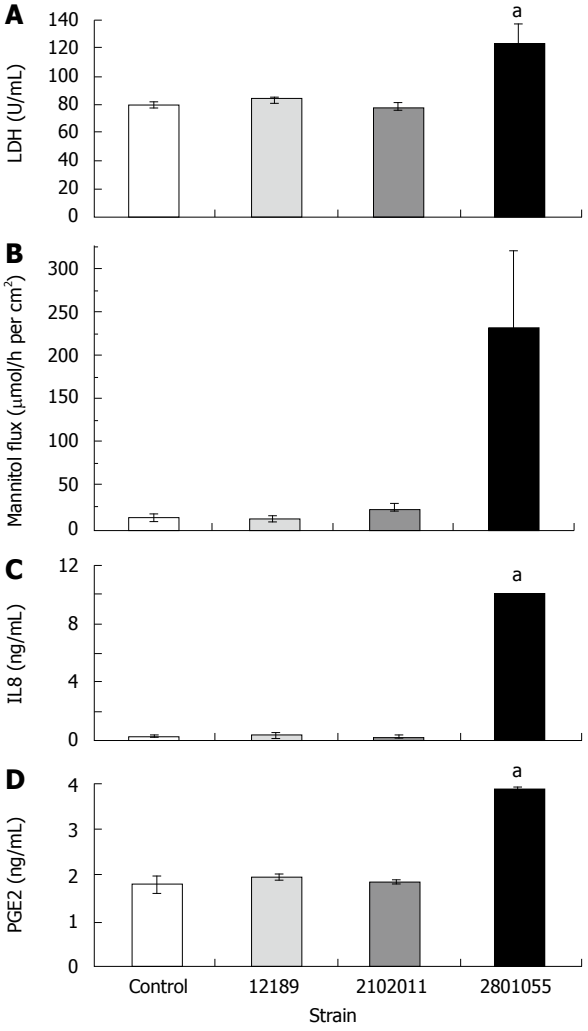

Figure 3 HCA-7 monolayer release of LDH, [^{3}H] mannitol flux and release of IL8 and PGE2 after inoculation with different strains of *C. jejuni*. All data are mean ± SE at 4 h, (n = 4). A: Strain 2801055 showed a significantly higher LDH release after 4 h ($^aP < 0.05$) incubation compared to the other two strains, which were similar to control values; B: Strain 2801055 induced a significantly higher flux rate across the monolayer after 4 h ($^aP < 0.05$) incubation compared to the other two strains, which were similar to control values; C: Strain 2801055 showed a significantly higher IL8 release after 4 h ($^aP < 0.05$) incubation compared to the other two strains, which were similar to control values; D: Strain 2801055 showed a significantly higher PGE2 release after 4 h ($^aP < 0.05$) incubation compared to the other two strains, which were similar to control values.

IL-8 production

IL-8 release from HCA-7 monolayers increased in response to inoculation with the invasive strain 2801055, whereas IL-8 release with the non-invasive strains 12189 and 210211 was not significantly different from that seen in uninfected monolayers (Figure 3C).

PGE2 release

There was a two-fold rise in PGE2 released after inoculation with the invasive strain 2801055 (n = 4, $P < 0.05$). By contrast, PGE2 levels for strains 12189 and 2102011 were similar to spontaneous PGE2 production in control monolayers (Figure 3D).

Bradykinin- and carbachol-induced secretion

Bradykinin 10^{-6} mol/L administered basolaterally induced a ΔSCC of 19.83 ± 3.25 μAmp/cm^2. ΔSCC responses to bradykinin were significantly enhanced by 30% compared with control (uninfected) monolayers at 4 h (n = 4, $P < 0.05$ for all 3 strains Figure 5A), but were lost at later time points, when there was a significant reduction with strain 2801055. The early increase was not seen in response to carbachol (Figure 5B), and at 8 h was significantly decreased with strains 2801055 and 2102011 (n = 4, $P < 0.05$).

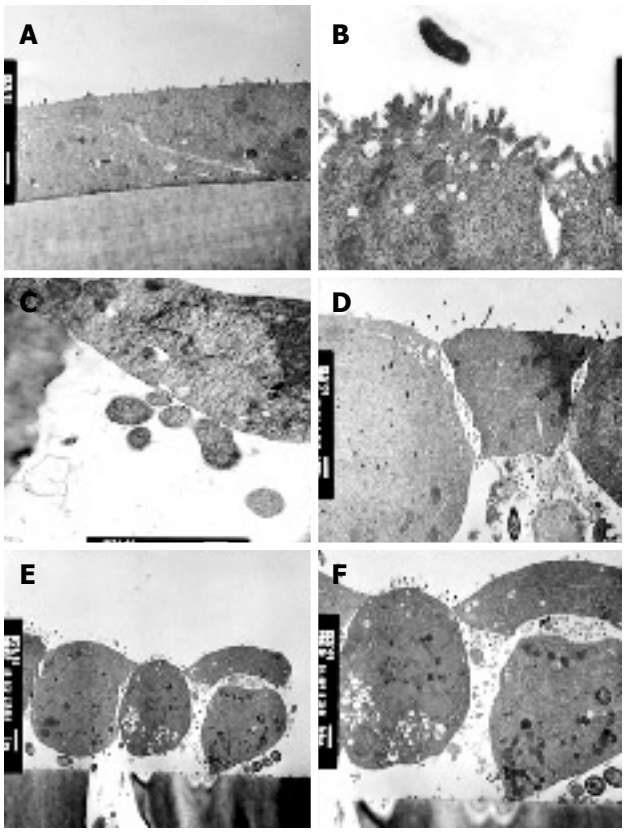

Figure 4 Comparison of the effects of strains 12189 and 2801055 on transmission electron micrograph appearance of monolayers of HCA-7 cells. A: Following inoculation of strain 12189, 10^7 bacteria/0.5 mL, on the apical side for 6 h, there was no disruption of monolayer integrity; B: There were normal tight junctions and normal apical microvilli; C: Close cell-to-cell contact is seen with occasional bacteria located in the pores of the filter membrane; D: Following inoculation of strain 12189, 10^7 bacteria/0.5 mL, on the apical side for 6 h, monolayer integrity was compromised with disruption of tight junctions; E: Monolayers showed condensation of the plasma membrane with lifting and rounding of cells off the supporting membrane. *C. jejuni* are also seen beneath a cell which is lifting off the membrane; F: Changes are seen at higher power.

DISCUSSION

In this study, we showed two distinct patterns of interaction between clinical isolates of *C. jejuni* and a colonic epithelial cell line. Strains that invaded epithelial cells were shown to destroy them, as demonstrated by a fall in transepithelial resistance and release of LDH. These processes were accompanied by release of IL-8 and PGE2. Strains that did not invade epithelial cells did not affect barrier properties or increase mediator production.

Translocation to the lamina propria[2,33] and a consequent interaction between bacterial antigens and antigen presenting cells, immunocytes and macrophages in the

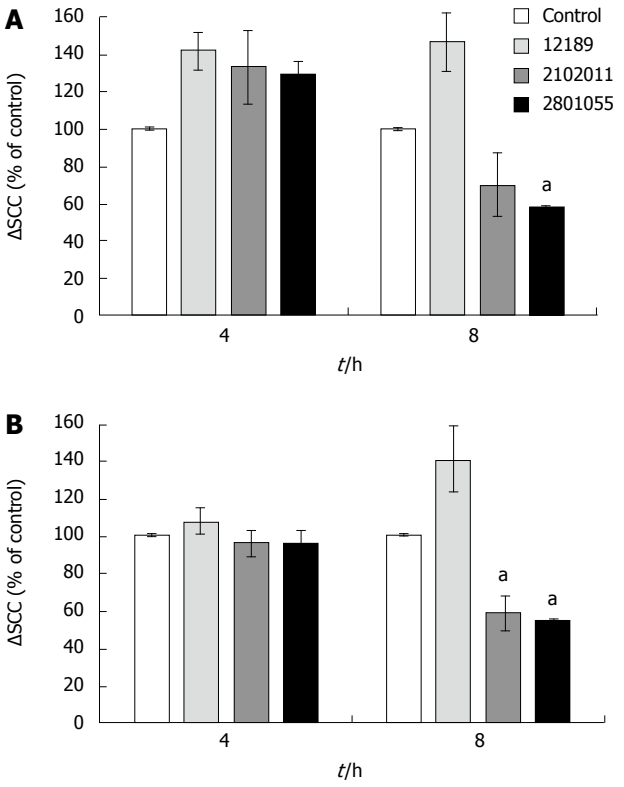

Figure 5 ΔSCC after stimulation with bradykinin (10^{-6} mol/L) and carbachol (10^{-4} mol/L) in monolayers inoculated with different strains of *C. jejuni*. Data are mean ± SE (n = 4) and are shown as percentage of control. A: Reduced chloride secretory response (represented by SCC) to bradykinin after 8 h inoculation with strain 2801055. $^aP < 0.05$; B: Reduced chloride secretory response to carbachol after 8 h inoculation with strains 2801055 and 2102011. $^aP < 0.05$.

lamina propria[36], is likely also to result in production of cytokines and chemokines. However our data show that epithelial cells can themselves be a source of mediators that could influence inflammatory and secretory processes in the case of strains that invade epithelial cells.

Although a paracellular route has been invoked to explain the ability of *C. jejuni* to invade the mucosa and achieve systemic infection, intracellular bacteria have been reported and a paracellular route of invasion inferred[37]. Using a modified gentamicin protection assay that allows for cell death, we were able to confirm cellular invasion by *C. jejuni*, and showed that this was associated with cytotoxicity and elaboration of pro-inflammatory and pro-secretory molecules. As yet it is uncertain whether intracellular invasion directly causes the associated release of IL-8 and PGE2, or whether this is a secondary consequence of cell death. A direct specific effect is possible since we have reported that IL-8 synthesis and PGE2 release from epithelial monolayers also occur in response to treatment with a boiled cell-free extract of *C. jejuni*[38], with induction of COX-2[39] and activation of nuclear factor-κB (NF-κB) and other relevant signaling pathways[40].

Enhanced release of IL-8 and other chemokines would play an important role in chemoattraction of neutrophils that characterizes some clinical infections with *C. jejuni*. Whether the increased prostaglandin synthesis that we observed with cellular invasion is sufficient under some circumstances to induce secretory diarrhea is more difficult to evaluate. We showed an early increase in bradykinin-induced secretion, as indicated by changes in SCC, with all strains tested, including non-invasive strains that did not stimulate prostaglandin synthesis. This increase in bradykinin-induced chloride secretion may therefore occur by prostaglandin-independent mechanisms. Direct epithelial action, *via* cAMP, cGMP, calcium mobilization, or induction of galanin or inducible nitric oxide are alternative mechanisms that are activated directly by bacterial enterotoxins or *via* signaling mechanisms that include NF-κB, which we and others have shown are upregulated by components of *C. jejuni*[24-27,40-43]. Destruction of the monolayer by strains that did invade epithelial cells and stimulate prostaglandin synthesis makes it difficult to evaluate whether enhanced prostaglandin synthesis by epithelial cells contributed to secretory diarrhea in these cases.

Campylobacter, like *Salmonella*, *Yersinia*, *Shigella* and *Listeria*, is an organism capable of translocation, as demonstrated by the current and previous monolayer studies and clinical features that include septicemia, Guillain-Barre syndrome and meningitis[16,33,34,41]. Previous studies have left unclear whether the main route is transcellular or paracellular. Both have been described. Our data suggest the translocation across the monolayer is common since all of the 19 clinical strains isolated from patients and one laboratory strain showed this property, regardless of whether they invaded epithelial cells and/or destroyed the monolayer. This suggests a dominant paracellular route of translocation, which is supported by our EM observations, which showed bacteria in the paracellular space. This appeared to occur efficiently, as judged by the rate of accumulation of bacteria on the basolateral side, and without gross disruption of tight or adherence junctions, as judged by the unchanged transepithelial resistance and mannitol permeability, seen with strains that translocated without epithelial cell invasion.

In the case of bacteria that invaded and destroyed the epithelial monolayer, translocation could be a crude consequence of its destruction, although bacteria that destroyed the monolayer did not appear to translocate faster than those that did not. Translocation results may differ according to the cell type used for epithelial monolayers. In previous studies that used differentiated CaCo2 cells, cellular invasion was not accompanied by the complete abrogation of monolayer resistance seen in our study and not all strains translocated[34]. We chose to use HCA7 cells in preference to CaCo2 cells because they have a differentiated large rather than small bowel phenotype and because they are capable of expressing COX-2 under induction conditions. Our data suggest that this is an informative model and cell line to study disease pathogenesis and signaling mechanisms[40].

COMMENTS

Background

Campylobacter jejuni (*C. jejuni*) is the commonest cause of bacterial diarrhea worldwide but its mode of pathogenesis is not clear.

Research frontiers

Since this work was done, the *Campylobacter* genome has been sequenced. Work following from the current study has investigated gene expression and has shown that chemokines play a central role.

Innovations and breakthroughs

The paper underlines the importance of allowing for cell destruction when doing gentamicin assays. Unlike many previous studies, this one used clinical isolates, which showed that there were two distinct patterns for the effect of *C. jejuni* on colonic epithelial cells. The cells themselves have the capacity to generate chemoattractant molecules, without necessary involvement of immune and other cells in the lamina propria.

Applications

Showing that *Campylobacter* spp. are cell invasive and stimulate production of chemoattractant mediators points to possible targets for treatment.

Terminology

Ussing chamber: Monolayers are grown on a permeable filter. When cells form tight junctions they cause resistance to an electrical current passed through the monolayer.

Peer review

The majority of previous studies regarding *C. jejuni* have been performed using a laboratory strain NCT11168. This manuscript is considered to contain attractive information for enhancing the understanding the mechanism of *C. jejuni* infection. It's an interesting paper.

REFERENCES

1 **Snelling WJ**, Matsuda M, Moore JE, Dooley JS. Campylobacter jejuni. *Lett Appl Microbiol* 2005; **41**: 297-302
2 **Young KT**, Davis LM, Dirita VJ. Campylobacter jejuni: molecular biology and pathogenesis. *Nat Rev Microbiol* 2007; **5**: 665-679
3 **Dorrell N**, Wren BW. The second century of Campylobacter research: recent advances, new opportunities and old problems. *Curr Opin Infect Dis* 2007; **20**: 514-518
4 **Jorgensen F**, Bailey R, Williams S, Henderson P, Wareing DR, Bolton FJ, Frost JA, Ward L, Humphrey TJ. Prevalence and numbers of Salmonella and Campylobacter spp. on raw, whole chickens in relation to sampling methods. *Int J Food Microbiol* 2002; **76**: 151-164
5 **Yao R**, Burr DH, Guerry P. CheY-mediated modulation of Campylobacter jejuni virulence. *Mol Microbiol* 1997; **23**: 1021-1031
6 **Guerry P**. Campylobacter flagella: not just for motility. *Trends Microbiol* 2007; **15**: 456-461
7 **Guerry P**, Ewing CP, Schirm M, Lorenzo M, Kelly J, Pattarini D, Majam G, Thibault P, Logan S. Changes in flagellin glycosylation affect Campylobacter autoagglutination and virulence. *Mol Microbiol* 2006; **60**: 299-311
8 **Konkel ME**, Kim BJ, Rivera-Amill V, Garvis SG. Bacterial secreted proteins are required for the internaliztion of Campylobacter jejuni into cultured mammalian cells. *Mol Microbiol* 1999; **32**: 691-701
9 **Ziprin RL**, Young CR, Byrd JA, Stanker LH, Hume ME, Gray SA, Kim BJ, Konkel ME. Role of Campylobacter jejuni potential virulence genes in cecal colonization. *Avian Dis* 2001; **45**: 549-557
10 **Poly F**, Ewing C, Goon S, Hickey TE, Rockabrand D, Majam G, Lee L, Phan J, Savarino NJ, Guerry P. Heterogeneity of a Campylobacter jejuni protein that is secreted through the flagellar filament. *Infect Immun* 2007; **75**: 3859-3867
11 **Johnson WM**, Lior H. A new heat-labile cytolethal distending toxin (CLDT) produced by Campylobacter spp. *Microb Pathog* 1988; **4**: 115-126
12 **Lara-Tejero M**, Galan JE. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. *Science* 2000; **290**: 354-357
13 **Hickey TE**, Majam G, Guerry P. Intracellular survival of Campylobacter jejuni in human monocytic cells and induction of apoptotic death by cytholethal distending toxin. *Infect Immun* 2005; **73**: 5194-5197
14 **Hickey TE**, McVeigh AL, Scott DA, Michielutti RE, Bixby A, Carroll SA, Bourgeois AL, Guerry P. Campylobacter jejuni cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. *Infect Immun* 2000; **68**: 6535-6541
15 **Hu L**, Hickey TE. Campylobacter jejuni induces secretion of proinflammatory chemokines from human intestinal epithelial cells. *Infect Immun* 2005; **73**: 4437-4440
16 **Yu RK**, Usuki S, Ariga T. Ganglioside molecular mimicry and its pathological roles in Guillain-Barre syndrome and related diseases. *Infect Immun* 2006; **74**: 6517-6527
17 **Perera VN**, Nachamkin I, Ung H, Patterson JH, McConville MJ, Coloe PJ, Fry BN. Molecular mimicry in Campylobacter jejuni: role of the lipo-oligosaccharide core oligosaccharide in inducing anti-ganglioside antibodies. *FEMS Immunol Med Microbiol* 2007; **50**: 27-36
18 **Oelschlaeger TA**, Guerry P, Kopecko DJ. Unusual microtubule-dependent endocytosis mechanisms triggered by Campylobacter jejuni and Citrobacter freundii. *Proc Natl Acad Sci USA* 1993; **90**: 6884-6888
19 **Harvey P**, Battle T, Leach S. Different invasion phenotypes of Campylobacter isolates in Caco-2 cell monolayers. *J Med Microbiol* 1999; **48**: 461-469
20 **Kopecko DJ**, Hu L, Zaal KJ. Campylobacter jejuni--microtubule-dependent invasion. *Trends Microbiol* 2001; **9**: 389-396
21 **Van Deun K**, Haesebrouck F, Heyndrickx M, Favoreel H, Dewulf J, Ceelen L, Dumez L, Messens W, Leleu S, Van Immerseel F, Ducatelle R, Pasmans F. Virulence properties of Campylobacter jejuni isolates of poultry and human origin. *J Med Microbiol* 2007; **56**: 1284-1289
22 **Wassenaar TM**. Toxin production by Campylobacter spp. *Clin Microbiol Rev* 1997; **10**: 466-476
23 **Kalischuk LD**, Inglis GD, Buret AG. Strain-dependent induction of epithelial cell oncosis by Campylobacter jejuni is correlated with invasion ability and is independent of cytolethal distending toxin. *Microbiology* 2007; **153**: 2952-2963
24 **Eckmann L**, Stenson WF, Savidge TC, Lowe DC, Barrett KE, Fierer J, Smith JR, Kagnoff MF. Role of intestinal epithelial cells in the host secretory response to infection by invasive bacteria. Bacterial entry induces epithelial prostaglandin h synthase-2 expression and prostaglandin E2 and F2alpha production. *J Clin Invest* 1997; **100**: 296-309
25 **Laurent F**, Kagnoff MF, Savidge TC, Naciri M, Eckmann L. Human intestinal epithelial cells respond to Cryptosporidium parvum infection with increased prostaglandin H synthase 2 expression and prostaglandin E2 and F2alpha production. *Infect Immun* 1998; **66**: 1787-1790
26 **Resta-Lenert S**, Barrett KE. Enteroinvasive bacteria alter barrier and transport properties of human intestinal epithelium: role of iNOS and COX-2. *Gastroenterology* 2002; **122**: 1070-1087
27 **Berkes J**, Viswanathan VK, Savkovic SD, Hecht G. Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. *Gut* 2003; **52**: 439-451
28 **Everest PH**, Cole AT, Hawkey CJ, Knutton S, Goossens H, Butzler JP, Ketley JM, Williams PH. Roles of leukotriene B4, prostaglandin E2, and cyclic AMP in Campylobacter jejuni-induced intestinal fluid secretion. *Infect Immun* 1993; **61**: 4885-4887
29 **Beltinger J**, Hawkey CJ, Stack WA. TGF-alpha reduces bradykinin-stimulated ion transport and prostaglandin release in human colonic epithelial cells. *Am J Physiol* 1999; **276**: C848-C855
30 **Ussing HH**, Zerahn K. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Reprinted from Acta. Physiol. Scand. 23: 110-127, 1951. *J Am Soc Nephrol* 1999; **10**: 2056-2065
31 **Adams RB**, Guerrant RL, Zu S, Fang G, Roche JK. Cryptosporidium parvum infection of intestinal epithelium: morphologic and functional studies in an in vitro model. *J*

Infect Dis 1994; **169**: 170-177
32 **Terres AM**, Pajares JM, Hopkins AM, Murphy A, Moran A, Baird AW, Kelleher D. Helicobacter pylori disrupts epithelial barrier function in a process inhibited by protein kinase C activators. *Infect Immun* 1998; **66**: 2943-2950
33 **Konkel ME**, Mead DJ, Hayes SF, Cieplak W Jr. Translocation of Campylobacter jejuni across human polarized epithelial cell monolayer cultures. *J Infect Dis* 1992; **166**: 308-315
34 **Bras AM**, Ketley JM. Transcellular translocation of Campylobacter jejuni across human polarised epithelial monolayers. *FEMS Microbiol Lett* 1999; **179**: 209-215
35 **Carew MA**, Thorn P. Carbachol-stimulated chloride secretion in mouse colon: evidence of a role for autocrine prostaglandin E2 release. *Exp Physiol* 2000; **85**: 67-72
36 **Jones MA**, Totemeyer S, Maskell DJ, Bryant CE, Barrow PA. Induction of proinflammatory responses in the human monocytic cell line THP-1 by Campylobacter jejuni. *Infect Immun* 2003; **71**: 2626-2633
37 **van Spreeuwel JP**, Duursma GC, Meijer CJ, Bax R, Rosekrans PC, Lindeman J. Campylobacter colitis: histological immunohistochemical and ultrastructural findings. *Gut* 1985; **26**: 945-951
38 **Mellits KH**, Mullen J, Wand M, Armbruster G, Patel A, Connerton PL, Skelly M, Connerton IF. Activation of the transcription factor NF-kappaB by Campylobacter jejuni. *Microbiology* 2002; **148**: 2753-2763
39 **Mellits KH**, Mullen J, Wand M, Smith J, Connerton I, Hawkey CJ. Activation of cellular genes by Campylobacter jejuni. *Gastroenterol* 2003; A1097
40 **Mellits KH**, Connerton IF, Loughlin MF, Clarke P, Smith J, Dillon E, Connerton PL, Hawkey CJ. Induction of a chemoattractant transcriptional response by campylobacter jejuni extract in colonocytes. *BMC Microbiology* 2009; **9:** 28
41 **Rinella ES**, Eversley CD, Carroll IM, Andrus JM, Threadgill DW, Threadgill DS. Human epithelial-specific response to pathogenic Campylobacter jejuni. *FEMS Microbiol Lett* 2006; **262**: 236-243
42 **Scott RO**, Thelin WR, Milgram SL. A novel PDZ protein regulates the activity of guanylyl cyclase C, the heat-stable enterotoxin receptor. *J Biol Chem* 2002; **277**: 22934-22941
43 **Matkowskyj KA**, Danilkovich A, Marrero J, Savkovic SD, Hecht G, Benya RV. Galanin-1 receptor up-regulation mediates the excess colonic fluid production caused by infection with enteric pathogens. *Nat Med* 2000; **6**: 1048-1051

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BASIC RESEARCH

Magnolol attenuates sepsis-induced gastrointestinal dysmotility in rats by modulating inflammatory mediators

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Abstract

AIM: To investigate the protective effects of magnolol on sepsis-induced inflammation and intestinal dysmotility.

METHODS: Sepsis was induced by a single intraperitoneal injection of lipopolysaccharide (LPS). Male Wistar rats were randomly assigned to one of three treatment groups: magnolol prior to LPS injection (LPS/Mag group); vehicle prior to LPS injection (LPS/Veh group); vehicle prior to injection of saline (Control/Veh). Intestinal transit and circular muscle mechanical activity were assessed 12 h after LPS injection. Tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1) and inducible nitric oxide synthase (iNOS) mRNA in rat ileum were studied by RT-PCR 2 h after LPS injection. Nuclear factor-κB (NF-κB) activity in the intestine was also investigated at this time using electrophoretic mobility shift assay. In addition, antioxidant activity was determined by measuring malondialdehyde (MDA) concentration and superoxide dismutase (SOD) activity in the intestine 2 h after LPS injection.

RESULTS: Magnolol significantly increased intestinal transit and circular muscle mechanical activity in LPS-treated animals. TNF-α, MCP-1 and iNOS mRNA expression in the small intestine were significantly reduced after magnolol treatment in LPS-induced septic animals, compared with untreated septic animals. Additionally, magnolol significantly increased IL-10 mRNA expression in septic rat ileum. Magnolol also significantly suppressed NF-κB activity in septic rat intestine. In addition, magnolol significantly decreased MDA concentration and increased SOD activity in rat ileum.

CONCLUSION: Magnolol prevents sepsis-induced suppression of intestinal motility in rats. The potential mechanism of this benefit of magnolol appears to be modulation of self-amplified inflammatory events and block of oxidative stress in the intestine.

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Key words: Sepsis; Motility; Cytokines; Magnolol; Lipopolysaccharide

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INTRODUCTION

Sepsis frequently occurs after trauma, burns, hemorrhage or abdominal surgery. It is a leading cause of morbidity and mortality in critically ill patients[1]. During sepsis, the most frequent complications within the gastrointestinal (GI) tract are ileus and mucosal barrier dysfunction[2]. Ileus plays an important role in the pathophysiology of sepsis by promoting bacterial stasis, bacterial overgrowth and bacterial translocation, which lead to the development of secondary infections and multiple organ failure[3].

Although common during sepsis, the etiology of ileus is still unclear. Current evidence supports the hypothesis that lipopolysaccharide (LPS) rapidly activates resident intestinal macrophages, which subsequently initiate a molecular and cellular inflammatory response that causes intestinal dysmotility[4-6]. Additionally, oxidative stress during sepsis may also be involved in this process[7]. Currently, there is no accepted pharmacological prevention or management of sepsis-induced intestinal

dysmotility. Blocking oxidative stress and modulating the inflammatory events might be helpful.

Magnolia officinalis, a traditional Chinese herb, is commonly used in the treatment of abdominal distention and vomiting associated with many clinical conditions. It has been reported to attenuate L-arginine-induced GI dysmotility in rodents[8], and to improve the electrical activity of GI smooth muscle during endotoxemia[9]. Recently, magnolol (5,5'-di-2-propenyl-1,1'-biphenyl-2,2'-diol), a principal constituent isolated from the bark of *Magnolia officinalis*, has been showed to attenuate peroxidative damage and to improve survival of rats with sepsis[10]. Treatment with magnolol after hemorrhagic shock can suppress the tumor necrosis factor-α (TNF-α) level and preserve interleukin-10 (IL-10) production in rats[11].

Thus, we have developed the hypothesis that through modulation of inflammatory cytokines during sepsis, magnolol may be helpful for treatment of sepsis-induced ileus. Therefore, the objective of the present study was to examine the capacity of magnolol pretreatment to prevent sepsis-induced intestinal dysmotility and to determine its effects on pro- and anti-inflammatory molecular responses in the local intestine.

MATERIALS AND METHODS

Animal preparation and experimental design

Male Wistar rats (250-300 g body weight) were obtained from the Academy of Military Medicine Sciences (Beijing, China). The rats were exposed to 12 h light and 12 h darkness each day, with free access to food and water. All experiments were performed in accordance with the institutional criteria for the care and use of laboratory animals in research. Sepsis was induced by a single intraperitoneal injection of LPS (*Escherichia coli*, O55: B5; Sigma, St Louis, MO, USA) at 20 mg/kg. Controls received intraperitoneal injections of saline.

Magnolol (National Institute for the Control of Pharmaceutical and Biological Products, China) was dissolved in 40% (v/v) propylene glycol and diluted to the desired concentration in normal saline. Final concentration of propylene glycol in the injected solution was < 4.0×10^{-3}% (v/v). The single dose used for the magnolol instillation was 10^{-5} g/kg, which was previously shown to be helpful for increasing survival of surgically induced sepsis[10]. Normal saline with 4.0×10^{-3}% (v/v) propylene glycol served as a vehicle.

Animals were randomly assigned to one of three treatment groups. LPS/Mag group: rats received magnolol (10^{-5} g/kg, intravenous bolus *via* the tail vein) 30 min before LPS injection; LPS/Veh group: rats received vehicle 30 min before LPS injection; Control/Veh: rats received vehicle 30 min before injection of saline. Preliminary results showed that intraperitoneal injection of LPS caused a profound suppression of intestine muscle contractile activity, which was both dose- and time-dependent. Furthermore, the effects of LPS are always rat strain specific and relate to the serotype of LPS[12]. In this study, we chose the 12-h time point for measurement of intestinal smooth muscle function. To elucidate the potential mechanism for magnolol preventing sepsis-induced ileus, we also evaluated changes in the chemokines and cytokines in the intestine 2 h after LPS injection, because the inflammatory response in the local intestine rapidly initiated by LPS is always responsible for GI dysmotility[4-6].

Intestinal transit

Twelve hours after LPS (or saline) was administered, the animals received an intragastric injection of 0.1 mL Evans blue (50 mg in 1 mL 0.9% NaCl). Then, the rats were killed by exsanguination 1 h later. Intestinal transit was determined by measuring the distance between the gastric pylorus and distal small intestine that was stained blue[13].

Measurement of muscle contractility

Circular muscle mechanical activity was assessed using full-thickness strips obtained from the ileum of each animal 12 h after LPS (or saline) injection. Muscle strips (2 × 10 mm) were placed in a mechanical organ chamber that was continuously perfused with pre-oxygenated Krebs-bicarbonate solution maintained at 37℃. One end of each strip was tied to a fixed post, and the other was attached to an isometric force transducer. After an equilibration period of 30 min, spontaneous mechanical contractions were recorded. The contractile responsiveness of muscle strips to the muscarinic receptor agonist bethanechol was also evaluated. Dose-response curves were generated by exposing the muscles to increasing concentrations of bethanechol (0.1-100 μmol/L) for 10 min; with intervening 20-min wash periods. Contractions were recorded, measured, and stored in a computer using a commercially available hardware and software package (TaiMeng Technology, Chengdu, China).

RT-PCR

To elucidate the potential mechanism of magnolol treatment blunting sepsis-induced intestinal dysmotility, mRNA for TNF-α, IL-10, monocyte chemoattractant protein 1 (MCP-1) and inducible nitric oxide synthase (iNOS) in rat ileum was assessed by RT-PCR.

Total mRNA was extracted with TRIZOL Reagent (GIBCO BRL, USA). Reverse transcription was performed using a Reverse Transcription System Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Primers were designed and purchased from AuGCT Biotechnology (Beijing, China). β-actin was used as an endogenous control. The sequences of the RT-PCR primers are listed in Table 1. PCR was performed with 25 μL reaction mixture of 1 μL RT product, 2 mmol/L $MgCl_2$, 0.03 U/L Taq DNA polymerase, 0.4 mmol/L dNTP, 0.1 μmol/L primer (endogenous control, target genes), and 1 × Taq DNA polymerase magnesium-free buffer. Then, the reaction

Table 1 Primer sequences

Gene	Primer sequences	Product size (bp)
β-actin	F 5' GAAATCGTGCGTGACATTA 3' R 5' TAGGAGCCAGGGCAGTAA 3'	349
TNF-α	F 5' GTAGCAAACCACCAAGCAG 3' R 5' GGTATGAAATGGCAAATCG 3'	211
IL-10	F 5' GCTATGTTGCCTGCTCTT 3' R 5' ATGCTCCTTGATTTCTGG 3'	307
MCP-1	F 5' ACTTGACCCATAAATCTGA 3' R 5' TGGAAGGGAATAGTGTAAT 3'	168
iNOS	F 5' TTGGGTCTTGTTAGCCTAGTC 3' R 5' TGTGCAGTCCCAGTGAGGAAC 3'	264

mixture was overlaid with two drops of mineral oil and incubated in a thermocycler (Eppendorf, Germany) programmed to pre-denature at 94℃ for 2 min, denatured at 94℃ for 30 s, annealed at 55℃ for 30 s, and extended at 72℃ for 30 s for a total of 30 cycles. The last cycle was followed by a final incubation at 72℃ for 6 min and cooling to 4℃. PCR products were electrophoresed on a 1.2% agarose gel and saved as digital images. Relative quantities of target gene mRNA were analyzed by Quantity One software (Bio-Rad Laboratories, USA), normalized with β-actin expression.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein of rat ileum was prepared by hypotonic lysis followed by high salt extraction[14,15]. Nuclear factor-κB (NF-κB) activity in the nuclear extract was analyzed using the EMSA kit according to the manufacturer's protocol (Gel Shift Assay System; Promega). In brief, an NF-κB oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') was end-labeled with [γ-32P] ATP and T4-polynucleotide kinase. Binding assays were performed in 10 μL binding reaction mixture that contained 10 μg nuclear proteins and [γ-32P]-labeled NF-κB oligonucleotides. The binding reaction mixture was incubated at room temperature for 20 min and then electrophoresed on 4% non-denaturing PAGE. After PAGE, the gels were dried and exposed to X-ray film. The autoradiograms were quantified by scanning densitometry, using Quantity One software (Bio-Rad US).

Detection of superoxide dismutase (SOD) and malondialdehyde (MDA) in ileum

To evaluate the antioxidative capacity of magnolol, MDA concentration and SOD activity in rat ileum were measured 2 h after LPS injection. Intestinal tissue samples were thawed, weighed and homogenized 1:9 (w/v) in 0.9% saline. The homogenates were centrifuged at 3000 r/min for 10 min at 4℃, and the supernatant was removed for the assay of MDA content, SOD activity and total protein.

Total intestinal protein concentration was determined using the Coomassie blue method, with bovine serum albumin as a standard. SOD activity and MDA level were detected with kits, according to the manufacturer's instructions (Jiancheng Bioengineering Ltd, Nanjing, China). Results were expressed as N/mg protein and nmol/mg protein, respectively.

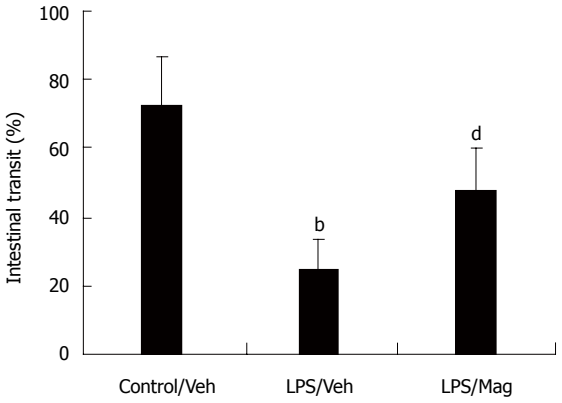

Figure 1 Magnolol prevents delayed small intestinal transit caused by LPS-induced sepsis. Data are shown as mean ± SE; $n = 6$. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{d}P < 0.01$ LPS/Mag *vs* LPS/Veh, LPS/Mag *vs* Control/Veh.

Statistical analysis

Data were expressed as mean ± SE. Statistical significance was determined by one-way ANOVA using SPSS 11.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Intestinal transit

As shown in Figure 1, LPS significantly delayed small intestinal transit from 74% ± 14% in control rats to 25% ± 9% in LPS rats. Pretreatment with magnolol significantly increased the transit in LPS animals, although this increase did not return to the control distribution pattern.

Changes in muscle contractility

The second series of experiments was designed to determine the effect of intravenous magnolol pretreatment on the intestinal musculature by measuring *in vitro* ileal circular muscle contractility from septic animals after LPS injection. Figure 2A shows the typical spontaneous contractility of circular muscle strips from three different animals. Analysis of the frequency of spontaneous contraction showed that muscle contractility in LPS-treated intestines was significantly lower than that in control tissues. Pretreatment with magnolol partly restored the spontaneous contractile pattern (Figure 2B).

Next, we evaluated the contractile response of muscle strips to the muscarinic receptor agonist bethanechol (0.1-100 μmol/L) using isometric force measurements.

As shown in Figure 3A, ileal circular muscle strips from LPS-treated animals showed significant impairment in the dose-response curve of bethanechol-stimulated muscle contraction. Magnolol treatment partly prevented LPS-induced impairment of ileal circular smooth muscle contractility. Figure 3B shows that, compared with

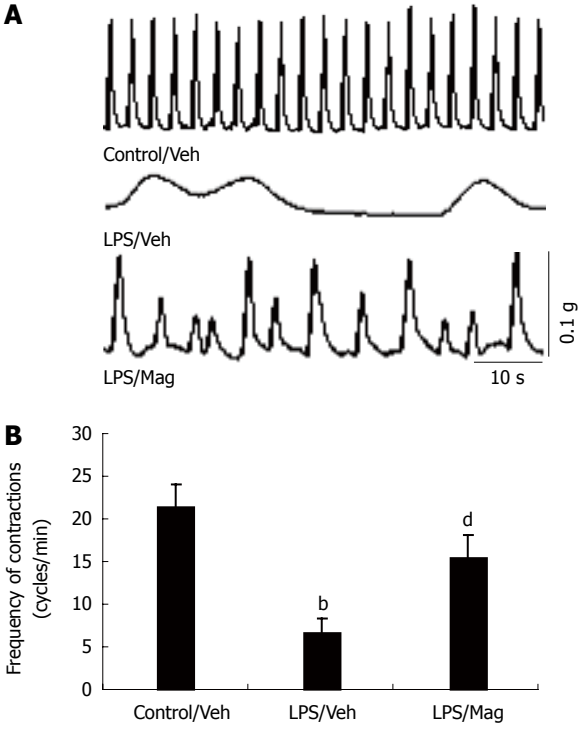

Figure 2 Change in spontaneous rhythmic contractions. A: Original traces of ileal circular muscle strip rhythmic contractions; B: Frequency of spontaneous rhythmic contractions. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{d}P < 0.01$ (LPS/Mag *vs* LPS/Veh) ($n = 6$).

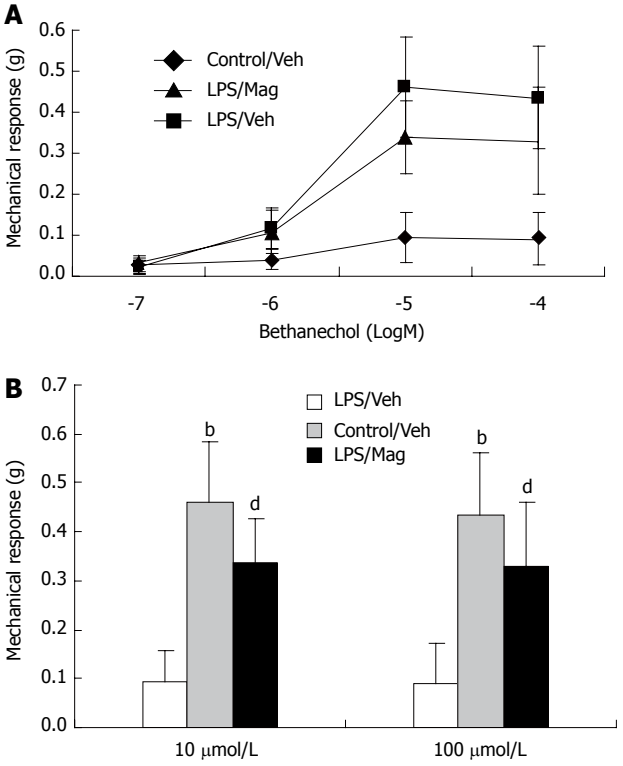

Figure 3 The contractile responsiveness of circular muscle strips to bethanechol. A: Bethanechol-stimulated dose-response curve; B: Circular muscle contractions at bethanechol concentration of 10 and 100 μmol/L. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{d}P < 0.01$ (LPS/Mag *vs* LPS/Veh) ($n = 6$).

controls, LPS significantly suppressed bethanechol-induced circular muscle contractions at bethanechol concentrations of 10 and 100 μmol/L. Magnolol treatment significantly increased the mechanical response of ileal circular muscles in LPS-treated animals.

The effect of magnolol treatment on GI motility of control rats was also evaluated. Neither intestinal transit nor circular muscle strip contractility was altered by magnolol (data not shown).

Molecular inflammatory responses

As shown in Figure 4, LPS induced a significant increase in TNF-α, IL-10 and MCP-1 mRNA levels in the ileum. Magnolol treatment significantly decreased LPS-induced TNF-α and MCP-1 mRNA expression. As for the anti-inflammatory mediator IL-10, magnolol significantly increased IL-10 mRNA expression in the ileum of LPS-treated animals.

iNOS has been shown to be the most important mediator of smooth muscle contraction during sepsis. Therefore, we also explored the effect of magnolol on iNOS mRNA expression in the ileum. Magnolol significantly suppressed LPS-induced iNOS mRNA expression.

NF-κB activity in rat intestine

NF-κB comprises a family of transcription factors that act as regulators of pro-inflammatory mediators[16]. We hypothesized that magnolol could potentially produce the above beneficial effects through decreased expression of NF-κB. As shown in Figure 5, LPS significantly induced activated NF-κB above control levels, and as hypothesized, magnolol significantly suppressed this response.

SOD and MDA in the small intestine

As shown in Figure 6, the MDA concentration in rat ileum, an index of lipid peroxidation, was significantly increased after LPS challenge compared with controls. Pretreatment with magnolol significantly decreased the MDA concentration. SOD activity in intestinal tissue decreased markedly in LPS-treated animals. Magnolol pretreatment caused a significant increase in SOD activity in rat ileum.

DISCUSSION

This study demonstrated the ability of magnolol, an antioxidant isolated from a Chinese herb, to prevent intestinal dysmotility in LPS-induced septic rats. It also provided evidence that the potential mechanism of action of magnolol results from both attenuation of peroxidative damage and modulation of the inflammatory response during sepsis.

Sepsis-induced ileus after complicated abdominal surgery, hemorrhagic shock, trauma and burns still causes morbidity and mortality in critically ill patients. Accumulating evidence has indicated that overwhelming pro-inflammatory and oxidative stress responses combined with diminished anti-inflammatory pathways are responsible for GI dysmotility during sepsis[4-7]. Unfortunately, there is no accepted pharmacological

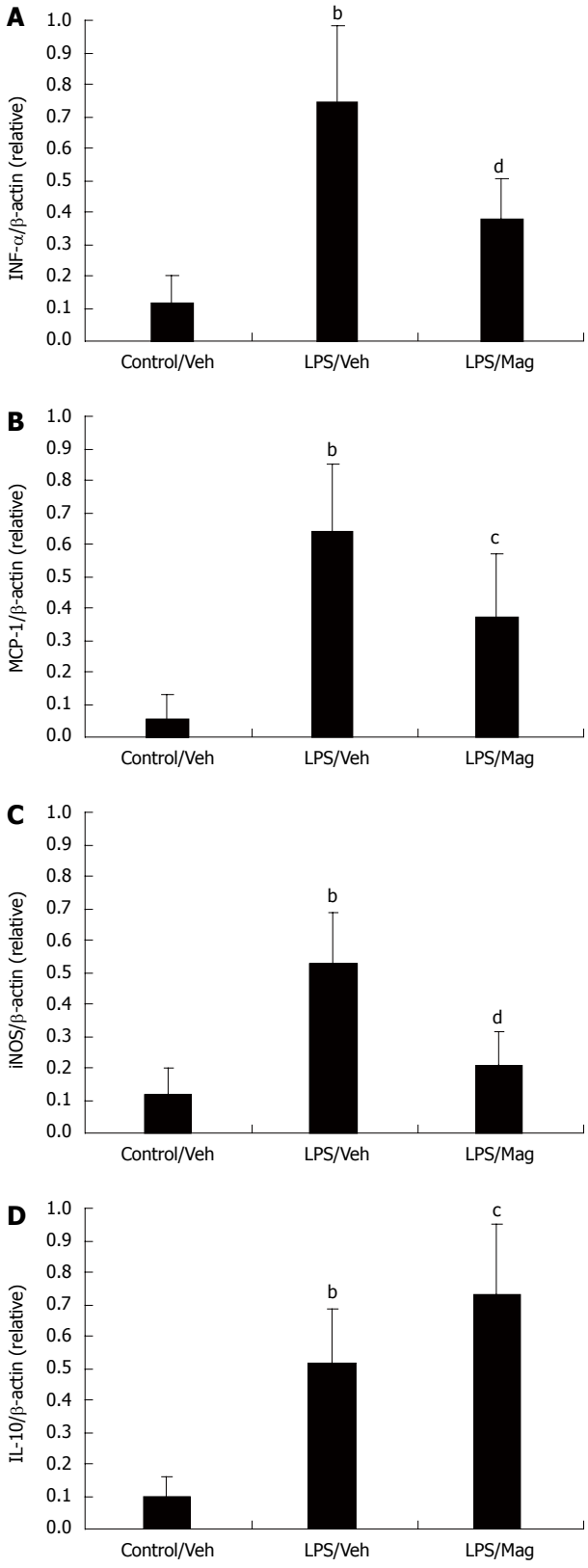

Figure 4 RT-PCR analysis of (A) TNF-α, (B) IL-10, (C) MCP-1, and (D) iNOS mRNA expression in rat ileum. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{c}P < 0.05$ (LPS/Mag *vs* LPS/Veh) ($n = 6$); $^{d}P < 0.01$ (LPS/Mag *vs* LPS/Veh).

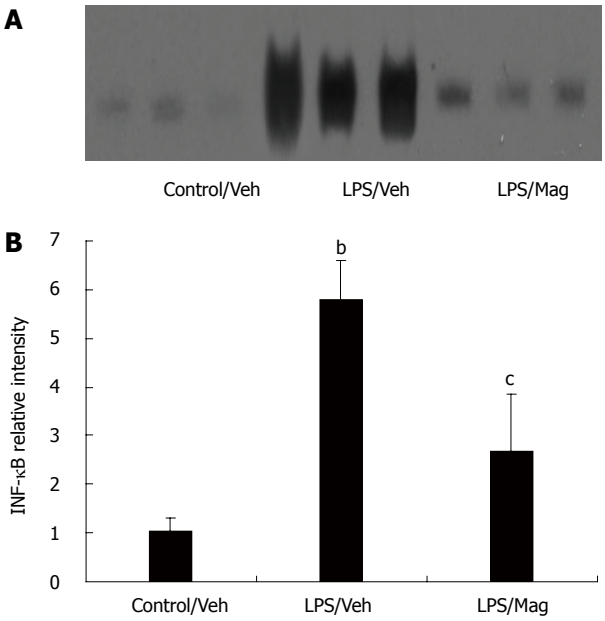

Figure 5 NF-κB activity in rat intestine. A: Representative EMSA gel; B: Scanning densitometry analysis of NF-κB activity. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{c}P < 0.05$ (LPS/Mag *vs* LPS/Veh) ($n = 3$).

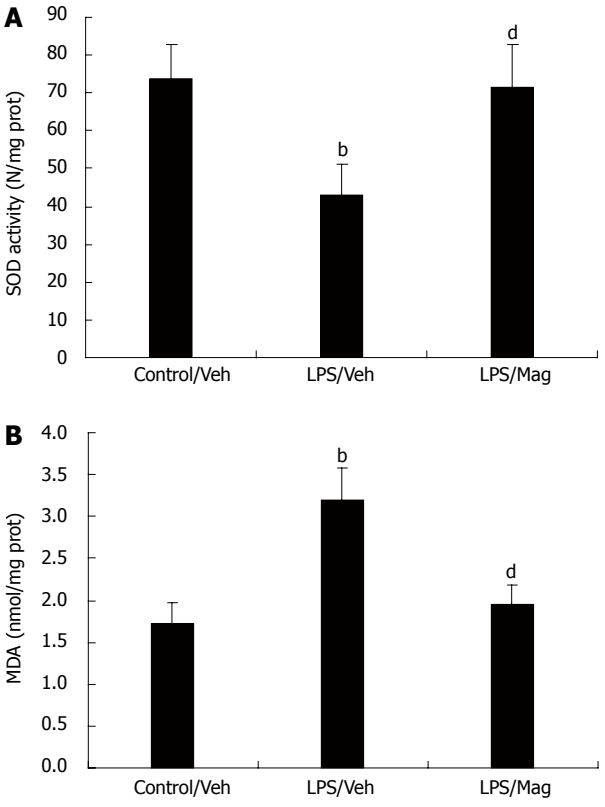

Figure 6 SOD activity (A) and MDA concentration (B) in rat ileum. $^{b}P < 0.01$ (LPS/Veh *vs* Control/Veh); $^{d}P < 0.01$ (LPS/Mag *vs* LPS/Veh) ($n = 6$).

prevention or management of sepsis-induced ileus at present. The present study demonstrated that magnolol can partly restore the delayed intestinal transit caused by LPS. Additionally, magnolol treatment can prevent LPS-induced impairment of ileal circular smooth muscle contractility.

Numerous studies have demonstrated that exaggerated production of oxygen-derived free radicals in the face of defective antioxidative protection occurs in animals and humans with sepsis[17-19]. This

imbalance between pro- and anti-oxidants may produce oxidative stress, which ultimately leads to cellular injury and necrosis, *via* several mechanisms including lipid peroxidation, protein denaturation, and DNA damage. *Magnolia officinalis* has been used as a blood-quickening and stasis-dispelling agent in traditional Chinese medicine. Magnolol, a compound purified from the bark of *Magnolia officinalis*, has been shown to be 1000 times more potent than α-tocopherol in inhibiting lipid peroxidation in rat heart mitochondria[20], and 50 000 times more potent than glutathione, a well-known antioxidant. It can also exhibit free radical scavenging activity. Moreover, it has been reported to suppress superoxide anion production in myocardium exposed to ischemia and reperfusion[21]. In accordance with these studies, we found that magnolol significantly attenuated the intensity of lipid peroxidation and increased SOD activity in rat ileum during sepsis. The antioxidant properties of magnolol are proposed to underlie its beneficial effects during sepsis.

We considered that prevention of sepsis-induced intestinal dysmotility by magnolol was partly through interruption of the cycle of inflammatory events in the local intestine. To confirm this hypothesis, we performed semi-quantitative RT-PCR on ileal tissue for inflammatory cytokines TNF-α, MCP-1 and iNOS, which have been shown to participate in leukocyte recruitment and functional muscle impairment[4,5,22]. The anti-inflammatory mediator IL-10 was also evaluated in our experiment. We found that magnolol significantly suppressed the initial surge of TNF-α at the gene level and increased IL-10 expression in septic rat intestine. Pro-inflammatory cytokines, such as TNF-α, have been shown to be released early after an inflammatory stimulus[23]. The increase in pro-inflammatory cytokines is followed by an increase in anti-inflammatory cytokines, such as IL-10, which reflect the compensatory anti-inflammatory response syndrome[24]. It has been reported that IL-10 can inhibit cytokine production in monocytes by blocking LPS-induced NF-κB activation[25]. Additionally, IL-10 modulates the production of various chemokines (such as MCP-1) and prevents generation of NO by LPS-activated monocytes/macrophages[26-28].

MCP-1 is a potent chemoattractant that is capable of promoting monocyte recruitment into an inflammatory site, as well as activating monocytes and macrophages[29,30]. It has previously been shown that regulation of leukocyte recruitment and subsequent intestinal smooth muscle dysmotility during endotoxemia is mediated through MCP-1, and that a major source of MCP-1 is the dense network of resident muscularis macrophages[13]. In this study, we used MCP-1 mRNA as our marker of chemokine activity. As mentioned above, magnolol significantly reduced intestinal MCP-1 mRNA expression during LPS-induced sepsis.

NO is known to be the main inhibitory neurotransmitter of the GI tract, caused by the activity of the constitutive isoform of neural NO synthase (cNOS) within the enteric nervous system[31]. Besides, NO is produced at almost all sites of inflammation by leukocytes through the activity of iNOS[32]. Evidence indicates that NO from iNOS plays a pivotal role in mediating LPS-induced suppression of intestinal smooth muscle activity[4], and that up-regulation of iNOS activity is mediated by TNF-α and IL-1[5]. Additionally, NO and superoxide anions can join to form the toxic metabolite $ONOO^-$[33,34], which is also involved in the pathogenesis of sepsis-induced motility disturbances[7]. Magnolol has been reported to suppress the overproduction of NO and TNF-α in LPS-activated macrophages[35]. The results obtained in the present study provide support for this view. Pretreatment with magnolol significantly decreased iNOS mRNA expression in the intestine of the LPS-treated animals.

NF-κB is an inducible nuclear transcription factor that plays a central role in regulating the transcription of many pro-inflammatory cytokines[16], including TNF-α and IL-1β. Furthermore, intricate negative and positive feedback loops exist within NF-κB activation and cytokine expression. Pro-inflammatory cytokines activate NF-κB, but IL-10 deactivates NF-κB[36]. In the present study, we found that magnolol significantly suppressed NF-κB activation in the intestine of septic rats, which suggests that magnolol modulates inflammatory cytokines may be through intervention in the NF-κB signal transduction system. In addition, magnolol might also inhibit NF-κB activation through increasing IL-10 gene expression.

During sepsis, oxidative stress causes direct damage to cells and tissues and is involved with inflammatory cytokine production[17]. Suppression of cytokines by antioxidants has been demonstrated in previous studies. *N*-acetyl-cysteine has been shown to prevent the priming of increased expression of TNF-α mRNA after LPS[37]. Also, it has been reported that the free-radical-trapping compound phenyl *N-tert*-butylnitrone administered in LPS-induced sepsis promotes enhanced production of endogenous IL-10[38]. Additionally, the involvement of oxidative stress or oxygen free radicals in NF-κB activation has been suggested[39]. Therefore, we assume that magnolol modulation of cytokine synthesis may be related to its antioxidant properties. This is in agreement with previous studies that have shown that gut injury is partly prevented by antioxidants[40]. However, this has not been proven experimentally.

Although the findings of the present study predict a role for magnolol in a clinical setting, several problems should be mentioned. We did not use the cecal ligation and puncture (CLP) sepsis model in our study, which appears to be a reliable and clinically relevant animal model of the human septic condition, because abdominal surgery can also initiate an inflammatory cascade and ultimately lead to impairment of intestinal smooth muscle activity. More intricate pathophysiological mechanisms may be involved in the development of gut dysmotility in the CLP sepsis model[41]. Additionally, Zhang *et al*[42] previously reported that, *in vitro*, magnolol exerted an inhibitory effect on isolated ileum of guinea pigs. However, we found in our study that *in vivo,* magnolol treatment could prevent LPS-induced suppression of intestinal motility but had no

effect on control animals. These discrepancies suggest that the pharmacological properties of magnolol on GI motility might change when it is administered at different doses or *via* different routes. At the dose and route that we used in our study, the antioxidant effect of magnolol could be the important mechanism through which it ameliorates the severity of sepsis. Under other pathophysiological conditions, whether magnolol could exert a similar effect is still not known. Other well-designed experiments are needed to further determine the clinical usefulness and safety of magnolol.

In conclusion, the data presented in this study suggest a protective role of magnolol in preventing sepsis-induced suppression of intestinal motility. The potential mechanism of this beneficial effect of magnolol appears to be modulation of the self-amplified inflammatory events and block of oxidative stress in the intestine.

COMMENTS

Background

During sepsis, gastrointestinal (GI) dysmotility occurs frequently. Accumulating evidence has indicated that overwhelming pro-inflammatory and oxidative stress responses combined with diminished anti-inflammatory pathways are responsible. Recently, magnolol, an antioxidant isolated from a traditional Chinese herb, has been showed to attenuate peroxidative damage and to improve survival of rats with sepsis. It can also suppress the TNF-α level and preserve IL-10 production in hemorrhagic shock in rats. Thus, the authors presumed that through modulation of inflammatory cytokines during sepsis, magnolol might be helpful for treatment of sepsis-induced ileus.

Research frontiers

Sepsis-induced GI dysmotility is a major problem in critically ill patients. The pharmacological intervention is difficult for the clinician to handle. In addition, there is a lack of controlled studies on which to build an evidence-based treatment concept for critically ill patients.

Innovations and breakthroughs

Currently, there is no accepted pharmacologic prevention or management of sepsis-induced GI dysmotility. Therefore, management remains largely supportive. Insights gained in this preliminary study might be helpful in producing an effective pharmacological intervention strategy.

Applications

This study provides the evidence that pretreatment with magnolol could attenuate sepsis-induced GI dysmotility. The potential mechanism of this benefit of magnolol appears to be modulation of the self-amplified inflammatory events and block of oxidative stress in the intestine.

Terminology

Sepsis is defined as infection plus systemic manifestations of infection. Cytokines: non-antibody proteins secreted by inflammatory leukocytes and some non-leukocytic cells, which act as intercellular mediators. Magnolol (5,5' -di-2-propenyl-1,1'-biphenyl-2,2'-diol), a principal constituent isolated from a traditional Chinese herb. Lipopolysaccharides (LPS) are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, act as endotoxins and elicit strong immune responses in animals.

Peer review

This preliminary study provides us with a new insight into management of sepsis-induced GI dysmotility. However, the pharmacological properties of magnolol may change when it is administered at different doses or *via* different routes. Other well-designed experiments are needed to further determine its clinical utility and safety.

REFERENCES

1 **Angus DC**, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 2001; **29**: 1303-1310

2 **Carrico CJ**, Meakins JL, Marshall JC, Fry D, Maier RV. Multiple-organ-failure syndrome. *Arch Surg* 1986; **121**: 196-208

3 **MacFie J**, O'Boyle C, Mitchell CJ, Buckley PM, Johnstone D, Sudworth P. Gut origin of sepsis: a prospective study investigating associations between bacterial translocation, gastric microflora, and septic morbidity. *Gut* 1999; **45**: 223-228

4 **Eskandari MK**, Kalff JC, Billiar TR, Lee KK, Bauer AJ. LPS-induced muscularis macrophage nitric oxide suppresses rat jejunal circular muscle activity. *Am J Physiol* 1999; **277**: G478-G486

5 **Lodato RF**, Khan AR, Zembowicz MJ, Weisbrodt NW, Pressley TA, Li YF, Lodato JA, Zembowicz A, Moody FG. Roles of IL-1 and TNF in the decreased ileal muscle contractility induced by lipopolysaccharide. *Am J Physiol* 1999; **276**: G1356-G1362

6 **Torihashi S**, Ozaki H, Hori M, Kita M, Ohota S, Karaki H. Resident macrophages activated by lipopolysaccharide suppress muscle tension and initiate inflammatory response in the gastrointestinal muscle layer. *Histochem Cell Biol* 2000; **113**: 73-80

7 **de Winter BY**, van Nassauw L, de Man JG, de Jonge F, Bredenoord AJ, Seerden TC, Herman AG, Timmermans JP, Pelckmans PA. Role of oxidative stress in the pathogenesis of septic ileus in mice. *Neurogastroenterol Motil* 2005; **17**: 251-261

8 **Wang HL**, Bai H, Wang XQ, Li Y. Experimental study of effect of Magnolia officinalis cotex on improving rat gastrointestinal motility. *Shiyong Yaowu Yu Linchuang* 2007; **10**: 65-66

9 **Ci XL**, Wang BE, Guo CY, Chen L. Experimental study of effect of Magnolia officinalis on improving the electricity activity of gastrointestinal smooth muscle in septic rats. *Zhongguo Zhongyiyao Keji* 1999; **6**: 154-156

10 **Kong CW**, Tsai K, Chin JH, Chan WL, Hong CY. Magnolol attenuates peroxidative damage and improves survival of rats with sepsis. *Shock* 2000; **13**: 24-28

11 **Shih HC**, Wei YH, Lee CH. Magnolol alters cytokine response after hemorrhagic shock and increases survival in subsequent intraabdominal sepsis in rats. *Shock* 2003; **20**: 264-268

12 **Eskandari MK**, Kalff JC, Billiar TR, Lee KK, Bauer AJ. Lipopolysaccharide activates the muscularis macrophage network and suppresses circular smooth muscle activity. *Am J Physiol* 1997; **273**: G727-G734

13 **Wirthlin DJ**, Cullen JJ, Spates ST, Conklin JL, Murray J, Caropreso DK, Ephgrave KS. Gastrointestinal transit during endotoxemia: the role of nitric oxide. *J Surg Res* 1996; **60**: 307-311

14 **Zhou W**, Jiang ZW, Tian J, Jiang J, Li N, Li JS. Role of NF-kappaB and cytokine in experimental cancer cachexia. *World J Gastroenterol* 2003; **9**: 1567-1570

15 **Gong JP**, Liu CA, Wu CX, Li SW, Shi YJ, Li XH. Nuclear factor kB activity in patients with acute severe cholangitis. *World J Gastroenterol* 2002; **8**: 346-3492

16 **Abraham E**. NF-kappaB activation. *Crit Care Med* 2000; **28**: N100-N104

17 **Goode HF**, Webster NR. Free radicals and antioxidants in sepsis. *Crit Care Med* 1993; **21**: 1770-1776

18 **Cuzzocrea S**, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacol Rev* 2001; **53**: 135-159

19 **Albuszies G**, Bruckner UB. Antioxidant therapy in sepsis. *Intensive Care Med* 2003; **29**: 1632-1636

20 **Lo YC**, Teng CM, Chen CF, Chen CC, Hong CY. Magnolol and honokiol isolated from Magnolia officinalis protect rat heart mitochondria against lipid peroxidation. *Biochem Pharmacol* 1994; **47**: 549-553

21 **Lee YM**, Hsiao G, Chen HR, Chen YC, Sheu JR, Yen MH.

Magnolol reduces myocardial ischemia/reperfusion injury via neutrophil inhibition in rats. *Eur J Pharmacol* 2001; **422**: 159-167

22 **Turler A**, Schwarz NT, Turler E, Kalff JC, Bauer AJ. MCP-1 causes leukocyte recruitment and subsequently endotoxemic ileus in rat. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G145-G155

23 **Hesse DG**, Tracey KJ, Fong Y, Manogue KR, Palladino MA Jr, Cerami A, Shires GT, Lowry SF. Cytokine appearance in human endotoxemia and primate bacteremia. *Surg Gynecol Obstet* 1988; **166**: 147-153

24 **Molloy RG**, Mannick JA, Rodrick ML. Cytokines, sepsis and immunomodulation. *Br J Surg* 1993; **80**: 289-297

25 **Wang P**, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *J Biol Chem* 1995; **270**: 9558-9563

26 **de Waal Malefyt R**, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991; **174**: 1209-1220

27 **Fiorentino DF**, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991; **147**: 3815-3822

28 **Ikeda T**, Sato K, Kuwada N, Matsumura T, Yamashita T, Kimura F, Hatake K, Ikeda K, Motoyoshi K. Interleukin-10 differently regulates monocyte chemoattractant protein-1 gene expression depending on the environment in a human monoblastic cell line, UG3. *J Leukoc Biol* 2002; **72**: 1198-1205

29 **Leonard EJ**, Yoshimura T. Human monocyte chemoattractant protein-1 (MCP-1). *Immunol Today* 1990; **11**: 97-101

30 **Fuentes ME**, Durham SK, Swerdel MR, Lewin AC, Barton DS, Megill JR, Bravo R, Lira SA. Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1. *J Immunol* 1995; **155**: 5769-5776

31 **Stark ME**, Bauer AJ, Szurszewski JH. Effect of nitric oxide on circular muscle of the canine small intestine. *J Physiol* 1991; **444**: 743-761

32 **Billiar TR**. Nitric oxide. Novel biology with clinical relevance. *Ann Surg* 1995; **221**: 339-349

33 **Kruidenier L**, Verspaget HW. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Aliment Pharmacol Ther* 2002; **16**: 1997-2015

34 **Szabo C**. The pathophysiological role of peroxynitrite in shock, inflammation, and ischemia-reperfusion injury. *Shock* 1996; **6**: 79-88

35 **Son HJ**, Lee HJ, Yun-Choi HS, Ryu JH. Inhibitors of nitric oxide synthesis and TNF-alpha expression from Magnolia obovata in activated macrophages. *Planta Med* 2000; **66**: 469-471

36 **Blackwell TS**, Christman JW. The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol* 1997; **17**: 3-9

37 **Fan J**, Kapus A, Li YH, Rizoli S, Marshall JC, Rotstein OD. Priming for enhanced alveolar fibrin deposition after hemorrhagic shock: role of tumor necrosis factor. *Am J Respir Cell Mol Biol* 2000; **22**: 412-421

38 **Kotake Y**, Sang H, Tabatabaie T, Wallis GL, Moore DR, Stewart CA. Interleukin-10 overexpression mediates phenyl-N-tert-butyl nitrone protection from endotoxemia. *Shock* 2002; **17**: 210-216

39 **Schreck R**, Baeuerle PA. Assessing oxygen radicals as mediators in activation of inducible eukaryotic transcription factor NF-kappa B. *Methods Enzymol* 1994; **234**: 151-163

40 **Deitch EA**. Multiple organ failure. Pathophysiology and potential future therapy. *Ann Surg* 1992; **216**: 117-134

41 **Overhaus M**, Tögel S, Pezzone MA, Bauer AJ. Mechanisms of polymicrobial sepsis-induced ileus. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G685-G694

42 **Zhang WW**, Li Y, Wang XQ, Tian F, Cao H, Wang MW, Sun QS. Effects of magnolol and honokiol derived from traditional Chinese herbal remedies on gastrointestinal movement. *World J Gastroenterol* 2005; **11**: 4414-4418

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RAPID COMMUNICATION

Adverse events with bismuth salts for *Helicobacter pylori* eradication: Systematic review and meta-analysis

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Abstract

AIM: To assess the safety of bismuth used in *Helicobacter pylori* (*H pylori*) eradication therapy regimens.
METHODS: We conducted a systematic review and meta-analysis. MEDLINE and EMBASE were searched (up to October 2007) to identify randomised controlled trials comparing bismuth with placebo or no treatment, or bismuth salts in combination with antibiotics as part of eradication therapy with the same dose and duration of antibiotics alone or, in combination, with acid suppression. Total numbers of adverse events were recorded. Data were pooled and expressed as relative risks with 95% confidence intervals (CI).
RESULTS: We identified 35 randomised controlled trials containing 4763 patients. There were no serious adverse events occurring with bismuth therapy. There was no statistically significant difference detected in total adverse events with bismuth [relative risk (RR) = 1.01; 95% CI: 0.87-1.16], specific individual adverse events, with the exception of dark stools (RR = 5.06; 95% CI: 1.59-16.12), or adverse events leading to withdrawal of therapy (RR = 0.86; 95% CI: 0.54-1.37).
CONCLUSION: Bismuth for the treatment of *H pylori* is safe and well-tolerated. The only adverse event occurring significantly more commonly was dark stools.

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Key words: Bismuth; Eradication therapy; *Helicobacter pylori*; Adverse events; Systematic review; Meta-analysis

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INTRODUCTION

Bismuth salts have been used for centuries in medicine. From a gastroenterology perspective these drugs have been used to treat peptic ulcer disease, dyspepsia, parasitic infections, microscopic colitis, and infectious diarrhoea[1]. The discovery of *Helicobacter pylori* (*H pylori*) in 1983 by Warren and Marshall revolutionised the management of peptic ulcer disease[2], and led to a renewed interest in bismuth compounds, largely because bismuth was found to inhibit the growth of *H pylori* and was effective in eradicating the organism (when combined with antibiotics or in combination with antibiotics and acid suppression therapy[3,4]).

The first randomised controlled trial (RCT) of bismuth in *H pylori*-positive individuals suggested that bismuth was superior to erythromycin monotherapy in eradicating the infection[5]. A further RCT of 6 wk of colloidal bismuth subcitrate versus cimetidine, in *H pylori*-positive duodenal ulcer patients, demonstrated that bismuth successfully eradicated the bacterium in up to 50% of patients[6]. Subsequently, an RCT of both colloidal bismuth subcitrate and cimetidine, alone or in combination with tinidazole, confirmed that colloidal bismuth subcitrate and tinidazole cleared the infection in almost 75% of patients[7]. With the addition of a second antibiotic, tetracycline or amoxicillin, eradication rates in later RCTs exceeded 80%[8-10]. However, there were some problems associated with bismuth-based triple therapy, which included the number of tablets patients were required to take, the duration of therapy, and side effects such as altered taste, nausea, and diarrhoea.

There are a variety of bismuth salts currently available on the market. All are inorganic, poorly soluble and therefore less than 1% is typically absorbed systemically[11]. Blood concentrations of bismuth do rise when these compounds are ingested however, and there is therefore the potential for toxicity, though levels less than 50 μg/mL are unlikely to be associated with any meaningful toxicity in man[11]. In the 1970s, high doses of bismuth salts were used for long periods and were associated with neurotoxicity. In France, there were almost 1000 cases of bismuth-associated encephalopathy of which 72 were fatal[1]. The doses of bismuth used in *H pylori* eradication are administered for a much shorter duration, typically 1 to 2 wk. In a recent bioavailability study, where bismuth salts were given in combination with omeprazole for 6 d[12], plasma levels of bismuth remained well below 50 μg/mL, but a review of their safety profile would provide additional evidence that such low doses of bismuth, given for a short period of time, do not expose patients to undue risks. We have therefore conducted a systematic review and meta-analysis of available published literature to assess the magnitude of the risk of adverse events experienced when bismuth salts are used, either alone or in combination with one or more antibiotics, to eradicate *H pylori*.

MATERIALS AND METHODS

Outcomes assessed

Primary outcomes: The primary aim of this systematic review and meta-analysis was to assess the total number of adverse events occurring following treatment for *H pylori* with bismuth compounds, either alone, or in combination with antibiotics and/or acid suppression therapy, compared to treatment with antibiotics alone, acid suppression therapy alone, a combination of the two, or no treatment/placebo.

Secondary outcomes: The secondary aims were to evaluate the number of specific individual adverse events occurring and the number of withdrawals of therapy due to adverse events, and to assess the effect of long-term (defined as 1 mo or more) therapy on number of adverse events (both total number and by specific category) and withdrawals due to adverse events.

Eligibility criteria

Types of studies: In order to best estimate adverse events that were directly attributable to the use of bismuth, studies were only eligible for inclusion in this systematic review if they were RCTs that compared bismuth monotherapy with either acid suppression therapy alone, placebo, or no treatment, or compared bismuth compounds in combination with either antibiotics, or antibiotics and acid suppression therapy as part of a recognised efficacious eradication regimen with an identical dose and duration of antibiotics either alone or in combination with acid suppression therapy. We defined an efficacious bismuth-containing eradication regimen as any one of: bismuth triple therapy (bismuth in combination with two antibiotics); bismuth quadruple therapy (as for triple therapy, but with the addition of acid suppression therapy); or ranitidine bismuth citrate dual (with one antibiotic) or triple (with two antibiotics) therapy.

Types of participants: Patients were required to be *H pylori*-positive adults (over the age of 16 years) taking any bismuth compound for more than 1 d with a comparison group of *H pylori*-positive patients who were not taking bismuth.

Types of assessment: Bismuth toxicity had to be assessed and recorded using one or more of the following methods: medical databases; face-to-face interviews; telephone interviews; symptom diaries; or questionnaire in order for studies to be eligible for inclusion. The questionnaire used was not required to be previously validated but, if there were sufficient studies using questionnaires, we aimed to assess the impact of this in a sensitivity analysis.

Types of outcome measures: The proportion of patients that reported any adverse event and the proportion experiencing specific individual adverse events were assessed wherever trial reporting allowed this.

Search strategy and identification of eligible studies

Search strategy: Two authors performed searches of the medical literature to identify articles from MEDLINE (from 1966 up to October 2007), EMBASE (from 1988 up to October 2007), and the Cochrane Library and Current Contents electronic databases. RCTs using bismuth salts were identified using the medical subject heading term "bismuth". These studies were combined using the set operator and with papers that used a variety of free text terms including "Denol", "Pepto-Bismol", "bismuth", "subsalicylate", "tripotassium dicitrato bismuthate", "subnitrate", "subgallate", "ranitidine bismuth citrate", "pylorid", "quadruple therapy", "pylera", and "bismuth subcitrate potassium". There were no language restrictions, and papers published in abstract form only were also eligible for inclusion in the review. The abstracts of all papers identified by the initial search were evaluated for appropriateness to the study question, and all potentially relevant studies were retrieved and examined in greater detail to determine whether or not they met all eligibility criteria. The bibliographies of identified studies were then used to perform a recursive search of the literature to identify other potentially eligible studies. In addition, Digestive Disease Week, United European Gastroenterology Week, and European *H pylori* Study Group conference abstract books between 2000 and 2007 were hand-searched.

Selection of studies: Two reviewers screened all titles and abstracts of trials that were identified by the search strategy as being potentially eligible for inclusion in the systematic review to confirm or refute eligibility. This was performed using pre-designed eligibility forms. A third reviewer adjudicated where any disagreements arose, and a consensus view was taken.

Assessment of study quality: The quality of studies was assessed according to the following pre-defined criteria: method of assessment of occurrence of adverse events (interview, diary, and questionnaire), generation of randomisation schedule, method of allocation of concealment, and blinding of assessor as to patient allocation to therapy.

Data extraction

Data concerning total number of adverse events and number of specific individual adverse events were extracted on to specially developed forms by two reviewers and all data extraction was checked by a third reviewer. These verified data were then entered onto a Microsoft Excel spreadsheet (XP professional edition; Microsoft Corp, Redmond, WA, USA), and again this was double-checked by a third reviewer. Trial characteristics including setting (population-based, primary care, secondary care), country of origin, number of centres involved, duration of bismuth therapy and dosage schedule, type of bismuth compound, mean age of included patients, and proportion of male patients were recorded to allow exploration of potential reasons for any heterogeneity detected between trial results.

Data synthesis and statistical analysis

Data were extracted as dichotomous outcomes and pooled using a random effects model[13], where sufficient data were available. The impact of bismuth therapy on the incidence of total and specific individual adverse effects *versus* comparison regimen was expressed as a combined relative risk (RR) with a 95% confidence interval (CI). The number needed to harm with bismuth therapy to cause one adverse event, and a 95% CI, were calculated as the reciprocal of the risk difference from the meta-analysis, and where this was statistically significant the results were reported.

Due to differences in methodology, patient populations, and outcome measures between eligible trials, the results of individual studies can be very diverse and therefore when they are included in the same meta-analysis this may affect the accuracy of the overall result. This inconsistency within a single meta-analysis can be quantified with a statistical test of heterogeneity, to assess whether the variation across trials is due to true heterogeneity, or chance. This quantity is termed I^2, and its value ranges from 0 to 100 percent, with 0 percent representing no observed heterogeneity, and larger values indicating increasing heterogeneity. A value below 25 percent is arbitrarily chosen to represent low levels of heterogeneity[14]. Where the degree of statistical heterogeneity is greater than this, clinical reasons within individual trials that may account for some of this inconsistency can be explored. Wherever statistically significant heterogeneity existed between trial results in this systematic review, possible explanations were investigated informally using sensitivity analyses. These are exploratory only, and may explain some of the observed variability, but the results should be interpreted with caution.

All statistical analyses were performed using Stats Direct version 2.2.4 (Stats Direct Ltd, Sale, Cheshire, UK), which was used to generate Forest plots of pooled relative risks for total adverse event rates and specific individual adverse event rates by category, as well as funnel plots to assess for evidence of publication bias.

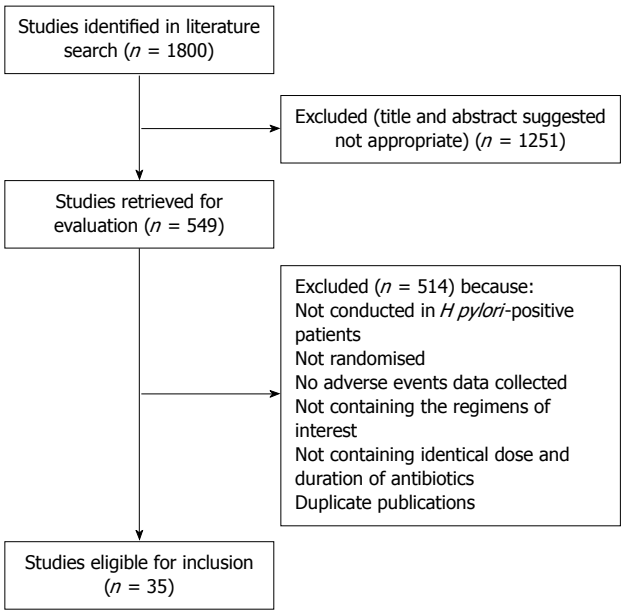

Figure 1 Flow diagram of assessment of studies identified in the systematic review.

RESULTS

Selection of eligible studies

The search strategy identified 1800 studies, of which 549 were possibly eligible. After reviewing the abstracts of these it became clear that 209 were RCTs of bismuth, and these were retrieved for further assessment. Of these, 35 were eligible for inclusion in the meta-analysis[7,15-48], reporting on 4763 *H pylori*-positive patients, 2435 of whom received bismuth or bismuth-based regimen, and 2328 received a comparison regimen (Figure 1). Thirty-three of the trials were found in fully published form, and two were only published as abstracts[29,44]. Seven of the RCTs used more than one bismuth-containing regimen[7,20,31,34,35,41,47].

Trial characteristics

Detailed trial characteristics are provided in Table 1. Nineteen of the trials were conducted in Europe[15-18,20,23-25,27-30,37,40,42-45,47], eight in the Far East[21,22,32,35,36,38,46,48], four in the USA[31,33,34,41], one in the Middle East[26], one in South America[19], one in Australia[7], and one was a multi-national study[39]. Eleven of the studies were multicentre RCTs[15,21,25,29,31,32,34,39,41,44,45]. Duration of bismuth therapy ranged from 7 to 56 d, with a total daily dose of between 400 mg and 2100 mg. Nineteen studies used ranitidine bismuth citrate[15,17,20,22,24,28-32,34,36,39-41,43,45,46,48], ten studies colloidal bismuth subcitrate[7,16,18,21,26,27,37,38,42,44], two studies tripotassium dictrato bismuthate[23,35], two studies bismuth subsalicylate[25,33], one study bismuth subnitrate[19], and one study both bismuth subnitrate and

Table 1 Characteristics of included studies

Study	Country	No. of centres	Bismuth compound used[1]	Duration of bismuth therapy (days)	Total dose (mg/d) used	Method of collection of adverse event data	Generation of randomization schedule provided	Method of concealment of allocation provided	Double-blind
Bujanda 2001[15]	Spain and Portugal	Multi-centre	RBC	7	800	Unclear	Yes	No	Yes
Burette 1992[16]	Belgium	1	CBS	10	480	Unclear	No	No	No
Buzas 2001[17]	Hungary	1	RBC	7	800	Unclear	No	No	No
Carpintero 1997[18]	Spain	1	CBS	42	480	Unclear	Yes	No	No
Carvalho 1998[19]	Brazil	1	BSN	14	1200	Unclear	No	No	No
Catalano 2000[20]	Italy	1	RBC	10	800	Questionnaire	Yes	No	Yes
Chuang 2001[22]	Taiwan	1	RBC	7	800	Unclear	No	No	Yes
Dal Bo 1998[23]	Italy	1	TDB	14	480	Unclear	No	No	No
Danese 2001[24]	Italy	1	RBC	7	800	Validated questionnaire	No	No	No
Eberhardt 1990[25]	Germany	4	BSS	28	1800	Unclear	No	No	No
Fakheri 2004[26]	Iran	1	CBS	14	480	Unclear	Yes	No	No
Forne 1995[27]	Spain	1	CBS	7	480	Diary cards	No	Yes	No
Gasbarrini 2000[28]	Italy	1	RBC	7	800	Validated questionnaire	No	No	No
Georgopoulos 1999[29]	Greece	3	RBC	7	800	Unclear	No	No	No
Gisbert 2000[30]	Spain	1	RBC	7	800	Unclear	Yes	No	No
Graham 1998[31]	USA	111	RBC	28	800	Unclear	No	No	Yes
Hung 2002[32]	Hong Kong	3	RBC	7	800	Diary	Yes	Yes	No
Lanza 1989[33]	USA	1	BSS	21	2100	Unclear	No	No	Yes
Lanza 1998[34]	USA	47	RBC	28	800	Diary cards	No	No	Yes
Liu 1999[35]	China	1	TDB	7	480	Diary	No	No	No
Mao 2000[36]	Vietnam	1	RBC	10	400	Diary	No	No	No
Marshall 1988[7]	Australia	1	CBS	56	480	Unclear	No	No	Yes
Masci 1995[37]	Italy	1	CBS	28 to 56	480	Unclear	Yes	No	Yes
Nafeeza 1992[38]	Malaysia	1	CBS	28	480	Unclear	No	No	Yes
Pare 1999[39]	Multi-national	Multi-centre	RBC	28	800	Unclear	Yes	No	Yes
Perri 2002[40]	Italy	1	RBC	7	800	Questionnaire	No	No	No
Peterson 1996[41]	USA	38	RBC	28	800	Unclear	No	No	Yes
Rokkas 1988[42]	UK	1	CBS	56	480	Unclear	No	No	Yes
Spadaccini 1998[43]	Italy	1	RBC	7	800	Face-to-face interview	No	No	No
Spiliadis 1998[44]	Greece	3	CBS	14	1200	Unclear	No	No	No
Spinzi 2000[45]	Italy	6	RBC	7	800	Face-to-face interview	No	No	No
Sung 1998[46]	Hong Kong	1	RBC	7	800	Telephone interview	No	No	No
Whitehead 2000[47]	UK	1	CBS and BSN	28	Unclear	Unclear	Yes	Yes	Yes
Wong 2001[48]	Hong Kong	1	RBC	7	800	Diary	Yes	Yes	No
Xiao 2001[21]	China	Multi-centre	CBS	7	480	Diary	No	No	No

[1]BSN: Bismuth subnitrate; BSS: Bismuth subsalicylate; CBS: Colloidal bismuth subcitrate; RBC: Ranitidine bismuth citrate; TDB: Tripotassium dictrato bismuthate.

colloidal bismuth subcitrate[47]. Comparison regimens were proton pump inhibitor or H_2-receptor antagonist (H_2-RA)-based eradication therapy in 23 studies[15,17-24,26-30,32,35,36,39,40,43,45,46,48], antibiotics alone in four studies[16,38,44,47], antibiotics or placebo in three studies[31,34,41], H_2-RA alone in two studies[25,37], placebo alone in two studies[33,42], and H_2-RA in combination with either one antibiotic or placebo in one study[7]. The mean age of individuals in included studies ranged from 36.7 years to 50.5 years, and the proportion of male patients varied between 32 percent and 78 percent. The number of participants in each RCT ranged from 20 to 530 individuals.

Trial quality

Thirteen of the trials were double-blind randomised studies[7,15,20,22,31,33,34,37-39,41,42,47], the remainder being either single-blind or open. Five of the single-blind trials specifically stated that assessors were blinded to treatment allocation[21,24,45,46,48]. Ten of the studies reported the method of generation of the randomization schedule[15,18,20,26,30,32,37,39,47,48], but only four the method of concealment of allocation[27,32,47,48]. Four of the studies recorded adverse events using a questionnaire[20,24,28,40], but only two of these stated that the questionnaire was validated[24,28]. Seven studies collected information concerning adverse events using a diary or diary cards[21,27,32,34-36,48], two *via* face-to-face interview[43,45], and one *via* telephone interview[46]. The remainder of trials did not state how they collected adverse events data.

Total number of adverse events with bismuth or bismuth-containing regimen versus comparison regimen

There were no serious adverse events such as death or neurotoxicity in either arm of any of the included

Table 2 Crude adverse event rates, and relative risk of adverse events

Adverse event	Number of trials	Total number of patients	Number of patients in bismuth arms	Number of patients in comparison arms	Number of adverse events in bismuth arms (%)	Number of adverse events in comparison arms (%)	Relative risk of adverse events with bismuth versus comparison regimen (95% CI)
Any	25	3180	1585	1595	431 (27.2)	419 (26.3)	1.01 (0.87-1.16)
Abdominal pain	13	2439	1221	1218	63 (5.2)	61 (5.0)	1.06 (0.64-1.74)
Dark stools	4	467	233	234	39 (16.7)	5 (2.1)	5.06 (1.59-16.12)
Diarrhoea	22	3406	1761	1645	124 (7.0)	113 (6.9)	1.01 (0.72-1.42)
Dizziness	8	1630	867	763	54 (6.2)	49 (6.4)	1.18 (0.81-1.72)
Headache	14	2433	1276	1157	41 (3.2)	28 (2.4)	1.31 (0.81-2.11)
Metallic taste	14	2475	1260	1215	124 (9.8)	116 (9.6)	1.02 (0.81-1.28)
Nausea and/or vomiting	20	3417	1767	1650	111 (6.3)	86 (5.2)	1.16 (0.89-1.52)
Leading to withdrawal of therapy	28	3951	2033	1918	33 (1.6)	38 (2.0)	0.86 (0.54-1.37)

RCTs. Twenty-five trials reported the total number of individuals experiencing any adverse event with bismuth or bismuth-containing regimens *versus* comparison regimen[15-20,23-27,30,32,33,35-40,42,44,45,47,48]. Three of these studies utilized more than one regimen[20,35,47], allowing 28 comparisons to be made. The relative risk of an adverse event with bismuth or bismuth-containing regimens versus comparison regimen was 1.01 (95% CI: 0.87 to 1.16) (Figure 2 and Table 2). There was statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 30.3\%$). The Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.16$). Sensitivity analysis according to trial setting, country of origin, dose of bismuth salt used, type of bismuth salt used, mean age of patients included in the study, and proportion of males included in the study failed to reveal any obvious explanation for the observed heterogeneity.

Number of specific individual adverse events with bismuth or bismuth-containing regimen versus comparison regimen

Abdominal pain: Thirteen trials reported the total number of individuals experiencing abdominal pain with bismuth or bismuth-containing regimens versus comparison regimen[17,18,20,21,24,26,28,30,34,39,40,46,47]. Three of these studies utilized more than one regimen[20,34,47], allowing 16 comparisons to be made. The relative risk of abdominal pain with bismuth or bismuth-containing regimens versus comparison regimen was 1.06 (95% CI: 0.64 to 1.74) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 22.0\%$), and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.15$).

Dark stools: Four trials reported the total number of individuals experiencing dark stools with bismuth or bismuth-containing regimens versus comparison regimen[17,42,46,48]. The relative risk of dark stools with bismuth or bismuth-containing regimens versus comparison regimen was 5.06 (95% CI: 1.59 to 16.12) (Figure 3 and Table 2). There was marginal statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 25.2\%$), but no obvious causes were found, and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.28$). The number of patients needed to harm with bismuth or bismuth-containing regimen versus comparison regimen to cause one case of dark stools was 7.5 (95% CI: 4 to 71).

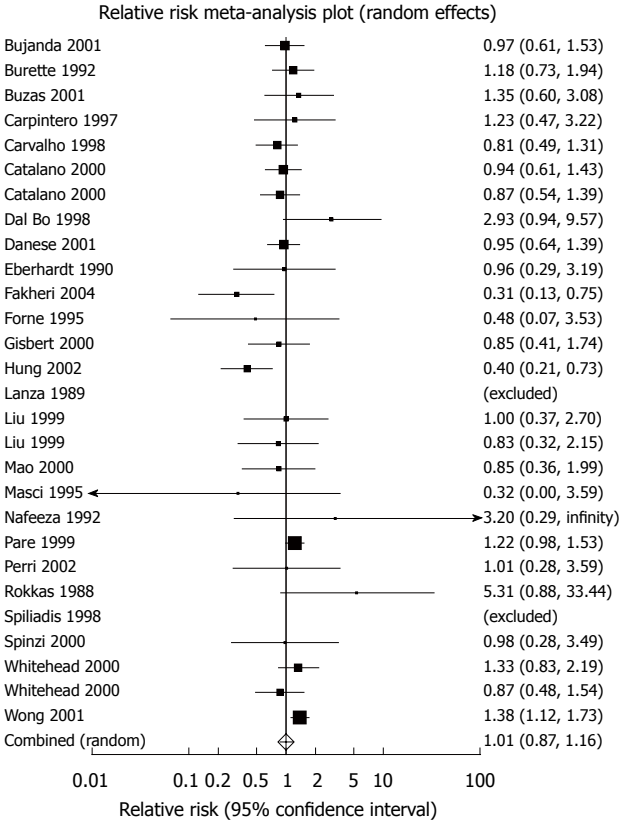

Figure 2 Forest plot of trials of bismuth or bismuth-containing regimens versus comparison regimen examining the effect on relative risk of any adverse event.

Diarrhoea: Twenty-two trials reported the total number of individuals experiencing diarrhoea with bismuth or bismuth-containing regimens versus comparison regimen[7,17,18,20,24-28,30-32,34,36,39-42,45-48]. Six of these studies utilized more than one regimen[7,20,31,34,41,47], allowing 28 comparisons to be made. The relative risk of diarrhoea with bismuth or bismuth-containing regimens versus comparison regimen was 1.01 (95% CI: 0.72 to 1.42) (Table 2). There was marginal statistically significant heterogeneity detected between trial results (heterogeneity

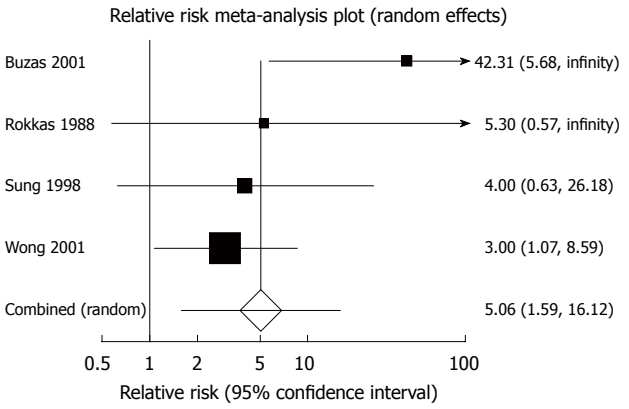

Figure 3 Forest plot of trials of bismuth or bismuth-containing regimens *versus* comparison regimen examining the effect on relative risk of dark stools.

test: $I^2 = 26.2\%$), but no obvious causes were found, and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.75$).

Dizziness: Eight trials reported the total number of individuals experiencing dizziness with bismuth or bismuth-containing regimens versus comparison regimen[21,26,31,32,35,41,46,48]. Three of these studies utilized more than one regimen[31,35,41], allowing 11 comparisons to be made. The relative risk of dizziness with bismuth or bismuth-containing regimens versus comparison regimen was 1.18 (95% CI: 0.81 to 1.72) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.20$).

Headache: Fourteen trials reported the total number of individuals experiencing headache with bismuth or bismuth-containing regimens versus comparison regimen[17,18,20,24-26,30,31,34,39-41,46,47]. Five of these studies utilized more than one regimen[20,31,34,41,47], allowing 19 comparisons to be made. The relative risk of headache with bismuth or bismuth-containing regimens *versus* comparison regimen was 1.31 (95% CI: 0.81 to 2.11) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), and the Egger test did not suggest any trend for funnel plot asymmetry ($P = 0.83$).

Metallic taste: Fourteen trials reported the total number of individuals experiencing metallic taste with bismuth or bismuth-containing regimens versus comparison regimen[17,20,24,27,30,34,35,39-42,45,46,48]. Four of these studies utilized more than one regimen[20,34,35,41], allowing 18 comparisons to be made. The relative risk of metallic taste with bismuth or bismuth-containing regimens versus comparison regimen was 1.02 (95% CI: 0.81 to 1.28) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), though the Egger test suggested there was funnel plot asymmetry ($P = 0.01$).

Nausea and/or vomiting: Twenty trials reported the total number of individuals experiencing nausea and/or vomiting with bismuth or bismuth-containing regimens versus comparison regimen[17,18,20,21,24-28,30-32,34,35,39-42,46,47]. Six of these studies utilized more than one regimen[20,31,34,35,41,47], allowing 26 comparisons to be made. The relative risk of nausea and/or vomiting with bismuth or bismuth-containing regimens versus comparison regimen was 1.16 (95% CI: 0.89 to 1.52) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), and the Egger test did not suggest that there was any evidence of funnel plot asymmetry ($P = 0.85$).

Withdrawal of therapy due to adverse events with bismuth or bismuth-containing regimen versus comparison regimen

Twenty-eight trials reported the total number of individuals who terminated therapy due to experiencing adverse events with bismuth or bismuth-containing regimens versus comparison regimen[16-18,20,22-32,34-37,39-43,45-48]. Six of these studies utilized more than one regimen[20,31,34, 35,41,47], allowing 34 comparisons to be made. The relative risk of withdrawal of therapy due to adverse events with bismuth or bismuth-containing regimens versus comparison regimen was 0.86 (95% CI: 0.54 to 1.37) (Table 2). There was no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$), though the Egger test suggested that there was evidence of funnel plot asymmetry ($P = 0.05$).

Effect of duration of bismuth therapy on incidence of adverse events

The duration of bismuth therapy was one month or more in eleven of the included studies[7,18,25,31,34,37-39,41,42,47]. There were sufficient trials to pool data to examine the effect of duration of therapy on total number of adverse events, some of the specific individual adverse events (including diarrhoea, headache, and nausea and/or vomiting), and withdrawal of therapy due to adverse events.

Total number of adverse events: Seven trials provided data on total number of adverse events in 945 individuals (467 of whom were assigned to bismuth)[18,25,37-39,42,47], and one study utilized more than one regimen allowing eight comparisons to be made[47]. The relative risk of an adverse event with bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 1.20 (95% CI:1.00 to 1.44), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

Diarrhoea: Nine studies provided data on the incidence of diarrhoea with one month or more of bismuth in 1601 patients (859 of whom were assigned to bismuth)[7,18,25,31,34,39,41,42,47], with five of the studies utilizing more than one regimen allowing fourteen comparisons to be made[7,31,34,41,47]. The relative risk of diarrhoea with

bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 1.72 (95% CI: 1.14 to 2.60), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

Headache: Seven studies provided data on the incidence of headache with one month or more of bismuth in 1435 patients (778 of whom were allocated to bismuth)[18,25,31,34,39,41,47], with four of the studies utilizing more than one regimen allowing eleven comparisons to be made[31,34,41,47]. The relative risk of headache with bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 1.39 (95% CI: 0.76 to 2.53), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

Nausea and/or vomiting: Eight studies provided data on the incidence of nausea and/or vomiting with one month or more of bismuth in 1501 patients (810 of whom were allocated to bismuth)[18,25,31,34,39,41,42,47], with four of the studies utilizing more than one regimen allowing twelve comparisons to be made[31,34,41,47]. The relative risk of nausea and/or vomiting with bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 1.47 (95% CI: 0.87 to 2.48), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

Withdrawal of therapy due to adverse events: Nine studies provided data on the incidence of withdrawal of therapy due to adverse events with one month or more of bismuth in 1554 patients (837 of whom were allocated to bismuth)[18,25,31,34,37,39,41,42,47], with four of the studies utilizing more than one regimen allowing thirteen comparisons to be made[31,34,41,47]. The relative risk of withdrawal of therapy due to adverse events with bismuth or bismuth-containing regimens used for one month or more versus comparison regimen was 0.86 (95% CI: 0.47 to 1.57), with no statistically significant heterogeneity detected between trial results (heterogeneity test: $I^2 = 0\%$).

DISCUSSION

This is, to our knowledge, the first systematic review and meta-analysis to examine the safety profile of bismuth compounds used either alone or in combination with antibiotics for the treatment of *H pylori* infection, or *H pylori*-related diseases. This information is very important because there have been previous concerns surrounding the issue of potential for toxicity with use of the drug in some countries, particularly in France, where severe neurological adverse events related to the prolonged use of bismuth, given in large quantities, led to the complete withdrawal of all bismuth compounds. This is in contrast to much of the rest of the world, particularly North America, where these drugs are still available without prescription over the counter.

Serfontein *et al*[11], when reviewing blood bismuth levels in patients following administration of therapeutic bismuth formulations, concluded that levels less than 50 μg/mL were highly unlikely to be associated with any meaningful toxicity in man. The authors also reported site-specific toxicity issues depending on whether the complexes of bismuth were water or lipid soluble, the former being associated with renal toxicity, the latter with neurotoxicity. In both cases the doses used and the duration of treatment leading to such adverse effects were much greater than the ones used in the context of *H pylori* eradication therapy. When bismuth-based compounds are used in the treatment of *H pylori* they are usually only given for 1 to 2 wk at low doses, so it would be expected that in this situation the incidence of severe adverse events such as death or neurotoxicity would be lower. These data, with no reported deaths or neurotoxicity in any of the included RCTs, would support this hypothesis. Less serious adverse events are still important though, particularly from the patient's perspective. These may affect compliance with therapy, which is important as successful eradication of *H pylori* is likely to lead to successful ulcer healing and prevention of ulcer relapse[49], and may also improve symptoms in a small but significant proportion of those with functional dyspepsia[50,51]. In a previous analysis of factors that determine the likely success of *H pylori* eradication with bismuth-based triple therapies, patient compliance was shown to be the most important predictor of response[52].

No statistically significant difference was detected in the total number of side-effects between those receiving bismuth-based therapy and comparison regimen in this meta-analysis. In addition, there was no statistically significant difference detected in individual adverse events such as abdominal pain, diarrhoea, dizziness, headache, metallic taste, and nausea and/or vomiting with bismuth compounds versus comparison regimen. Finally, there was no statistically significant increase detected in the number of individuals requiring cessation of therapy as a direct result of adverse events with bismuth-based therapy versus comparison regimen. The number of individuals reporting dark stools with bismuth was significantly higher, though there were fewer studies reporting this adverse event, which probably explains the wider confidence interval. This is unlikely to have any serious consequences related to patient safety, but it is still important to warn patients that this is an expected side-effect of therapy. This observation also has implications for the successful blinding of patients allocated to bismuth therapy in double-blind RCTs.

Total number of side-effects did appear to increase slightly when only those trials that used one month or more of bismuth therapy were included in the meta-analysis, though this did not achieve statistical significance. Diarrhoea was significantly more common with bismuth compounds when only studies using more than one month of therapy were included, but no statistically significant difference was detected in the incidence of other adverse events reported, where there were sufficient trials to examine this issue. Again, there was no increase in cessation of therapy in individuals

assigned to bismuth-based therapy, even if treatment was for one month or more. As mentioned earlier, most current bismuth-based *H pylori* eradication regimens are given for 1 or 2 wk only, so these observations related to longer duration of bismuth therapy are unlikely to have any significant implications in the majority of patients.

The strengths of this systematic review and meta-analysis are that it has been conducted using rigorous methodology and contains a large number of RCTs, which have provided data from in excess of 2000 patients for most of the analyses. In addition, the fact that the data of interest to this meta-analysis were not the primary endpoint of any of the included trials means that the results of the current study are likely to be free from publication bias, as evidenced by the funnel plots for many of the outcomes we assessed. Disadvantages, as with any systematic review, arise from the methodology of the trials included. Many studies were not double-blind and few reported that assessors were blinded to the treatment allocation of the patients either, and this may have led to under-reporting of adverse events in those assigned to "active treatment" with bismuth therapy. Most studies also failed to report either the method of generation of the randomization schedule or the method of concealment of allocation. Finally, only four of the studies used a questionnaire to collect adverse event data, and only two stated that this questionnaire was validated. This may mean that adverse event data were inaccurate in many of the trials and we cannot exclude the possibility that this may have biased the results of the current meta-analysis towards the null hypothesis.

In summary, this systematic review and meta-analysis provides strong evidence that bismuth compounds used either alone, or in combination with antibiotics and acid-suppression therapy, for the treatment of *H pylori* are safe and well-tolerated. The only observation of note was that dark stools were significantly more common in those assigned to bismuth-based therapies.

COMMENTS

Background

Bismuth compounds are often used as part of eradication therapy for *Helicobacter pylori* (*H pylori*). There are concerns about toxicity of these compounds in some countries, particularly as a result of their potential neurological sequelae.

Research frontiers

Data concerning toxic effects of bismuth compounds are mainly derived from studies that have used these compounds at a high dose for a prolonged period of time. We conducted a meta-analysis of adverse events resulting from a 1 to 2-wk course of bismuth based *H pylori* eradication therapy.

Innovation and breakthroughs

The current study demonstrated that bismuth compounds, when used short-term for 1 to 2 wk in *H pylori* eradication therapy, are safe. The only adverse event that occurred more frequently in patients receiving bismuth was dark stools.

Applications

Potential adverse events from bismuth compounds in a 1 to 2-wk course of *H pylori* eradication therapy are now quantified. Gastroenterologists can be assured that these compounds are safe to use.

Terminology

The number needed to harm is the number of patients that would need to be treated with bismuth compounds for one patient to experience an adverse event.

Peer review

There are now problems in obtaining satisfactory eradication rates of *H pylori* with PPI-based triple therapies, so the use of bismuth containing regimens has been recommended as a potential first line therapy in the Maastricht guidelines. Furthermore, there are now new bismuth combinations commercially available. For these reasons it is important to be sure of the safety of bismuth compounds.

REFERENCES

1 **Tillman LA**, Drake FM, Dixon JS, Wood JR. Review article: safety of bismuth in the treatment of gastrointestinal diseases. *Aliment Pharmacol Ther* 1996; **10**: 459-467
2 **Warren JR**, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983; **321**: 1273-1275
3 **Wolle K**, Malfertheiner P. Treatment of Helicobacter pylori. *Best Pract Res Clin Gastroenterol* 2007; **21**: 315-324
4 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315
5 **McNulty CA**, Gearty JC, Crump B, Davis M, Donovan IA, Melikian V, Lister DM, Wise R. Campylobacter pyloridis and associated gastritis: investigator blind, placebo controlled trial of bismuth salicylate and erythromycin ethylsuccinate. *Br Med J* (Clin Res Ed) 1986; **293**: 645-649
6 **Coghlan JG**, Gilligan D, Humphries H, McKenna D, Dooley C, Sweeney E, Keane C, O'Morain C. Campylobacter pylori and recurrence of duodenal ulcers--a 12-month follow-up study. *Lancet* 1987; **2**: 1109-1111
7 **Marshall BJ**, Goodwin CS, Warren JR, Murray R, Blincow ED, Blackbourn SJ, Phillips M, Waters TE, Sanderson CR. Prospective double-blind trial of duodenal ulcer relapse after eradication of Campylobacter pylori. *Lancet* 1988; **2**: 1437-1442
8 **Graham DY**, Lew GM, Evans DG, Evans DJ Jr, Klein PD. Effect of triple therapy (antibiotics plus bismuth) on duodenal ulcer healing. A randomized controlled trial. *Ann Intern Med* 1991; **115**: 266-269
9 **Graham DY**, Lew GM, Klein PD, Evans DG, Evans DJ Jr, Saeed ZA, Malaty HM. Effect of treatment of Helicobacter pylori infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. *Ann Intern Med* 1992; **116**: 705-708
10 **Rauws EA**, Tytgat GN. Cure of duodenal ulcer associated with eradication of Helicobacter pylori. *Lancet* 1990; **335**: 1233-1235
11 **Serfontein WJ**, Mekel R. Bismuth toxicity in man II. Review of bismuth blood and urine levels in patients after administration of therapeutic bismuth formulations in relation to the problem of bismuth toxicity in man. *Res Commun Chem Pathol Pharmacol* 1979; **26**: 391-411
12 **Spenard J**, Aumais C, Massicotte J, Tremblay C, Lefebvre M. Influence of omeprazole on bioavailability of bismuth following administration of a triple capsule of bismuth biskalcitrate, metronidazole, and tetracycline. *J Clin Pharmacol* 2004; **44**: 640-645
13 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188
14 **Higgins JP**, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003; **327**: 557-560
15 **Bujanda L**, Herrerias JM, Ripolles V, Pena D, Chaves da Cruz ATC, Fueyo A. Efficacy and tolerability of three regimens for Helicobacter pylori eradication: A multicentre, double-blind, randomised clinical trial. *Clin Drug Investig* 2001; **21**: 1-7
16 **Burette A**, Glupczynski Y, De Prez C. Evaluation of various multi-drug eradication regimens for Helicobacter pylori. *Eur J Gastroenterol Hepatol* 1992; **4**: 817-823
17 **Buzas GM**, Illyes G, Szekely E, Szeles I. Six regimens for the

eradication of Helicobacter pylori (Hp) in duodenal ulcer patients: three consecutive trials (1995-1999). *J Physiol Paris* 2001; **95**: 437-441

18 **Carpintero P**, Blanco M, Pajares JM. Ranitidine versus colloidal bismuth subcitrate in combination with amoxicillin and metronidazole for eradicating Helicobacter pylori in patients with duodenal ulcer. *Clin Infect Dis* 1997; **25**: 1032-1037

19 **Carvalho AF**, Fiorelli LA, Jorge VN, Da Silva CM, De Nucci G, Ferraz JG, Pedrazzoli J. Addition of bismuth subnitrate to omeprazole plus amoxycillin improves eradication of Helicobacter pylori. *Aliment Pharmacol Ther* 1998; **12**: 557-561

20 **Catalano F**, Branciforte G, Catanzaro R, Cipolla R, Bentivegna C, Brogna A. Helicobacter pylori-positive duodenal ulcer: three-day antibiotic eradication regimen. *Aliment Pharmacol Ther* 2000; **14**: 1329-1334

21 **Xiao SD**, Liu WZ, Hu PJ, Ouyang Q, Wang JL, Zhou LY, Cheng NN. A multicentre study on eradication of Helicobacter pylori using four 1-week triple therapies in China. *Aliment Pharmacol Ther* 2001; **15**: 81-86

22 **Chuang CH**, Sheu BS, Yang HB, Wu JJ, Lin XZ. Ranitidine bismuth citrate or omeprazole-based triple therapy for Helicobacter pylori eradication in Helicobacter pylori-infected non-ulcer dyspepsia. *Dig Liver Dis* 2001; **33**: 125-130

23 **Dal Bo' N**, Di Mario F, Battaglia G, Buda A, Leandro G, Vianello F, Kusstatscher S, Salandin S, Pilotto A, Cassaro M, Vigneri S, Rugge M. Low dose of clarithromycin in triple therapy for the eradication of Helicobacter pylori: one or two weeks? *J Gastroenterol Hepatol* 1998; **13**: 288-293

24 **Danese S**, Armuzzi A, Romano A, Cremonini F, Candelli M, Franceschi F, Ojetti V, Venuti A, Pola P, Gasbarrini G, Gasbarrini A. Efficacy and tolerability of antibiotics in patients undergoing H. pylori eradication. *Hepatogastroenterology* 2001; **48**: 465-467

25 **Eberhardt R**, Kasper G. Effect of oral bismuth subsalicylate on Campylobacter pylori and on healing and relapse rate of peptic ulcer. *Rev Infect Dis* 1990; **12** Suppl 1: S115-S119

26 **Fakheri H**, Merat S, Hosseini V, Malekzadeh R. Low-dose furazolidone in triple and quadruple regimens for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2004; **19**: 89-93

27 **Forne M**, Viver JM, Espinos JC, Coll I, Tresserra F, Garau J. Impact of colloidal bismuth subnitrate in the eradication rates of Helicobacter pylori infection-associated duodenal ulcer using a short treatment regimen with omeprazole and clarithromycin: a randomized study. *Am J Gastroenterol* 1995; **90**: 718-721

28 **Gasbarrini A**, Ojetti V, Pitocco D, Armuzzi A, Silveri NG, Pola P, Ghirlanda G, Gasbarrini G. Efficacy of different Helicobacter pylori eradication regimens in patients affected by insulin-dependent diabetes mellitus. *Scand J Gastroenterol* 2000; **35**: 260-263

29 **Georgopolous S**, Karatapanis S, Ladas S, Papamrkos D, Vretou N, Artikis V, Mentis A, Raptis S. Lansoprazole vs ranitidine bismuth citrate based short-term triple therapies for Helicobacter pylori (H. pylori) eradication: A randomised study with 6-month follow-up. *Gut* 1999; **44** (suppl 1): A120-A121

30 **Gisbert JP**, Carpio D, Marcos S, Gisbert JL, Garcia Gravalos R, Pajares JM. One-week therapy with pantoprazole versus ranitidine bismuth citrate plus two antibiotics for Helicobacter pylori eradication. *Eur J Gastroenterol Hepatol* 2000; **12**: 489-495

31 **Graham DY**, Breiter JR, Ciociola AA, Sykes DL, McSorley DJ. An alternative non-macrolide, non-imidazole treatment regimen for curing Helicobacter pylori and duodenal ulcers: ranitidine bismuth citrate plus amoxicillin. The RBC H. pylori Study Group. *Helicobacter* 1998; **3**: 125-131

32 **Hung WK**, Wong WM, Wong GS, Yip AW, Szeto ML, Lai KC, Hu WH, Chan CK, Xia HH, Yuen MF, Fung FM, Tong TS, Ho VY, Lam SK, Wong BC. One-week ranitidine bismuth citrate, amoxicillin and metronidazole triple therapy for the treatment of Helicobacter pylori infection in Chinese. *Aliment Pharmacol Ther* 2002; **16**: 2067-2072

33 **Lanza FL**, Skoglund ML, Rack MF, Yardley JH. The effect of bismuth subsalicylate on the histologic gastritis seen with Campylobacter pylori: a placebo-controlled, randomized study. *Am J Gastroenterol* 1989; **84**: 1060-1064

34 **Lanza FL**, Sontag SJ, Ciociola AA, Sykes DL, Heath A, McSorley DJ. Ranitidine bismuth citrate plus clarithromycin: a dual therapy regimen for patients with duodenal ulcer. *Helicobacter* 1998; **3**: 212-221

35 **Liu WZ**, Xiao SD, Shi Y, Wu SM, Zhang DZ, Xu WW, Tytgat GN. Furazolidone-containing short-term triple therapies are effective in the treatment of Helicobacter pylori infection. *Aliment Pharmacol Ther* 1999; **13**: 317-322

36 **Mao HV**, Lak BV, Long T, Chung NQ, Thang DM, Hop TV, Chien NN, Hoan PQ, Henley KS, Perez-Perez GI, Connor BA, Stone CD, Chey WD. Omeprazole or ranitidine bismuth citrate triple therapy to treat Helicobacter pylori infection: a randomized, controlled trial in Vietnamese patients with duodenal ulcer. *Aliment Pharmacol Ther* 2000; **14**: 97-101

37 **Masci E**, Colombo E, Testoni PA, Fanti L, Guslandi M, Tittobello A. Colloid bismuth versus famotidine in the treatment and prevention of duodenal ulcer relapse: results of a double-blind, double dummy randomized study. *Fundam Clin Pharmacol* 1995; **9**: 280-283

38 **Nafeeza MI**, Shahimi MM, Kudva MV, Ahmad H, Isa MR, Sood IM, Mazlam MZ, Jamal F, Suboh Y. Evaluation of therapies in the treatment of Helicobacter pylori associated non-ulcer dyspepsia. *Singapore Med J* 1992; **33**: 570-574

39 **Pare P**, Farley A, Romaozinho JM, Bardhan KD, French PC, Roberts PM. Comparison of ranitidine bismuth citrate plus clarithromycin with omeprazole plus clarithromycin for the eradication of Helicobacter pylori. *Aliment Pharmacol Ther* 1999; **13**: 1071-1078

40 **Perri F**, Festa V, Merla A, Quitadamo M, Clemente R, Andriulli A. Amoxicillin/tetracycline combinations are inadequate as alternative therapies for Helicobacter pylori infection. *Helicobacter* 2002; **7**: 99-104

41 **Peterson WL**, Ciociola AA, Sykes DL, McSorley DJ, Webb DD. Ranitidine bismuth citrate plus clarithromycin is effective for healing duodenal ulcers, eradicating H. pylori and reducing ulcer recurrence. RBC H. pylori Study Group. *Aliment Pharmacol Ther* 1996; **10**: 251-261

42 **Rokkas T**, Pursey C, Uzoechina E, Dorrington L, Simmons NA, Filipe MI, Sladen GE. Non-ulcer dyspepsia and short term De-Nol therapy: a placebo controlled trial with particular reference to the role of Campylobacter pylori. *Gut* 1988; **29**: 1386-1391

43 **Spadaccini A**, De Fanis C, Sciampa G, Russo L, Silla M, Pantaleone U, Di Virgilio M, Pizzicanella G. Triple regimens using lansoprazole or ranitidine bismuth citrate for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 1998; **12**: 997-1001

44 **Spiliadis C**, Georgopolous S, Stambolos P, Mentis A, Gianikaki L, Manika Z, Skandalis N. Evaluation of the efficacy of clarithromycin in the eradication of Helicobacter pylori. *Gut* 1998; **43** (suppl 2): A85

45 **Spinzi GC**, Boni F, Bortoli A, Colombo E, Ballardini G, Venturelli R, Minoli G. Seven-day triple therapy with ranitidine bismuth citrate or omeprazole and two antibiotics for eradication of Helicobacter pylori in duodenal ulcer: a multicentre, randomized, single-blind study. *Aliment Pharmacol Ther* 2000; **14**: 325-330

46 **Sung JJ**, Leung WK, Ling TK, Yung MY, Chan FK, Lee YT, Cheng AF, Chung SC. One-week use of ranitidine bismuth citrate, amoxycillin and clarithromycin for the treatment of Helicobacter pylori-related duodenal ulcer. *Aliment Pharmacol Ther* 1998; **12**: 725-730

47 **Whitehead MW**, Phillips RH, Sieniawska CE, Delves HT, Seed PT, Thompson RP, Powell JJ. Double-blind

comparison of absorbable colloidal bismuth subcitrate and nonabsorbable bismuth subnitrate in the eradication of Helicobacter pylori and the relief of nonulcer dyspepsia. *Helicobacter* 2000; **5**: 169-175

48 **Wong BC**, Wong WM, Wang WH, Fung FM, Lai KC, Chu KM, Yuen ST, Leung SY, Hu WH, Yuen MF, Lau GK, Chan CK, Lam SK. One-week ranitidine bismuth citrate-based triple therapy for the eradication of Helicobacter pylori in Hong Kong with high prevalence of metronidazole resistance. *Aliment Pharmacol Ther* 2001; **15**: 403-409

49 **Ford AC**, Delaney BC, Forman D, Moayyedi P. Eradication therapy in Helicobacter pylori positive peptic ulcer disease: systematic review and economic analysis. *Am J Gastroenterol* 2004; **99**: 1833-1855

50 **Moayyedi P**, Soo S, Deeks J, Forman D, Mason J, Innes M, Delaney B. Systematic review and economic evaluation of Helicobacter pylori eradication treatment for non-ulcer dyspepsia. Dyspepsia Review Group. *BMJ* 2000; **321**: 659-664

51 **Moayyedi P**, Deeks J, Talley NJ, Delaney B, Forman D. An update of the Cochrane systematic review of Helicobacter pylori eradication therapy in nonulcer dyspepsia: resolving the discrepancy between systematic reviews. *Am J Gastroenterol* 2003; **98**: 2621-2626

52 **Graham DY**, Lew GM, Malaty HM, Evans DG, Evans DJ Jr, Klein PD, Alpert LC, Genta RM. Factors influencing the eradication of Helicobacter pylori with triple therapy. *Gastroenterology* 1992; **102**: 493-496

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RAPID COMMUNICATION

Changing patterns of hepatitis A prevalence within the Saudi population over the last 18 years

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Abstract

AIM: To determine the seroprevalence of Hepatitis A (HAV) amongst Saudi children and compare it with previously reported prevalence data from the same population.

METHODS: A total of 1357 students were randomly selected between the ages of 16 and 18 years (689 males and 668 females) from three different regions of Saudi Arabia (Madinah, Al-Qaseem, and Aseer) and tested for anti-HAV-IgG.

RESULTS: The overall prevalence of anti-HAV-IgG among the study population was 18.6%. There was no difference between males and females but there was a significant difference in the seroprevalence (P = 0.0001) between the three different regions, with Madinah region showing the highest prevalence (27.4%). When classified according to socioeconomic status, lower class students had a prevalence of 36.6%, lower middle class 16.6%, upper middle class 9.6%, and upper class 5.9% (P = 0.0001). Comparing the current study results with those of previous studies in 1989 and 1997 involving the same population, there was a marked reduction in the overall prevalence of HAV from 52% in 1989, to 25% in 1997, to 18.6% in 2008 (P < 0.0001).

CONCLUSION: Over the last 18 years, there has been a marked decline in the prevalence of HAV in Saudi children and adolescents. The current low prevalence rates call for strict adherence to vaccination policies in high-risk patients and raises the question of a universal HAV vaccination program.

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Key words: Hepatitis A; Saudi Arabia; Epidemiology; Prevalence; Serology

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INTRODUCTION

Hepatitis A (HAV) is a major health problem worldwide and, like other enteric infectious diseases, is classically an infection of childhood. Although acute infection commonly passes unnoticed, a significant proportion of patients may have fulminant liver failure, especially patients with liver cirrhosis or immune deficiencies. Generally, its prevalence pattern varies from one population to the other and is closely related to the socioeconomic conditions of sanitation and hygiene. An improvement in sanitation and living standards in many areas of the world has caused the epidemiology of HAV to rapidly evolve. As such, with an improvement in living conditions, more clinical cases are being diagnosed owing to the increased age of those susceptible, which is paradoxical to childhood infection where the majority of infections are subclinical[1-5]. The availability of safe and efficacious vaccines against HAV has made it

increasingly important to understand the epidemiology of HAV in a given area before a strategy for the use of the vaccine is advised or implemented[6-8]. In particular, the epidemiological data from the previous decade may no longer be valid now.

Understanding the epidemiological shift in HAV seropositivity is of strategic importance to a nation's healthcare system. In countries that dramatically improve their socioeconomic status and standards of living, the susceptible pool may increase rapidly to such an extent that HAV becomes a major public health concern. The epidemiology of HAV in developed countries is characterized by low prevalence rates among children with a large group of susceptible adults being negative for anti-HAV.

Two decades ago, studies performed in Saudi Arabia indicated the HAV prevalence rate to be in the range of 90%-100% amongst the adult population[9-13]. However, later studies showed a consistent decline in anti-HAV prevalence rate[14,15]. In the years 1989 and 1997, community-based studies revealed that the overall prevalence rate of HAV infection among children of age 1-12 years had reduced dramatically from 52% (1989) to 25% (1997)[16,17].

We aimed to evaluate the epidemiological shift in HAV serostatus within the adolescent population of three predefined regions of Saudi Arabia and compare it to previously published data.

MATERIALS AND METHODS

Study population

We selected our sample from a population of 10-12th grade school students (corresponding to the age of 16-18 years) in three regions. These regions were as follows: (1) The Aseer region: school population of 25 512, 13 996 males and 11 516 females; (2) Madinah: school population of 23 852, 12 133 males and 11 719 females; and (3) The Al-Qaseem region: school population of 16 067, 7974 males and 8093 females. These regions were selected because they represented low, medium, and high prevalence rates in our previous studies. The sample was selected using a stratified random sampling technique where the Kingdom was stratified into three strata according to the previous endemicity of infection. The proportional allocation method was used to determine the recruited number of students in each stratum. Within the stratum, the sample was proportionally allocated according to gender. In every region, schools served as the sampling units. From the list of schools in the region, one or more male schools and one or more female schools that satisfied the required sample size were randomly selected.

A total of 1357 students (689 males, 668 females) from these three regions of the Kingdom of Saudi Arabia (KSU), were randomly selected. The socioeconomic status of this population was also stratified (low, middle and high class). King Abdulaziz City for Science and Technology approved the protocol of this study, and informed consent was obtained from the parents as well as from the students participating in the study.

Data collection, blood sampling and testing

Fieldwork for this study was undertaken in December 2007 and January 2008. Demographic data were recorded, and a venous blood sample (5-10 mL) was taken from each student. Serum was separated by centrifugation, coded, and stored at -70℃ until tested. Blood samples were tested for anti-HAV-IgG using EIA kits ADVIA Centaur system.

The socioeconomic status of each student was taken to be representative of that of the father and/or the mother and classified using a socioeconomic status 3-point scoring system derived according to the type of house (mud-built = 1, apartment or ordinary house = 2, villa = 3), number of rooms in the house (1-2 rooms = 1, 3-4 rooms = 2, 5 and more = 3), number of family members (4 or less = 3, 5 = 3, 6 = 2, 7 or more = 1), father and mother education (primary grade or less = 1, secondary/high school = 2, university or equivalent = 3), parent occupation (laborer = 1, farmer or office clerk = 2, trader = 3). An overall score of less than 10 from a maximum of 21 was classified as representative of a low socioeconomic status, 10-15 as low middle, 15-17 as high middle and above 17 as high class.

Statistical analyses

Data was entered in MS Excel and analyzed using SPSS Pc+ version 16.0 statistical software. Descriptive statistics (proportional) were used to summarize the categorical variables. χ^2 test followed by analysis of residuals was used to calculate the statistical association between two categorical variables. χ^2 test for trend was used to calculate the significance of proportions of categorical variables at different time points. P value of < 0.05 was considered statistically significant.

RESULTS

The blood samples of 1357 students (aged 16 to 18 years; 689 males and 668 females) were collected and analyzed. The overall prevalence rate of anti-HAV-IgG among the population study was 18.6%.

Association between anti-HAV-IgG values and gender

A significant association between gender and anti-HAV serostatus was seen. The proportion of males who were anti-HAV positive (21%) was significantly higher when compared with females (16.2%) ($P = 0.021$). The adjusted residuals of the frequencies were also statistically significant when compared with the 5% standard normal deviate value (1.96) (Table 1).

Association between anti-HAV-IgG values and regions

A significant association was observed between the area (Aseer, Madinah, and Al-Qaseem) and anti-HAV serostatus. The proportion of subjects from Madinah who were anti-HAV positive (27.4%) was significantly higher compared with samples from other areas (Aseer: 13.5% and Al-Qaseem: 13.9%; $P < 0.0001$). The adjusted residuals of the frequencies of Madinah area (6.2)

Table 1 Prevalence of anti-HAV within the study population and its association with gender

Gender	Anti-HAV (%)		*P* value
	Positive	Negative	
Male (*n* = 689)	145 (21)	544 (79)	0.021
Female (*n* = 668)	108 (16.2)	560 (83.8)	

Table 2 Prevalence of anti-HAV and its association with the socioeconomic status of study population

Socioeconomic status	Anti-HAV(%)		*P* value
	Positive	Negative	
Low class (*n* = 239)	88 (36.8)	151 (63.2)	< 0.0001
Lower middle class (*n* = 880)	146 (16.6)	734 (83.4)	
Upper middle class (*n* = 136)	13 (9.6)	123 (90.4)	
High class (*n* = 102)	6 (5.9)	96 (94.1)	

were also statistically significantly higher when compared with the 5% standard normal deviate value (1.96) (Table 2).

Association between anti-HAV-IgG values and socioeconomic status

A significant association between social status of the sampled population and anti-HAV status was found. The proportion of low class subjects who were anti-HAV positive (36.8%) was significantly higher compared with subjects of other classes (lower middle class: 16.6%; upper middle class: 9.6% and high class: 5.9%; $P < 0.0001$). The adjusted residuals of the frequencies of low class (7.9) were also statistically significantly higher when compared with the 5% standard normal deviate value (1.96) (Table 3).

Comparison of anti-HAV-IgG of previous studies performed in 1989[16] and 1997[17], with the present study

There was a high statistically significant trend for decreased prevalence of HAV infection in all three areas over the three time points. Among the three areas (Al-Qaseem, Aseer and Madinah), the Al-Qaseem area had a significantly decreased prevalence of HAV infection (61.1% in 1989, 31.5% in 1997 and 13.8% in 2007-2008). The decrease in prevalence of HAV infection in Aseer area from 1997 to 2007-2008 was only 5.4% and in Madinah for the same period was only 1.2%, whereas in Al-Qaseem the decrease was 17.7% during the same period (Table 4).

DISCUSSION

The results of this study show a marked decline in the endemicity of HAV within the Saudi population in the age range of 16-18 years. This trend is highlighted by the dramatic linear decline from 53% in 1987 to 25% in 1997 and finally to 18.6% in the present study. Other studies from the region have shown a similar trend.

Table 3 Prevalence of anti-HAV and its association with the region of study population

Region	Anti-HAV(%)		*P* value
	Positive	Negative	
Aseer (*n* = 532)	72 (13.5)	460 (86.5)	< 0.0001
Al-Qaseem (*n* = 332)	46 (13.9)	286 (86.1)	
Madinah (*n* = 493)	135 (27.4)	358 (72.6)	

Table 4 Prevalence of HAV infection in KSA over the last 18 years (1989[16], 1997[17], and 2008)

Region	1989 prevalence (%)	1997 prevalence (%)	2007-2008 prevalence (%)	*P* value
Al-Qaseem	126/201 (61.1)	71/225 (31.5)	46/332 (13.8)	< 0.00001
Aseer	212/476 (44.5)	78/411 (18.9)	72/532 (13.5)	< 0.00001
Madinah	208/350 (59.4)	83/317 (26.2)	135/493 (27.4)	< 0.0001
Total	546/1027 (53.1)	232/953 (24.3)	253/1357 (18.6)	

Al Muneef and colleagues found a hepatitis A seroprevalence of 28.9% in 2399 Saudi children two years ago[18]. However, the present study which was performed on the same population cohort three times over the past 18 years showed a graded but dramatic decline in prevalence. In addition, this study was performed in three areas of different endemicity within the country, representing different levels of socioeconomic development.

HAV endemicity is closely linked to improvements in sanitation and living conditions in the population. In the case of Saudi Arabia, the Saudi government's real-estate bank has helped to build 851 000 housing units through a government loan from 1974 to 2003[19].

According to the official human development report in 2003, the Saudi per Capita GDP increased from 1145 USD in 1970 to 10 853 USD in 2002 and the life expectancy at birth has changed from 53.9 years (1970-1975) to 70.9 in 2000[19].

We believe therefore, that the vast improvement in the socioeconomic status of the Saudi population is the factor most likely to be responsible for this decline. Furthermore, the overall reduction in illiteracy within the Saudi population from greater than 90% in the 1960s to 13.4% in 2007-2008 (7% in males and 19.8% in females)[20] is likely to have contributed to this decline.

A difference was also observed in the prevalence rates of anti-HAV between the three different regions of the KSA in our study, in effect reflecting the different stages of economic development of these regions. The role of socioeconomic status in the study population in determining the level of HAV prevalence was also demonstrated in this study (Table 3) similar to previous publications[16-17].

Previous community-based studies conducted in the Saudi population have shown differing gender-based results in HAV seropositivity. Al-Rashed showed no difference in the anti-HAV prevalence rates between male and female populations[17], while in another study, Khalil *et al*[21] showed a higher seropositivity for Saudi males. The dif-

ference between male and female prevalence rates among this age group is likely to be related to the greater exposure of the male population to HAV infection sources in the community. For instance, the eating habits of the Saudi male population are certainly more gregarious compared to the female population. Similarly, the local culture of less female co-habitation and social interaction may also play a role in reducing their exposure to infection sources.

Finally, this study indicated that more than 82% of the adolescent population of Saudi Arabia is susceptible to symptomatic HAV infection. This could occur either by exposure to infected persons, either where they live or upon travel to high endemic areas either within or outside the country. Outbreaks of symptomatic acute HAV infection have been recently reported within increasing pools of susceptible populations within the country[22-24]. This high susceptibility of the young population represents a continuous challenge to the healthcare system of the country.

Recently, an HAV vaccine has been introduced in many countries as part of an Extended Program of Immunization (EPI). Several studies have demonstrated the efficacy and safety of the vaccine[25-28] and some authorities have recommended its universal implementation in certain populations[29]. Therefore, the recent decision by the Saudi Ministry of Health to introduce the HAV vaccine as part of the EPI program starting from 2008, to children of 18-24 mo of age is certainly timely. The effect of this strategy needs to be studied in future community-based studies, where the results of the present study could well serve as a reference point for comparative analysis.

COMMENTS

Background

Hepatitis A (HAV) is a major health problem worldwide. Generally, its prevalence pattern varies from one population to the other and is closely related to the socioeconomic conditions of sanitation and hygiene. An improvement in sanitation and living standards in many areas of the world has caused the epidemiology of HAV to rapidly evolve.

Research frontiers

This research group took blood samples from school students aged 16-18 years in three different regions of the country after consent of the parents and students. It was found that the prevalence of hepatitis A in this population was 18.6% compared to 25% in 1997 and 52% in 1989. There was also a link between hepatitis A and socioeconomic status with children from a lower socioeconomic status having a higher prevalence.

Innovation and breakthroughs

This study confirmed the findings of other studies in Saudi Arabia and other developing countries showing a reduced rates of hepatitis A with improved socioeconomic status.

Applications

This study is important because it compares current prevalence rates with previous rates from the same community. The reported low rates in the current study calls for strict adherence to vaccination policies in high-risk patients and raises the question of a universal HAV vaccination program.

Peer review

HAV infection is an important topic, and continues to be a source of morbidity and mortality. Al Faleh elegantly describes how the HAV seroprevalance has decreased in Saudi Arabia, and appropriately raises concerns about an increasingly susceptible population.

REFERENCES

1 **Gust ID**. Epidemiological patterns of hepatitis A in different parts of the world. *Vaccine* 1992; **10** Suppl 1: S56-S58
2 **Feinstone SM**. Hepatitis A: epidemiology and prevention. *Eur J Gastroenterol Hepatol* 1996; **8**: 300-305
3 **Halliday ML**, Kang LY, Zhou TK, Hu MD, Pan QC, Fu TY, Huang YS, Hu SL. An epidemic of hepatitis A attributable to the ingestion of raw clams in Shanghai, China. *J Infect Dis* 1991; **164**: 852-859
4 **Innis BL**, Snitbhan R, Hoke CH, Munindhorn W, Laorakpongse T. The declining transmission of hepatitis A in Thailand. *J Infect Dis* 1991; **163**: 989-995
5 **Purcell RH**, Mannucci PM, Gdovin S, Gringeri A, Colombo M, Mele A, Schinaia N, Ciavarella N, Emerson SU. Virology of the hepatitis A epidemic in Italy. *Vox Sang* 1994; **67** Suppl 4: 2-7; discussion 24-26
6 **Nalin D**, Brown L, Kuter B, Patterson C, McGuire B, Werzberger A, Santosham M, Block S, Reisinger K, Watson B. Inactivated hepatitis A vaccine in childhood: implications for disease control. *Vaccine* 1993; **11** Suppl 1: S15-S17
7 **Shouval D**, Ashur Y, Adler R, Lewis JA, Armstrong ME, Davide JP, McGuire B, Kuter B, Brown L, Miller W. Single and booster dose responses to an inactivated hepatitis A virus vaccine: comparison with immune serum globulin prophylaxis. *Vaccine* 1993; **11** Suppl 1: S9-S14
8 **Innis BL**, Snitbhan R, Kunasol P, Laorakpongse T, Poopatanakool W, Kozik CA, Suntayakorn S, Suknuntapong T, Safary A, Tang DB. Protection against hepatitis A by an inactivated vaccine. *JAMA* 1994; **271**: 1328-1334
9 **Ashraf SJ**, Arya SC, Parande CM, Kristensen E. Hepatitis A virus among natives and expatriates in Saudia Arabia. *J Med Virol* 1986; **19**: 151-153
10 **Shobokshi O**, Serebour F, Abdulrahim SM. The prevalence and pattern of hepatitis A viral infection in the western region of Saudi Arabia. *Saudi Med J* 1986; **7**: 402-408
11 **Talukder MAS**, Walter DK, Nixon P, al Admoury AMO. Prevalence of expatriates from various parts of the world working in Saudi Arabia. *J Infect* 1983; **148**: 1167
12 **Ramia S**. Antibody against hepatitis A in Saudi Arabians and in expatriates from various parts of the world working in Saudi Arabia. *J Infect* 1986; **12**: 153-155
13 **El-Hazmi MA**F, Al-Faleh FZ, Warsy AS. Epidemiology of viral hepatitis among Saudi population. A study of viral markers in Khober. *Saudi Med J* 1986; **7**: 122-129
14 **Arif M**, Al-Faleh FZ, Al-Frayh AR, Ramia S. Reduction in the prevalence of antibody to hepatitis A virus among Saudi adults: implications for hepatitis A vaccine. *Saudi J Gastroenterol* 1995; **1**: 93-96
15 **Al-Faleh FZ**. Changing pattern of hepatitis viral infection in Saudi Arabia in the last two decades. *Ann Saudi Med* 2003; **23**: 367-371
16 **Al-Faleh FZ**. Hepatitis A in Saudi Arabia: A comparative sero-epidemiological study. *Saudi Med J* 1999; **20**: 678-681
17 **Al Rashed RS**. Prevalence of hepatitis A virus among Saudi Arabian children: A community-based study. *Ann Saudi Med* 1997; **17**: 200-203
18 **Almuneef MA**, Memish ZA, Balkhy HH, Qahtani M, Alotaibi B, Hajeer A, Qasim L, Al Knawy B. Epidemiologic shift in the prevalence of Hepatitis A virus in Saudi Arabia: a case for routine Hepatitis A vaccination. *Vaccine* 2006; **24**: 5599-5603
19 **Human development report 2003, ministry of economy and planning, Saudi Arabia**
20 **Illiteracy report 2008, Ministry of Education, Saudi Arabia**
21 **Khalil M**, Al-Mazrou Y, Al-Jeffri M, Al-Howasi M. Childhood epidemiology of hepatitis A virus in Riyadh, Saudi Arabia. *Ann Saudi Med* 1998; **18**: 18-21
22 **AlSaleh E**, Turkistani A, Nooh R. Hepatitis A outbreak at Al-Berk, Asir region, 2004. *Saudi Epidemical Bull* 2005; **12**: 3-5

23 **Basurrah M**, Turkistami A., Hepatitis (A) outbreak in Besha 2003. *Saudi Epidemical Bull* 2003; **10**: 29
24 **Danish AA**, Fountaine RE., hepatitis A from unsafe water. *Saudi Epidemical Bull* 1977; **4**: 19-26
25 **Dagan R**, Leventhal A, Anis E, Slater P, Ashur Y, Shouval D. Incidence of hepatitis A in Israel following universal immunization of toddlers. *JAMA* 2005; **294**: 202-210
26 **Van Damme P**, Van Herck K. Effect of hepatitis A vaccination programs. *JAMA* 2005; **294**: 246-248
27 **Martin A**, Lemon SM. Hepatitis A virus: from discovery to vaccines. *Hepatology* 2006; **43**: S164-S172
28 **Temte JL**. Should all children be immunised against hepatitis A? *BMJ* 2006; **332**: 715-718
29 **Advisory Committee on Immunization Practices (ACIP)**. Fiore AE, Wasley A, Bell BP. Prevention of hepatitis A through active or passive immunization: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2006; **55**: 1-23

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RAPID COMMUNICATION

Prevalence of celiac disease in Iranian children with idiopathic short stature

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Abstract

AIM: To determine the prevalence of celiac disease (CD) in children with idiopathic short stature (ISS) and the diagnostic value of immunoglobulin (Ig) A G antigliadin antibodies (AGA) and transglutaminase (TTG) antibodies for CD.

METHODS: A total of 104 children (49 male, 55 female) with ISS without a specific etiology were studied. Extensive endocrine investigations had shown no abnormalities in any subject. Anthropometric parameters and IgA AGA and IgA TTG antibodies were evaluated in this study group. These antibodies were measured by enzyme-linked immunosorbent assay. All patients were referred for an endoscopic intestinal biopsy. The biopsy samples were classified according to revised Marsh criteria (UEGW 2001).

RESULTS: We detected positive IgA TTG antibodies in 36 and IgA AGA in 35 of these patients. Thirty one IgA TTG antibody positive and 28 IgA AGA positive subjects showed histological abnormalities compatible with celiac disease (33.6%). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value for IgA AGA were found to be 80%, 88.4%, 77.8% and 89.7%, respectively. Sensitivity, specificity and PPV for IgA TTG antibodies were 88.6%, 94.2% and 88.6%, respectively.

CONCLUSION: We conclude that the prevalence of celiac disease is high in patients with ISS and it is important to test all children with ISS for celiac disease by measuring serologic markers and performing an intestinal biopsy.

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Key words: Celiac disease; Growth disorders; Transglutaminases; Antibodies; Gliadin

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INTRODUCTION

Short stature is one of the most common causes for referrals to pediatric endocrinologists. Many of these patients have no identifiable medical abnormality and are classified as idiopathic short stature (ISS). For most of these patients, it is believed that genetic variations are the underlying cause. Short stature is a well-known feature of pediatric celiac disease (CD)[1]. In recent studies, CD was considered to be a more common cause of short stature in otherwise healthy children than growth hormone deficiency[2,3]. In other studies, CD has been found without typical gastrointestinal symptoms in some cases of short stature[4-26]. Moreover, some studies suggested an association between CD and growth hormone deficiency[27]. Diagnosis of CD depends on the demonstration of a flat or almost flat jejunal mucosa in biopsy specimens from the small intestine and regeneration of the mucosa after a gluten-free diet[5]. It has been suggested that patients with untreated CD have circulating antibodies against gliadin, and antiendomysium antibodies (anti-EMA) have proven to be a reliable screening test for CD, even in asymptomatic patients[6,7]. The immunofluorescence test is technically difficult to

interpret, with large interobserver variability. In addition, esophageal tissue from monkeys is a common substrate, and the testing is time consuming. Transglutaminase (TTG) antibodies are also highly sensitive and specific and since IgA antibodies to TTG can be examined by enzyme-linked immunosorbent assay (ELISA), they are easier to use as screening antibodies compared with EMA testing[9]. The purpose of the present study was to evaluate prospectively the clinical, laboratory and histologic features of CD and the sensitivity, specificity and positive and negative predictive values (PPV and NPV, respectively) of antibodies against gliadin and TTG in 104 children with a diagnosis of ISS but with no specific etiology.

MATERIALS AND METHODS

A total of 104 children (55 female, 49 male) with ISS and height less than the 2nd percentile adjusted for age and sex, but without specific etiology were enrolled in the study from November 1, 2003 to September 1, 2005 at Ahwaz Jundishapour University Hospitals. Ages ranged from 2 to 18 years. The height, weight and weight for height measurements had been recorded for all patients at presentation and the patients and their parents answered a CD-specific questionnaire used for data collection. All children were being followed at the Department of Endocrinology of Golestan Hospital and had undergone an extensive negative endocrine investigation which included: concentrations of serum electrolytes and glucose, sweat test, total proteins and albumin, determination of immunoglobulin A (IgA), assessment of liver and renal function (determined by standard methods), and hormonal evaluation through the measurement of thyroid-stimulating hormone, free-thyroxin, and growth hormone.

All etiologic factors known to produce growth retardation had also been excluded, e.g, diabetes mellitus, hematological and liver disease, renal failure, fetal growth failure, diseases of bone metabolism, and chromosomal abnormalities. When no cause of the short stature was found, additional investigations were performed by measuring the serum levels of IgA anti-TTG antibodies and IgA antigliadin antibodies (AGA). AGA was measured by a commercial ELISA assay (ELISA-Biosystem, Madrid, Spain). A serum dilution of 1:100 was used and the results were reported in terms of arbitrary units (AU/mL). An IgA AGA $\geqslant$ 20 AU/mL was considered positive. A commercial ELISA. (Orgentec) kit was used to measure anti-TTG antibodies and a titer of more than 1/10 was considered positive. Intestinal biopsies were obtained from all 104 patients with endoscopic grasp forceps (who had negative or positive results for anti-TTG antibody). Four to six biopsy specimens were taken from the second and third parts of the duodenum. Formalin-fixed biopsy specimens stained with hematoxylin and eosin were studied with the use of light microscopy. The slides were examined and confirmed by a pathologist experienced in CD. Mucosal lesions were classified according to the criteria of Marsh[8] as: (1) type 0, normal mucosa, pre-infiltrative lesions; (2) type 1, normal mucosal architecture with epithelial lymphocyte infiltration, infiltrative lesions; (3) type 2, hypertrophic crypts with epithelial lymphocyte infiltration, hyperplastic lesions; and (4) type 3, typical flat mucosa, destructive lesions. The research protocol was reviewed and approved by the Medical Ethics Committee of the Ahwaz Jundishapour University Hospitals. Written informed consent was obtained from the children's parents.

Table 1 The age, weight, height, short stature and BMI of patients (mean ± SD)

Group	Age	Weight	Height	Short stature (*n*)		BMI
				> 2 SD	> 3 SD	
Without CD	16.6 ± 6.5	38.7 ± 13.4	140 ± 17.1	54	15	19 ± 3.5
With CD	16.9 ± 7.1	37.9 ± 13.1	137.6 ± 13.1	30	5	19.1 ± 3.1
Total	16.8 ± 6.7	38.5 ± 13.2	139.2 ± 17.3	84	20	19.1 ± 3.5

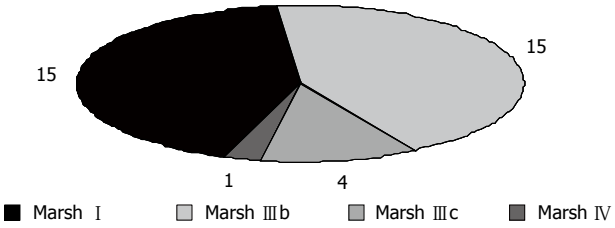

Figure 1 Histological findings of celiac disease.

Statistical analysis

The results are reported as mean ± SD. Statistical analysis was performed by the unpaired Student *t*-test (GraphPad Prism Software Incorporated), with the level of significance set at $P < 0.05$.

RESULTS

The most frequent symptom was diarrhea ($n = 13$) followed by abdominal pain and distention ($n = 3$) in patients with CD and the patients affected by CD did not differ from those without CD in any of the symptoms. A family history of CD was detected in two patients (5.7%). At diagnosis, in the CD patient group, mean weight was 37.9 ± 13.1 and mean height was 137.6 ± 13.1. In this group, short stature of > 2 SD and > 3 SD was found in 30 patients (85.7%) and 5 patients (14.3%), respectively ($P > 0.05$, Table 1).

Small intestine biopsies were performed in all 104 patients with ISS. Duodenal mucosal histopathology was normal in 69 patients. Histopathologic analysis showed evidence of abnormalities compatible with CD in 35 cases (33.6%).

The following histological findings were obtained: (a) 15 of 35 patients had normal mucosal architecture with epithelial lymphocyte infiltration and (b) 15 cases had hypertrophic crypts with epithelial lymphocyte infiltration and partial villous atrophy and (c) five cases showed subtotal or total villous atrophy (Figure 1).

Therefore, the prevalence of properly diagnosed CD

Table 2 Relationship between positive and negative IgA AGA, and IgA TTG antibodies and histological evidence of celiac disease (*n*)

Lab group	IgA AGA		IgA TTG antibodies	
	Positive	Negative	Positive	Negative
Without CD	8	61	4	65
With CD	28	7	31	4
Total	36	68	35	69

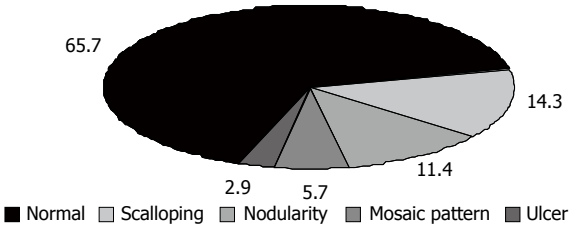

Figure 2 Endoscopic features.

among patients with ISS in this study was 33.6% (35 of 104 patients). IgA AGA, and I IgA TTG antibodies were found in 80% (n = 28), and 88.6% (n = 31) of patients with ISS, respectively. Specificity and the positive predictive value (PPV) for TTG antibodies were found to be 94.2% and 88.6% for CD in the group of patients with ISS in this study. Table 2 shows the relationship between positive and negative IgA AGA, and IgA TTG antibodies and histological evidence of CD. IgA AGA: sensitivity 80%, specificity 88.4%, PPV: 77.8%, negative predictive value (NPV) 89.7%; IgA TTG antibodies: sensitivity 88.6%, specificity 94.2%, PPV 88.6%, NPV 94.2%. The endoscopic features are summarized in Figure 2.

DISCUSSION

Screening for CD in the general population indicates a prevalence of 1:300 to 1:100. About 50% of these children are completely symptomless but because of these figures some experts suggest CD screening for all adults[28] and children[29]. In a British population-based study on short stature, where CD was not specifically investigated, the prevalence of CD was 2:180. In children with short stature and no gastrointestinal symptoms who were investigated for CD, the prevalence increased to 2%-8%. When other (endocrine) causes for short stature are excluded, the prevalence might rise as high as 59%[30,31]. Although CD was once thought to be rare in Iran, several recent reports have cleared this misconception[23,24]. Dr. Shahbazkhani reported that the minimum prevalence of gluten sensitivity among apparently healthy urban Iranian blood donors is 1/166[25]. However, all of these reports deal with typical presentation of CD.

We performed a prospective study on newly diagnosed cases of CD in a group of short stature patients in the south-West of Iran in 2003-2005. Despite the presence of clinical signs of CD during childhood in more than one-third of the patients, the disease remained undiagnosed for many years. This late diagnosis may lead to short stature and low female fertility.

In the last 20 years the clinical picture of CD has changed considerably. The classic form of CD now accounts for a small and systematically shrinking percentage of cases, while atypical forms that present with few or no symptoms are the majority[11]. Short stature is a well-recognized complication of CD[12] although Cacciari *et al*[13] found that adult height is normal in patients who experienced their first symptoms of CD during adulthood. Adult height was shorter only in patients who had symptoms during childhood. This study demonstrated the prevalence and clinical features of CD (n = 35) in a group of 104 patients with ISS. Ages ranged from 2 to 18 years and the mean age of diagnosis was 16.9 years, similar to the results of the study by Mäki *et al*[14]. The age at onset of symptoms appeared to modify the clinical picture. Patients with an earlier onset of CD have a typical clinical picture, whereas patients with delayed onset have atypical presentation, such as short stature.

According to our findings, the prevalence of biopsy-proven CD was 33.6% in the group of ISS children, thereby justifying screening for this disease in all children with short stature. The proportion of CD in cases with ISS ranged from 18.6% to 59.1% in other studies[15,16]. The mechanism of growth retardation is not clearly understood in patients with CD; nutritional deficiencies especially zinc deficiency, low serum somatomedin activity and defects in growth hormone secretion have been proposed as underlying mechanisms[17-19]. An association between CD and autoimmune disorders, such as type I diabetes, autoimmune thyroid disease, and Sjögren's syndrome, has been well documented in the literature[20]. These conditions were not detected in patients in the present study. Susceptibility to CD is determined by genetic factors, which is confirmed by the occurrence of multiple cases of CD in the same family. The prevalence of CD found among first degree relatives is approximately 10%[21]. Screening of siblings in the present study showed that only two siblings (5.7%) had CD. The tests used for CD in this study were IgAAGA and IgA TTG antibodies. The total IgA level was determined also, because CD is associated with IgA deficiency. Antiendomysial antibody and anti-TTG antibodies have been shown to have a high sensitivity and specificity for the diagnosis of CD and correlate well with villous atrophy in untreated patients[32], but false-negative results have been obtained for patients with IgA deficiency[22]. A jejunal biopsy remains the gold standard for the diagnosis of CD but both serologic and histopathologic parameters of CD were investigated in the patients in this study. The sensitivity and specificity of serologic tests were variable. The TTG antibody test has been shown to have a higher sensitivity and specificity for the diagnosis of CD in our patients. Our patients were also tested for IgA deficiency and all were found to have normal IgA values. However, negative results for these tests would not exclude CD. Shamir *et al*[10], using multiple serological strategies

to diagnose silent CD, demonstrated that using any serological marker alone, including EMA antibodies detected by immunofluorescence, would underestimate the prevalence of CD[10]. We found that seven cases with partial villous atrophy had normal AGA, and four of this subgroup had normal anti-TTG antibodies also. Anti-TTG antibodies seem to be more specific but both measurements had limitations in the diagnosis of CD. Our data support the view that there is no single test or measurement that can identify all subjects with CD and ISS. Histological findings of CD showed a spectrum ranging from type 1 mucosal lesions to total villous atrophy type 4 in our study. Fifteen of 35 CD patients (42.9% of CD cases) had mild mucosal abnormality without villous atrophy, 15 (42.9% of CD cases) had partial villous atrophy, four (10.3% of CD cases) had subtotal villous atrophy and one (2.9% of CD cases) had total villous atrophy. According to histological findings, if we limited the diagnosis of CD to cases with villous atrophy, only 20 of 106 ISS cases in our study would have CD (19.3% of all cases) which is obviously more common than the general population.

In conclusion, the possibility of CD should be kept in mind as the prevalence of CD is high in patients with ISS. The patients affected by CD did not differ from those without CD in any of the symptoms. Patients with ISS should be evaluated for CD even in the absence of typical clinical symptoms. It is important to test all children with ISS for CD by measuring anti-EMA IgA or anti-TTG antibodies and performing an intestinal biopsy.

Clinical bottom line: In 33.6% patients with idiopathic short stature, CD may be the underlying cause. Investigation of CD is recommended in the diagnostic assessment of a short child with no endocrinological abnormality.

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COMMENTS

Background

Short stature is one of the most common causes for referrals to pediatric endocrinologists. Many of these patients have no identifiable medical abnormality and are classified as idiopathic short stature (ISS). For most of these patients, it is believed that genetic variations are the underlying cause. Short stature is a well known feature of pediatric celiac disease (CD).

Research frontiers

The prevalence of CD is high in patients with ISS and it is important to test all children with ISS for CD by measuring serologic markers and performing an intestinal biopsy.

Peer review

This is a well written manuscript with a good abstract. In the text they mentioned jejunal biopsies as the gold standard for diagnosis.

REFERENCES

1 **Pasquino AM**, Albanese A, Bozzola M, Butler GE, Buzi F, Cherubini V, Chiarelli F, Cavallo L, Drop SL, Stanhope R, Kelnar CJ. Idiopathic short stature. *J Pediatr Endocrinol Metab* 2001; **14** Suppl 2: 967-974

2 **Cacciari E**, Salardi S, Volta U, Biasco G, Lazzari R, Corazza GR, Feliciani M, Cicognani A, Partesotti S, Azzaroni D. Can antigliadin antibody detect symptomless coeliac disease in children with short stature? *Lancet* 1985; **1**: 1469-1471

3 **Cacciari E**, Salardi S, Lazzari R, Cicognani A, Collina A, Pirazzoli P, Tassoni P, Biasco G, Corazza GR, Cassio A. Short stature and celiac disease: a relationship to consider even in patients with no gastrointestinal tract symptoms. *J Pediatr* 1983; **103**: 708-711

4 **Visakorpi JK**, Maki M. Changing clinical features of coeliac disease. *Acta Paediatr Suppl* 1994; **83**: 10-13

5 **Misra S**, Ament ME. Diagnosis of coeliac sprue in 1994. *Gastroenterol Clin North Am* 1995; **24**: 133-143

6 **Grodzinsky E**, Franzen L, Hed J, Strom M. High prevalence of celiac disease in healthy adults revealed by antigliadin antibodies. *Ann Allergy* 1992; **69**: 66-70

7 **George EK**, Mearin ML, Bouquet J, von Blomberg BM, Stapel SO, van Elburg RM, de Graaf EA, Hertzberger-ten Cate R, van Suijlekom-Smith LW, Reeser HM, Oostdijk W. Screening for coeliac disease in Dutch children with associated diseases. *Acta Paediatr Suppl* 1996; **412**: 52-53

8 **Oberhuber G**, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999; **11**: 1185-1194

9 **Vitoria JC**, Arrieta A, Arranz C, Ayesta A, Sojo A, Maruri N, García-Masdevall MD. Antibodies to gliadin, endomysium, and tissue transglutaminase for the diagnosis of celiac disease. *J Pediatr Gastroenterol Nutr* 1999; **29**: 571-574

10 **Shamir R**, Lerner A, Shinar E, Lahat N, Sobel E, Bar-or R, Kerner H, Eliakim R. The use of a single serological marker underestimates the prevalence of celiac disease in Israel: a study of blood donors. *Am J Gastroenterol* 2002; **97**: 2589-2594

11 **Visakorpi JK**, Maki M. Changing clinical features of coeliac disease. *Acta Paediatr Suppl* 1994; **83**: 10-13

12 **Bonamico M**, Scire G, Mariani P, Pasquino AM, Triglione P, Scaccia S, Ballati G, Boscherini B. Short stature as the primary manifestation of monosymptomatic celiac disease. *J Pediatr Gastroenterol Nutr* 1992; **14**: 12-16

13 **Cacciari E**, Corazza GR, Salardi S, Pascucci MG, Tacconi M, Cicognani A, Tassinari D, Biasco G, Volta U, Lazzari R. What will be the adult height of coeliac patients? *Eur J Pediatr* 1991; **150**: 407-409

14 **Mäki M**, Holm K. Incidence and prevalence of coeliac disease in Tampere. Coeliac disease is not disappearing. *Acta Paediatr Scand* 1990; **79**: 980-982

15 **de Lecea A**, Ribes-Koninckx C, Polanco I, Calvete JF. Serological screening (antigliadin and antiendomysium antibodies) for non-overt coeliac disease in children of short stature. *Acta Paediatr Suppl* 1996; **412**: 54-55

16 **Tumer L**, Hasanoglu A, Aybay C. Endomysium antibodies in the diagnosis of celiac disease in short-statured children with no gastrointestinal symptoms. *Pediatr Int* 2001; **43**: 71-73

17 **Vanderschueren-Lodeweyckx M**, Wolter R, Molla A, Eggermont E, Eeckels R. Plasma growth hormone in coeliac disease. *Helv Paediatr Acta* 1973; **28**: 349-357

18 **Lecornu M**, David L, Francois R. Low serum somatomedin activity in celiac disease. A misleading aspect in growth failure from asymptomatic celiac disease. *Helv Paediatr Acta* 1978; **33**: 509-516

19 **Naveh Y**, Lightman A, Zinder O. A prospective study of serum zinc concentration in children with celiac disease. *J Pediatr* 1983; **102**: 734-736

20 **Swinson CM**, Slavin G, Coles EC, Booth CC. Coeliac disease and malignancy. *Lancet* 1983; **1**: 111-115

21 **Mäki M**, Holm K, Lipsanen V, Hällström O, Viander M, Collin P, Savilahti E, Koskimies S. Serological markers and HLA genes among healthy first-degree relatives of patients with coeliac disease. *Lancet* 1991; **338**: 1350-1353

22 **Hin H**, Bird G, Fisher P, Mahy N, Jewell D. Coeliac disease in primary care: case finding study. *BMJ* 1999; **318**: 164-167

23 **Shahbazkhani B**, Faezi T, Akbari MR, Mohamadnejad M, Sotoudeh M, Rajab A, Tahaghoghi S, Malekzadeh R. Coeliac disease in Iranian type I diabetic patients. *Dig Liver Dis* 2004; **36**: 191-194

24 **Malekzadeh R**, Sachdev A, Fahid Ali A. Coeliac disease in developing countries: Middle East, India and North Africa. *Best Pract Res Clin Gastroenterol* 2005; **19**: 351-358

25 **Shahbazkhani B**, Malekzadeh R, Sotoudeh M, Moghadam KF, Farhadi M, Ansari R, Elahyfar A, Rostami K. High prevalence of coeliac disease in apparently healthy Iranian blood donors. *Eur J Gastroenterol Hepatol* 2003; **15**: 475-478

26 **Queiroz MS**, Nery M, Cancado EL, Gianella-Neto D, Liberman B. Prevalence of celiac disease in Brazilian children of short stature. *Braz J Med Biol Res* 2004; **37**: 55-60

27 **Bozzola M**, Giovenale D, Bozzola E, Meazza C, Martinetti M, Tinelli C, Corazza GR. Growth hormone deficiency and coeliac disease: an unusual association? *Clin Endocrinol* (Oxf) 2005; **62**: 372-375

28 **Collin P**. Should adults be screened for celiac disease? What are the benefits and harms of screening? *Gastroenterology* 2005; **128**: S104-S108

29 **Hoffenberg EJ**. Should all children be screened for celiac disease? *Gastroenterology* 2005; **128**: S98-S103

30 **van Rijn JC**, Grote FK, Oostdijk W, Wit JM. Short stature and the probability of coeliac disease, in the absence of gastrointestinal symptoms. *Arch Dis Child* 2004; **89**: 882-883

31 **Dieterich W**, Laag E, Schopper H, Volta U, Ferguson A, Gillett H, Riecken EO, Schuppan D. Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 1998; **115**: 1317-1321

32 **Ahmed ML**, Allen AD, Sharma A, Macfarlane JA, Dunger DB. Evaluation of a district growth screening programme: the Oxford Growth Study. *Arch Dis Child* 1993; **69**: 361-365

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RAPID COMMUNICATION

Gluten sensitive enteropathy in patients with iron deficiency anemia of unknown origin

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Abstract

AIM: To determine the prevalence of gluten sensitive enteropathy (GSE) in a large group of patients with iron deficiency anemia (IDA) of obscure origin.

METHODS: In this cross-sectional study, patients with IDA of obscure origin were screened for GSE. Anti-endomysial antibody (EMA) and tissue transglutaminase antibody (tTG) levels were evaluated and duodenal biopsies were taken and scored according to the Marsh classification. The diagnosis of GSE was based on a positive serological test and abnormal duodenal histology. Gluten free diet (GFD) was advised for all the GSE patients.

RESULTS: Of the 4120 IDA patients referred to our Hematology departments, 206 (95 male) patients were found to have IDA of obscure origin. Thirty out of 206 patients (14.6%) had GSE. The mean age of GSE patients was 34.6 ± 17.03 (range 10-72 years). The female to male ratio was 1.3:1. Sixteen patients had Marsh 3, 12 had Marsh 2, and 2 had Marsh 1 lesions. The severity of anemia was in parallel with the severity of duodenal lesions. Twenty-two GSE patients (73.3%) had no gastrointestinal symptoms. Fourteen GSE patients who adhered to GFD without receiving iron supplementation agreed to undergo follow up visits. After 6 mo of GFD, their mean hemoglobin levels (Hb) increased from 9.9 ± 1.6 to 12.8 ± 1.0 g/dL ($P < 0.01$). Interestingly, in 6 out of 14 patients who had Marsh 1/2 lesions (e.g. no villous atrophy) on duodenal biopsy, mean Hb increased from 11.0 ± 1.1 to 13.1 ± 1.0 g/dL ($P < 0.01$) while they did not receive any iron supplementation.

CONCLUSION: There is a high prevalence (e.g. 14.6%) of GSE in patients with IDA of obscure origin. Gluten free diet can improve anemia in GSE patients who have mild duodenal lesions without villous atrophy.

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Key words: Gluten sensitive enteropathy; Iron deficiency anemia; Anti-Tissue transglutaminase antibody; Anti-endomysial antibody; Gluten free diet

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INTRODUCTION

Gluten sensitive enteropathy (GSE) is an autoimmune enteropathy due to food gluten intolerance in genetically predisposed people[1]. While GSE was thought to be a rare disease in the past and was believed to be essentially a disease of Europeans[2-5], recent screening studies showed that GSE is one of the most frequent genetically based diseases which occurs worldwide, with a prevalence ranging from 1:85 to 1:500 in different populations[6-9].

Several categories of GSE have recently emerged, including: monosymptomatic, oligosymptomatic, atypical (without gastrointestinal symptoms), silent, potential and latent form[10,11]. Iron deficiency anemia (IDA) is a commonly observed sign in GSE and is the only abnormality in 40% of patients[12]. In fact, only a minority of GSE patients present with classical malabsorption symptoms of diarrhea and weight loss, whereas most patients have subclinical or silent forms in which IDA can be the sole presentation[13].

In an extensive evaluation of the gastrointestinal tract in patients with IDA in order to identify a source of bleeding, the origin of bleeding cannot be detected in a significant minority of patients. In some of these patients IDA could be the result of diseases that impair iron absorption in the absence of bleeding[14,15]. Gluten sensitive enteropathy is one of these disorders which causes chronic inflammation in the bowel surface, leading to infiltration of T-lymphocytes, hyperplasia of crypts, villous atrophy and reduction of the bowel absorption surface for various nutrients such as iron[16].

Considering the broad spectrum of clinical manifestations of GSE, including anemia, osteoporosis, dermatitis herpetiformis, neurologic disorders and life-threatening complications such as non Hodgkin's lymphoma, small intestinal adenocarcinoma, esophageal cancer, and melanoma, early diagnosis of GSE is essential[17-20].

The present study was conducted to estimate the prevalence of GSE in a large group of patients with IDA of unknown origin by use of two highly sensitive and specific serological tests. We also present the follow-up data of those GSE patients who adhered strictly to a GFD and agreed to undergo follow up visits.

MATERIALS AND METHODS

Subjects

In this prospective study we evaluated all 4120 patients with IDA referred to the Hematology departments of Shariati Hospital, and Firoozgar Hospital from April 2003 to September 2007. Iron deficiency anemia was defined as: hemoglobin concentration less than 13.5 g/dL in men and less than 11.5 g/dL in women; mean corpuscular volume (MCV) less than 80 fl; and ferritin level less than 30 ng/mL.

Methods

Patients were evaluated in 6 steps. In step 1, patients with the following conditions were excluded from the study: known malignancies, hematological diseases (hemolytic anemia, aplastic anemia, thalassemia and myelodysplasia), known chronic diseases (e.g. chronic renal failure, chronic infectious disease, severe cardiac and respiratory disease, collagen vascular disease and chronic liver disease), pregnancy, heavy menstrual flow (cycles $\geqslant$ 7 d), menometrorrhagia, drug addiction, alcoholism, gastric surgery, and obvious blood loss (e.g. melena, hematochezia, hematuria, recurrent epistaxis). In this step 3559 patients were excluded and 561 were entered into the next step.

In step 2, patients were offered the chance to participate in the study, and a questionnaire was completed by each patient. Ninety-four patients declined to enter the study, and 467 patients entered into the next step. Informed consent was obtained from each patient and documented under institutional guidelines and oversight.

In step 3 all patients underwent colonoscopy. Patients with likely sources of blood loss, including any mass lesions, polyps greater than 1.5 cm, five or more vascular ectasias, histologically-proven inflammatory bowel disease, ischemic colitis, or solitary rectal ulcer were excluded. In this step 108 patients were excluded, and 359 patients were entered into the fourth step.

In step 4, all remaining patients underwent upper gastrointestinal endoscopy to exclude sources of blood loss, including varices, peptic ulcer, mass lesions, polyps greater than 1.5 cm in diameter, five or more vascular ectasias, or erosive gastritis. If none of the above lesions were detected, three biopsy specimens were taken from the second part of the duodenum. One hundred and forty seven patients were excluded in step 4.

In step 5, the remaining 212 patients underwent small bowel barium study. Six patients with abnormal small bowel series were excluded in this step.

Thus, from the 4120 patients with IDA who entered the Hematology department, 206 patients were found to have IDA of obscure origin.

In step 6, venous blood samples for tissue transglutaminase antibody (tTG) and endomysial antibody (EMA) were obtained from the 206 remaining patients with IDA of obscure origin. The duodenal biopsy specimens were fixed immediately in formalin solution for 4-8 h at room temperature and were routinely processed for conventional histological evaluation. The biopsy specimens were read by one expert histopathologist and the histological damage of duodenum was expressed based on Modified Marsh classification[3]: 0: Normal mucosal structure without significant lymphocytic infiltration; 1: Lymphocytic enteritis (more than 30 lymphocytes/100 epithelial cells); 2: Lymphocytic enteritis and crypt hyperplasia; 3A: Partial villous atrophy; 3B: Subtotal villous atrophy.

The levels of antibodies against IgA tTG were determined by ELISA using human recombinant tTG as the antigen (Orgentec Diagnostika GmbH, Mainz, Germany). Serum samples were diluted to 1:100 with distilled water, incubated with antigen for 30 min at room temperature, washed three times, and subsequently incubated for another 30 min with antihuman IgA. Optical density was read at 450 nm. Results were expressed in arbitrary units (AU) according to the reference calibrator. The cut-off value for a positive outcome was considered to be 10 AU, according to the instructions on the kit. IgA EMA assay was performed using an indirect immunofluorescence technique. The result was considered positive when bright green reticular fluorescence of smooth muscle was detected by fluorescence microscopy. Total serum IgA was measured in patients with negative tTG and EMA results to exclude IgA deficiency as a cause of false-negative tTG and EMA.

Table 1 Hemoglobin (Hb), Mean Corpuscular Volume (MCV) and Ferritin in GSE patients as compared with other anemic patients

	GSE	Other IDA patients[1]
Hb (g/dL)	9.8 ± 1.7	9.3 ± 2.0
MCV	74.0 ± 9.2	69.1 ± 12.6
Ferritin (ng/mL)	12.4 ± 9.8	11.2 ± 24.2

[1]*P* value was not significant compared to GSE patients (independent *t*-test).

Table 2 Mean hemoglobin level among patients with various degrees of duodenal lesions

MARSH classification	No. of GSE patients	Mean Hemoglobin level
1	2	11.2 ± 1.6
2	12	10.9 ± 1.2[b]
3	16	8.68 ± 1.5[b,d]

[b]*P* < 0.001 compared to Marsh 1 group (independent t-test), [d]*P* < 0.001 compared to Marsh 2 group (independent *t*-test).

The presence of positive tTG or EMA plus abnormal duodenal histology (e.g. Marsh 1, 2 or 3) was defined as gluten sensitivity enteropathy (GSE). All GSE patients were referred to our nutrition clinic and advised to follow a strict gluten free diet, but iron supplementation was withheld. Patients were followed up after 6 mo. Adherence to GFD was assessed in the follow up visit.

Statistical analysis

Data are presented as mean ± SD or percentage. Statistical analysis was performed using SPSS software version 15 and *t*-test for comparison of the means of quantitative variables. $P < 0.05$ was considered statistically significant.

RESULTS

From the 206 patients with IDA of obscure origin, 95 were men with a mean age of 37.6 ± 19.8 years, and 111 were women with a mean age of 39.1 ± 14.4 years.

Serological findings

Serological screening tests showed 31 patients had one or two positive tests. Twenty eight patients had positive tTG, and 23 had positive EMA. In 20 patients both tests were positive. None of the patients with negative serological tests was IgA-deficient.

Biopsy findings

Thirty-eight patients had abnormal duodenal histology. Sixteen patients had Marsh 3, 15 had Marsh 2 and 7 had Marsh 1. Among 38 patients with abnormal duodenal histology, 8 patients (3 with Marsh 2, and 5 with Marsh 1) had negative serologic tests. Eight patients who had abnormal duodenal histology but negative serological tests were not considered to have GSE.

GSE patients

Thirty out of 206 (14.6%) of the patients had GSE. The mean age of these patients was 34.6 ± 17.03 (range 10-72 years). The female/male ratio was 1.3:1. Thirty-one patients were positive for one or two serologic tests, but one of the tTG-positive patients had normal duodenal histology. Among 30 GSE patients, three had negative tTG, and seven had negative EMA. The mean duration of anemia before the diagnosis of GSE was 3.6 ± 1.4 years. These patients had been treated with oral iron for a mean duration of 1.9 years. Anemia improved in only 8 patients (26.8%) treated with oral iron supplementation before GSE diagnosis. Four patients (13.3%) had a family history of prolonged anemia of unknown cause in first degree relatives.

Six patients (20%) mentioned flatulence, two (6.7%) had intermittent diarrhea and one (3.3%) had dermatitis herpetiformis. There were no gastrointestinal symptoms in 22 GSE patients (73.3%).

The mean age of the GSE patients was not significantly different from other IDA of obscure origin patients (34.6 ± 17.0 *vs* 39.3 ± 17.1 years, respectively).

In Table 1, mean Hb, MCV and ferritin in GSE patients are compared with other patients with IDA of obscure origin. There were no statistically significant differences between the patient groups.

In GSE patients, the decrement in hemoglobin level was parallel to the severity of duodenal lesion. Patients with Marsh 3 lesions had more severe anemia (Table 2).

Sensitivity and specificity of the serologic tests

We calculated the sensitivity and specificity of the serological tests based on our definition of GSE (e.g. positive tTG or EMA, plus abnormal duodenal histology). The sensitivity and specificity of IgA tTG-Ab for diagnosing GSE were 90% and 98.5% respectively. Also, the sensitivity and specificity of EMA for diagnosing GSE were 76.6% and 100%, respectively.

Follow up

All the GSE patients were referred to our Nutrition Clinic, and GFD was advised for all of them. Iron supplementation was not started in the patients. The patients were invited for a follow up visit 6 mo after the diagnosis.

Four GSE patients were lost to follow up. Seven patients did not strictly adhere to GFD. For five other patients, iron supplementation was started at other clinics during the follow up period. Thus, we present the follow up data of 14 patients who strictly adhered to GFD and did not use iron supplementation during the 6 mo follow up period.

Mean hemoglobin increased from 9.9 ± 1.6 to 12.8 ± 1.0 g/dL ($P < 0.001$), and mean serum ferritin level increased from 12.0 ± 6.0 to 22.1 ± 7.9 ng/mL.

Interestingly, in 6 patients with Marsh 1/2 lesions (e.g. without villous atrophy) mean Hb increased from 11.0 ± 1.1 to 13.1 ± 1.0 g/dL ($P = 0.002$), and mean serum ferritin level increased from 16.5 ± 4.3 to 25.9 ± 6.2 ng/ml ($P = 0.014$). Demographic and clinical data are presented in Table 3.

Table 3 Demographic and clinical data of the 6 patients with Marsh 1/2 lesions that adhered to GFD

Patient No.	Gender	Age	Marsh classification	Hb level before GFD (g/dL)	Hb level 6 mo after GFD (g/dL)	Ferritin level before GFD (ng/mL)	Ferritin level 6 mo after GFD (ng/mL)
1	Male	29	1	12.8	14.0	19	24.6
2	Male	10	2	10.5	13.5	13.7	34.0
3	Female	17	2	9.5	12.2	20	23.5
4	Male	22	2	11.5	13.0	18	31.0
5	Male	30	2	11.1	14.2	9	16.2
6	Female	38	2	10.4	11.8	19	26.0

DISCUSSION

In this prospective study, we found GSE as the cause of IDA of obscure origin in a significant proportion (14.6%) of patients. Various rates of prevalence of GSE in IDA patients have been reported among different studies[21-24]. This discrepancy may be explained by patient selection criteria in the different studies.

In our study, the prevalence of GSE is amongst the highest rates reported. One reason is that we evaluated GSE among highly selected patients in whom the cause of IDA could not be identified after extensive evaluation. Also, we considered patients with positive serological tests and milder degrees of duodenal mucosal lesions (e.g. Marsh 1 or 2) as having GSE.

Physicians may fail to consider GSE as a cause of IDA when gastrointestinal symptoms are absent or nonspecific. In this study, most GSE patients (73.3%) did not report any gastrointestinal symptoms. In our study, there were no differences in demographic characteristics or hematological indices between GSE patients and other patients with anemia of obscure origin to help to distinguish them (Table 1). In GSE patents, the hemoglobin level was inversely correlated with the severity of the histological injury. Patients with Marsh 3 lesions had the most severe anemia, consistent with the role of impaired intestinal absorption in the pathogenesis of IDA. We found marked improvement of anemia in 14 patients who adhered to GFD but did not use iron supplementation. Many authors consider the presence of villous atrophy (e.g. Marsh 3) as one of the major criteria for diagnosing celiac disease (CD)[25,26]. In order to avoid this controversy in the definition of CD, we used the term "gluten sensitive enteropathy" rather than celiac disease to describe patients with any degree of intestinal damage together with positive serologic tests. In this study, we showed a significant objective improvement in hemoglobin level with GFD alone in patients with positive serology but no villous atrophy (e.g. Marsh 1 or 2). Our study suggests that restriction of the diagnosis of CD to patients with overt villous atrophy will exclude some patients who might benefit from GFD.

In this study, we used a human recombinant protein-based tTG test, which has higher sensitivity and accuracy than a guinea pig protein-based tTG test[27]. However, neither tTG nor EMA was 100% sensitive. We found 7 patients with positive tTG, negative EMA and intestinal damage. On the other hand, we found 3 patients with negative tTG, positive EMA, and duodenal lesions. While some guidelines suggest that either EMA or tTG is sufficient for identifying patients with CD[27,28], our study provides evidence that both tTG and EMA should be used for diagnosing CD.

In this study we did not evaluate GSE in all patients with IDA. One may speculate that some patients who were excluded before step 5, may have had GSE as well as another cause of IDA. The prevalence of GSE in IDA has been reported in previous studies[21-23]. In fact, the aim of our study was to evaluate GSE in a large population of patients with IDA of obscure origin. In a population based study done in Iran, the female to male ratio of GSE was 1 to 1.1[9]. Thus, the female to male ratio found in our study represents the ratio of the disease found in the general population of the country.

In conclusion, celiac disease should be considered in any patient with unexplained IDA, even if they do not have any gastrointestinal symptoms. Furthermore, GFD can improve anemia in IDA patients who have positive tTG/EMA and mild duodenal lesions without villous atrophy.

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COMMENTS

Background

Iron deficiency anemia (IDA) is the only abnormality in 40% of patients diagnosed with gluten sensitive enteropathy (GSE). The majority of patients with GSE don't present with classical malabsorption symptoms such as diarrhea and weight loss. In this study we determined the prevalence of GSE in patients with IDA in Iran.

Research frontiers

Recent screening studies shows that GSE is one of the most commongenetic based diseases which occurs worldwide, with a prevalence of ranging from 1:85 to 1:500 in different populations. Various rates of prevalence of GSE in IDA patients have been reported among different studies probably due to different patient selection criteria in these studies.

Innovation and breakthroughs

Iron deficiency anemia is a common presentation of GSE and can be the sole presentation of the disease. Gluten free diet (GFD) might improve mild duodenal damage (e.g. Marsh 1 or 2) without villous atrophy.

Applications

According to our results, identification of anemic patients with underlying GSE is of great importance. Since IDA can be the sole manifestation of GSE, by diagnosing GSE and giving GFD to patients, we can both prevent the complications of GSE and probably cure IDA without iron supplementation.

Terminology

GSE is an autoimmune enteropathy triggered by the ingestion of gluten-containing grains in susceptible individuals.

Peer review

This is a well-written, well analyzed and scientifically accurate manuscript. The paper is balanced in every part, the reference list is adequate and the conclusions are clear.

REFERENCES

1 **Lima VM**, Gandolfi L, Pires JA, Pratesi R. Prevalence of celiac disease in dyspeptic patients. *Arq Gastroenterol* 2005;

42: 153-156
2 **Davidson LS**, Fountain JR. Incidence of the sprue syndrome; with some observations on the natural history. *Br Med J* 1950; **1**: 1157-1161
3 **Marsh MN**. The natural history of gluten sensitivity: defining, refining and re-defining. *QJM* 1995; **88**: 9-13
4 **Fasano A**, Catassi C. Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. *Gastroenterology* 2001; **120**: 636-651
5 **Goggins M**, Kelleher D. Celiac disease and other nutrient related injuries to the gastrointestinal tract. *Am J Gastroenterol* 1994; **89**: S2-S17
6 **Kolho KL**, Farkkila MA, Savilahti E. Undiagnosed coeliac disease is common in Finnish adults. *Scand J Gastroenterol* 1998; **33**: 1280-1283
7 **Catassi C**, Fabiani E, Ratsch IM, Coppa GV, Giorgi PL, Pierdomenico R, Alessandrini S, Iwanejko G, Domenici R, Mei E, Miano A, Marani M, Bottaro G, Spina M, Dotti M, Montanelli A, Barbato M, Viola F, Lazzari R, Vallini M, Guariso G, Plebani M, Cataldo F, Traverso G, Ventura A. The coeliac iceberg in Italy. A multicentre antigliadin antibodies screening for coeliac disease in school-age subjects. *Acta Paediatr Suppl* 1996; **412**: 29-35
8 **Accomando S**, Cataldo F. The global village of celiac disease. *Dig Liver Dis* 2004; **36**: 492-498
9 **Akbari MR**, Mohammadkhani A, Fakheri H, Javad Zahedi M, Shahbazkhani B, Nouraie M, Sotoudeh M, Shakeri R, Malekzadeh R. Screening of the adult population in Iran for coeliac disease: comparison of the tissue-transglutaminase antibody and anti-endomysial antibody tests. *Eur J Gastroenterol Hepatol* 2006; **18**: 1181-1186
10 **Martucci S**, Biagi F, Di Sabatino A, Corazza GR. Coeliac disease. *Dig Liver Dis* 2002; **34** Suppl 2: S150-S153
11 **Biagi F**, Corazza GR. Clinical features of coeliac disease. *Dig Liver Dis* 2002; **34**: 225-228
12 **Unsworth DJ**, Lock RJ, Harvey RF. Improving the diagnosis of coeliac disease in anaemic women. *Br J Haematol* 2000; **111**: 898-901
13 **Brandimarte G**, Tursi A, Giorgetti GM. Changing trends in clinical form of celiac disease. Which is now the main form of celiac disease in clinical practice? *Minerva Gastroenterol Dietol* 2002; **48**: 121-130
14 **Annibale B**, Capurso G, Chistolini A, D'Ambra G, DiGiulio E, Monarca B, DelleFave G. Gastrointestinal causes of refractory iron deficiency anemia in patients without gastrointestinal symptoms. *Am J Med* 2001; **111**: 439-445
15 **Rockey DC**, Cello JP. Evaluation of the gastrointestinal tract in patients with iron-deficiency anemia. *N Engl J Med* 1993; **329**: 1691-1695
16 **Silano M**, Volta U, Mecchia AM, Dessi M, Di Benedetto R, De Vincenzi M. Delayed diagnosis of coeliac disease increases cancer risk. *BMC Gastroenterol* 2007; **7**: 8
17 **Hernandez L**, Green PH. Extraintestinal manifestations of celiac disease. *Curr Gastroenterol Rep* 2006; **8**: 383-389
18 **Somech R**, Spirer Z. Celiac disease: extraintestinal manifestations, associated diseases, and complications. *Adv Pediatr* 2002; **49**: 191-201
19 **Tursi A**, Giorgetti G, Brandimarte G, Rubino E, Lombardi D, Gasbarrini G. Prevalence and clinical presentation of subclinical/silent celiac disease in adults: an analysis on a 12-year observation. *Hepatogastroenterology* 2001; **48**: 462-464
20 **Green PH**, Fleischauer AT, Bhagat G, Goyal R, Jabri B, Neugut AI. Risk of malignancy in patients with celiac disease. *Am J Med* 2003; **115**: 191-195
21 **Ackerman Z**, Eliakim R, Stalnikowicz R, Rachmilewitz D. Role of small bowel biopsy in the endoscopic evaluation of adults with iron deficiency anemia. *Am J Gastroenterol* 1996; **91**: 2099-2102
22 **Karnam US**, Felder LR, Raskin JB. Prevalence of occult celiac disease in patients with iron-deficiency anemia: a prospective study. *South Med J* 2004; **97**: 30-34
23 **Grisolano SW**, Oxentenko AS, Murray JA, Burgart LJ, Dierkhising RA, Alexander JA. The usefulness of routine small bowel biopsies in evaluation of iron deficiency anemia. *J Clin Gastroenterol* 2004; **38**: 756-760
24 **Patterson RN**, Johnston SD. Iron deficiency anaemia: are the British Society of Gastroenterology guidelines being adhered to? *Postgrad Med J* 2003; **79**: 226-228
25 **Feighery C**, Conlon N, Jackson J. Adult population screening for coeliac disease: comparison of tissue-transglutaminase antibody and anti-endomysial antibody tests. *Eur J Gastroenterol Hepatol* 2006; **18**: 1173-1175
26 **Abrams JA**, Brar P, Diamond B, Rotterdam H, Green PH. Utility in clinical practice of immunoglobulin a anti-tissue transglutaminase antibody for the diagnosis of celiac disease. *Clin Gastroenterol Hepatol* 2006; **4**: 726-730
27 **Hill ID**. What are the sensitivity and specificity of serologic tests for celiac disease? Do sensitivity and specificity vary in different populations? *Gastroenterology* 2005; **128**: S25-S32
28 **James SP**. This month at the NIH: Final statement of NIH Consensus Conference on celiac disease. *Gastroenterology* 2005; **128**: 6

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RAPID COMMUNICATION

Inhibitory effect of modified citrus pectin on liver metastases in a mouse colon cancer model

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Abstract

AIM: To discuss the expression of glactin-3 in liver metastasis of colon cancer and its inhibition by modified citrus pectin (MCP) in mice.

METHODS: Seventy-five Balb/c mice were randomly divided into negative control group (n = 15), positive control group (n = 15), low MCP concentration group (n = 15), middle MCP concentration group (n = 15) and high MCP concentration group (n = 15). CT26 colon cancer cells were injected into the subcapsule of mouse spleen in positive control group, low, middle and high MCP concentrations groups, except in negative control, to set up a colon cancer liver metastasis model. The concentration of MCP in drinking water was 0.0%, 0.0%, 1.0%, 2.5% and 5.0% (wt/vol), respectively. Liver metastasis of colon cancer was observed after 3 wk. Enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of galectin-3 in serum. Expression of galectin-3 in liver metastasis was detected by immunohistochemistry.

RESULTS: Except for the negative group, the percentage of liver metastasis in the other 4 groups was 100%, 80%, 73.3% and 60%, respectively. The number of liver metastases in high MCP concentration group was significantly less than that in positive control group (P = 0.008). Except for the negative group, the median volume of implanted spleen tumor in the other 4 groups was 1.51 cm^3, 0.93 cm^3, 0.77 cm^3 and 0.70 cm^3, respectively. The volume of implanted tumor in middle and high MCP concentration groups was significantly smaller than that in positive control group (P = 0.019; P = 0.003). The concentration of serum galectin-3 in positive control and MCP treatment groups was significantly higher than that in the negative control group. However, there was no significant difference between them. Except for the negative control group, the expression of galectin-3 in liver metastases of the other 4 groups showed no significant difference.

CONCLUSION: Expression of galectin-3 increases significantly in liver metastasis of colon cancer, which can be effectively inhibited by MCP.

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Key words: Pectin; Colonic neoplasms; Metastasis; Liver; Mice

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INTRODUCTION

Liver metastasis is the main cause that impacts the therapeutic effect and postoperative prognosis of colorectal cancer. Inhibiting liver metastasis is beneficial to the therapeutic effect and postoperative prognosis of colorectal cancer[1]. Galectin-3, a carbohydrate-binding protein on tumor cell surface, is closely related to cell to cell adhesion, aggregation of cancer cells *in vitro*, tumor growth and metastasis *in vivo*[2,3]. Galectin-3 is highly expressed in a variety of metastatic cancer cells[4]. Galactosyl, a main component of modified citrus pectin (MCP), can specifically inhibit tumor growth and metastasis *in vivo* and galectin-3-mediated functions *in vitro*[5]. Few studies are available dealing with the inhibitory effects of MCP on cancer metastasis. The aim of this study was to discuss the inhibitory effect of MCP on liver metastasis in a rat colon cancer model.

MATERIALS AND METHODS

Cell lines

Mouse colon adenocarcinoma cell line (CT-26), preserved

and passaged in our biotechnology laboratory, was cultivated in RPMI-1640 culture medium containing 10% new born calf serum, penicillin G and streptomycin at 37℃ in an 5% CO_2 incubator containing 50 mL/L CO_2.

Animals

Seventy-five 6-8 wk old Balb/c female mice, offered by Guangdong Medical Laboratory Animal Center (certification No. 2006A019), weighing 20-25 g, were used in this study. The mice were free from specified-pathogens. Experiments were performed in the SPF Animal Laboratory.

Drugs and reagents

MCP was provided by Centraxinc International, Inc (Francisco, USA). Mouse galectin-3 ELISA kit was provided by R&D Company (Minneapolis, USA). Mouse galectin-3 affinity purified pol was purchased from Jingmei Biotech Co, Lid (Shanghai, China).

Main equipments

U.S Beecher tissue microarray meter, ST360 auto ELIASA were purchased from Kehua (Shanghai, China).

Establishment of mouse model of liver metastasis of colon cancer

Seventy-five Balb/c mice were randomly divided into negative control group, positive control group, low MCP concentration group, middle MCP concentration group and high MCP concentration group. The concentration of MCP in drinking water was 0.0%, 0.0%, 1.0%, 2.5% and 5.0% (wt/voL), respectively. CT26 cells in exponential growth with sufficient NS were used to mix up into a suspension (1×10^6/mL). The mice were anesthetized with 4% chloral hydrate (10 mL/kg) by injecting into their abdominal cavity and an abdominal wall incision paralleling the left subcostal margin was then made. Laparotomy was performed and 0.05 mL of CT-26 suspension was injected into the spleen. A same volume of NS was injected into the abdominal cavity of mice in the negative control group. The incision was closed with #1 suture. All mice continuously received MCP dissolved in drinking water from the 2nd d after operation, to the necropsy day 21. A same volume of distilled water was given in negative control group. All mice had free access to food and water during the experiment.

Observation

After a 3-wk observation, the eyeball of mice was removed to collect 0.5-1.0 mL peripheral blood. All mice were killed by decapitation. The abdominal cavity was opened to observe primary neoplasms of the spleen and record the volume and number of neoplasms (volume = $ab^2/2$, a = max diameter, b = min diameter). The total volume was recorded if there were more than 2 neoplasms. The number of liver metastases was calculated. All neoplasms were identified with HE staining. Liver metastasis was divided into 4 grades as previously described[6]: grade 0: no liver metastases; grade Ⅰ: 1-5 liver metastases; grade Ⅱ: 6-10 liver metastases; grade Ⅲ: more than 10 liver metastases.

ELISA analysis of galectin-3

Blood sample was centrifuged at 3000 r/min for 5 min to separate serum. The isolated serum was stored at ≤ -20℃. The serum sample was diluted in a diluent at 1: 20. In brief, 100 μL of a diluent was added into each well of a plate and incubated for 2 h at room temperature, and the plate was washed with a washing buffer. One hundred μL of detection antibody was added into each well of a plate, incubated for 2 h at room temperature, and the plate was washed with a washing buffer. One hundred μL of a working diluent of streptavidin-HRP was added into each well of a plate, incubated for 20 min at room temperature in the dark, the plate was washed. Finally, 100 μL of a substrate solution was added into each well of a plate, incubated for 20 min at room temperature in the dark, and 50 μL of a stop solution was then added into each well of the plate. A microplate reader was used to read the absorbance at 450 nm, then a standard curve was plotted and a formula was used to fit the OD of standard samples.

Liver metastasis tissue microarray

Liver tissue sections were stained with HE to select typical nidi, such as a region rich of neoplasms but lack of necrosed areas and bleeding. A tissue microarray meter was used to perforate into a paraffin block (25 mm × 25 mm × 20 mm). The diameter of each hole was 1.2 mm, and the distance between two holes was 1.0 mm. Fifty holes were arranged in 10 lines and 5 arrays. A 1.2-mm long puncture needle was used to draw out the marked typical tissue core and to transfer it to a certain location on the paraffin block. Forty-seven metastasis samples were arranged into 2 paraffin blocks. Each sample included 2 marked cores. The tissue array paraffin was kept on a 55℃ copper board for 30 min. The paraffin block was pressed gently to array the tissue cores and cooled at room temperature. The arrays were sliced quickly after pre-cooled at 4℃ for 4 h.

Immunohistochemistry analysis

The tissue sample sections were stained with galectin-3 immunohistochemistry following the instructions provided with galectin-3 affinity purified polyclonal antibody. The sections were deparaffined and hydrated. After washed with PBS, the sections were incubated with 3% hydrogen dioxide for 10 min at room temperature, with antibody for 10 min at room temperature, with EnVision for 30 min at room temperature, finally with DAB for color development. The results were judged double-blindly by 2 pathologists. The level of galectin-3 expression was classified into negative (-), weakly positive (+), positive (++) and strong positive (+++) as previously described[7].

Statistical analysis

All the data were analyzed by SPSS10.0 Software. Tumor

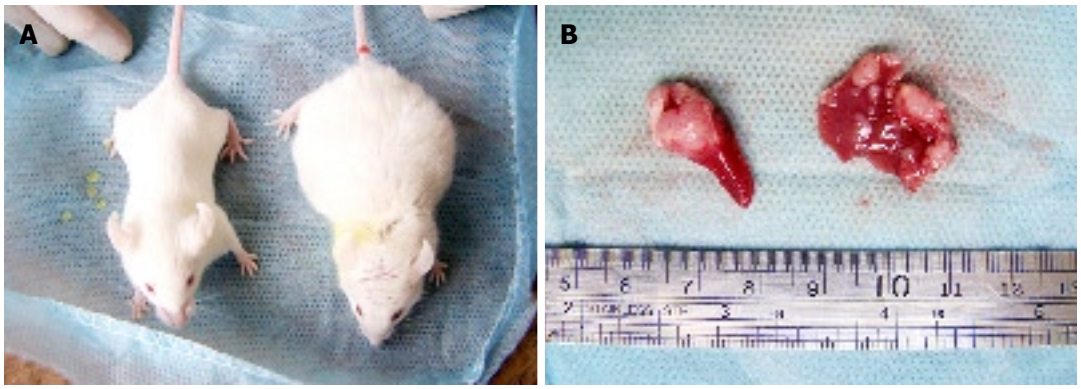

Figure 1 Mouse model of liver metastastic colon cancer. A: Tumor-bearing and healthy mice; B: Primary spleen tumor and liver metastasis.

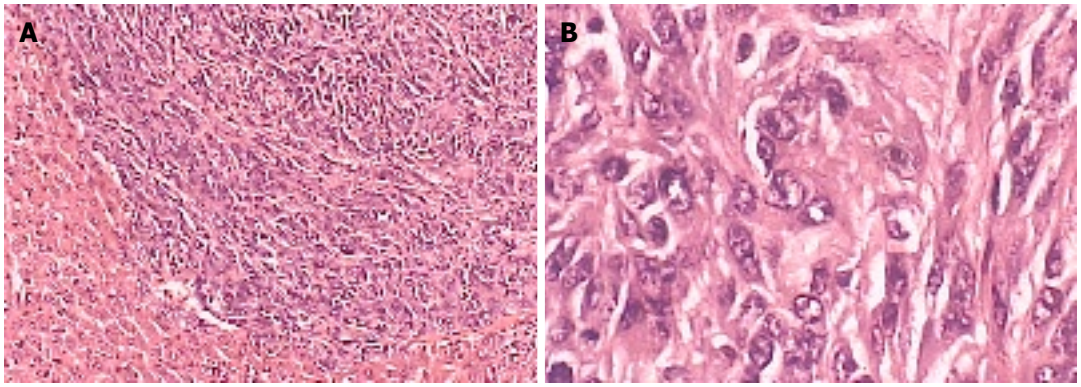

Figure 2 Liver metastastic colon cancer tissue sections stained with HE (A × 200, B × 400).

Table 1 Liver metastases

Groups	*n*	Numbers of liver metastases				χ^2	*P*
		0 (0)	I (1-5)	II (6-10)	III (> 10)		
Positive control group	15	0	2	7	6		
1.0% MCP group	15	3	6	2	4	3.996	> 0.05
2.5% MCP group	15	4	7	1	3	7.069	> 0.05
5.0% MCP group	15	6	4	2	3	8.052	< 0.05[1]

[1]The number of liver metastases in high MCP concentration (5.0%) group was significantly less than that in positive control group ($P = 0.008$).

Table 2 Volume of primary spleen tumor (cm^3)

Groups	*n*	*M*	mean ± SD	χ^2	*P*
Positive control group	15	1.51	1.71 ± 1.29		
1.0% MCP group	15	0.93	1.28 ± 0.68	2.955	> 0.05
2.5% MCP group	15	0.77	0.90 ± 0.55	8.083	< 0.05[1]
5.0% MCP group	15	0.70	0.76 ± 0.30	7.989	< 0.05[1]

[1]The volume of primary spleen tumor in middle and high MCP concentration groups was significantly smaller than that in positive control group ($P = 0.003$).

volume, number of liver metastases, concentration of galectin-3 in serum and tissue were analyzed by non-parametric test.

RESULTS

Mouse living status

No mouse died during the 3-wk experiment period. Some mice were found to have tumor mass bulging on the abdominal wall. Some of the cancer-carrying mice appeared signs of mental depression, such as reduced activity, slow response, gloomy hair color, loss of appetite (Figure 1).

Metastatic liver cancer

Except for the negative control group, the liver metastatic rate for the other 4 groups treated with high, middle and low MCP concentrations was 100%, 80%, 73.3% and 60%, respectively. The number of liver metastases in high MCP concentration group was significantly less than that in low and middle MCP concentration groups ($P < 0.05$) (Table 1).

Volume of primary spleen tumor

The median volume of implanted spleen tumor in high, middle and low MCP concentration groups was 1.51 cm^3, 0.93 cm^3, 0.77 cm^3 and 0.70 cm^3, respectively. No tumor was found in negative control group. The volume of tumor in high MCP concentration group was significantly lower than that in middle and low MCP concentration groups ($P < 0.05$) (Table 2, Figure 2).

Concentration of galectin-3 in serum

The concentration of galectin-3 in serum samples calculated according to the standard regression formula was (14.63 ± 10.08) ng/mL in negative control group, (91.01 ± 22.94) ng/mL in positive control group, (82.75 ± 20.33) ng/mL in low MCP concentration group, (79.01 ± 17.64) ng/mL in middle MCP concentration group and (85.94 ± 15.52) ng/mL in high MCP concentration

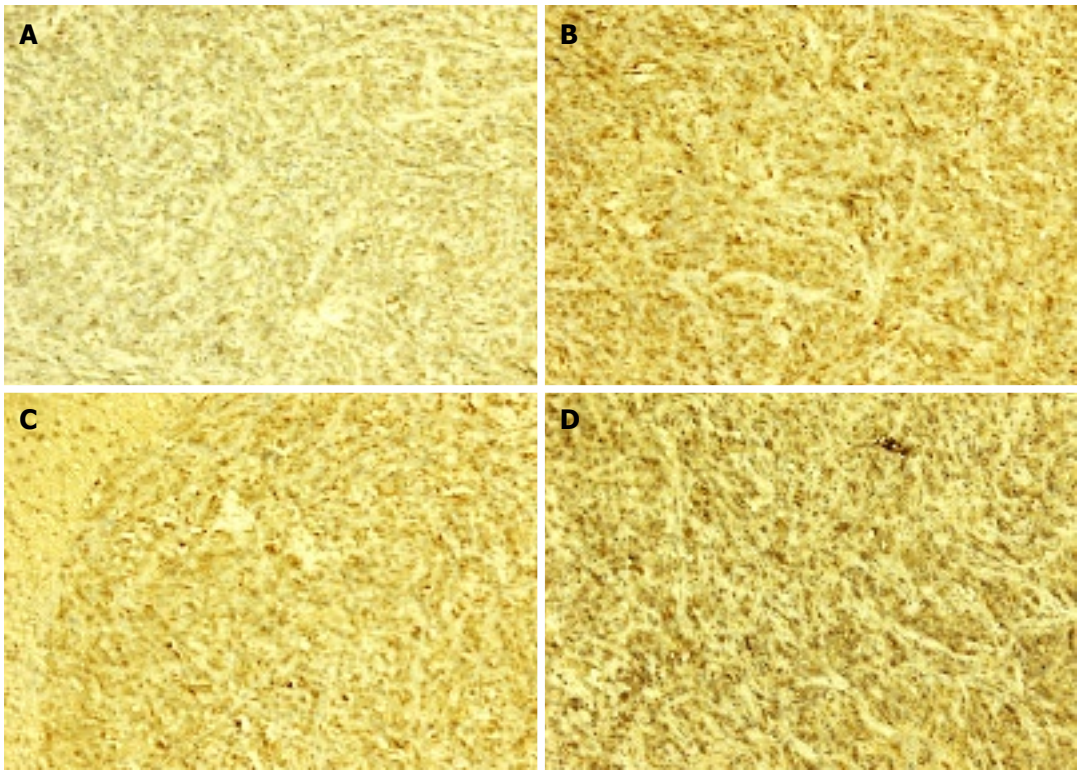

Figure 3 Expression of galectin-3 (× 200) in positive control group (A),1.0% MCP concentration (B), 2.5% MCP concentration group (C), and 5.0% MCP concentration group (D).

Table 3 Serum concentration of galectin-3 (μg/mL)

Groups	*n*	mean ± SD	*H*	*P*
Negative control group	15	14.63 ± 10.08	9.37	< 0.01[1]
Positive control group	15	91.01 ± 12.94		
1.0% MCP group	15	82.75 ± 20.33		
2.5% MCP group	15	79.01 ± 17.64	4.34	
5.0% MCP group	15	85.94 ± 15.52		

[1]The serum concentration of galectin-3 in negatives control group was significantly lower than that in positive control group ($P < 0.01$).

Table 4 Expression of galectin-3 in liver metastasis

Groups	*n*	Expression of galectin-3				*H*	*P*
		(-)	(+)	(++)	(+++)		
Positive control group	15	5	6	2	2	0.52	*P* = 0.170
1.0% MCP group	12	4	3	2	3		
2.5% MCP group	11	2	6	2	1		
5.0% MCP group	9	3	4	1	1		

group, respectively. The results indicate that the concentration of serum galectin-3 in positive control group and MCP treatment groups was significantly higher than that in negative control group ($P < 0.01$, Table 3).

Expression of galectin-3 in liver metastasis

Brown cells in cytolymph under microscope were considered positive cells. The percentage of positive cells in metastatic liver tissue showed that galectin-3 had no significant difference in liver metastases positive control and MCP treatment groups (Figure 3, Table 4).

DISCUSSION

Liver metastasis of colon cancer includes tumor cell infiltration, exfoliation, adhesion, aggregation and invasion, which involve carbohydrate-mediated recognition proteins, such as the galectins. Adhesion of tumor cells to tumor embolus and anchorage of tumor cells to blood vessel endothelium or basement membrane are the two crucial steps of liver metastasis of colon cancer. Different galectins expressed in different steps of metastasis cascade might play a crucial role in tumor progression[8]. Galectin-3, a member of the lectin family, is a multifunctional oncogenic protein which regulates cell growth, adhesion[9], proliferation and apoptosis, as well as cell-cell interaction and angiogenesis[10-13]. A large body of evidence has confirmed that metastatic cancer cells significantly express galectin-3, and high expression of galectin-3 can be detected in both primary and metastatic lesions[14], even in blood[15], showing a strong relation with cancer growth and metastasis[16-18]. Moreover, the expression of galectin-3 can be used as a diagnostic and prognostic marker of colorectal cancer[19-21]. Therefore, if the function of galectin-3 is blocked, the progression of adhesion and aggregation can be intercepted, which may stimulate the development of novel drugs for the targeted treatment of colorectal cancer and other cancers[22].

MCP is a non-digestible, water-soluble polysaccharide fiber derived from citrus fruits, and also a complex polysaccharide rich in galactosyl residues. MCP can specifically inhibit carbohydrate-binding protein as a high affinitive ligand[23]. When the concentration of MCP reaches an adequate level, galectin-3 protein on the surface of cancer cells would be almost completely blocked by MCP molecules. As a result, the procession of adhesion

and aggregation between cancer cells will be intercepted. In addition, MCP can inhibit morphogenesis of endothelial cells and angiogenesis by blocking galectin-3, thus intercepting cancer cells to absorb nutrition from vessels and cancer progression[24,25]. However, there is no evidence that MCP attacks cancer cells directly or indirectly with or without toxicity and side effects[26]. *In vitro* experiments have shown that MCP is able to inhibit adhesion of cancer cells to laminin and homotypic aggregation[27]. Animal experiments also showed that oral MCP can inhibit the growth and metastases of rat prostate cancer cells[28], human breast cancer[5] and melanoma cells[29-31].

The results of our study show that MCP could effetely inhibit the growth and metastasis of implanted colon cancer in mouse spleen. The number of liver metastases and tumor volume in high MCP concentration group were significantly less and smaller than those in control group, indicating that MCP can inhibit the growth and metastasis of colon cancer in a dose-dependent manner, which is consistent with the reported data[5,28-30]. In contrast, low MCP concentration group showed no significant difference in colon cancer growth and liver metastasis, which may be due to the lack of samples and the low sensitivity of non-parametric statistics. Further studies are needed to clarify the role of MCP concentration in this regard.

ELISA and immunohistochemistry analysis have shown that MCP does not impact glactin-3 concentration and expression in liver metastatic cancer cells, but inhibits liver metastasis *in vitro*[30]. The possible mechanism is that MCP only blocks out galectin-3 molecules on the surface of cancer cells, but does not intercept the expression or secretion of cancer cells. It was recently reported that galectin-3 can be used as a reliable diagnostic marker of colorectal cancer and is one of the target proteins in cancer treatment[22].

In conclusion, MCP can effectively inhibit the growth of colon cancer and liver metastasis by intercepting the adhesion and aggregation of cancer cells. MCP, as a natural polysaccharide derived from fruits and a nontoxic drug, may pave a new way in controlling the growth and metastasis of colon cancer and other cancers. The role of MCP and chemotherapy in controlling and curing liver metastatic colon cancer needs further study.

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COMMENTS

Background

Galectin-3 is a carbohydrate-binding protein closely related with cancer growth and metastasis. Studies have shown that galectin-3 is over-expressed in different types of cancer. Dietary components play an important role in cancer progression and metastasis, carbohydrate-mediated recognition processes participate in cancer progression. Modified citrus pectin (MCP), a non-digestible and water-soluble polysaccharide fiber derived from citrus fruits, can inhibit galectin-3-mediated function *in vivo* and *in vitro*.

Research frontiers

The role of galectin-3 in cancer growth and metastasis is an important field in tumor research. Several experimental studies have reported the specific inhibitory effect of MCP on different cancer cells in xenograft models. The present study investigated the effect of MCP on colon cancer growth and liver metastasis *in vivo*. In addition, the expression of galectin-3 was also studied to describe the possible anticancer mechanism of MCP.

Innovations and breakthroughs

Few experiments have been conducted to observe the effect of MCP on preventing cancer growth and metastasis *in vivo*. This study showed the inhibitory effect of MCP on liver metastasis of colon cancer in a mouse model. Besides, the expression of galectin-3 in tumor tissue and serum was tested to describe the galectin-3 status during MCP treatment.

Applications

This study demonstrated that galectin-3 was over-expressed in liver metastasis of colon cancer and oral MC could inhibit cancer growth and metastasis in a mouse model. The results show that galectin-3, as a potential marker and therapeutic target of colorectal cancer, played an important role in prevention and treatment of cancer.

Terminology

Citrus pectin is a complex polysaccharide fiber derived from the pulp and peel of citrus fruits. Citrus pectin is rich in galactosyl, a ligand for galectin-3, when it is modified by high-pH and temperature. MCP inhibits galectin-3 function when citrus fruits are combined with galectin-3.

Peer review

In this manuscript, the authors studied the inhibitory effect of MCP on liver metastasis in a mouse colon cancer model. The study demonstrated that MCP inhibited liver metastasis by suppressing the function of galectin-3. The study is well designed and the results are reliable.

REFERENCES

1 **Kindler HL**, Shulman KL. Metastatic colorectal cancer. *Curr Treat Options Oncol* 2001; **2**: 459-471
2 **Dumic J**, Dabelic S, Flogel M. Galectin-3: an open-ended story. *Biochim Biophys Acta* 2006; **1760**: 616-635
3 **Krzeslak A**, Lipinska A. Galectin-3 as a multifunctional protein. *Cell Mol Biol Lett* 2004; **9**: 305-328
4 **Califice S**, Castronovo V, Van Den Brole F. Galectin-3 and cancer (Review). *Int J Oncol* 2004; **25**: 983-992
5 **Nangia-Makker P**, Hogan V, Honjo Y, Baccarini S, Tait L, Bresalier R, Raz A. Inhibition of human cancer cell growth and metastasis in nude mice by oral intake of modified citrus pectin. *J Natl Cancer Inst* 2002; **94**: 1854-1862
6 **Zhou ZW**, Wan DS, Wang GQ, Ren JQ, Lu ZH, Lin SX, Tang SX, Ye YL, Chen G. [Inhibitory effect of angiogenesis inhibitor YH-16 on liver metastases from colorectal cancer] *Ai Zheng* 2006; **25**: 818-822
7 **Sanjuan X**, Fernandez PL, Castells A, Castronovo V, van den Brule F, Liu FT, Cardesa A, Campo E. Differential expression of galectin 3 and galectin 1 in colorectal cancer progression. *Gastroenterology* 1997; **113**: 1906-1915
8 **Grassadonia A**, Tinari N, Iurisci I, Piccolo E, Cumashi A, Innominato P, D'Egidio M, Natoli C, Piantelli M, Iacobelli S. 90K (Mac-2 BP) and galectins in tumor progression and metastasis. *Glycoconj J* 2004; **19**: 551-556
9 **Hughes RC**. Galectins as modulators of cell adhesion. *Biochimie* 2001; **83**: 667-676
10 **Nakahara S**, Raz A. Regulation of cancer-related gene expression by galectin-3 and the molecular mechanism of its nuclear import pathway. *Cancer Metastasis Rev* 2007; **26**: 605-610
11 **Fukumori T**, Kanayama HO, Raz A. The role of galectin-3 in cancer drug resistance. *Drug Resist Updat* 2007; **10**: 101-108
12 **Takenaka Y**, Fukumori T, Raz A. Galectin-3 and metastasis. *Glycoconj J* 2004; **19**: 543-549
13 **Zou J**, Glinsky VV, Landon LA, Matthews L, Deutscher SL. Peptides specific to the galectin-3 carbohydrate recognition domain inhibit metastasis-associated cancer cell adhesion. *Carcinogenesis* 2005; **26**: 309-318

14 **Iurisci I**, Tinari N, Natoli C, Angelucci D, Cianchetti E, Iacobelli S. Concentrations of galectin-3 in the sera of normal controls and cancer patients. *Clin Cancer Res* 2000; **6**: 1389-1393
15 **Greco C**, Vona R, Cosimelli M, Matarrese P, Straface E, Scordati P, Giannarelli D, Casale V, Assisi D, Mottolese M, Moles A, Malorni W. Cell surface overexpression of galectin-3 and the presence of its ligand 90k in the blood plasma as determinants in colon neoplastic lesions. *Glycobiology* 2004; **14**: 783-792
16 **Bresalier RS**, Mazurek N, Sternberg LR, Byrd JC, Yunker CK, Nangia-Makker P, Raz A. Metastasis of human colon cancer is altered by modifying expression of the beta-galactoside-binding protein galectin 3. *Gastroenterology* 1998; **115**: 287-296
17 **Tsuboi K**, Shimura T, Masuda N, Ide M, Tsutsumi S, Yamaguchi S, Asao T, Kuwano H. Galectin-3 expression in colorectal cancer: relation to invasion and metastasis. *Anticancer Res* 2007; **27**: 2289-2296
18 **Zhang N**, Ding YQ, Liang L. [Association of galectin-3 expression with biological behaviors of human colorectal carcinoma] *Nanfang Yikedaxue Xuebao* 2006; **26**: 1685-1689
19 **Endo K**, Kohnoe S, Tsujita E, Watanabe A, Nakashima H, Baba H, Maehara Y. Galectin-3 expression is a potent prognostic marker in colorectal cancer. *Anticancer Res* 2005; **25**: 3117-3121
20 **Bresalier RS**, Byrd JC, Tessler D, Lebel J, Koomen J, Hawke D, Half E, Liu KF, Mazurek N. A circulating ligand for galectin-3 is a haptoglobin-related glycoprotein elevated in individuals with colon cancer. *Gastroenterology* 2004; **127**: 741-748
21 **Nakamura M**, Inufusa H, Adachi T, Aga M, Kurimoto M, Nakatani Y, Wakano T, Nakajima A, Hida JI, Miyake M, Shindo K, Yasutomi M. Involvement of galectin-3 expression in colorectal cancer progression and metastasis. *Int J Oncol* 1999; **15**: 143-148
22 **Shi Y**, He B, Kuchenbecker KM, You L, Xu Z, Mikami I, Yagui-Beltran A, Clement G, Lin YC, Okamoto J, Bravo DT, Jablons DM. Inhibition of Wnt-2 and galectin-3 synergistically destabilizes beta-catenin and induces apoptosis in human colorectal cancer cells. *Int J Cancer* 2007; **121**: 1175-1181
23 **Modified citrus pectin-monograph.** *Altern Med Rev* 2000; **5**: 573-575
24 **Nangia-Makker P**, Honjo Y, Sarvis R, Akahani S, Hogan V, Pienta KJ, Raz A. Galectin-3 induces endothelial cell morphogenesis and angiogenesis. *Am J Pathol* 2000; **156**: 899-909
25 **Liu F**T, Rabinovich GA. Galectins as modulators of tumour progression. *Nat Rev Cancer* 2005; **5**: 29-41
26 **Chen CH**, Sheu MT, Chen TF, Wang YC, Hou WC, Liu DZ, Chung TC, Liang YC. Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways. *Biochem Pharmacol* 2006; **72**: 1001-1009
27 **Inohara H**, Raz A. Effects of natural complex carbohydrate (citrus pectin) on murine melanoma cell properties related to galectin-3 functions. *Glycoconj J* 1994; **11**: 527-532
28 **Pienta KJ**, Naik H, Akhtar A, Yamazaki K, Replogle TS, Lehr J, Donat TL, Tait L, Hogan V, Raz A. Inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin. *J Natl Cancer Inst* 1995; **87**: 348-353
29 **Hayashi A**, Gillen AC, Lott JR. Effects of daily oral administration of quercetin chalcone and modified citrus pectin on implanted colon-25 tumor growth in Balb-c mice. *Altern Med Rev* 2000; **5**: 546-552
30 **Platt D**, Raz A. Modulation of the lung colonization of B16-F1 melanoma cells by citrus pectin. *J Natl Cancer Inst* 1992; **84**: 438-442
31 **Johnson KD**, Glinskii OV, Mossine VV, Turk JR, Mawhinney TP, Anthony DC, Henry CJ, Huxley VH, Glinsky GV, Pienta KJ, Raz A, Glinsky VV. Galectin-3 as a potential therapeutic target in tumors arising from malignant endothelia. *Neoplasia* 2007; **9**: 662-670

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RAPID COMMUNICATION

Melatonin protects liver from intestine ischemia reperfusion injury in rats

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Abstract

AIM: To investigate the protective effect of melatonin on liver after intestinal ischemia-reperfusion injury in rats.

METHODS: One hundred and fifty male Wistar rats, weighing 190-210 g, aged 7 wk, were randomly divided into melatonin exposure group, alcohol solvent control group and normal saline control group. Rats in the melatonin exposure group received intraperitoneal (IP) melatonin (20 mg/kg) 30 min before intestinal ischemia-reperfusion (IR), rats in the alcohol solvent control group received the same concentration and volume of alcohol, and rats in the normal saline control group received the same volume of normal saline. Serum samples were collected from each group 0.5, 1, 6, 12, and 24 h after intestinal IR. Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with an auto-biochemical analyzer. Serum TNF-α was tested by enzyme-linked immunosorbent assay (ELISA). Malondialdehyde (MDA) in liver was detected by colorimetric assay. Pathological changes in liver and immunohistochemical straining of ICAM-1 were observed under an optical microscope.

RESULTS: The levels of ALT measured at various time points after intestinal IR in the melatonin exposure group were significantly lower than those in the other two control groups ($P < 0.05$). The serum AST levels 12 and 24 h after intestinal IR and the ICAM-1 levels (%) 6, 12 and 24 h after intestinal IR in the melatonin exposure group were also significantly lower than those in the other two control groups ($P < 0.05$).

CONCLUSION: Exotic melatonin can inhibit the activity of ALT, AST and TNF-α, decrease the accumulation of MDA, and depress the expression of ICAM-1 in liver after intestinal IR injury, thus improving the liver function.

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Key words: Melatonin; Intestinal ischemia-reperfusion injury; Liver; TNF-α

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INTRODUCTION

Bowel transplantation is still the only definite therapy for bowel dysfunction although it is more difficult than other organ transplantations at the final stage of bowel dysfunction[1]. Small intestine with high immunogenicity has the highest reject reaction rate in all organ transplantations[2]. Although more and more powerful nonspecific immunosuppressive agents have been used in recent years, the rate of graft rejecting reaction is still higher than 50% in intestine[3]. Current clinical data show that combined liver and small intestine transplantation decreases both acute and chronic rejection compared with simple small intestine transplantation[4]. Small intestine transplantation, the best therapy for bowel dysfunction at present, cannot replace total parenteral nutrition (TPN) due to its severe rejecting reaction. Long term

TPN therapy may induce liver injury and some patients with intestinal dysfunction can accompany hepatic diseases. Since combined liver and intestine transplantation can prevent further liver injuries[5], systematic research should be done to identify the mechanism of hepatic injury caused by intestinal ischemia-reperfusion (IR) and detect the possible intervention which can lessen the injury. Melatonin can protect liver from intestinal IR injury due to its powerful ability to clear free radicals[6]. The objective of this study was to determine the possible protective effect and mechanisms of melatonin in intestinal IR models and provide evidence for its clinical application in spare-part surgery.

MATERIALS AND METHODS

Materials

One hundred and fifty healthy male Wistar rats, weighing 190 to 210 g, aged 6-7 wk, were randomly allocated into melatonin disposure group, alcohol solvent control group and normal saline (NS) control group. All rats were raised for at least 1 wk before operation in a 12 h dark and 12 h light cycle, with free access to food and water. One gram of melatonin (Sigma Company, USA) was dissolved in alcohol solvent (40%) and kept at a subambient temperature. Rats in the melatonin exposure group received 20 mg/kg intra-peritoneal melatonin diluted by NS to 1/10 of the incipient concentration 30 min before intestinal IR. Rats in the alcohol solvent control group received the same concentration of alcohol and NS. Thiopental sodium (40 mg/kg) was injected into biceps femoris of rats 45 min before the intestinal IR model was established. A model of pan-small intestinal IR injury was established by occlusion of the superior mesenteric artery (SMA) for 30 min. Serum samples were collected from rats in each group after reperfusion for 0.5, 1, 6, 12 and 24 h. A paraformaldehyde (PFA, 40 g/L) phosphate buffer (0.1 mol/L, pH = 7.3) was provided by Laboratory of Shengjing Hospital, China Medical University (Shenyang, China). Correlated biochemical agents were bought from Boehringer Mannheim Company in Germany. MDA kits were purchased from Nanjing Jiancheng Biological Engineering Research Center. TNF-α kit was bought from Shenzhen Jingmei Biological Engineering Limited Company (China). ICAM-1 kit was purchased from Serotech Company in UK. OLYMPUS-BX60 optical microscope and photograph system, HITACHI-7600A automatic biochemistry analyzer and SAKURA paraffin section cutter, were provided by Laboratory of China Medical University (Shenyang, China).

Methods

Blood was obtained by trans-diaphragmatic cardiac puncture, each sample was centrifuged for 5 min at 3000 r/min with the clear supernatant remained. ALT and AST were detected with an automatic biochemistry analyzer. Serum TNF-α was determined by ELISA. MDA in liver tissue homogenate obtained from the central part of medial lobes was assayed by colorimetry. ICAM-1 in liver cells was tested by immunohistochemistry and observed under an optical microscope. Cells (including endotheliocytes in liver sinusoid and hepatocytes) with fine yellow particles in cytoplasm were defined as positive. The number of ICAM-1 positive cells per one high power field was calculated, the mean value was expressed as mean ± SD. All parameters were analyzed by variance analysis and SNK test using SPSS 13.0.

RESULTS

Serum ALT

After reperfusion for 12 and 24 h, the levels of ALT in the exposure group at each time point were significantly lower than those in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between alcohol solvent and NS control groups (Table 1).

Serum AST

After reperfusion for 12 and 24 h, the levels of AST in the melatonin exposure group were significantly lower than those in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between the alcohol solvent and NS control groups (Table 1).

Serum TNF-α

After reperfusion for 12 and 24 h, the levels of TNF-α in the melatonin exposure group were significantly lower than those in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between the alcohol solvent and NS control groups (Table 1).

MDA in liver tissue homogenate

After reperfusion for 6, 12 and 24 h, MDA in the melatonin exposure group was significantly lower than that in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between the alcohol solvent and NS control groups (Table 1).

ICAM-1 stained cells in liver tissue

After reperfusion for 6, 12 and 24 h, the positive rate of ICAM-1-stained cells in the melatonin exposure group was significantly lower than that in the alcohol solvent and NS control groups ($P < 0.05$), while there was no obvious difference between the alcohol solvent and NS control groups. The positive cells were mainly liver parenchymal cells located near the sinus hepaticus and sinusoid endothelial cells of liver (Figure 1A-F). The number of positive cells in the alcohol solvent and NS control groups increased gradually and reached its peak at 12 h, and then decreased. The similar trend occurred in the melatonin exposure group, but the extent was much lower than that in the other two control groups (Table 1).

DISCUSSION

Serum ALT and AST levels are generally accepted as the most sensitive indexes of acute hepatic injury and AST appears at the later phase[7]. After reperfusion, the ALT levels in the three groups gradually decreased with the time and were lower in the melatonin exposure group than in the other two control groups ($P < 0.05$),

Table 1 ALT, AST, TNF-α, MDA and ICAM-1 in rats with ischemia-reperfusion injury (mean ± SD)

Group	Time (h)	Serum ALT (μkat/L)	Serum AST (μkat/L)	Serum TNF-α (pg/mL)	MDA in liver (nmol/g)	ICAM-1 in liver (%)
Melatonin	0.5	1.8 ± 0.6[a]	1.8 ± 0.6[a]	8.2 ± 2.9	23.7 ± 4.3	2.9 ± 1.2
	1	1.6 ± 0.5[a]	1.6 ± 0.5[a]	10.9 ± 3.0	28.3 ± 5.1	3.0 ± 0.9
	6	1.5 ± 0.5[a]	1.5 ± 0.5[a]	14.8 ± 3.8[a]	42.0 ± 5.2[a]	4.0 ± 1.4[a]
	12	1.4 ± 0.4[a]	1.4 ± 0.4[a]	18.0 ± 3.3[a]	55.2 ± 5.4[a]	11.8 ± 3.3[a]
	24	1.3 ± 0.4[a]	1.3 ± 0.4[a]	15.7 ± 3.3	82.7 ± 6.1[a]	6.9 ± 2.7[a]
Alcohol	0.5	3.1 ± 0.3	3.1 ± 0.3	8.8 ± 2.5	27.7 ± 5.9	2.4 ± 1.3
	1	3.0 ± 0.6	3.0 ± 0.6	11.8 ± 3.7	33.2 ± 6.3	2.5 ± 0.7
	6	2.5 ± 0.4	2.5 ± 0.4	20.9 ± 4.3	54.3 ± 6.5	7.0 ± 2.3
	12	2.4 ± 0.4	2.4 ± 0.4	23.5 ± 4.3	79.1 ± 6.2	22.4 ± 4.3
	24	2.1 ± 0.5	2.1 ± 0.5	17.7 ± 3.9	106.2 ± 7.1	16.0 ± 3.2
NS	0.5	3.3 ± 0.4	3.3 ± 0.4	9.4 ± 2.8	29.5 ± 6.3	2.8 ± 0.8
	1	2.9 ± 0.5	2.9 ± 0.5	11.6 ± 3.3	34.1 ± 5.4	2.6 ± 1.3
	6	2.6 ± 0.4	2.6 ± 0.4	21.4 ± 5.0	53.8 ± 6.1	6.8 ± 2.2
	12	2.5 ± 0.4	2.5 ± 0.4	24.4 ± 4.9	83.3 ± 5.2	21.4 ± 5.4
	24	2.2 ± 0.4	2.2 ± 0.4	16.3 ± 3.6	108.5 ± 9.8	14.3 ± 2.8

[a]$P < 0.05$ *vs* alcohol solvent and NS control groups.

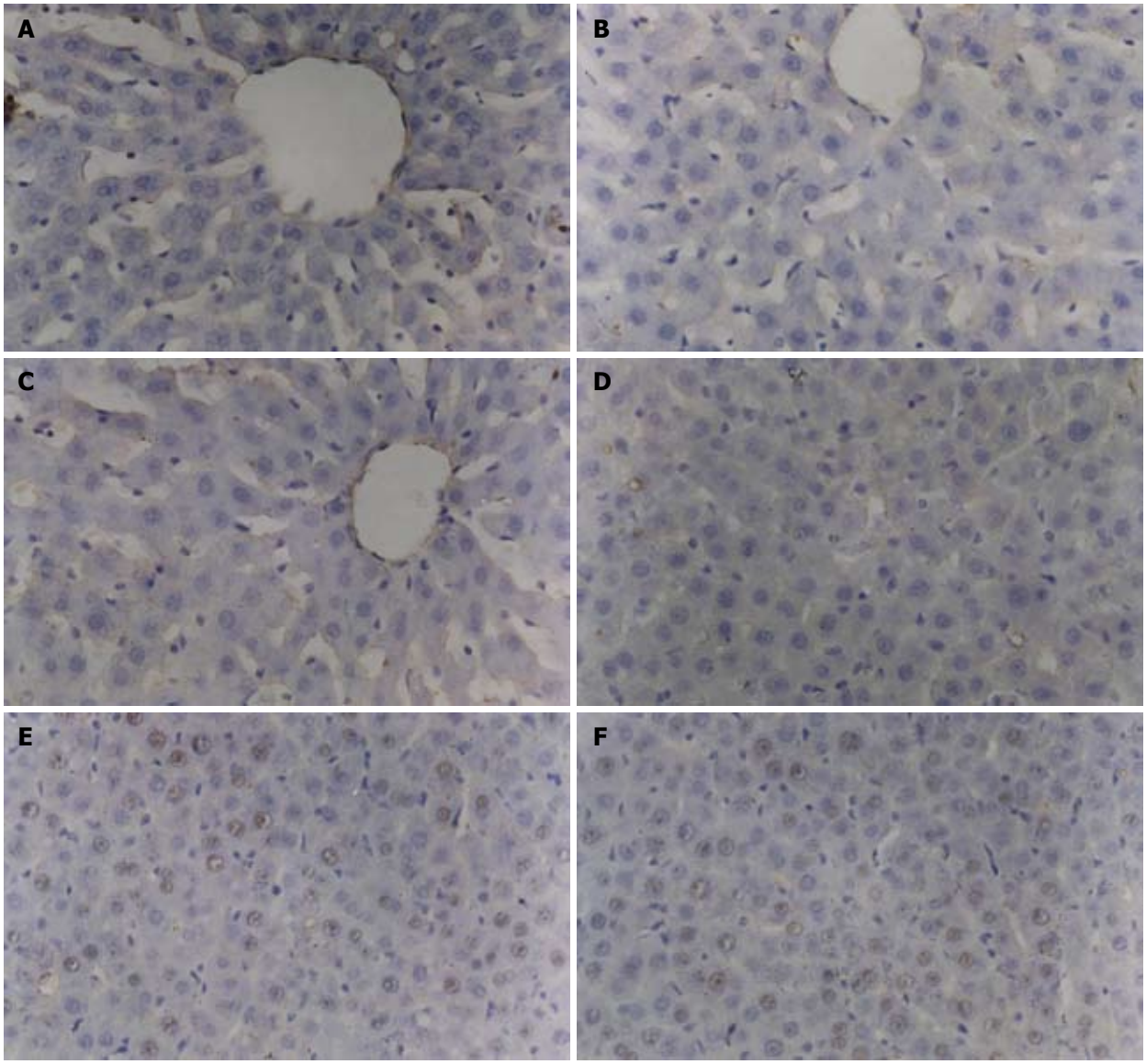

Figure 1 Positive cells are mainly hepatic parenchymal cells located near the sinus hepaticus and sinusoid endothelial cells of liver in melatonin exposure group (A, D), alcohol solvent control group (B, E), NS control group (C, F). The positive cell rate of two control groups increased gradually and reached its peak 12 h after reperfusion, then decreased. This condition also occurred in melatonin exposure group, but the extent was much lower than that in the control groups.

while AST level gradually increased with the time and maintained at its original level in the melatonin exposure group ($P < 0.05$, Table 1). Hepatic injury occurred at the early stage of pan-intestinal IR SMA was blocked for 30 min. The possible reason is that indirect clipping SMA reduced the portal blood flow. The accrescence of ALT indicates the hepatic injury, but the ischemic time is too short to lead to prominent AST changes. After the block of SMA was released, the hepatic injury due to reduced blood flow was ameliorated, but a great amount of active substances, such as free radicals, TNF-α, ICAM-1, *etc*, entered hepatic tissues *via* superior mesenteric and portal veins leading to secondary hepatic injuries, which can explain the variable curves of ALT and AST in the other two control groups. Compared with the other two control groups, exogenous melatonin could ameliorate the hepatic function after intestinal IR injury at the early or late phase. Recent studies about the protective effect of melatonin on IR injury in various organs showed that melatonin can eliminate free radicals, inhibit release of inflammatory media and apoptosis[8-10]. On the other hand, many factors can result in IR injury, such as free radicals[11], overload of calcium[12], inflammation of white blood cells and vascular endothelial cells[13] and apoptosis, *etc*[14]. Therefore, we detected serum TNF-α, MDA and ICAM-1-stained cells in liver tissues in this study. TNF-α gradually increased during the first 12 h after reperfusion and then decreased when it reached its peak between 12 and 24 h in the three groups. However, the TNF-α level was lower in the melatonin exposure group than in the other two control groups 12 and 24 h after reperfusion ($P < 0.05$), suggesting that TNF-α increases in systemic circulation after pan-intestinal IR, which can be effectively inhibited by exogenous melatonin[15-17]. Furthermore, TNF-α is generally accepted as an important inflammatory medium which has the same position as the interleukin family, and participates in almost all inflammatory reactions[18,19]. According to the curves of TNF-α and AST in the first 12 h systemic circulation after reperfusion, the secondary hepatic injury after pan-intestinal reperfusion is related to the concentration of TNF-α in systemic circulation. MDA is a final product of lipid peroxide, and its concentration in tissues can directly reflect the extent of lipid peroxide injury[20]. The concentration of MDA in each group gradually increased after reperfusion, which is generally considered the result of generous release of free radicals and accumulation of lipid peroxide products after pan-intestinal IR[21]. However, the concentration of MDA was obviously lower in the melatonin exposure group than in the other two control groups 6, 12 and 24 h after reperfusion, indicating that melatonin can relieve the IR injury by eliminating free radicals[22]. It was reported that ICAM-1 is directly correlated with the inflammation of white blood cells and vascular endothelial cells[23]. In this study, ICAM-1 gradually increased after reperfusion and decreased after reaching its peak between 12 and 24 h, which was in accordance with the concentration of TNF-α in systemic circulation. However, ICAM-1 was obviously lower in the melatonin exposure group than in the other two control groups. Analysis of the data showed that after clipping SMA for 30 min, (1) the first hepatic injury was relatively mild and reversible while the secondary injury was permanent and severe; (2) a large number of free radicals induced the expression of inflammatory factors such as TNF-α and ICAM-1 leading to hepatic injury through the portal vein, and free radicals directly caused peroxide injury after reperfusion; (3) exogenous melatonin protected liver from intestinal IR injury by inhibiting the production of free radicals, reducing the concentration of TNF-α in systemic circulation, and suppressing the expression of ICAM-1 in liver, *etc*. It has been recently shown that liver is the final metabolic place of melatonin and melatonin protects liver from intestinal IR injury[24-26]. Studies have shown that melatonin has no side effect when a large dose is used[27,28]. Intestine can absorb nutrients and has immune functions, and its vascular anatomy is specifically related with liver, the biggest digestive gland in human body. Since the blood in intestinal vein goes through the portal vein to the liver, intestinal IR inevitably affects the normal physical function of liver. During IR, a great amount of xanthic dehydrogenase in intestinal mucosa would change into xanthic oxydase, and xanthic oxydase can induce the production of free radicals which enhance the expression of adherence factors such as ICAM-1. These adherence factors are the main etiological agents of the secondary hepatic injury[29]. Up to now, rejecting reaction, graft-*versus*-host disease (GVHD) and infection are the restrained factors for intestine transplantation[30].

In conclusion, IR is the key mechanism underlying liver injuries at the early stage of organ transplantation.

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COMMENTS

Background

Common intestinal ischemic diseases, intestinal resection caused by various etiologies, intestinal transplantation due to short bowel syndrome and long-term intravenous nutrition induce various hepatic injuries. On the other hand, combined liver and intestine transplantation has some immune dominance compared with simple intestinal transplantation, and acute or chronic rejecting reaction occurred in combined liver and intestine transplantation obviously decreases. Thus, it is necessary to study the mechanism of hepatic injury caused by intestine ischemia-reperfusion (IR) and discuss the injury relieving therapies.

Research frontiers

Melatonin is an effective free radical scavenger in liver, kidney, pancreas, digestive tract, *etc*. It is generally accepted that melatonin plays a role in protecting liver from intestinal IR injury by eliminating free radicals in cytoplasm. Thus, melatonin secreted by conarium is an endocrine hormone and has a very complicated mechanism of action and its receptors are distributed in almost all human organs. At present, researchers are paying their attention to the modulating function of melatonin in various inflammatory media.

Innovations and breakthroughs

Melatonin interferes with hepatic injury after intestinal reperfusion. The possible protective mechanism of melatonin by producing and releasing inflammatory agents was discussed. Melatonin was found to be able to reduce the concentration of serum TNF-α and inhibit the expression of ICAM-1.

Applications

Exogenous melatonin can protect liver from intestinal IR injury after intestinal

ischemic-reperfusion by eliminating free radicals, reducing the production and release of inflammatory media, *etc*. As an effective and safe clinical medicine, it can be extensively used in surgery.

Terminology

Melatonin is an endocrine hormone secreted by conarium. Endogenous melatonin plays an important role in organic biorhythm, developing cycle, internal environmental stabilization and anti-caducity. TNF-α is an important inflammatory medium and participates in almost all inflammatory reactions. ICAM-1 is a member of the adhesive molecule family and is closely related to adhesion of neutrophile granulocytes and vascular endothelial cells in inflammatory reaction.

Peer review

This is a well written and interesting manuscript. The authors demonstrated that melatonin can protect liver from intestinal IR injury by abating the concentration of serum TNF-α and inhibiting the expression of ICAM-1 in liver cells.

REFERENCES

1 **DeLegge M**, Alsolaiman MM, Barbour E, Bassas S, Siddiqi MF, Moore NM. Short bowel syndrome: parenteral nutrition versus intestinal transplantation. Where are we today? *Dig Dis Sci* 2007; **52**: 876-892

2 **Harada E**, Ito H, Murakami M, Li TS, Enoki T, Noshima S, Hamano K. Small bowel transplantation tolerance achieved by costimulatory blockade leading to mixed chimerism. *Front Biosci* 2007; **12**: 3017-3023

3 **O'Keefe SJ**, Emerling M, Koritsky D, Martin D, Stamos J, Kandil H, Matarese L, Bond G, Abu-Elmagd K. Nutrition and quality of life following small intestinal transplantation. *Am J Gastroenterol* 2007; **102**: 1093-1100

4 **Buchman AL**, Iyer K, Fryer J. Parenteral nutrition-associated liver disease and the role for isolated intestine and intestine/liver transplantation. *Hepatology* 2006; **43**: 9-19

5 **Troxell ML**, Higgins JP, Kambham N. Evaluation of C4d staining in liver and small intestine allografts. *Arch Pathol Lab Med* 2006; **130**: 1489-1496

6 **Cay A**, Imamoglu M, Unsal MA, Aydin S, Alver A, Akyol A, Sarihan H. Does anti-oxidant prophylaxis with melatonin prevent adverse outcomes related to increased oxidative stress caused by laparoscopy in experimental rat model? *J Surg Res* 2006; **135**: 2-8

7 **Zhang WH**, Li JY, Zhou Y. Melatonin abates liver ischemia/reperfusion injury by improving the balance between nitric oxide and endothelin. *Hepatobiliary Pancreat Dis Int* 2006; **5**: 574-579

8 **Wang WZ**, Fang XH, Stephenson LL, Khiabani KT, Zamboni WA. Melatonin reduces ischemia/reperfusion-induced superoxide generation in arterial wall and cell death in skeletal muscle. *J Pineal Res* 2006; **41**: 255-260

9 **Duan Q**, Wang Z, Lu T, Chen J, Wang X. Comparison of 6-hydroxylmelatonin or melatonin in protecting neurons against ischemia/reperfusion-mediated injury. *J Pineal Res* 2006; **41**: 351-357

10 **Munoz-Casares FC**, Padillo FJ, Briceno J, Collado JA, Munoz-Castaneda JR, Ortega R, Cruz A, Tunez I, Montilla P, Pera C, Muntane J. Melatonin reduces apoptosis and necrosis induced by ischemia/reperfusion injury of the pancreas. *J Pineal Res* 2006; **40**: 195-203

11 **He SQ**, Zhang YH, Venugopal SK, Dicus CW, Perez RV, Ramsamooj R, Nantz MH, Zern MA, Wu J. Delivery of antioxidative enzyme genes protects against ischemia/reperfusion-induced liver injury in mice. *Liver Transpl* 2006; **12**: 1869-1879

12 **Li SZ**, Wu F, Wang B, Wei GZ, Jin ZX, Zang YM, Zhou JJ, Wong TM. Role of reverse mode Na+/Ca2+ exchanger in the cardioprotection of metabolic inhibition preconditioning in rat ventricular myocytes. *Eur J Pharmacol* 2007; **561**: 14-22

13 **Hsieh YH**, Huang SS, Wei FC, Hung LM. Resveratrol attenuates ischemia - reperfusion-induced leukocyte - endothelial cell adhesive interactions and prolongs allograft survival across the MHC barrier. *Circ J* 2007; **71**: 423-428

14 **Kovacevic M**, Simic O, Jonjic N, Stifter S. Apoptosis and cardiopulmonary bypass. *J Card Surg* 2007; **22**: 129-134

15 **Rodriguez-Reynoso S**, Leal C, Portilla E, Olivares N, Muniz J. Effect of exogenous melatonin on hepatic energetic status during ischemia/reperfusion: possible role of tumor necrosis factor-alpha and nitric oxide. *J Surg Res* 2001; **100**: 141-149

16 **Gitto E**, Romeo C, Reiter RJ, Impellizzeri P, Pesce S, Basile M, Antonuccio P, Trimarchi G, Gentile C, Barberi I, Zuccarello B. Melatonin reduces oxidative stress in surgical neonates. *J Pediatr Surg* 2004; **39**: 184-189; discussion 184-189

17 **Gitto E**, Reiter RJ, Cordaro SP, La Rosa M, Chiurazzi P, Trimarchi G, Gitto P, Calabro MP, Barberi I. Oxidative and inflammatory parameters in respiratory distress syndrome of preterm newborns: beneficial effects of melatonin. *Am J Perinatol* 2004; **21**: 209-216

18 **Lopez-Neblina F**, Toledo-Pereyra LH. Anti-ischemic effect of selectin blocker through modulation of tumor necrosis factor-alpha and interleukin-10. *J Surg Res* 2007; **138**: 275-283

19 **Cavriani G**, Domingos HV, Oliveira-Filho RM, Sudo-Hayashi LS, Vargaftig BB, de Lima WT. Lymphatic thoracic duct ligation modulates the serum levels of IL-1beta and IL-10 after intestinal ischemia/reperfusion in rats with the involvement of tumor necrosis factor alpha and nitric oxide. *Shock* 2007; **27**: 209-213

20 **Bolcal C**, Yildirim V, Doganci S, Sargin M, Aydin A, Eken A, Ozal E, Kuralay E, Demirkilic U, Tatar H. Protective effects of antioxidant medications on limb ischemia reperfusion injury. *J Surg Res* 2007; **139**: 274-279

21 **Ozacmak VH**, Sayan H, Igdem AA, Cetin A, Ozacmak ID. Attenuation of contractile dysfunction by atorvastatin after intestinal ischemia reperfusion injury in rats. *Eur J Pharmacol* 2007; **562**: 138-147

22 **Kiarostami V**, Samini L, Ghazi-Khansari M. Protective effect of melatonin against multistress condition induced lipid peroxidation via measurement of gastric mucosal lesion and plasma malondialdehyde levels in rats. *World J Gastroenterol* 2006; **12**: 7527-7531

23 **Monson KM**, Dowlatshahi S, Crockett ET. CXC-chemokine regulation and neutrophil trafficking in hepatic ischemia-reperfusion injury in P-selectin/ICAM-1 deficient mice. *J Inflamm* (Lond) 2007; **4**: 11

24 **Chegaev K**, Lazzarato L, Rolando B, Marini E, Tosco P, Cena C, Fruttero R, Bertolini F, Reist M, Carrupt PA, Lucini V, Fraschini F, Gasco A. NO-donor melatonin derivatives: synthesis and in vitro pharmacological characterization. *J Pineal Res* 2007; **42**: 371-385

25 **Sutken E**, Aral E, Ozdemir F, Uslu S, Alatas O, Colak O. Protective role of melatonin and coenzyme Q10 in ochratoxin A toxicity in rat liver and kidney. *Int J Toxicol* 2007; **26**: 81-87

26 **Xu J**, Sun S, Wei W, Fu J, Qi W, Manchester LC, Tan DX, Reiter RJ. Melatonin reduces mortality and oxidatively mediated hepatic and renal damage due to diquat treatment. *J Pineal Res* 2007; **42**: 166-171

27 **Cheung RT**, Tipoe GL, Tam S, Ma ES, Zou LY, Chan PS. Preclinical evaluation of pharmacokinetics and safety of melatonin in propylene glycol for intravenous administration. *J Pineal Res* 2006; **41**: 337-343

28 **Pignone AM**, Rosso AD, Fiori G, Matucci-Cerinic M, Becucci A, Tempestini A, Livi R, Generini S, Gramigna L, Benvenuti C, Carossino AM, Conforti ML, Perfetto F. Melatonin is a safe and effective treatment for chronic pulmonary and extrapulmonary sarcoidosis. *J Pineal Res* 2006; **41**: 95-100

29 **Xiaoqiao Z**, Rong M, Zhigang Y, Yong D, Xihong F, Jingzhong S. Protective effect of ulinastatin against ischemia-reperfusion injury in rat small bowel transplantation. *Transplant Proc* 2004; **36**: 1564-1566

30 **Ruiz P**, Kato T, Tzakis A. Current status of transplantation of the small intestine. *Transplantation* 2007; **83**: 1-6

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CASE REPORT

Peutz-Jeghers syndrome with small intestinal malignancy and cervical carcinoma

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Abstract

We report a case of 30-year-old woman with Peutz-Jeghers syndrome (PJS). Because of small intestinal obstruction, she received the small intestinal polypectomy in 2001, and the pathological diagnosis was Peutz-Jeghers polyp canceration (mucinous adenocarcinoma, infiltrating full-thickness of the intestine). The patient did not feel uncomfortable after 6 mo of chemotherapy and other management. We kept a follow-up study on her and found that she suffered from cervical cancer in 2007, with a pathological diagnosis of cervical adenosquamous carcinoma.The patient presented with typical features of PJS, but without a family history. The PJS accompanied with both small intestinal and cervical malignancies has not been reported so far in the world.

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Key words: Peutz-Jeghers syndrome; Polypectomy; Small intestine malignancy; Cervix cancer; Multiple organ neoplasms

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INTRODUCTION

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disease characterized by hamartomatous polyps in the gastrointestinal tract and mucocutaneous melanin pigmentation. Patients with PJS are at increased risk for common and unusual types of gastrointestinal and extra-gastrointestinal tumors[1].This report describes the clinicopathological characteristics of PJS complicating multiple organ neoplasms. It provides evidence not only for the risk of malignancy in this disorder, but also for a hamartoma-adenoma-carcinoma sequence.

CASE REPORT

We report here a case of a 30-year old woman who suffered from multiple organ neoplasms with PJS. The melanin pigmentation around lips appeared when she was 3 mo old, after that, melanin pigmentation increased and also appeared on the palm and planta. She presented with iterative abdominal pain and vomit in 1997, and was found having multiple polyps in colon by colonoscopy. The polyps had been resected several times, but the symptoms still existed.

The patient presented to us in April 2001. Physical examination revealed the pigmentation on the oral lips, the buccal mucosa (Figure 1A), and the hands and feet (Figure 1B), about 1-3 mm in diameter. Endoscopy and gastrointestinal tract contrast examination revealed multiple polyps in sinus ventriculi, small intestine and colon. Most polyps were resected and demonstrated hamartomatous polyps, some of which showed adenomatous changes (Figure 2A), and the one in small intestine revealed carcinomatous changes (mucinous adenocarcinoma, infiltrating full-thickness of the intestine) (Figure 2B). The patient received FOLFOX4 chemotherapy after surgery. Tumor markers and routine blood tests were mainly normal during the treatment. The patient gradually recovered, then we kept follow-up visit on her.

In November 2007, the patient complained of abnormal vaginal discharge for 2 mo, without vaginal

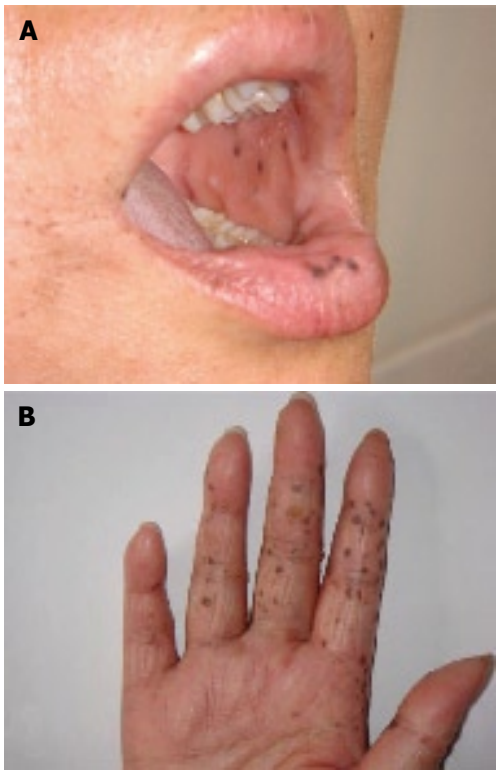

Figure 1 Melanin pigmentation. A: oral lips and buccal mucosa; B: Palm.

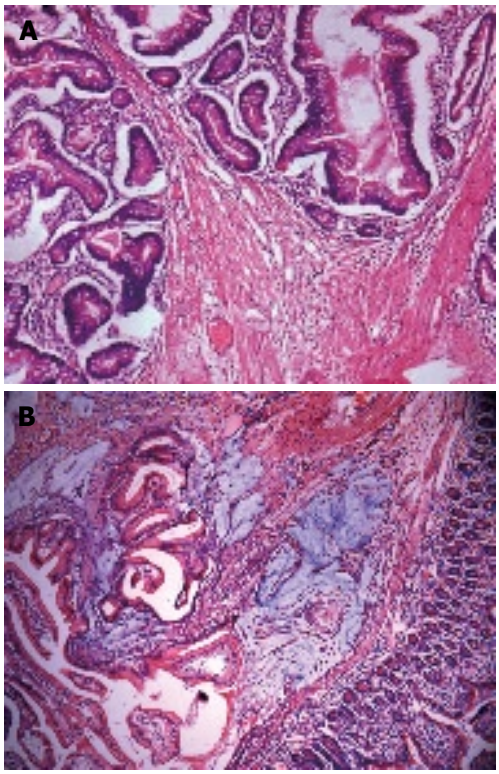

Figure 2 Polyp (HE × 100). A: Adenomatous changes in Peutz-Jeghers polyp; B: Focal mucinous adenocarcinoma in the small intestinal polyp.

bleeding and abdominal pain. Gynecological B-mode ultrasonography found occupying lesion located between inferior segment of cervix and vagina. Gynecologic examination revealed thick cervix, and cervical scraping

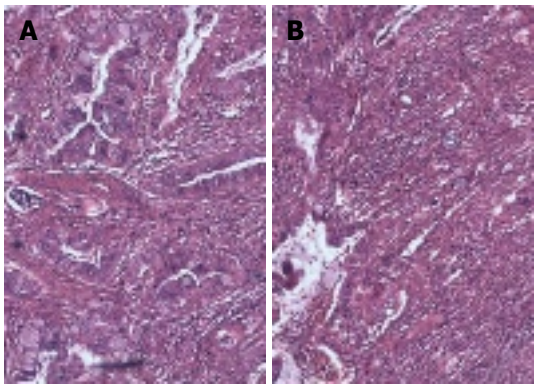

Figure 3 Cervical adenosquamous carcinoma. A: Moderately differentiated adenocarcinoma; B: Well-differentiated squamous carcinoma (HE × 100).

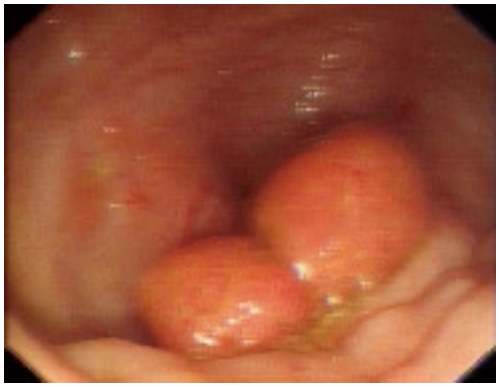

Figure 4 Endoscopic view of multiple polyps in sigmoid colon (12 mm in diameter).

smear confirmed cervical adenocarcinoma. After neoadjuvant chemotherapy (Paclitaxel 120 mg/dL, Carboplatin 350 mg/dL, one day per wk for 6 wk), total hysterectomy was performed in February 2008. Final pathological diagnosis was cervical adenosquamous carcinoma (Figure 3), and cancerometastasis of lymph nodes in left external iliac (1/1). The patient also received pelvic radiotherapy after surgery (50 GY in total).

The tumor markers (CA125, CA19-9, CEA) were normal when the patient paid a return visit in May 2008. She was found to have multiple polyps in her stomach, terminal ileum and sigmoid colon by endoscopy (Figure 4), polyps were resected and pathology still confirmed hamartomatous polyps. In addition, none of her lineal relations has the symptoms and physical signs of PJS, and no tumor patient was found in her family history.

DISCUSSION

PJS patients have an increased risk for several malignancies including small intestine, stomach, pancreas, colon, esophagus, ovary, uterus, lung, and breast cancer. PJS is associated with a markedly increased risk of malignancy that is not confined to the gastrointestinal tract. A metaanalysis found that,

compared with the general population, patients with PJS have a relative risk (RR) of 15 times higher than for developing many kinds of cancer. And the cumulative risk for all cancers was 93% from age 15 to 64. Very high RRs for the development of cancer were observed in the small intestine (520), stomach (213), pancreas (132), colon (84) and esophagus (57), and RRs are greater than 10 for the development of breast, lung and ovarian cancer[2]. Several gonadal malignancies occur in PJS patients. In female patients, sex cord tumors with annular tubes (SCTAT) are found in the ovaries of many individuals examined. Patients with these tumors can present with menoxenia, hyperestrogenism or sexual precocity[3]. Minimal deviation of cervical adenocarcinoma has been reported in PJS patients. Presenting symptoms include abnormal vaginal bleeding or a mucoid vaginal discharge. It is an extremely well differentiated adenocarcinoma of the cervix. It usually shows poor malignant behavior and poor prognosis with mucinous type of adenocarcinoma[4].

STK11/LKB1 was identified strongly relative to the PJS, which is a tumor-suppressor involved in intracellular signal transduction and cellular polarity[5]. Some studies provided molecular evidences of a hamartoma-adenoma-carcinoma sequence in PJS. The second hit in *LKB1* causing loss of heterozygosity (LOH) in adenomatous and carcinomatous lesions in PJS polyps was noted by several investigators[6,7]. In addition, LOH of *p53*, *K-Ras* and β-*catenin* mutations were found in adenomas developing in hamartomatous polyps, indicating that molecular alterations in these genes drive carcinogenesis in PJS as well[8]. However, the precise frequency of LOH of *LKB1* in PJS polyps in human remains unclear, and studies in mice showed that loss of the wild-type *LKB1* allele is not a prerequisite for the formation of hamartomatous polyps[9]. Therefore, the need for the second-hit in *LKB1* during polyp development in PJS, and the role of *LKB1* as a typical 'Knudson' two-hit tumor-suppressor gene, is questioned. One theory suggests mucosal prolapse as a pathogenic mechanism underlying the development of typical hamartomatous polyps in PJS. In this hypothesis, PJS hamartomatous polyps represent an epiphenomenon to the cancer-prone condition and the hamartoma-adenoma-carcinoma sequence as such does not exist[10]. The important role of *LKB1* in cellular polarity may provide new insights into the molecular mechanism of polyp and carcinoma development in PJS. Loss of polarity function may also affect asymmetric stem cell division in PJS and lead to expansion of the stem cell pool[11]. It could contribute to polyp formation and explain the increased cancer risk as well. A recent study found *STK11*-deficient mesenchymal cells produced less TGF-β, and defective TGF-β signaling to epithelial cells coincided with epithelial proliferation. TGF-β signaling defects in polyps of individuals with PJS, suggesting that the identified stromal-derived mechanism of tumor suppression is also relevant to PJS[12].

We report the unique case of a patient with the Peutz-Jeghers syndrome who developed intestinal malignancy and cervical cancer in 7 years. As far as we know, no similar case has been reported to date. The pathological changes of polyps would support the development of hamartoma-adenoma-carcinoma; and the carcinomatous change of cervix would add the risk of extra-gastrointestinal tumors in this disorder. However, the pathogenic mechanism of these changes is still unknown. It should be studied progressively on whether germline mutation of *STK11/LKB1* exists or other factors participate in the process of malignant changes. We also suggest that the patient and her family members should be followed up with endoscopy.

REFERENCES

1 **Giardiello FM**, Trimbath JD. Peutz-Jeghers syndrome and management recommendations. *Clin Gastroenterol Hepatol* 2006; **4**: 408-415

2 **Giardiello FM**, Brensinger JD, Tersmette AC, Goodman SN, Petersen GM, Booker SV, Cruz-Correa M, Offerhaus JA. Very high risk of cancer in familial Peutz-Jeghers syndrome. *Gastroenterology* 2000; **119**: 1447-1453

3 **Young RH**, Welch WR, Dickersin GR, Scully RE. Ovarian sex cord tumor with annular tubules: review of 74 cases including 27 with Peutz-Jeghers syndrome and four with adenoma malignum of the cervix. *Cancer* 1982; **50**: 1384-1402

4 **Chen KT**. Female genital tract tumors in Peutz-Jeghers syndrome. *Hum Pathol* 1986; **17**: 858-861

5 **Baas AF**, Smit L, Clevers H. LKB1 tumor suppressor protein: PARtaker in cell polarity. *Trends Cell Biol* 2004; **14**: 312-319

6 **Miyaki M**, Iijima T, Hosono K, Ishii R, Yasuno M, Mori T, Toi M, Hishima T, Shitara N, Tamura K, Utsunomiya J, Kobayashi N, Kuroki T, Iwama T. Somatic mutations of LKB1 and beta-catenin genes in gastrointestinal polyps from patients with Peutz-Jeghers syndrome. *Cancer Res* 2000; **60**: 6311-6313

7 **Wang ZJ**, Ellis I, Zauber P, Iwama T, Marchese C, Talbot I, Xue WH, Yan ZY, Tomlinson I. Allelic imbalance at the LKB1 (STK11) locus in tumours from patients with Peutz-Jeghers' syndrome provides evidence for a hamartoma-(adenoma)-carcinoma sequence. *J Pathol* 1999; **188**: 9-13

8 **Gruber SB**, Entius MM, Petersen GM, Laken SJ, Longo PA, Boyer R, Levin AM, Mujumdar UJ, Trent JM, Kinzler KW, Vogelstein B, Hamilton SR, Polymeropoulos MH, Offerhaus GJ, Giardiello FM. Pathogenesis of adenocarcinoma in Peutz-Jeghers syndrome. *Cancer Res* 1998; **58**: 5267-5270

9 **Miyoshi H**, Nakau M, Ishikawa TO, Seldin MF, Oshima M, Taketo MM. Gastrointestinal hamartomatous polyposis in Lkb1 heterozygous knockout mice. *Cancer Res* 2002; **62**: 2261-2266

10 **Jansen M**, de Leng WW, Baas AF, Myoshi H, Mathus-Vliegen L, Taketo MM, Clevers H, Giardiello FM, Offerhaus GJ. Mucosal prolapse in the pathogenesis of Peutz-Jeghers polyposis. *Gut* 2006; **55**: 1-5

11 **Clevers H**. Stem cells, asymmetric division and cancer. *Nat Genet* 2005; **37**: 1027-1028

12 **Katajisto P**, Vaahtomeri K, Ekman N, Ventelä E, Ristimäki A, Bardeesy N, Feil R, DePinho RA, Mäkelä TP. LKB1 signaling in mesenchymal cells required for suppression of gastrointestinal polyposis. *Nat Genet* 2008; **40**: 455-459

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
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Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

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FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

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Strasbourg, France
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N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

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E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

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Gastro 2009 UEGW/World Congress of Gastroenterology
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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

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]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious 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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