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EDITORIAL

Antiproliferative effect of somatostatin analogs in gastroenteropancreatic neuroendocrine tumors

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Abstract

Somatostatin analogs were initially developed for the control of hormonal syndromes associated with neuroendocrine tumors (NETs). In recent years, accumulating data has supported their role as antiproliferative agents, capable of stabilizing tumor growth in patients with metastatic neuroendocrine malignancies, including carcinoid and pancreatic endocrine tumors. A phase Ⅲ, randomized, placebo-controlled trial has now demonstrated that octreotide long-acting repeatable (LAR) 30 mg can significantly prolong time to tumor progression among patients with metastatic midgut NETs regardless of functional status, chromogranin A level or age. In addition to significantly lengthening time to tumor progression in the overall study population, subset analysis suggests that patients with low tumor burden are most likely to experience disease stabilization with octreotide LAR 30 mg, supporting the early use of octreotide LAR in patients with metastatic disease. Further research efforts are underway to evaluate the use of somatostatin analogs as antiproliferative agents in other types of gastroenteropancreatic-NETs. Ongoing studies are also evaluating novel somatostatin analogs and somatostatin analogs in combination with other anti-tumor therapies.

INTRODUCTION

The human hormone somatostatin was first isolated in 1973 and identified as a hypothalamic inhibitor of growth hormone[1-4]. It was subsequently discovered in multiple tissues, including the central nervous system, endocrine system and gastrointestinal tract[3]. Somatostatin has been characterized as a universal endocrine "off-switch" due to its exocrine, endocrine, paracrine and autocrine inhibitory effects[5-7]. In the digestive tract, it reduces secretion and motility, decreases portal blood flow, inhibits gallbladder contraction and reduces the secretion of other gastrointestinal hormones[8]. The effects of somatostatin are mediated through interaction with five somatostatin receptors (sst_{1-5})[9], belonging to a family of G-protein coupled receptors with seven transmembrane domains.

The clinical utility of native human somatostatin is limited by its short half life of approximately two minutes. Both bioactive forms of the hormone, the fourteen-peptide somatostatin-14 and a C-terminally extended form, somatostatin-28, contain multiple enzymatic cleavage sites resulting in rapid circulatory degradation[6]. In order to improve the pharmacokinetic profile, synthetic somatostatin

analogs (SSAs) have been developed by shortening the polypeptide chain while retaining binding affinity to somatostatin receptors (Figure 1)[10]. The two commercially available analogs, octreotide and lanreotide, are octapeptides that bind with high affinity to somatostatin receptor subtype 2 (sst_2) and with moderate affinity to sst_5 (Table 1).

Octreotide has been used in clinical practice since data emerged in the 1980s confirming its ability to palliate carcinoid syndrome[14], as well as other hormonal syndromes caused by metastatic gastroenteropancreatic neuroendocrine tumors (GEP-NETs). Octreotide was initially available in an immediate-release formulation suitable for deep subcutaneous or intravenous administration[15]. Octreotide subcutaneous (sc) has been tested primarily at doses ranging from 100 to 500 μg two to three times daily. During the past decade, a long-acting repeatable (LAR) depot formulation of octreotide (Sandostatin LAR®) has been available, which allows monthly intramuscular dosing. Octreotide LAR has demonstrated similar efficacy to octreotide sc in the control of flushing and diarrhea associated with carcinoid syndrome[16]. The dose of octreotide sc and octreotide LAR should be titrated per symptom control for optimal patient care[17]. A second somatostatin analog, lanreotide, was licensed in Europe in 1998 for the treatment of symptoms associated with neuroendocrine (particularly carcinoid) tumors. A long-acting formulation of lanreotide (Somatuline Autogel®)[18] has also been developed as a deep subcutaneous injection.

Early on, clinical trials of SSAs tested their ability to inhibit the release of neuroendocrine hormones such as serotonin, glucagon, insulin, gastrin and vasoactive intestinal peptide (VIP)[14,19-22]. These trials formed the basis for the approval of octreotide and lanreotide as antisecretory agents indicated for treatment of hormonally active GEP-NETs. It was not until several years after the approval of octreotide that evidence of antineoplastic activity emerged. Although objective radiographic responses associated with SSAs were rare, many cases of prolonged disease stability were documented in the literature, leading to the hypothesis that SSAs exert an inhibitory effect on tumor growth. Recently, this hypothesis was tested in a Phase Ⅲ, randomized, placebo-controlled clinical trial evaluating octreotide LAR 30 mg. This review summarizes the preclinical and clinical evidence supporting the role of SSAs as antiproliferative agents in the treatment of patients with GEP-NETs. To date, most data (including the results from the only phase Ⅲ randomized, placebo-controlled trial) have been generated in studies evaluating octreotide sc and LAR.

BIOLOGICAL BASIS FOR THE ANTIPROLIFERATIVE EFFECTS OF SSAS

Over the past two decades there has been significant progress in our understanding of the molecular basis for the antiproliferative effects of somatostatin and its analogs. Antitumoral activity appears to be mediated *via* direct and indirect mechanisms. Direct mechanisms involve the activation of somatostatin receptors on tumor cells leading to modulation of intracellular signaling transduction pathways. Multiple *in vitro* studies using cell lines transfected with somatostatin receptors indicate that all receptor subtypes (sst_{1-5}) may mediate inhibition of cell proliferation[23], whereas specific receptor subtypes ($sst_{2,3}$) may mediate apoptosis (Table 2)[24-26]. These actions appear to be regulated primarily *via* the MAP-kinase signaling pathway and through activation of phosphotyrosine phosphatases (Figure 2)[27-29]. Indirect antiproliferative mechanisms include inhibition of mitogenic growth factors such as insulin-like growth factor (IGF), as well as inhibition of tumor angiogenesis through interaction with somatostatin receptors on endothelial cells and monocytes[30].

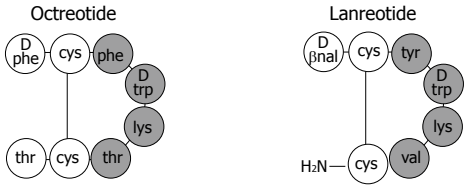

Figure 1 Chemical structure of the synthetic somatostatin analogs octreotide and lanreotide (adapted from[11]).

Table 1 Receptor binding affinities of somatostatin, octreotide and lanreotide

	sst_1	sst_2	sst_3	sst_4	sst_5
Receptor binding affinity (IC_{50} nmol/L)					
Somatostatin[12]	0.93	0.15	0.56	1.50	0.29
Octreotide[12]	280.00	0.38	7.10	> 1000	6.30
Lanreotide[13]	> 1000	0.80	107	> 1000	5.20

Table 2 Receptor mediation of cell proliferation

	sst_1	sst_2	sst_3	sst_4	sst_5
Induction of G1 cell cycle arrest	+	+		+	+
Induction of apoptosis		+	+		

Activation of phosphotyrosine phosphatases

Several phosphotyrosine phosphatases (PTPs), including SHP-1 and SHP-2, have emerged as important regulators of intracellular signaling pathways[27]. Somatostatin receptor-mediated activation of SHP-1 results in arrest of cell proliferation in various cell lines, including cells derived from pancreatic, breast and prostate carcinomas[31,32]. In pituitary adenoma cells, activation of sst_2 inhibits PI3 kinase activity and causes cell growth arrest *via* stimulation of SHP-1[33]. The enzymatic activity of SHP-1 has also been implicated in sst_3-dependent apoptosis in transfected Chinese Hamster Ovary (CHO) cells[34]. Stimulation of SHP-1 in sst_2-expressing CHO cells has led to G1 cell cycle arrest *via* induction of the cyclin-dependent kinase inhibitor p27[35]. SHP-2 has also been identified as a mediator of the antiproliferative effects of somatostatin receptors,

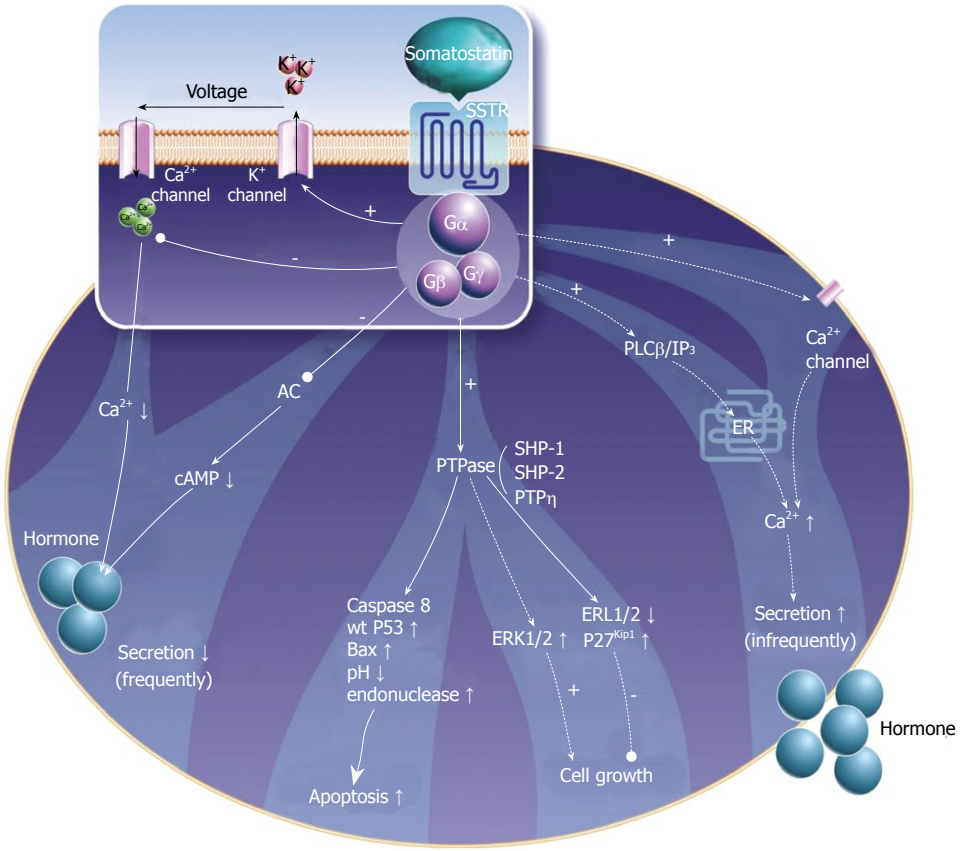

Figure 2 Somatostatin receptor-mediated effects on neuroendocrine cells (adapted from[23]).

primarily through inactivation of tyrosine kinase receptors for insulin and epidermal growth factors[36]. Moreover, activation of PTPs has been shown to down-regulate Raf-1[37] and block the MAP-kinase pathway[38].

Modulation of the mitogen activated protein-kinase pathway

Both inhibition and stimulation of the mitogen activated protein (MAP)-kinase pathway have been linked to the antiproliferative effects of somatostatin and its analogs. In a glioma cell line, the receptor-like PTP, PTPeta, mediated the antiproliferative effects of somatostatin through inhibition of ERK1/2[39]. Conversely, another study of sst_1-expressing CHO cells demonstrated that somatostatin robustly activated MAP-kinase, which in turn enhanced the expression of the cyclin-dependent kinase inhibitor p21, thereby inhibiting cell proliferation[40]. Another study in CHO cells demonstrated that activation of p38 MAP-kinase *via* sst_2 and sst_4 mediated the inhibitory effects of somatostatin on fibroblast growth factor induced proliferation[41].

Indirect antiproliferative mechanisms

Suppression of tumor growth may occur *via* inhibition of various circulating growth factors, including insulin-like growth factor (IGF), epidermal growth factor (EGF) and growth hormone (GH). Inhibition of GH is thought to be mediated primarily *via* sst_2 and sst_5, which are strongly expressed in the anterior pituitary[42-44]. Octreotide has been shown to suppress circulating levels of IGF-1, both *via* suppression of pituitary secretion of GH as well as through direct inhibition of IGF-1 production in the liver[45,46].

The antiangiogenic effects of octreotide have been demonstrated in multiple *in vitro* tumor models[47,48]. Octreotide has been shown to inhibit proliferating endothelial cells that over-express sst_2 and sst_5[49]. The primary mechanism of angiogenesis inhibition may be suppression of endothelial nitric oxide release[50]. Inhibition of circulating vascular-endothelial growth factor (VEGF) appears to also play a role in suppression of peritumoral vessel growth[51,52].

EARLY CLINICAL EVIDENCE FOR THE ANTIPROLIFERATIVE EFFECTS OF SSAS

Since the introduction of SSAs, multiple phase Ⅱ trials and retrospective series have demonstrated that SSA treatment is associated with prolonged survival and disease stabilization in a large proportion of patients. For example, a single-institution retrospective study of 146 patients with metastatic mid-gut NETs, 91% of whom received long term octreotide treatment, demonstrated a 5-year survival rate of 75% (compared to 19% historically)[53]. Additionally, an analysis of the US-based Surveillance, Epidemiology and End Results (SEER) database found a significant increase in survival from 1988 to 2004 compared with 1973 to 1987, coinciding with the introduction of octreotide[54].

In general, early clinical studies evaluating the dis-

ease-stabilizing effect of SSAs in patients with GEP-NETs are characterized by their lack of randomized design and enrollment of heterogeneous populations of patients with GEP-NETs. Although objective radiographic response rates have been rare (generally < 5%), the rate of tumor stabilization observed in most studies has ranged from 40%-60%, with higher rates observed in patients without documented disease progression at onset of treatment[55].

Among the first prospective studies documenting the antiproliferative effects of SSAs in GEP-NETs was one conducted by the German Sandostatin Study Group[56]. In this study, 103 patients with metastatic carcinoid and pancreatic endocrine tumors were treated with octreotide 200 μg thrice daily until evidence of radiographic progression. Among patients who had disease progression documented at treatment outset, the rate of disease stability lasting at least 3 mo was 37%, whereas among patients with documented stable disease at treatment outset, disease stability lasting at least 12 mo was documented in 54% of patients[57]. No objective tumor responses were observed. Another phase Ⅱ clinical trial testing octreotide as an antiproliferative agent in 34 patients with progressive metastatic NETs demonstrated a disease stabilization rate of 50% lasting a median of 5 mo[58].

The antiproliferative effect of intramuscular lanreotide SR 30 mg every 10 or 14 d was evaluated in a phase Ⅱ trial of 46 patients with carcinoid and pancreatic endocrine tumors. Two patients (4%) achieved an objective radiographic response while 19 patients (41%) experienced stable disease for a mean duration of 9.5 mo[59]. In another phase Ⅱ study of lanreotide SR 30 mg in 55 patients with GEP-NETs (48 with carcinoid tumors, six with gastrinomas and one with a VIPoma), 7% of 31 assessable patients achieved a partial response and 81% experienced disease stability[60]. In one study of patients with progressive tumors, participants received either octreotide LAR 30 mg or lanreotide SR 60 mg (this study considered all patients as a single cohort). Among 31 assessable patients, 14 (45%) achieved disease stability *vs* 55% who continued to progress radiographically[61]. Overall survival was considerably prolonged among patients with stable *vs* progressive disease. In multivariate analysis, pancreatic endocrine tumors appeared significantly less likely to achieve disease stabilization compared to intestinal carcinoid tumors. Extra-hepatic metastases were also associated with a poor prognosis. Table 3 summarizes the results of multiple non-randomized clinical trials evaluating the antineoplastic effects of octreotide and lanreotide in GEP-NETs.

Table 3 Summary of non-randomized clinical trials evaluating the antiproliferative effect of somatostatin analogs *n* (%)

Analog	Author	*n*	CR/PR	SD	PD
Patients with documented tumor progression					
Lanreotide	Faiss *et al*[62], 2003	22	1 (4)	7 (32)	14 (64)
Lanreotide	Aparicio *et al*[63], 2001	35	1 (3)	20 (57)	14 (40)
Octreotide	Arnold *et al*[64], 1993	52	0 (0)	19 (36)	33 (63)
Octreotide	Saltz *et al*[58], 1993	34	0 (0)	17 (50)	17 (50)
Octreotide	di Bartolomeo *et al*[19], 1996	58	2 (3)	27 (46)	29 (50)
		201	4 (1)	90 (45)	107 (53)
Patients without documented tumor progression					
Lanreotide	Wymenga *et al*[60], 1999	31	2 (6)	25 (80)	4 (13)
Lanreotide	Ducreux *et al*[59], 2000	39	2 (5)	21 (54)	16 (41)
Lanreotide	Eriksson *et al*[65], 1997	19	1 (5)	12 (63)	6 (32)
Lanreotide	Tomasetti *et al*[66], 1998	18	0 (0)	14 (77)	4 (22)
Octreotide	Tomasetti *et al*[67], 2000	16	0 (0)	14 (87)	2 (12)
Octreotide	Ricci *et al*[68], 2000	15	1 (6)	6 (40)	8 (53)
		138	6 (4)	92 (67)	40 (29)

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

THE PROMID TRIAL

Although providing initial evidence for the antitumor effects of SSAs, studies described in the previous section have a number of features that prevent them from providing conclusive evidence. Examples of these features include relatively small patient cohorts, lack of a randomized placebo control group, and analysis of heterogeneous populations. As such, to prove or to disprove an antiproliferative effect of octreotide LAR 30 mg, the PROMID (Placebo-controlled, Prospective, Randomized study in patients with metastatic neuroendocrine midgut tumors) study was initiated. This randomized, double-blind, placebo-controlled, phase Ⅲ trial, was among the very few randomized trials performed in patients with this rare tumor type. To avoid confounding variables, only patients with well-differentiated inoperable or metastatic midgut tumors were included. Additionally, octreotide LAR 30 mg was the only dose of octreotide LAR evaluated.

High-level evidence of the antiproliferative effects of octreotide emerged after publication of the PROMID trial[69]. Eighty-five participants with well-differentiated carcinoid tumors originating in the distal intestine and proximal colon were randomized to receive either octreotide LAR 30 mg or placebo until radiographic evidence of progression or death. The primary endpoint was time to tumor progression. Most patients (75%) had evidence of somatostatin receptor expression as evidenced by radiotracer uptake on Octreoscan. Nearly half of patients (38%) manifested the carcinoid syndrome (flushing and/or diarrhea associated with elevation in urine 5-HIAA). Only patients with mild carcinoid syndrome who tolerated flushing without intervention or responded to treatment with loperamide and/or cholestyramine in cases of diarrhea were included.

Median time to tumor progression was 14.3 mo in the octreotide LAR 30 mg group *vs* 6.0 mo in the placebo group (P = 0.000072, Figure 3). This significantly lengthened time-to-tumor progression was seen in the overall study population, regardless of tumor functionality, chromogranin A level or age. At 6 mo, tumor progression was observed in 24% of patients on the octreotide LAR 30 mg arm *vs* 66% of patients receiving placebo (P = 0.0079). Serious adverse events were nearly evenly balanced (11 patients in the octreotide LAR 30 mg arm and 10 patients in the placebo arm). On multivariate analysis, the highest rates of disease stabilization were observed in patients

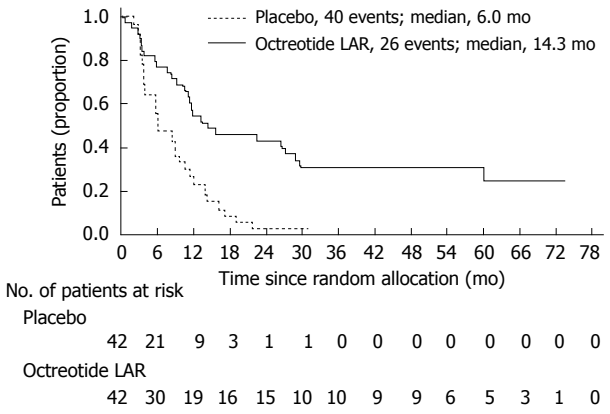

Figure 3 Kaplan Meier curve demonstrating time to tumor progression in patients treated with octreotide long-acting repeatable (LAR) *vs* placebo[69]. Log-rank test stratified by functional activity: P = 0.000072, HR = 0.34 (95% CI: 0.20-0.59).

with low hepatic tumor load (< 10%) and resected primary tumor, however both of these subgroups contained the majority of study patients. Even patients with higher hepatic tumor burden (> 10%) experienced a near doubling in time to progression on the octreotide LAR arm of the study. The small number of deaths in both treatment arms (seven in the octreotide LAR 30 mg arm; nine in the placebo arm) precluded any analysis of differences in survival.

FUTURE DIRECTIONS

Novel somatostatin analogs

NETs generally express multiple somatostatin receptors[13,70], all of which may mediate the antiproliferative effects of SSAs. These receptor subtypes can undergo heterodimerization with each other and with other receptor families (such as the dopamine receptor family), enhancing their binding affinities and internalization[71,72]. Thus, novel SSAs that bind to multiple receptor subtypes as well as analogs capable of binding to different families of receptors may prove to be effective antisecretory and antiproliferative agents in patients refractory to octreotide or lanreotide.

Pasireotide is one such novel somatostatin analog; it binds avidly to four of the five somatostatin receptors ($sst_{1,2,3}$ and sst_5). Compared with octreotide, pasireotide has a 40-, 30- and 5-fold higher binding affinity for sst_5, sst_1 and sst_3, and a slightly lower affinity for sst_2[73]. Pasireotide also has a 2-times higher binding affinity for sst_5 than endogenous somatostatin[73]. In an *in vitro* study evaluating the use of octreotide and pasireotide on HEK293 cells expressing somatostatin receptor subtype sst_2 on the cell membrane, treatment with octreotide resulted in an internalization of sst_2 receptors at 30 min whereas treatment with pasireotide did not lead to sst_2 internalization. Such findings may suggest that a persistent and more durable efficacy could be obtained with pasireotide[74].

An open-label trial evaluated the activity of pasireotide sc in patients with carcinoid syndrome whose symptoms (flushing and diarrhea) were inadequately controlled with octreotide LAR[75]. Preliminary data indicated activity in this refractory population. Future clinical trials are being designed to test the antiproliferative effects of pasireotide in neuroendocrine carcinomas. Other compounds capable of interacting with sst_2 as well as with the dopamine D2 receptor (DAD2) are in clinical development[76].

Radioactive labeling of SSAs is another promising approach to treatment of neuroendocrine malignancies which express high levels of somatostatin receptors. Early clinical trials employing ^{111}In-pentetreotide produced limited objective responses, probably due to the small particle range and short tissue penetration of Auger electrons emitted by the ^{111}In isotope[77]. The next generation of radiolabled SSAs used ^{90}Y-DOTATOC, a β-particle emitter with a tissue range of approximately 12 mm[78-80]. Objective response rates of 10%-30% were reported in phase Ⅰ and Ⅱ clinical trials. Dose-limiting side effects included bone marrow and renal toxicity.

The latest research efforts in radiolabeled SSAs have focused on ^{177}Lu octreotate, a β-and γ-emitting radionuclide with a shorter range of tissue penetration (2 mm) than ^{90}Y. A recent phase Ⅱ clinical trial reported an objective radiographic response rate of 30% among 310 patients with GEP-NETs, and a median progression-free survival duration of 40 mo[81].

CONCLUSION

SSAs were initially developed as antisecretory agents used primarily for the control of hormonal syndromes associated with NETs. In recent years, accumulating laboratory and clinical data has supported their role as antiproliferative agents, capable of stabilizing tumor growth in a large proportion of patients with metastatic carcinoid and pancreatic endocrine tumors. The recently-published PROMID study provides high-level evidence validating the role of octreotide LAR 30 mg as an antiproliferative agent in patients with metastatic carcinoid tumors of the midgut. Subset analysis suggests that patients with low tumor burden are most likely to experience disease stabilization, supporting the early use of octreotide LAR 30 mg in patients with metastatic disease. Further research efforts are underway to evaluate the use of novel SSAs, SSAs as antiproliferative agents in other types of GEP-NETs, and SSAs in combination with other anti-tumor agents.

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EDITORIAL

Gastrointestinal involvement in systemic lupus erythematosus: Insight into pathogenesis, diagnosis and treatment

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease characterized by the presence of a plethora of autoantibodies and immune complex formation. Virtually every system and organ can be affected by SLE. Gastrointestinal symptoms are common in SLE patients, and more than half of them are caused by adverse reactions to medications and viral or bacterial infections. Though not as common as lupus nephritis, SLE-related gastrointestinal involvement is clinically important because most cases can be life-threatening if not treated promptly. Lupus mesenteric vasculitis is the most common cause, followed by protein-losing enteropathy, intestinal pseudo-obstruction, acute pancreatitis and other rare complications such as celiac disease, inflammatory bowel diseases, *etc*. No specific autoantibody is identified as being associated with SLE-related gastroenteropathy. Imaging studies, particularly abdominal computed tomography scans, are helpful in diagnosing some SLE-related gastroenteropathies. Most of these complications have good therapeutic responses to corticosteroids and immunosuppressive agents. Supportive measures such as bowel rest, nutritional support, antibiotics and prokinetic medications are helpful in facilitating functional recovery and improving the outcome.

Key words: Systemic lupus erythematosus; Systemic; Vasculitis; Gastroenteropathy

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune inflammatory disease with protean clinical manifestations. The gastrointestinal tract is one of the most commonly affected systems in SLE. However, most gastrointestinal manifestations are caused by adverse reactions from therapeutic agents and infections, while the symptoms related to the disease *per se* are not as common as other organ involvement such as lupus nephritis[1-6]. On the other hand, the incidence of gastrointestinal manifestations may be underestimated clinically because some of them are indistinct and may not have abdominal symptoms. One autopsy study found that 60%-70% of SLE patients had evidence of peritonitis, whereas only around 10% of them were recognized clinically[1]. It is noteworthy

that gastrointestinal vasculitis and thrombosis can lead to life-threatening ischemia, perforation and infarction, and surgical interventions are usually needed if not treated promptly with immunosuppressant. In this paper, clinically important gastrointestinal complications of SLE are reviewed for the purpose of improving treatment efficacy and outcome.

LUPUS MESENTERIC VASCULITIS

Epidemiology and pathogenesis

A number of terms have been used to describe lupus mesenteric vasculitis (LMV), including mesenteric arteritis, lupus enteritis, lupus arteritis, lupus vasculitis, gastrointestinal vasculitis, intra-abdominal vasculitis and acute gastrointestinal syndrome. LMV is one of the main causes of acute abdominal pain in SLE patients. It can be classified into an acute ischemic enteritis that involves mainly small intestine and chronic multiple ulcers occurring mainly in the colon[2]. About 8%-40% of SLE patients have acute abdominal pain during the stage of active disease[3], but LMV is generally an uncommon condition in SLE patients. In Asia, the reported overall prevalence of LMV in patients with SLE is 2.2%-9.7%[4-6]. However, the prevalence of LMV in America seems to be much lower: 0.9%[7]. Ju *et al*[8] reported that the global prevalence of LMV ranges from 0.2% to 9.7% among all SLE patients and from 29% to 65% in patients who had acute abdominal pain. LMV occurs almost always in patients with active disease[6].

The predisposing factors for LMV are unknown. The proposed trigger factors include bacterial infections that lead to changes of intestinal flora, cytomegalovirus infection, eosinophilia, non steroidal anti-inflammatory drugs, chemicals, metallic particulates, animal viruses, helminth infection, caffeine, phosphodiesterase-4-inhibitors, adenosine diphosphate, certain foods and herbal medicines[6].

Inflammatory vasculitis secondary to immune complex deposition and thrombosis of the intestinal vessels secondary to circulating anti-phospholipid antibodies are the proposed pathogenic mechanisms of LMV[9-12]. Both types of microvasculopathy can activate each other reciprocally, resulting in worsening cascades of vasculitis and thrombosis. Autoantibodies, such as lupus anticoagulant, anti-cardiolipin antibody and anti-β2-glycoprotein antibody, are associated with LMV. A study suggests that some cryptic antigens which can stimulate the production of anti-endothelial antibodies are exposed when endothelial cells are disrupted[10]. In a study by Kwok *et al*[6], the authors found that the serum levels of anti-endothelial cell IgG were significantly higher in SLE patients with LMV than in those without LMV or in healthy controls. Macroscopically, the appearance of LMV varies from segmental edema to ulceration, gangrene and perforation[8]. Both small arteritis and venulitis are found in LMV. Microscopically, fibrinoid necrosis of subserosal vessels and leukocytoclasis on the vascular wall, as well as edematous submucosa with mild diffuse inflammatory infiltration of mononuclear cells, can be observed. In the muscular layer, intravascular fibrin thrombus and hemorrhage around the small veins can be found.

Clinical features

Both the inflammatory and thrombotic mesenteric vasculopathy of LMV can cause mesenteric ischemia. LMV can cause very severe abdominal symptoms and signs and sometimes is diagnosed as acute surgical abdomen. Typically, the abdominal pain caused by LMV is diffuse in pattern, in some cases accompanied by rebound tenderness and abdominal muscle guarding. The symptoms of LMV vary from mild, nonspecific abdominal pain, bloating or loose stool, to necrosis and intestinal perforation which manifest as severe extensive gastrointestinal bleeding or acute surgical abdomen. It is noteworthy that in LMV patients with bowel perforation, the typical signs can be absent. Other manifestations of LMV include anorexia, nausea, vomiting, dysphagia, hematemesis, postprandial fullness, diarrhea and melena. Kishimoto *et al*[13] reported two cases of "acute gastrointestinal distress syndrome". Patients with this syndrome have recurrent lupus enteritis on the basis of mesenteric vasculopathy characterized by reversible intestinal wall edema accompanied by significant hypocomplementemia. These attacks are not complicated by bowel infarction, perforation or hematachezia and respond quickly to corticosteroid treatment.

Diagnosis

Accurate diagnosis of LMV is critical to allow prompt treatment to avoid unnecessary surgical intervention. As the clinical symptoms and laboratory parameters are non-specific, and bowel specimens are not always available, the diagnosis of LMV relies on abdominal computed tomography (CT) scan which allows both the bowel wall and the abdominal vasculature to be visualized. Advances in CT technology have been extremely helpful in detecting ischemia and for evaluating the causes of abdominal pain.

Common CT findings in patients with LMV include dilated bowel, focal or diffuse bowel wall thickening, abnormal bowel wall enhancement which is also called "target sign", mesenteric edema, stenosis or engorgement of mesenteric vessels which is also called "the comb sign" and ascites (Figure 1). Segmental or multifocal involvement of the small and large bowel loops with intervening normal bowel segments indicates ischemic change, which is almost always indicative of vasculitis.

Ultrasonography is also useful for both the diagnosis and follow-up of LMV. Small intestinal wall edema and thickening can be visualized under ultrasonography. Irregular thickening and projection of folds in multiple segments of the duodenum and the terminal ileum accompanied by "thumb print" signs in double-contrast radiography suggest ischemic changes. Gastroscopy and colonoscopy can show ischemia and ulcerative changes. However, endoscopy-guided biopsy might not yield a definitive diagnosis of LMV because the affected vessels are usually located in an inaccessible area. Laparoscopy can be used in the diagnosis of LMV[8,9].

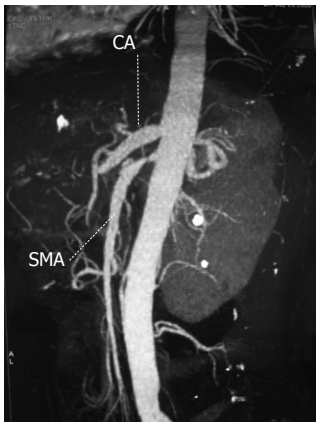

Figure 1 Stenosis of artery mesenterica superior (SMA) in a patient with systemic lupus erythematosus.

Management

Early diagnosis and appropriate intervention can avoid potentially fatal complications of LMV. Because the primary lesions of LMV are inflammatory ischemic vasculitis, immediate and aggressive anti-inflammatory immunosuppressive treatment should be initiated as soon as the diagnosis of LMV is made. The treatments include high dose intravenous infusion of methylprednisolone or an equivalent agent and complete bowel rest. For patients with recurrent LMV and those who do not have adequate response to intravenous prednisolone alone, intravenous cyclophosphamide should be initiated. The initial dosage of cyclophosphamide is 1 mg/kg daily and the dosage is gradually tapered when LMV is stabilized[6]. In Kim's study, they found that a bowel wall thickness greater than 9 mm usually indicated the presence of recurrent LMV and should be considered a high risk factor for recurrence[14]. They suggested that for patients with high risk of recurrence, immunosuppressive agents should be initiated as early as possible.

When a rapid response to immunosuppressive therapy is not achieved, surgical intervention for possible bowel perforation or large area of ischemia should be considered. Early laparotomy within 24 to 48 h is critical for improving the prognosis of LMV patients. Medina *et al*[15] found that 10 of 11 LMV patients who underwent surgery after 48 h died, while none of 33 patients who were operated on within 24-48 h died.

Prognosis

The prognosis of LMV varies in reports from different areas of the world. This might be due to genetic differences. Reports from Europe and North America indicate that the prognosis of LMV is poor and some fatalities can occur. Some reports claimed that the mortality of LMV could be as high as 50%[6,15]. The prognosis of LMV depends on the extent of vascular involvement, the prompt implementation of immunosuppressive therapy, and the time of surgical intervention. On the basis of published reports, the prognosis of LMV can be improved if abdominal CT is used to aid the diagnosis and if prompt immunosuppressive therapy is implemented.

PROTEIN-LOSING GASTROENTEROPATHY

Epidemiology and pathogenesis

Protein-losing gastroenteropathy (PLGE) is a condition characterized by profound edema and severe hypoalbuminemia secondary to excessive loss of serum protein from the gastrointestinal tract, clinically indistinguishable from nephrotic syndrome. Clinically significant PLGE seems uncommon in SLE. Besides two large series reported by Mok and our group with 16 and 15 patients respectively, most are isolated case reports or small series[16,17]. So far, less than 60 patients with SLE-related PLGE have been reported in the literature, and the majority of these patients are Asians; whether this is due to genetics or environmental factors remains to be elucidated. In Mok's study, the point prevalence of their Chinese patients is 3.2% for PLGE, while in our series the prevalence is 1.9%. PLGE may occur in SLE patients of any age and occurs with a female predominance. In most circumstances, the typical features of PLGE develop before the diagnosis of SLE. In our study, 53.3% of patients had PLGE as the initial presentation of SLE, but PLGE could also occur 17 years after the establishment of SLE[17]. Gornisiewicz *et al*[18] have concluded that PLGE often happens in patients with clinically severe SLE with multiple system involvement.

The exact pathogenesis of SLE-related PLGE is still unclear. Mucosal ulceration, non-necrotizing mesenteric or intestinal blood vessel vasculitis, increase in capillary permeability caused by intravascular activation and conversion of complement, cytokine- (such as tumor necrosis factor-α and interleukin-6) or complement-mediated vascular or mucosal damage, and intestinal lymphangiectasia have been postulated as the pathogenic mechanisms[16,19].

Clinical features

The most predominant clinical manifestation of PLGE is profound peripheral pitting edema. Patients may have pleural, pericardial effusion and ascites due to severe hypoalbuminemia. Nausea, vomiting and diarrhea are also common in these patients. Diarrhea is present in 50% of cases, which is generally liquid in nature and can be as frequent as 20 times a day, but steatorrhea is absent[17,20]. No particular autoantibodies have been found to be associated with PLGE. Severe hypoalbuminemia and hypocomplementemia are the most predominant laboratory findings of PLGE. Kim *et al*[19] found in their study that hypercholesterolemia was common in SLE-related PLGE, but rare in idiopathic PLGE[16]. This might be due to the leakage of cholesterol-rich lipoprotein particles in the intestinal lymph. Most PLGE patients also have hypoglobulinemia because immunoglobulin can also leak into the intestinal lumen. The presence of anti-dsDNA and anti-ENA antibodies is not significantly different from patients without PLGE. Eighty percent of PLGE in our series occurred at the stage of active lupus, and

non-specific chronic inflammation were detected in all pathological specimens obtained.

Diagnosis

The diagnosis of SLE-related PLGE mainly relies on the exclusion of other causes of hypoalbuminemia such as lupus nephritis, abnormal liver function, decreased protein synthesis or malabsorption. In recent years, Tc-99m albumin scintigraphy has become the most frequently used diagnostic method. It is noninvasive and safe in demonstrating gastrointestinal loss of blood albumin to intestinal lumen. It also has the potential to localize protein leakage and can be used to monitor the efficacy of treatment. Gastroenteroscopy is usually not diagnostic, as 50% of SLE-related PLGE patients present with non-specific intestinal wall edema and 10% of patients have no abnormalities under endoscopy examination[21]. Histologically, lymphangiectesia, edematous villi and non-specific inflammation can be found in the intestine, but histology can also be normal because of the inaccessibility of the involved area under gastroendoscopy. An elevated α-1 antitrypsin can be used as a diagnostic alternative[22].

Management

Due to limited number of patients, there is no controlled clinical trial to demonstrate the efficacy of treatment for SLE-related PLGE. Corticosteroid is the mainstay of treatment. Response to corticosteroid alone is excellent in more than 60% of patients. For those refractory to steroid, immunosuppressive agents such as azathioprine, cyclosporine A and cyclophosphamide could be added into the therapeutic regimen. Albumin infusion, nutritional support and diuretics are used as supplemental measures for the treatment. Octreotide can reduce intestinal microvasculature blood flow, decrease local lymph formation and ameliorate lymphatic dilatation, therefore can also be used in the treatment of PLGE. In addition, octreotide has immunomodulatory effects since it specifically binds with the somatostatin receptor. Prophylaxis for thromboembolic complications with warfarin should be considered in patients with severe and persistent protein loss, especially if antiphospholipid antibodies are present[16-18,20].

Prognosis

The outcome of most SLE-related PLGE cases is generally good since most patients respond well to steroid therapy. Relapse of PLGE occurs in 20%-30% of patients, particularly in patients undergoing maintenance therapy with low-dose prednisolone alone, but they respond again to an increased dosage of steroid. Mok *et al*[16] suggested that long-term maintenance treatment with low dose prednisolone plus azathioprine could reduce the rate of recurrence.

INTESTINAL PSEUDO-OBSTRUCTION

Prevalence and pathogenesis

SLE-related intestinal pseudo-obstruction (IPO) is a rare but well-recognized clinical syndrome that reflects the dysfunction of the visceral smooth muscle, the enteric nerve and/or the visceral automatic nervous system[23]. It usually coincides with ureterohydronephrosis and/or interstitial cystitis, rarely with biliary dilatation (megacholedochus)[24]. IPO is associated with ureterohydronephrosis in 63.3% of cases[25]. IPO may appear during the course of SLE, but it can also be the initial presentation of SLE[26]. It usually occurs in patients with active lupus. So far, only 28 cases have been reported in the English literature; half of the cases were Oriental and female patients predominated[23].

The pathogenesis of SLE-related IPO is unknown. Histopathological evidence of intestinal leiomyocyte damage suggests a systemic autoimmune process targeting smooth muscle cells[24]. Vasculitis leading to chronic ischemia of the bowel smooth muscle, which in turn leads to muscular damage and hypomotility, has been postulated as one of the possible mechanisms. Another possible mechanism is an intrinsic muscle dysmotility affecting the muscularis propria. The apparently high association between pseudo-obstruction and ureterohydronephrosis suggests a possible common smooth muscle dysmotility due to primary myopathy or neurogenic pathology, secondary to either immune complex-mediated vasculitis or common circulating autoantibodies against smooth muscle. Biopsied specimens have shown fibrotic process and atrophy in the muscularis layer, decreased number of smooth muscle cells and inflammatory cell infiltration associated with fibrinoid deposits, indicative of vasculitis[26].

Clinical features

The characteristic clinical manifestation of IPO is ineffective intestinal propulsion with the presence of clinical features of intestinal obstruction without an identifiable organic obstructive lesion and an abdominal distension with a very sluggish or absent peristalsis. Antroduodenal manometry demonstrates intestinal hypomotility and esophageal aperistalsis. Symptoms of IPO include a subacute onset of abdominal pain, nausea and vomiting, abdominal distension, constipation, diarrhea and weight loss. Laboratory findings are non-specific. Anti-proliferative cell nuclear antigen antibody (anti-PCNA) was found to be more frequently detected than in lupus patients without IPO in one study[27]. Mok *et al*[28] reported that SLE-related IPO patients had higher frequency of positive anti-Ro and anti-RNP antibodies compared to lupus patients without IPO.

Radiological examination can detect dilated fluid-filled bowel loops, with thickened bowel wall and multiple fluid levels. Bilateral ureter dilatation with a reduced urinary bladder capacity can be found if ureterhydronephrosis is the concurrent situation. Abdominal CT scan frequently reveals the presence of dilated small and large bowel with thickened intestinal wall.

Pathological examination of the gastrointestinal tract of SLE-related IPO patients can reveal widespread myocyte necrosis in the muscularis propria with active inflammatory cell infiltration, severe atrophy of muscularis, active serositis with serosal thickening and fibrosis, but little or no evidence of vasculitis and absence of thromboembolism.

Diagnosis

The diagnosis of this clinical syndrome is based on imaging findings consistent with the presence of dilated small and large bowel loops with thickened intestinal wall and multiple fluid levels. Other possible causes of intestinal obstruction should be excluded. Invasive diagnostic procedures should be avoided.

Management

Corticosteroids, immunosuppressive agents combined with supportive measures such as parenteral nutrition, oral broad-spectrum antibiotics to diminish bacteria overgrowth and pharmacological stimulation of small bowel motility are effective medical management options for SLE-related IPO. Timely diagnosis and early intervention are critical to rehabilitate the peristaltic activity of both the gastrointestinal tract and the genitourinary viscera; delayed treatment has been associated with failure to regain functional peristalsis and leads to histopathological progression to fibrosis and atrophy of the intestinal wall, and secondary impairment of the myenteric plexuses[24]. SLE-related IPO usually responds well to high dose corticosteroids. Erythromycin in particular is the appropriate antibiotic in this situation due to its prokinetic effect. Other prokinetic agents such as cisapride and octreotide are also helpful in stimulating small bowel motility. Octreotide appears to be effective in improving both clinical symptoms and manometric patterns[26]. Azathioprine, cyclophosphamide and cyclosporine A are the immunosuppressive agents that could be used as one component of a maintenance therapeutic regimen with oral corticosteroids. Other immunosuppressive agents against B cells, immune complex formation and pathogenic autoantibody formation could be the alternatives.

Prognosis

High dose intravenous corticosteroid treatment is effective in most patients, leading to clinical remission and disappearance of abnormal imaging findings. Long-term outcome of SLE-related IPO varies. Some patients may have recurrent attacks of IPO without other major organ involvement despite maintenance treatment with steroids and immunosuppressive agents. The reported mortality rate is 18%[26]. Early diagnosis and prompt treatment is critical to improve the overall outcome of SLE-related IPO patients.

PANCREATITIS

Epidemiology and pathogenesis

About 160 cases of SLE-related acute pancreatitis have been reported in the literature. Pancreatitis is a rare but life-threatening complication of SLE and our knowledge about this complication is mainly based on individual case reports. Based on the literature reports, the annual incidence of SLE-related pancreatitis is estimated to be 0.4-1.1/1000 lupus patients[29,30]. Reports from Europe and the United States showed that the rate of pancreatitis with SLE is between 0.7%-4%[31,32]. However, the rate of SLE-related pancreatitis may be underestimated because cases of subclinical pancreatitis with elevated pancreatic enzymes but no symptoms are not diagnosed or reported. It is estimated that 30.5% of asymptomatic SLE patients have hyperamylasemia[33].

Sixty percent of cases develop acute pancreatitis within 2 years of the diagnosis of lupus, and in 22% of patients, pancreatitis is the initial clinical presentation. In most cases, acute pancreatitis is associated with active lupus. In addition to traditional predisposing factors such as hypertriglyceridemia, steroid and azathioprine use were proposed as the possible causes of pancreatitis in lupus patients. However, in Pascual-Ramos's study, they could not prove the association between steroid and/or azathioprine administration and the development of pancreatitis even after they "rechallenged" the onset of pancreatitis with these medications[34].

The pathogenic mechanism of SLE-related pancreatitis is unclear. Vascular damage has been stressed as a cause of this problem. Necrotizing vasculitis, occlusion of arteries and arterioles by thrombi resulting from severe hypertension or antiphospholipid syndrome, intimal thickening and proliferation and immune complex deposition with complement activation in the wall of pancreatic arteries have been postulated[31]. Sixteen specimens of pancreatic tissue from SLE-related pancreatitis patients were examined in one series; evidence of inflammation and necrosis could be found in all specimens, but vasculitis could only be found in one case[31]. It is possible that an autoimmune reaction involving abnormal cellular immune response or antibody reaction, rather than vasculitis, is responsible for the inflammation reaction.

Clinical features

Eighty-eight percent of SLE-related pancreatitis cases have abdominal pain; in only 23% of them the pain radiates to the back. Two-thirds of patients have nausea and vomiting, and half of them have fever. Diarrhea is uncommon and a few patients have panniculitis.

Elevated serum amylase and lipase are the most commonly detected biochemical abnormalities. Additional biochemical abnormalities include hypoalbuminemia, abnormal liver function tests, elevated serum creatinine and hypocalcemia. Anti-La antibody is the only autoantibody reported to be associated with this particular complication[32].

Diagnosis

The diagnosis is based on the laboratory evidence of elevated serum amylase or lipase levels. Clinical symptoms and suggestive tomographic findings are helpful. However, lupus patients may develop acute pancreatitis secondary to other "non-SLE" causes such as mechanical (including cholelithiasis), toxic chemicals (such as alcohol ingestion, some medications), hypertriglycemia and hypocalcemia, as well as viral infections or sepsis. The diagnosis of SLE-related pancreatitis can only be confirmed when these possible causes are excluded.

Management

Corticosteroids should be used as the medical management of SLE-related acute pancreatitis as long as this drug can be excluded as the cause of pancreatitis. Immunosuppressive agents such as azathioprine or cyclophosphamide can be used in combination with corticosteroid. In severe cases, plasmapheresis and intravenous gamma-globulin infusion may be helpful.

Prognosis

As many as 57% of SLE-related acute pancreatitis cases may develop complications if not treated promptly[30]. Many of these complications can be fatal, with a mortality rate of 45%, whereas it is only 3% in patients without complications. Lupus activity is significantly associated with increased mortality. It is reported that SLE-related acute pancreatitis concurrent with central nervous system and cardiac involvement has the highest mortality rate. Increased serum creatinine, hypoalbuminemia, anti-DNA antibodies, thrombocytopenia, low complement, hypocalcemia, hyperglycemia and elevated liver enzymes are risk factors for increased mortality.

About 22% of patients may experience recurrent acute pancreatitis attacks, while 12% of patients develop pancreatic pseudocysts and 5%-14% become chronic[30,32].

OTHER SLE-RELATED GASTROINTESTINAL PROBLEMS

Celiac disease

The coexistence of SLE and celiac disease is rare. So far, only 17 cases have been reported in the literature. Both diseases have an autoimmune nature and share HLA-B8 and HLA-DR3 histocompatibility antigens[35]. Celiac disease can occur before or after the diagnosis of lupus[36]. Most patients have positive serum antigliadin antibodies and histological findings of duodenal biopsy which are consonant with celiac disease. Patient's response to steroid along with gluten-free diet is excellent. The prognosis is generally good.

Inflammatory bowel disease

The coexistence of SLE and inflammatory bowel disease (IBD) is difficult to diagnose since both diseases share some common gastrointestinal features and some medications used in treating IBD may cause drug-induced lupus. The estimated prevalence of ulcerative colitis (UC) in SLE patients is around 0.4%[37]. So far, 27 cases of SLE-related UC have been reported, and numbers of SLE-related Crohn's disease are even less. UC may occur either before or after the diagnosis of SLE. The majority of patients have excellent response to steroids combined with hydroxychloroquine or azathioprine. However, some patients with Crohn's disease may have life-threatening massive gastrointestinal bleeding and need high dosage methylprednisolone[38]. The prognosis of SLE-related IBD is usually good.

Eosinophilic enteritis

Eosinophilic enteritis is a rare clinical condition. SLE-related eosinophilic enteritis is even rarer. Only 3 cases of SLE-related eosinophilic enteritis have been reported in the literature[39]. The clinical symptoms include abdominal pain, nausea, vomiting and sometimes diarrhea. Peripheral hypereosinophilia presents in most of the patients. The diagnosis depends on clinical symptoms and intestinal biopsy which shows eosinophils in the deep layers of the intestinal wall[39]. The recommended treatment regimen is prednisone 0.5-1 mg/kg per day in divided doses for 7-10 d followed by slow tapering over 2 to 3 mo. Immunosuppressive agents can be used in patients with recurrence or corticosteroid non-responders.

Pneumatosis cystoides intestinalis

This is an uncommon disorder characterized by the presence of gas within the walls of the gastrointestinal tract. The most common rheumatic disease that is associated with pneumatosis cystoides intestinalis (PCI) is systemic sclerosis. Only 14 SLE-related PCI cases have been reported in the literature. It might be caused by injury of the mucosa and immune barrier due to lupus vasculitis and impaired healing due to corticosteroid therapy[40]. Pathological evidence of vasculitis can be found in at least half of the patients. Increased intraluminal pressure, mucosal injury and production of gas from bacteria in the mucosa may be involved in the pathogenesis of SLE-related PCI. The diagnosis depends on the abdomen findings on CT, which show thickened bowel walls with multiple linear and cystic radiolucencies. Therapeutic strategy for each PCI patient should be individualized. Oxygen inhalation or hyperbaric oxygen therapy is reported to promote removal of the gas from the cysts. Antibiotics are effective in reducing bacterial overgrowth and gas production. Bowel rest and prokinetic agents are the supportive measures in treating patients with SLE-related PCI.

CONCLUSION

Gastrointestinal manifestations are common in SLE patients, but most of them are due to adverse reactions to medications and infection. SLE-related gastrointestinal involvement is not rare, and sometimes can be life-threatening. Most SLE-related gastrointestinal complications are caused by vasculitis and immune complex deposition, and respond well to corticosteroids and immunosuppressive agents. Early diagnosis and timely treatment are critical to improve the prognosis. Supportive measures such as bowel rest are beneficial, and in some situations such as IPO and PCI antibiotics are helpful to facilitate functional restoration.

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TOPIC HIGHLIGHT

Marcela Kopacova, Associate Professor, MD, PhD, *Series Editor*

Small intestinal bacterial overgrowth syndrome

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Abstract

Human intestinal microbiota create a complex polymicrobial ecology. This is characterised by its high population density, wide diversity and complexity of interaction. Any dysbalance of this complex intestinal microbiome, both qualitative and quantitative, might have serious health consequence for a macro-organism, including small intestinal bacterial overgrowth syndrome (SIBO). SIBO is defined as an increase in the number and/or alteration in the type of bacteria in the upper gastrointestinal tract. There are several endogenous defence mechanisms for preventing bacterial overgrowth: gastric acid secretion, intestinal motility, intact ileo-caecal valve, immunoglobulins within intestinal secretion and bacteriostatic properties of pancreatic and biliary secretion. Aetiology of SIBO is usually complex, associated with disorders of protective antibacterial mechanisms (e.g. achlorhydria, pancreatic exocrine insufficiency, immunodeficiency syndromes), anatomical abnormalities (e.g. small intestinal obstruction, diverticula, fistulae, surgical blind loop, previous ileo-caecal resections) and/or motility disorders (e.g. scleroderma, autonomic neuropathy in diabetes mellitus, post-radiation enteropathy, small intestinal pseudo-obstruction). In some patients more than one factor may be involved. Symptoms related to SIBO are bloating, diarrhoea, malabsorption, weight loss and malnutrition. The gold standard for diagnosing SIBO is still microbial investigation of jejunal aspirates. Non-invasive hydrogen and methane breath tests are most commonly used for the diagnosis of SIBO using glucose or lactulose. Therapy for SIBO must be complex, addressing all causes, symptoms and complications, and fully individualised. It should include treatment of the underlying disease, nutritional support and cyclical gastro-intestinal selective antibiotics. Prognosis is usually serious, determined mostly by the underlying disease that led to SIBO.

Key words: Bacterial overgrowth; Breath test; Hydrogen; Methane; Small intestine

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INTRODUCTION

Human intestinal microbiota create a complex polymicrobial ecology. This is characterised by its high population density, wide diversity and complexity of interaction. The duodenum and proximal jejunum normally contain small numbers of bacteria, usually lactobacilli and enterococci, gram-positive aerobes or facultative anaerobes ($< 10^4$ organisms per mL). Coliforms may be transiently present ($< 10^3$ bacteria per mL) and anaerobic *Bacteroides* are not found in the jejunum in healthy people. Up to one third of jejunal aspirates might be sterile in healthy volunteers. The distal ileum is a transition zone between sparse populations of aerobic bacteria of the proximal small intestine and very dense populations of anaerobic micro-organisms in the large bowel[1-3]. The epithelial surface of the small intestine in a healthy human is not colonised. Occasional groups of bacteria can be found in low concentrations within the lumen. Bacteria do not form clusters and spatial structures, and the luminal contents are separated from the mucosa by a mucus layer[4].

Any dysbalance of this complex intestinal microbiome, both qualitative and quantitative, might have serious health consequences for a macro-organism, including small intestinal bacterial overgrowth syndrome (SIBO).

DEFINITION

SIBO is a very heterogeneous syndrome characterised by an increased number and/or abnormal type of bacteria in the small bowel. Most authors consider diagnostic of SIBO to be the finding of $\geqslant 10^5$ bacteria [i.e. colony-forming units (CFU)] per mL of proximal jejunal aspiration. The normal value is $\leqslant 10^4$ CFU/mL[3, 5-7].

PREVALENCE

The overall prevalence of SIBO in the general public is unknown. In general, SIBO is substantially underdiagnosed. There are several reasons for this fact. Some patients may not seek healthcare or SIBO may not be properly diagnosed by medical investigations. SIBO might be asymptomatic or with non-specific symptoms only, and last but not least, all symptoms might be incorrectly ascribed to the underlying disease (leading to SIBO). Of course, diagnostic yield also depends on the methods used for investigation. According to different studies with the investigation of small sets of clinically healthy people as a control, findings consistent with SIBO were found in 2.5% to 22%[8-17].

In particular diseases and disorders, literature data on prevalence differ substantially. For instance, the prevalence of SIBO in patients fulfilling diagnostic criteria for irritable bowel syndrome was 30%-85%[9-11,16,18,19]. The prevalence of SIBO in coeliac disease non-responding to a gluten-free diet was up to 50%[20]. In liver cirrhosis, SIBO was diagnosed in more than 50% of cases[21,22]. In a small group of elderly people (70 to 94 years old) with lactose malabsorption, SIBO was documented in 90%[23]. An interesting study was performed on asymptomatic morbidly obese subjects and SIBO was found in 17% (compared to 2.5% in non-obese persons)[15].

AETIOLOGY

There are several endogenous defence mechanisms for preventing bacterial overgrowth: gastric acid secretion, intestinal motility, intact ileo-caecal valve, immunoglobulins within intestinal secretion and bacteriostatic properties of pancreatic and biliary secretion[24].

The aetiology of SIBO is usually complex, associated with disorders of protective antibacterial mechanisms (e.g. achlorhydria, pancreatic exocrine insufficiency, immunodeficiency syndromes), anatomical abnormalities (e.g. small intestinal obstruction, diverticula, fistulae, surgical blind loop, previous ileo-caecal resections) and/or motility disorders (e.g. scleroderma, autonomic neuropathy in diabetes mellitus, post-radiation enteropathy, small intestinal pseudo-obstruction). In some patients more than one factor may be involved. "Aetiological" and "predisposing" factors cannot be separated in some patients. SIBO may occur in elderly people without any evident underlying small intestinal pathology.

In some cases, a vicious circle arises: an underlying disease is complicated by SIBO and then SIBO directly (as a morphological impact) or vicariously (by malabsorption or nutrient deficiency) causes further deterioration of the underlying disease.

Out of all diseases and disorders associated with SIBO (listed below in detail), 90% of cases comprise small intestinal motility disorders (of various aetiology) and chronic pancreatitis[2].

Achlorhydria

Achlorhydria (due to chronic atrophic gastritis) and long-term administration of proton pump inhibitors may cause bacterial overgrowth in the stomach and duodenum. Proton pump inhibitors not only increase duodenal bacterial colonisation but also accelerate intestinal transit[24].

Exocrine pancreatic insufficiency

Chronic pancreatitis is complicated by SIBO in 30%-40% of cases[7,25]. Multiple factors can be involved: exocrine pancreatic insufficiency (with absence of anti-bacterial effect of proteolytic enzymes), abnormal chyme in the small intestinal lumen, motility disorders, administration of painkillers and ongoing alcohol consumption in some of patients. Cystic fibrosis is also associated with increased risk of SIBO. Fridge *et al*[26] diagnosed SIBO in 14/25 (56%) patients with cystic fibrosis. SIBO may be a causative fac-

tor of diarrhoea in advanced pancreatic cancer[27] apart from pancreatic exocrine insufficiency, chemotherapy or previous surgery.

Immunodeficiency syndromes

Various immunodeficiency syndromes, such as IgA deficiency, common variable immunodeficiency, AIDS and others, are complicated by miscellaneous infection complications, including SIBO[28,29].

Small intestinal obstruction and stagnation

All anatomical pathology associated with small intestinal obstruction and stagnation could be associated with SIBO, e.g. strictures, adhesions, tumours of the small bowel. Large and/or multiple duodenal and jejunal diverticula are often complicated by SIBO. Sequelae of previous abdominal surgery (afferent loop syndrome after Billroth-Ⅱ gastric resection, Roux-en-Y stasis syndrome, bariatric bypass surgery) may also lead to SIBO (with metabolic and nutritional disarrangement)[7,30-32]. Small intestinal pseudo-obstruction and some neurological diseases (e.g. myotonic dystrophy, Parkinson disease, Chagasic enteropathy) can be complicated by SIBO that is responsible for malabsorption and weight loss[33-36]. Spinucci *et al*[37] described an interesting case of endogenous ethanol production in a patient with chronic intestinal pseudo-obstruction and SIBO.

Tursi *et al*[38] investigated bacterial overgrowth in the small bowel in patients with acute diverticulitis of the colon. Small intestinal overgrowth was found in 53/90 (59%) subjects. The authors assumed that the primary mechanism is a slow large bowel transit with stasis of faeces in the colon. This results in dysmicrobia in the large bowel with metabolic changes and induction of inflammation. Subsequent reverse peristalsis facilitates colonisation of the small intestine by bacteria coming from the large bowel. SIBO deteriorates symptoms of acute colonic diverticulitis, protracts the course of the disease and thus could be an independent risk factor for future relapses of acute diverticulitis of the large bowel. Rifaximin was effective in the treatment of both acute colonic diverticulitis and SIBO in these patients[38].

Irritable bowel syndrome

The aetio-pathogenesis of irritable bowel syndrome has not yet been satisfactorily clarified. Symptoms of SIBO and irritable bowel syndrome overlap to a large degree. As mentioned earlier, SIBO is frequently found in persons fulfilling criteria of irritable bowel syndrome (30%-85%)[9-11,16,18,19]. According to authors of the bacterial hypothesis, SIBO is the primary event and irritable bowel is secondary to SIBO. In some patients, the onset of irritable bowel is preceded by infective gastroenteritis (so-called post-dysenteric bowel disturbance)[39]. Analysis of the microbial genome found different faecal microbiota in healthy people and patients with irritable bowel (e.g. phylotypes *Coprococcus*, *Collinsella*, *Coprobacillus*)[40-42]. Believers in an opposite hypothesis stated that irritable bowel is a primary factor (with motor disturbance, visceral afferent hypersensitivity, psycho-social dysfunction) in which motility disorders enable "secondary" bacterial overgrowth[40,43]. A third group of authors recommend strict distinction between irritable bowel syndrome (the hydrogen breath test with lactulose must be negative) and SIBO (in such a case, it is not irritable bowel despite the diagnostic criteria having been met) in patients with identical symptoms[44]. The last authors stated an opinion that SIBO does not play any significant role in the pathogenesis of irritable bowel[45].

Pimentel *et al*[12] found abnormal lactulose breath test results in 93/111 (84%) patients with irritable bowel syndrome. Successful treatment of SIBO using neomycin (in 35% of patients) was associated with relief of subjective symptoms. There was another interesting finding in this study: a subgroup of persons with a methanogenic phenotype was associated with constipation in 100% (constipation-predominant irritable bowel syndrome)[12]. Another study found that methanogenic status was never associated in irritable bowel syndrome with diarrhoea and Crohn's disease or ulcerative colitis with diarrhoea[13]. The association of methanogenic phenotype and constipation was also revealed by other authors[8].

Coeliac disease

A wide range of 9% to 55% of patients have been diagnosed with SIBO as a complication of coeliac disease[20,46-48]. The prevalence of SIBO is high, especially in patients who do not respond to a gluten-free diet and/or have lactose intolerance[20,46,47].

Crohn's disease

SIBO is frequently found in Crohn's disease (in about 25%). Loss of the ileo-caecal valve (due to previous ileocaecal resection) and/or large entero-enteric and enterocolic fistulae are important predisposing factors[49-55]. Castiglione *et al*[56] found bacterial overgrowth more frequently in those who underwent surgery (30%) compared to non-operated patients (18%). Furthermore, SIBO may mimic an acute flare of Crohn's disease (including increased bowel movements and lower body weight)[57]. Smokers may exhibit increased H_2 production which could lead to false positive test results. However, in the study by Klaus *et al*[57] there was no difference in the proportion of smokers and their respective daily consumption of cigarettes between patients with Crohn's disease with and without SIBO.

Short bowel syndrome

The problem of short bowel syndrome is not limited only to the reduced absorptive surface area. The loss of the ileocaecal valve and the loss of the ileal break from resection of the distal small bowel would accelerate the transit of chyme throughout the entire gastrointestinal tract. Undigested food becomes a substrate for bacterial fermentation. Large intestinal bacterial flora colonise proximally into the small intestine to result in SIBO. Because digestion and absorption cannot be completed without adequate time, these patients face chronic postprandial diarrhoea.

These problems may be exacerbated by SIBO that further accelerates transit and worsens digestion, absorption and malnutrition[58].

SIBO is an independent negative factor deteriorating adaptation of the small intestine in children after excessive bowel resections. SIBO lengthens the dependence of these patients on total parenteral nutrition and deteriorates malabsorption and hepatopathy associated with short bowel syndrome[59,60]. SIBO may lead to intestinal failure in these patients[61].

Non-alcoholic steatohepatitis

Wigg *et al*[17] found a higher prevalence of SIBO (11/22, 50%) in non-alcoholic steatohepatitis (NASH) compared to healthy control subjects (5/23, 22%). Higher values for the xylose-lactulose test in patients with NASH correlated with higher serum levels of tumor necrosis factor-α (TNF-α). However, they were not associated with increased intestinal permeability or increased serum endotoxin[17]. In another study of NASH[62], SIBO was diagnosed in half of the patients (6/12) but only in one subject (1/11, 9%) in the healthy control group. Treatment with ciprofloxacin suppressed bacterial overgrowth, increased serum insulin and decreased endogenous ethanol production but did not influence serum acetylated ghrelin (half values compared to controls). Changes in fasting insulin and ethanol following ciprofloxacin suggest that these parameters may be influenced by small intestinal bacterial activity[62]. In an experimental model of NASH in rats, there was a slower transit time and higher quantity of coliform bacteria (*Escherichia coli*). Treatment with gentamicin (cidomycin) accelerated the transit time, decreased TNF-α levels and alleviated severity of liver involvement in experimental animals. Thus SIBO might play an important role in the pathogenesis of NASH[63].

Liver cirrhosis

Portal hypertension in liver cirrhosis substantially changes the intraluminal milieu of the gut. Liver cirrhosis is an independent risk factor for SIBO. Small intestinal motility disorder, especially slow transit in advanced liver disease (Child-Pugh C) may partake in SIBO[64,65]. SIBO was diagnosed in 50%-60% of patients with liver cirrhosis[66,67]. SIBO is a risk factor for the development of spontaneous bacterial peritonitis[22,68], however, its role in the pathogenesis has not yet been fully clarified[22]. Prevalence of SIBO was higher in those patients with liver cirrhosis who had spontaneous bacterial peritonitis (14/20, 70%) compared to those without it (4/20, 20%)[64]. However, this finding was not confirmed in other studies[66]. SIBO might correlate with systemic endotoxaemia[69]. It is necessary to remind ourselves that glucose hydrogen breath test in liver cirrhosis correlates only to a small degree with microbiological analysis of jejunal aspirates (sensitivity 27%-52%, specificity 36%-80%)[70].

Scleroderma

Scleroderma (systemic sclerosis) is a chronic connective tissue disease that affects the gastrointestinal tract in more than 80% of patients[71]. Severe small bowel involvement by scleroderma can present as chronic intestinal pseudo-obstruction and SIBO. The reported prevalence of SIBO in scleroderma was 43% to 56%[72,73]. In our series, SIBO was proved in 4/15 (27%) patients with systemic sclerosis by means of glucose hydrogen and methane breath tests. Half the cases of SIBO had neither diarrhoea nor other signs of malassimilation at the time of examination. There was a tendency towards a higher dose of systemic glucocorticosteroids in persons with positive hydrogen and methane breath tests[74].

Autonomic neuropathy in diabetes mellitus

Gastrointestinal symptoms are present in 50%-70% of patients with diabetes mellitus. Delayed gastric emptying (or even diabetic gastroparesis) and intestinal motility disorders are the most important findings (with an unfavourable impact on glycaemic control). Impaired intestinal motility is often followed by SIBO[75-77]. In diabetes mellitus, first and foremost all results must be interpreted according to the diagnostic method that was used. Cuoco *et al*[78] performed the lactulose hydrogen breath test and found that 21/74 (28%) of subjects were affected by SIBO and delayed oro-caecal transit time. After treatment with rifaximin, three patients still showed SIBO, five persistent delayed transit time without SIBO and 13 persons (62%) experienced a significant improvement in their oro-caecal time (without SIBO)[78]. Reddymasu *et al*[79] used hydrogen and methane breath tests after glucose challenge. Thirty out of fifty (60%) patients had a positive breath test result for SIBO on the basis of hydrogen (63%), methane (27%) or both criteria (10%). SIBO was more likely in diabetic patients with gastroparetic symptoms of longer duration[79].

In about one third of patients with diabetes, SIBO was associated with cardiovascular autonomic neuropathy[77]. SIBO in diabetes may rarely manifest itself as protein-losing enteropathy[80].

Radiation enteropathy

SIBO and lactose intolerance may occur during and/or after pelvic (or abdominal) radiotherapy[81-83].

Fibromyalgia

Pimentel *et al*[14] found that 42/42 (100%) patients with fibromyalgia had an abnormal lactulose hydrogen breath test. This was a significantly higher rate compared to patients with irritable bowel syndrome (93/111, 84%) and clinically healthy persons used as a control (3/15, 20%). Patients with fibromyalgia also had a higher hydrogenic profile that correlated with somatic pain[14].

Other disorders and diseases associated with SIBO

Various diseases and disorders have been described to be associated with or complicated by SIBO, such as lymphoproliferative diseases (lymphoma, chronic lymphocytic leukaemia), benign lymphoid hyperplasia of the ileum, metabolic bone disease, acromegaly, hypothyreosis, alcoholism and rosacea[7,84-87]. The prevalence of SIBO rises with age (about 50% in persons > 75 years old)[88].

MICROBIOLOGY, PATHOGENESIS, PATHOPHYSIOLOGY AND PATHOLOGY

The total bacterial count in the proximal jejunum is < 10^4 bacteria per mL of jejunal content in healthy people. In the ileum, enteric bacterial populations increase in amount (including coliforms) up to 10^9 CFU/mL in the terminal ileum. There are several beneficial effects of normal small intestinal bacteria to the host. They can be extrapolated from experimental studies in germ-free animals. The small intestinal villi of these animals are thin and unusually regular, with relatively shortened crypts. The enterocytes are cuboidal rather than columnar. In addition, the number and size of Peyer's patches, the degree of leukocyte infiltration in the lamina propria, and the rate of mucosal regeneration are reduced. The introduction of micro-organisms rapidly restores the normal morphologic appearance and physiologic function of the small bowel mucosa[7].

Normal autochthonous bacterial flora of the gastrointestinal tract is an important factor for preservation of its integrity and normal functioning in humans. They participate in the protection of macro-organisms against pathogenic micro-organisms, stimulate the human immune system and influence the metabolic and trophic function of the intestinal mucosa. Enteric bacteria produce some nutrients (e.g. short-chain fatty acids) and vitamins such as folates and vitamin K. Last but not least, they impact the sensor and motor function of the gut. On the other hand, intestinal bacteria are influenced by many factors, first of all by the amount and composition of food, but also by environmental (and geographic) effects, drugs, alcohol and probably by several other factors (lifestyle, psychosomatic stress, *etc.*)[5,89]. The prevalence of bacteria in different parts of the GI tract appears to be dependent on several factors, such as pH, peristalsis, redox potential, bacterial adhesion, bacterial co-operation and antagonism, mucin secretion, diet and nutrient availability[90].

There are several host defence mechanisms to prevent excessive colonisation of the small bowel by bacteria: antegrade peristalsis prevents attachment of ingested micro-organisms; gastric acid and bile destroy many micro-organisms before they leave the stomach; digestion by proteolytic enzymes helps destroy bacteria in the small intestine; the intestinal mucus layer traps bacteria; an intact ileo-caecal valve inhibits retrograde translocation of bacteria from the colon to the small bowel; the immune system plays a role as evidenced by the high prevalence of bacterial overgrowth in patients who have immunodeficiency; the largest fraction of immunoglobulins secreted in the human body is the secretory IgA originating in the gastrointestinal tract, which aids in preventing bacterial proliferation[7,91]. SIBO may develop if some of the natural defensive mechanisms of a macro-organism (listed above) are disrupted.

In most patients, SIBO is not caused by a single bacterial strain. In general, there is an extension of colonic bacteria into the small bowel. Less frequently, the "normal" amount of small intestinal bacteria increases. Bouhnik *et al*[92] investigated samples of jejunal juice in 63 patients with diarrhoea and/or malabsorption. The diagnostic criteria of SIBO were fulfilled in 55 persons (87%). The authors identified 141 micro-aerophilic strains (*Streptococcus* 60%, *Escherichia coli* 36%, *Staphylococcus* 13%, *Klebsiella* 11% and others) and 117 anaerobes (*Bacteroides* 39%, *Lactobacillus* 25%, *Clostridium* 20% and others)[92].

SIBO may be accompanied by both maldigestion and malabsorption. Bacteria in SIBO might significantly interfere with enzymatic, absorptive and metabolic actions of a macro-organism. Due to injury of the brush-border of enterocytes, the activity of disaccharideses may be decreased. If bacteria simultaneously metabolise fructose, lactose and sorbitol, malabsorption of saccharides may occur. Injured small intestinal mucosa can have undesirable consequences in increased intestinal permeability and/or protein-losing enteropathy. Deficiency of vitamin B_{12} results from the consumption of this vitamin by anaerobic micro-organisms. Bacteria may also utilise intraluminal protein in the small bowel, this may lead to protein deficiency for the macro-organism and excessive production of ammonia by bacteria. Deconjugation of bile acids by bacteria results in malabsorption of fat and liposoluble vitamins. Extensively formed lithocholic acid is poorly absorbable and acts enterotoxically[5,7,93-95].

Bacteria produce various toxic agents that may have surprising systemic effects. These agents are ammonia, D-lactate, endogenous bacterial peptidoglycans and others. SIBO is regularly associated with increased serum endotoxin and bacterial compounds stimulating production of (pro)inflammatory cytokines[7,96]. SIBO might be associated with endogenous production of ethanol (probably synthesised by *Candida albicans* and *Saccharomyces cerevisiae*). Serum ethanol disappears after successful treatment of SIBO[37].

Small intestinal bacterial overgrowth has a negative impact not only on the function but also on the morphological structure of the small bowel. Microscopic inflammatory changes (especially in the lamina propria) and villous atrophy are found regularly. In such a case, the villous atrophy in SIBO must be distinguished from that of coeliac disease. Macroscopic changes may also be visible in some patients. Hoog *et al*[97] found small intestinal mucosal breaks (erosions or ulcers) in 16/18 patients with chronic myopathic or neuropathic motility disorders of the small bowel by means of wireless capsule endoscopy.

In some patients with short bowel syndrome, bacterial overgrowth can, to some extent, paradoxically exert a favourable effect on the macro-organism. Bacteria may partly metabolise saccharides and thus form some further energy substrates more easily utilisable by a diseased human.

CLINICAL FEATURES

Clinical symptoms are expressed more or less according to the severity of involvement and they are modified by a primary underlying disease. SIBO may be clinically asymptomatic or can resemble irritable bowel syndrome with

non-specific symptoms (bloating, flatulence, abdominal discomfort, diarrhoea, abdominal pain). In more severe cases, there are signs of malabsorption (weight loss, steatorrhoea, malnutrition), liver lesion, skin manifestation (rosacea), arthralgias and deficiency syndromes (anaemia, tetany in hypocalcaemia induced by vitamin D deficiency, metabolic bone disease, polyneuropathy due to vitamin B_{12} deficiency, impaired barrier function of the gut, *etc.*). Anaemia is usually macrocytic (megaloblastic) due to vitamin B_{12} deficiency. It could also be microcytic iron deficiency (due to occult gastrointestinal blood loss) or normocytic (as anaemia of chronic disease)[3,5-7]. Serum folate and vitamin K levels are usually normal. Serum vitamin K can even be increased owing to its bacterial overproduction. Moreover, there are some concerns as to whether endogenous intestinal production of vitamin K by bacteria might interfere with warfarin treatment in SIBO[98-100]. In the case of oedema of lower extremities, the aetiology is usually more complex (anaemia, malnutrition, hypoproteinaemia, vitamin B_{12} deficiency).

D-lactic acidosis is a severe complication of patients with short bowel syndrome (with intact large bowel). It is caused by an excessive overgrowth of lactobacilli. Non-absorbed saccharides pass from the small intestine to the large bowel and they are fermented down to the D-isomer of lactic acid. There is no human pathway to metabolise D-lactic acid. D-lactic acid is absorbed from the large bowel; its serum concentration is regularly increased in these patients. Nevertheless, most patients remain asymptomatic. In clinically expressed cases, leading symptoms comprise characteristic neurologic abnormalities including confusion, cerebellar ataxia, slurred speech, and loss of memory. Patients exhibit some degree of altered mental status. They may complain of or appear to be drunk in the absence of ethanol intake. In the treatment, it is necessary to compensate metabolic acidosis and administer peroral antibiotics (metronidazole, rifaximin). To prevent this serious complication, it is important to reduce peroral intake of simple sugars, polysaccharides given in smaller amounts together with a higher intake of fat[101].

DIAGNOSTICS

It is mandatory to consider SIBO in all cases of complex non-specific dyspeptic complaints (bloating, abdominal discomfort, diarrhoea, abdominal pain), in motility disorders, anatomical abnormalities of the small bowel and in all malassimilation syndromes (malabsorption, maldigestion)[3,5-7].

Physical investigation usually provides non-specific findings and could be modified by a primary underlying disease. The abdomen may be distended and a small intestinal succussion splash might be identified. Physical investigation can further reveal latent tetany, polyneuropathy and skin manifestation (rosacea).

Laboratory tests usually find anaemia, low serum vitamin B_{12} levels and laboratory signs of malnutrition (lymphopenia, low serum prealbumin and transferrin).

The gold standard for diagnosing SIBO is still microbial investigation of jejunal aspirates. Such a sample can be obtained by a special sonde or by means of enteroscopy. Nowadays, there are commercially available special aspiration catheters (with a spiral pattern of holes at the distal tip) for contaminate-free collection of fluids. Microbial investigation places high demands on the quality of laboratory work (determination of quantitative proportion of anaerobes) and has several difficulties (low reproducibility and identifying cultivation-resistant bacteria). Distribution of bacterial overgrowth might be irregular and that is why a single investigation might not detect it. Bacterial overgrowth may be restricted to a particular, difficult-to-access area for aspiration (e.g. a blind loop)[7].

Hydrogen and methane breath tests are currently the most important diagnostic methods. The principles and methods of hydrogen and methane breath tests were described in detail elsewhere[102-105]. In humans, hydrogen and methane are exclusively produced by intestinal bacteria, namely in the large bowel in healthy people and also in the small intestine in the case of SIBO. About 80% of hydrogen and methane is expelled by flatus, 20% is exhaled by lungs and can be measured in breath[106]. Hydrogen and methane breath tests to diagnose SIBO are performed after peroral glucose or lactulose challenge. Most authors (including our Department) use gas chromatography for breath analysis. A parallel measurement of CO_2 and correction of hydrogen values to CO_2 concentration make the measurement more precise[7,74,103,104]. Low humidity (< 25%) must be maintained in the laboratory atmosphere to obtain consistent measurements. There is an early increase in breath hydrogen and/or methane (single early peak) after glucose administration due to bacterial glucose fermentation in the small bowel. There are two peaks in the lactulose breath test, the first one owing to bacterial activity in the small intestine, the second one after lactulose reaches the colon. Unfortunately, hydrogen and methane breath tests have not yet been standardised, particular protocols differ in dose (and concentration) of the test substrate, duration of tests, time intervals of breath sampling and basic and peak cut-off values. According to most authors, basal cut-off values of hydrogen and/or methane in positive breath tests are $\geqslant$ 20 parts per million (ppm), 10-20 ppm is a grey zone. After a glucose challenge, an increase $\geqslant$ 12 ppm at 120 min is a positive result for bacterial overgrowth. A lactulose breath test is assessed as positive if there is a biphasic course or an early plateau pattern with a hydrogen increase of $\geqslant$ 12 ppm is found (possibly with an increase in methane at the second peak)[104,107-110].

The hydrogen breath test is considered to be more accurate for the diagnosis of SIBO compared to the methane breath test according to most authors[111-114]. There is a sensitivity of 62.5% and specificity of 82% (diagnostic accuracy of 72%) after glucose and 52% and 86% (diagnostic accuracy of 55%) after lactulose administration[109]. Hydrogen alone, methane alone or both gases simultaneously might be found in breath samples. That is why it is important to always determine both gases in the breath samples. There are several advantages of hydrogen and methane breath tests. They are non-invasive, non-toxic,

relatively easily available and performed at a low cost.

However, hydrogen and methane breath tests have some drawbacks with possible false results and difficulties in their interpretation. Very rapid absorption of glucose in the proximal jejunum can be responsible for a false negative result. In the case of bacterial overgrowth in the terminal ileum, it might be difficult to distinguish a pathological ileal peak from a "normal peak" after the caecum is reached. In short bowel syndrome (with intact large bowel), the test substrate may reach the colon very quickly and cause a false positive result. In the case of a low density of anaerobes, breath can be false negative[109,113,115-117]. If there is only one peak of hydrogen recorded in the lactulose breath test and the second peak is absent for hydrogen and methane, this could be assessed as a combination of SIBO and fermentative colopathy[108]. Results of hydrogen and methane breath tests are usually difficult to interpret in the case of advanced lung disease. In the case of a high concentration of hydrogen and low concentration of methane, analytical precision for methane determination is less accurate[104].

All hydrogen and methane are produced by so called "hydrogenic and methanogenic" bacteria in humans[103,104,106,118]. However, most authors usually do not specify which particular bacteria constitute these producers. Nitrogen, oxygen, carbon dioxide, hydrogen and methane account for more that 99% of expelled intestinal gas. Hydrogen is produced by bacterial fermentation of saccharides in the intestinal lumen. Concurrently, hydrogen is consumed by other intestinal bacteria to synthesise methane, acetate and hydrogen sulphide. Methane is synthesised solely by bacteria in the intestine (four mmols of hydrogen and one mmol of carbon dioxide create one mmol of methane and water). This reaction reduces the volume of gas that would otherwise be present in the colon[119-124]. The question of intestinal methane producers has not been definitely solved yet. *Methanobrevibacter smithii*, *Methanosphaera stadtmanae* and other *Methanobacteriales* are able to synthesise methane and some authors consider *Methanobrevibacter* to be the major producer of methane in the gut of humans[125,126]. It was assumed (based on 16S ribosomal DNA studies) that *Methanobrevibacter smithii* could make up about one in ten of all the prokaryotes in the human gut[127]. However, there is no final proof available that *Archea* (*Methanobrevibacter* and others) would be the prevailing microorganisms in the methanogenic phenotype of the human gut. We hypothesised that common coliform bacteria could also synthesise methane[128], however, this assumption was not proved by our further studies[129,130]. McKay *et al*[131] found that several anaerobes (*Bacteroides*, *Clostridium* and others) produced hydrogen but rarely methane. Hydrogen is also produced by *Enterobacteriaceae*[128,132].

In adult Caucasians, only 30%-50% of persons produce methane while hydrogen is produced by 90%-98% of people[104]. Most subjects with lactose intolerance who do not produce hydrogen would form methane after lactulose administration instead of hydrogen[133]. Bile in the intestinal lumen is an important suppressor of methanogenesis in humans[134]. According to some authors, a methanogenic phenotype is associated with constipation[8,12]. Methane production has been related to more severe colonic impaction in children with encopresis[135]. Higher production of methane has been detected in colorectal adenomas and cancer[136]. However, there is a general agreement that constipation itself is not a risk factor for the development of colorectal cancer.

Hydrogen and methane breath tests can be combined with a simultaneous D-xylose breath test[107,110]. This combination increases the sensitivity of non-invasive diagnostics of SIBO[107,114]. The cholyl-1-^{13}C-glycine hydrolase breath test is another possible alternative for the diagnosis of SIBO. The principle of this test is based on the fact that bacterial overgrowth will cause more rapid deconjugation of cholyl-1-^{13}C-glycine[137]. The reported sensitivity for this test was 70%[138]. The lack of sensitivity was attributed to bacterial overgrowth with species lacking cholylglycine hydrolase[137]. On the other hand, patients with bile acids malabsorption in the ileum might have a false positive result in this breath test after rapid deconjugation of cholylglycine in the proximal colon[139]. Berthold *et al*[140] recently proposed a lactose-^{13}C-ureide breath test for the diagnosis of small bowel bacterial overgrowth. This test should have 100% specificity (and thus predict positive culture in SIBO) but lower sensitivity (66%)[140].

There are several other tests to diagnose SIBO, for instance evaluation of short-chain fatty acids in jejunal aspiration[141], serum non-conjugated bile acids, urinary output of p-aminobenzoic acid (after peroral administration of colil-PABA) or urinary indican[6]. However, none of these tests has yet acquitted itself well in routine clinical practice.

If it is impossible to perform any diagnostic test for SIBO (if no test is available for a particular department) on a patient with legitimate suspicion of SIBO, it is possible to consider the exceptional use of an empiric therapeutic test with rifaximin (for 7-10 d). Quick disappearance of symptoms supports a possible diagnosis of SIBO, however, this is not definite outright proof of SIBO. On the other hand, demonstration of SIBO is not 100% proof of causal association between bacterial overgrowth and clinical symptoms (or laboratory abnormal results).

In some patients with SIBO, secondary inflammatory changes might be found not only in the small bowel but also in the colon as a response to absorbed bacterial antigens. This inflammatory involvement can cause separate symptoms[7]. Successful treatment with 5-aminosalicylates and glucocorticosteroids supports this theory[142].

DIFFERENTIAL DIAGNOSIS

Diagnosis and differential diagnosis of SIBO is difficult if this possibility is not considered. It is necessary to distinguish functional disorders (of no organic cause) and chronic gastrointestinal infections (e.g. giardiasis).

The relationship between SIBO and irritable bowel syndrome was discussed above. Esposito *et al*[44] proposed use of the lactulose breath test to distinguish SIBO and

irritable bowel syndrome. Parodi *et al*[143] recommend differentiating patients fulfilling the diagnostic criteria of irritable bowel syndrome (IBS-like symptoms) from functional bloating. If SIBO is proved, the first group will profit from antibiotic treatment while the second group will not[143].

It is always necessary to consider SIBO in the case of unexplained deterioration of the clinical status of patients with Crohn's disease, chronic pancreatitis or scleroderma. SIBO must be taken into account in coeliac disease non-responding to adequate gluten-free diet. SIBO is a crucial point in the differential diagnosis of short bowel syndrome and all other malassimilation syndromes (both with maldigestion and malabsorption).

On the other hand, some other intestinal disorders might mimic SIBO and must be considered in the differential diagnosis. Flatulence, abdominal bloating and distension, and malabsorption of mono- or disaccharides (like fructose or lactose) must be taken into account. Pneumatosis cystoides intestinalis is usually asymptomatic but it may be associated with abdominal pain, bloating and/or diarrhoea[106].

PRINCIPLES OF TREATMENT

Therapy for SIBO must be complex (addressing all causes, symptoms and complications) and fully individualised. It should include treatment of the underlying disease, nutritional support and cyclical gastro-intestinal selective antibiotics.

The most important thing is always treatment of the basic underlying disease if possible. Nutritional support is mandatory in SIBO associated with malnutrition, weight loss and nutrient deficiency. We usually use individualised diet, enteral nutrition by fine-bore naso-jejunal tube or nutritional support by sipping of polymeric formulas. In several patients, it is necessary to exclude lactose from the diet, to reduce other simple sugars, to increase coverage of energy needs by fat and to administer MCT oils (medium-chain triacylglyceroles).

Antibiotic treatment should selectively target those bacterial strains that cause SIBO. The choice of antibiotics should be based on sensitivity testing to particular antibiotics. However, this requirement is difficult to achieve in clinical practice as various bacteria are usually found simultaneously, each with different sensitivity to antibiotics. There is no common agreement concerning choice, dosing and duration of antibiotic therapy. In general, long-term treatment with broad-spectrum antibiotics is not the optimal solution as such a therapy is associated with several problems (intolerance by the patient, dysmicrobia, diarrhoea, *Clostridium difficile* expansion, possible increased resistance to antibiotics, financial cost, *etc.*).

Tetracycline was considered the treatment of choice for a long time. Di Stefano *et al*[144] administered tetracycline to patients with SIBO for 7 d (1000 mg/d) and achieved normalisation of the hydrogen breath test together with relief of symptoms in only 3/11 (27%) subjects[144]. Various antibiotics were tried in other small clinical studies. Attar *et al*[145] administered a placebo, norfloxacin (800 mg/d), amoxicillin clavulanate (1500 mg/d) and *Saccharomyces boulardii* (1500 mg/d) successively in 7-d intervals in 10 patients. Norfloxacin and amoxicillin clavulanate significantly decreased the frequency of diarrhoea compared to the placebo (in 9/10 and 6/10 patients, respectively), but the hydrogen breath test was normalised in only 3 and 5 subjects[145]. In Crohn's disease, Castiglione *et al*[56] achieved normalisation of the hydrogen breath test in 13/15 (87%) persons treated with metronidazole (750 mg/d) and in 14/14 (100%) treated with ciprofloxacin (1000 mg/d)[56]. Di Stefano *et al*[146] divided 21 patients with a blind loop syndrome into three different treatment groups: (1) rifaximin followed by metronidazole, or (2) two courses of metronidazole, or (3) two courses of rifaximin. Both antibiotics were effective; metronidazole markedly reduced both hydrogen breath tests and patients' symptoms[146]. However, rifaximin was more effective than metronidazole in another study (63% *vs* 44%)[147]. A drawback in all of these studies was not only the small set of patients but also the absence of long-term follow-up. Pimentel *et al*[12] administered neomycin or a placebo to 111 patients with irritable bowel syndrome (84% abnormal lactulose breath test). Neomycin improved both symptoms and the breath test in 35% of persons compared with 11% in the placebo group[12]. Some other peroral antibiotics, such as cephalexin, trimethoprim-sulfamethoxazole, levofloxacin and gentamicin were used for the therapy of SIBO[7].

The greatest experience for treatment of SIBO was acquired with rifaximin[43,148-154]. Rifaximin is a semi-synthetic rifamycin-based non-systemic antibiotic, with a low gastrointestinal absorption and good bactericidal activity. The antibacterial action covers Gram-positive and Gram-negative organisms, both aerobes and anaerobes[155]. According to different studies, rifaximin improves symptoms in 33%-92% and eradicates small intestinal bacterial overgrowth in up to 80% of patients[151,152]. Most authors recommend administering rifaximin for 7-10 d as one treatment course or as a cyclic therapy. Higher doses (1200 or 1600 mg/d) are more effective than standard doses (600 or 800 mg/d)[148,154]. Rifaximin is probably the only antibiotic that is capable of achieving a long-term favourable clinical effect in patients with irritable bowel and SIBO[43].

Prebiotics and probiotics exert various beneficial effects in the macro-organism, they strengthen the barrier function of the gut, inhibit several pathogens, modify the inflammatory response of the bowel, and they also reduce visceral hypersensitivity[156-159]. They seem to be more effective in influencing the clinical symptoms of irritable bowel syndrome compared to a placebo[159,160]. Studies dealing with the therapeutic use of prebiotics or probiotics in SIBO (except irritable bowel syndrome) are limited[161-163], and it is not therefore possible to recommend them for general clinical use[157,158]. *Lactobacilli*-based probiotics are contraindicated in patients with a risk of D-lactic acidosis. Very little data are available from experimental studies. Short-term administration of the hydrogenic probiotic *Escherichia coli 1917 Nissle* (3.5×10^{10} bacteria per

day for 14 d) did not influence methanogenic phenotype of experimental non steroidal anti-inflammatory drug-enteropathy in pigs[129].

Prokinetics seem to be a logical therapeutic step in SIBO due to motility disorders. Several studies tried metoclopramide, cisapride (which was later withdrawn from the market), domperidone, erythromycin, itopride, tegaserod and octreotide. However, there are only limited data suggesting that this treatment would be effective over the long term[7,71]. Cyclic lavages of the small bowel (e.g. by polyethylene glycol) can be considered as supportive therapy in cases of relapsing SIBO[7].

Surgical treatment must always be considered where possible to correct gastrointestinal pathology (entero-colic fistulae, blind loops, bowel obstruction, multiple small intestinal diverticula, *etc.*). Specialised non-transplant surgery can provide interventions in short bowel syndrome improving intestinal motility (STEP - serial transverse enteroplasty), slowing intestinal transit (valves, reversed segments, colon interposition) or increasing mucosal surface area of the gut (creation of "neo-mucosa", sequential intestinal lengthening)[164].

PROGNOSIS

The prognosis of SIBO is determined mostly by the underlying disease leading to bacterial overgrowth. Ultimately SIBO might result in intestinal failure[61]. In scleroderma with gastrointestinal involvement (SIBO, intestinal pseudo-obstruction, malnutrition), the overall 5-year mortality is more than 50%[71].

The relapse rate of SIBO after successful treatment is high. Lauritano *et al*[165] found recurrence of SIBO in 44% (35/80) of patients nine months after successful treatment with rifaximin. Apart from the basic underlying disease, further risk factors for recurrence of SIBO have been identified including older age (OR 1.1), appendectomy in the patient's history (OR 5.9) and long-term treatment with proton pump inhibitors (OR 3.5)[165].

CONCLUSION

SIBO is defined as an increase in the number and/or alteration in the type of bacteria in the upper gastrointestinal tract. The aetiology of SIBO is usually complex, associated with disorders of protective antibacterial mechanisms (e.g. achlorhydria, pancreatic exocrine insufficiency, immunodeficiency syndromes), anatomical abnormalities (e.g. small intestinal obstruction, diverticula, fistulae, surgical blind loop, previous ileo-caecal resections) and/or motility disorders.

SIBO is often misdiagnosed and generally underdiagnosed. Clinical symptoms might be non-specific (dyspepsia, bloating, abdominal discomfort). Nevertheless, SIBO can cause severe malabsorption, serious malnutrition and deficiency syndromes. Non-invasive hydrogen and methane breath tests after glucose or lactulose challenge are most commonly used for the diagnosis of SIBO. Therapy of SIBO must be complex and should include treatment of the underlying disease, nutritional support and cyclical gastrointestinal selective antibiotics. Prognosis is usually serious, determined mostly by the underlying disease that led to SIBO.

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OBSERVATION

Mesenteric lymph node cavitation syndrome

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Abstract

The mesenteric lymph node cavitation syndrome consists of central necrosis of mesenteric lymph nodes and may occur with either celiac disease or a sprue-like intestinal disease that fails to respond to a gluten-free diet. Splenic hypofunction may also be present. The cause is not known but its development during the clinical course of celiac disease is usually indicative of a poor prognosis for the intestinal disorder, a potential for significant complications including sepsis and malignancy, particularly T-cell lymphoma, and significant mortality. Modern abdominal imaging modalities may permit earlier detection in celiac disease so that earlier diagnosis and improved understanding of its pathogenesis may result.

Key words: Mesenteric lymph node; Celiac disease; Yersinia infection; Tuberculosis; Kikuchi disease; Hyposplenism; Lymphoma

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INTRODUCTION

Over 50 years ago, mesenteric lymph node changes were described in celiac disease[1-3]. At that time, definition of most changes in abdominal lymphoreticular structures relied largely on surgical observation or postmortem evaluation. In addition, altered abdominal lymphoreticular function was also evident, reflected in reduced splenic function[4-6]. In recent years, refinements in abdominal imaging, i.e. ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography, have led to improved and earlier documentation of lymphoreticular changes in celiac disease. Neoplastic (i.e. lymphoma) and non-neoplastic changes, including mesenteric lymph node cavitation, have been recorded.

MESENTERIC LYMPH NODE CAVITATION SYNDROME

Detailed descriptions of this entity appeared largely in the French literature[7-9]. These reports described an unusual syndrome that seemed to complicate the clinical course of adult celiac disease. While typical small intestinal mucosal biopsy changes of celiac disease were present, the most intriguing observation was the definition of cavitated mesenteric lymph nodes. In addition, splenic atrophy or splenic hypofunction appeared to be present, raising the spectra of impaired immune function and a specific infectious cause.

Matuchansky and collegues[10], in a seminal report, noted details of 6 cases that included 4 females and 2 males. Within the jejunoileal mesenteric nodes, pseudocystic lesions were detected that consisted histologically of a large central cavity occupied by hyaline-type material surrounded by fibrous tissue and lymph node remnants. Evidence of mesenteric panniculitis or malignant lymphoma was not detected. Persistent diarrhea or childhood diarrhea along with malabsorption accompanied the small intestinal changes. An unequivocal mucosal response to a gluten-free diet was shown in some cases confirming that celiac disease was present. In others, a definitive response to a gluten-free diet could not be documented suggesting

that either a resistant form of celiac disease or sprue-like intestinal disease was present. Similar features have been noted with other complicating disorders (e.g. lymphoma) in both adult celiac disease or sprue-like intestinal disease[11]. Microscopic examination of the lymph nodes in these cases usually shows some common features. Atrophic cavitated lymph nodes are usually present without evidence of infection or malignancy in the lymph nodes. Central acidophilic lipid-containing fluid is usually present surrounded by a rim of residual lymph node tissue that includes follicles, sinus and capsular components. The changes historically have appeared to be confined to the mesenteric lymph node chain.

HYPOSPLENISM

Another important, although not essential[4-6], component of this clinical syndrome appears to be splenic atrophy or splenic hypofunction. While splenic changes have been previously noted in other intestinal disorders (e.g. inflammatory bowel disease)[12], mesenteric lymph node cavitation was seen only in those with pathological changes of celiac disease or sprue-like small intestinal disease, and not with inflammatory bowel disease[10]. In early studies, splenic atrophy was often defined structurally during postmortem evaluation or abdominal surgery. More recently, these changes tended to be defined with modern imaging methods. Hyposplenism, indicative of reduced splenic function, appeared to reflect this structural change in the spleen, and depending on its mode of measurement, seemed to be quite common in celiac disease[6]. The latter disorder may be suspected from a peripheral blood smear with Howell-Jolly bodies, monocytosis, lymphocytosis and increased platelet counts, and confirmed with pitted red blood cells counts or radiolabeled colloid scanning of the spleen[13-15]. In celiac disease, for example, 76.2% of patients were found to have pitted red blood cells[6]. These may also occur during the clinical course of many other disorders, apart from celiac disease or sprue-like intestinal disease[13-15]. These disorders include dermatitis herpiformis[16], collagenous sprue[17], collagenous colitis[18] and abdominal lymphoma[14,19], all disorders intimately linked to celiac disease[19-21]. Hyposplenic patients have been reported to be at increased risk for bacterial sepsis, especially with encapsulated organisms such as pneumococcus, sometimes with a fatal outcome[22], so vaccination using the pneumococcal conjugate has been recommended[14]. The risk of sepsis appears to be more significant if a diagnosis of celiac disease is established during adult years rather than in childhood[23]. There may also be a higher risk for vascular, autoimmune and thrombotic disorders along with solid tumors[14]. In celiac disease with associated autoimmune or malignant complications, IgM memory B-cells were reduced suggesting a possible mechanism leading to infections by encapsulated bacteria[14]. Resolution of reduced splenic function appears to be possible, but only after improvement of small intestinal mucosal changes has occurred[6].

LONG-TERM NATURAL HISTORY AND COMPLICATIONS

Most early reports indicated that cavitation of mesenteric lymph nodes was associated with a very poor prognosis with a mortality of about 50%[24]. Nevertheless, dramatic improvement has also been recorded with complete normalization of the mesenteric lymph nodes[25,26].

In celiac disease with associated dermatitis herpetiformis, recurrent diarrhea, steatorrhea and protein-losing enteropathy were defined followed later by hyposplenism, mesenteric lymph node cavitation and malignant lymphoma of the small intestine[27]. In another case of mesenteric lymph node cavitation, lymphoma also developed with a sprue-like intestinal lesion that did not respond to a gluten-free diet[28]. Cavitation of mesenteric lymph nodes may also occur without celiac or other small bowel disease[29]. Bacterial infections may be associated with necrosis of lymph nodes (e.g. mycobacteria, *Yersinia*) and lymph node cavitation has been reported with Whipple's disease[30]. Necrotizing lymphadenitis may also occur with systemic lupus erythematosus[31], and this appears to be pathologically similar to Kikuchi-Fujimoto disease, a self-limited form reported in young Asian adults[32]. In celiac disease, however, malignant lymphoma may also be the cause of necrotic mesenteric lymph node changes[33]. In one report, these necrotic lesions were seen in the liver and spleen due to hepatosplenic type lymphoma, a rare type of peripheral T-cell lymphoma with T-cell receptor rearrangement[33]. In a more recent case report, progressive encephalopathy and focal lesions of the cerebellum and brainstem were associated with abnormal T-cell clones in a sprue-like disorder with mesenteric and mediastinal lymph node cavitation[34]. Finally, a case of necrotizing hepatitis with celiac disease and mesenteric lymph node cavitation was recently described, but lymphoma was not detected[35].

CONCLUSION

This syndrome is an intriguing entity or group of entities frequently associated with celiac disease or sprue-like intestinal disease consisting of cavitated mesenteric lymph nodes and, often, splenic hypofunction. Cavitated mesenteric nodes should be differentiated from other causes of mesenteric cystic lesions, including lymphatic cysts or cystic lymphangioma. These have also been recorded with celiac disease and hyposplenism[36]. Although diagnosis is usually confirmed by pathological evaluation of mesenteric lymph nodes, imaging studies may be very helpful. Ultrasound may show anechoic cysts up to 8 cm in size. CT may reveal low attenuation (fluid or fat) lymphadenopathy suggesting celiac disease, Whipple's disease, infections such as tuberculosis, lymphoma or necrotic metastases, including germ cell tumors[37]. Occasionally, fluid-fat levels are appreciated in the lymph node, reported only in celiac disease[37]. MRI may be useful as the fluid and fat layer may be appreciated in T2- and T1-weighted axial images, even if fluid-fat levels are not seen on CT[38]. In some cases, image-guided fine-needle aspiration of the

cyst may be performed[38]. The cause of cavitation syndrome is usually not defined. The mesenteric lymph node cavitation changes have been hypothesized to represent excess antigen exposure via damaged small bowel mucosa causing lymphoid cell depletion in the lymph nodes and spleen. Alternatively, changes may reflect necrosis in the mesenteric nodes triggered by localized immune-mediated complement activation and intravascular coagulation[38]. Specific infections and malignant lymphoma may complicate its natural history and clinical course and contribute to its poor prognosis.

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ORIGINAL ARTICLE

Emodin enhances alveolar epithelial barrier function in rats with experimental acute pancreatitis

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Abstract

AIM: To investigate the effect of emodin on expression of claudin-4, claudin-5 and occludin, as well as the alveolar epithelial barrier in rats with pancreatitis induced by sodium taurocholate.

METHODS: Experimental pancreatitis was induced by retrograde injection of 5% sodium taurocholate into the biliopancreatic duct. Emodin was injected *via* the external jugular vein 3 h after induction of acute pancreatitis. Rats from sham operation group and acute pancreatitis group were injected with normal saline (an equivalent volume as emodin) at the same time point. Samples of lung and serum were obtained 6 h after drug administration. Pulmonary morphology was examined with HE staining. Pulmonary edema was estimated by measuring water content in lung tissue samples. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) level were measured by enzyme-linked immunospecific assay. Serum amylase and pulmonary myeloperoxidase (MPO) activity were detected by spectrophotometry. Alveolar epithelial barrier was assessed by pulmonary dye extravasation. Expression of claudin-4, claudin-5 and occludin in lung tissue samples was examined by immunohistology, quantitative real-time reverse transcription polymerase chain reaction and Western blotting analysis, respectively.

RESULTS: Pancreatitis-associated lung injury was characterized by pulmonary edema, leukocyte infiltration, alveolar collapse, and elevated serum amylase level. The pulmonary damage, pulmonary pathological scores, serum amylase and MPO activity, TNF-α and IL-6 levels, and wet/dry ratio were decreased in rats after treatment with emodin. Immunostaining of claudin-4, claudin-5 and occludin was detected in lung tissue samples from rats in sham operation group, which was distributed in alveolar epithelium, vascular endothelium, and bronchial epithelium, respectively. The mRNA and protein expression levels of claudin-4, claudin-5 and occludin in lung tissue samples were markedly decreased, the expression level of claudin-4, claudin-5 and occluding was increased, and the pulmonary dye extravasation was reduced in lung tissue samples from rats with acute pancreatitis after treatment with emodin.

CONCLUSION: Emodin attenuates pulmonary edema and inflammation, enhances alveolar epithelial barrier function, and promotes expression of claudin-4, claudin-5 and occludin in lung tissue samples from rats with acute pancreatitis.

Key words: Acute pancreatitis; Emodin; Lung injury; Claudin; Occludin; Epithelial barrier

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INTRODUCTION

Acute pancreatitis is a common disease with a considerable morbidity and mortality of 20%[1,2]. Its mortality is attributed to inflammation-related complications, such as pancreatitis-associated lung injury, clinically presenting as adult respiratory distress syndrome[2-4]. Intervention can reduce its morbidity and mortality, although its mechanism remains unclear[4].

Pancreatitis-associated lung injury is characterized by significant pulmonary edema, hyperemia and inflammatory infiltration in alveoli[5]. It has been established that pulmonary edema is related to increased permeability and loss of barrier function[2-5]. Although elevated levels of pancreatic enzymes and pro-inflammatory cytokines are attributed to pulmonary vasculature damage and increased endothelial permeability[6], the molecular basis for these damages remains largely undefined. Tight junctions are intimately involved in epithelial and endothelial permeability[7]. Fernandez *et al*[8] recently demonstrated that claudins, the key components of tight junctions, restrict the paracellular movement of water, proteins, and solutes across cellular barriers including alveolar epithelium. In mammals, the claudin family includes at least 24 members. With small interfering RNA and a blocking peptide, Wray *et al*[9] described that inhibition of claudin-4 decreases transepithelial electrical resistance in primary rat and human epithelial cells, as well as air space fluid clearance, resulting in pulmonary edema in mice, suggesting that claudin-4 plays an important role in alveolar epithelial barrier function. Moreover, claudin-5 and occludin are also decreased in models of acute lung injury accompanying increased paracellular permeability, indicating that claudin-5 and occludin may also play a role in alveolar epithelial barrier function[10-12]. However, the relation between expression of claudin-4, claudin-5, and occludin in lung tissues of patients with acute pancreatitis and pancreatitis-associated lung injury remains largely undefined.

It was reported that emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), an anthraquinone derivative from the Chinese herb *Radix et Rhizoma Rhei*, inhibits the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α)[13]. Our previous study demonstrated that emodin significantly reduces serum TNF-α and interleukin-6 (IL-6) levels, thus attenuating lung injury in rats with acute pancreatitis[14]. The effect of emodin on pulmonary tight junction expression and alveolar epithelial barrier function, however, needs to be further defined.

In the present study, the effect of emodin on pancreatitis-associated lung injury and alveolar epithelial barrier function was assessed by examining pulmonary morphology, myeloperoxidase (MPO) activity (indicator of inflammatory infiltration), expression of claudin-4, claudin-5 and occludin, as well as dye extravasation, in lung tissue samples from rats with acute pancreatitis.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawely rats, weighing 200-250 g, obtained from Animal Facility of Jinling Hospital (Nanjing, China), were housed under controlled temperature and humidity in a day-night cycle, with free access to standard laboratory foot and water. The study was approved by Animal Studies Ethics Committee of Jinling Hospital.

Experiment model

Acute pancreatitis was induced as previously described[15]. Briefly, animals were anesthetized with intraperitoneal ketamine (80 mg/kg) and acepromazine (2.5 mg/kg). The biliopancreatic duct was cannulated through the duodenum, and the hepatic duct was closed with a small bulldog clamp. Pancreatitis was induced by retrograde injection of 5% sodium taurocholate (Sigma, St. Louis, MO, USA) into the biliopancreatic duct (1 mL/kg body weight), at a constant infusion pressure of 20 mmHg. Rats in sham operation group received retrograde sterile saline infusion.

Experiment design

Effect of emodin on expression of claudin-4, claudin-5 and occludin, as well as on pulmonary dye extravasation, a marker to evaluate alveolar epithelial barrier, was detected in rats with acute pancreatitis. Time course of pulmonary edema and inflammation was recorded. Rats with acute pancreatitis were randomly allocated into pancreatitis group and emodin treatment group. Rats in pancreatitis group were injected with emodin (2.5 mg/kg body weight) *via* the external jugular vein 3 h after sodium taurocholate infusion. Rats in sham operation group were injected with normal saline (equivalent volume as emodin) at the same time point and served as a control group.

Lung tissue samples were obtained 6 h after emodin injection, and maintained at -80℃ until assay. Blood samples were obtained from the inferior cava vein by direct puncture. Lung tissue samples were fixed in 4% neutral phosphate-buffered formalin and embedded in paraffin wax for histology examination. Serum amylase activity was detected to confirm the appropriate induction of pancreatitis.

Measurement of serum amylase level

Serum amylase level was measured by incubating serum with 4,6-ethylidene (G7)-p-nitrophenyl (G1)-1-D-maltoheptoside for 2 min at 37℃, with its absorbance detected once a minute for 2 min at 405 nm by high through universal microplate assay (BMG Lab Technologies, Germany).

Histological examination

Lung tissue sections were stained with hematoxylin and eosin. An experienced pathologist and a pancreatic specialist assessed tissue alterations under light microscope in a blinded fashion and scored them with a grading system[16]. The grading involved measurements of inflammatory infiltration, pulmonary edema and alveolar collapse, each on a scale of 0-3, giving a maximum score of 9.

Measurement of pulmonary cytokine level and MPO activity

TNF-α and IL-6 levels in lung tissue samples were measured using a sandwich enzyme-linked immunospecific assay (Jingmei Biotech, Beijing, China) according to its manufacturer's instructions. Absorbance was measured at 450 nm by high through universal microplate assay. Tissue homogenate was corrected with the protein concentration and expressed as per protein in lung tissue (pg/mg protein).

Sequestration of neutrophils in lung tissue samples was evaluated by measuring tissue MPO activity[15,17]. Briefly, lung tissue samples were homogenized with 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, USA) in 50 mmol/L phosphate buffer (pH 6.0). Homogenate was sonicated for 10 s, freeze-thawed three times, and centrifuged at 14000 *g* for 15 min. The resulted suspension was used for assay. The assay mixture contained 20 μL of supernatant, 10 μL of tetramethylbenzidine (final concentration 1.6 mmol/L), and 70 μL of H_2O_2 (final concentration 3.0 mmol/L). MPO activity was assessed photometrically at 630 nm. The results were corrected with the protein concentration and expressed as the activity of per protein in lung tissue (U/mg protein).

Evaluation of pulmonary edema and alveolar epithelial barrier function

Severity of pulmonary edema was estimated by measuring water content in lung tissue samples. Freshly blotted lung tissue samples were weighed on an aluminum foil, dried for 24 h at 95℃, and reweighed. Difference in wet and dry tissue weights was calculated and expressed as wet/dry ratio.

Alveolar epithelial barrier function was evaluated by measuring Evans blue (Sigma, St. Louis, MO, USA) extravasation[18]. Briefly, Evans blue (20 mg/kg body weight) was injected into the jugular vein of rats, 30 min before duct infusion. Lung tissue samples were obtained 6 h after duct infusion, sectioned and immersed in a formamide solution, homogenized for 2 min. After incubation at room temperature for 24 h, the suspension was centrifuged at 4000 *g* for 30 min. The amount of dye extracted was determined spectrophotometrically at 620 nm and calculated from a standard curve established with a known amount of Evans blue. Results were corrected by the wet/dry lung tissue ratio and expressed as the dye content per dry weight of lung tissue (μg/g tissue).

Western blotting analysis

Western blotting analysis was performed as previously described[19]. Total protein (20 μg) was separated from each sample by electrophoresis on a 4%-20% SDS-polyacrylamide gel and electroblotted onto polyvinylidene difluoride membranes. Membranes were blocked in a blocking solution, incubated overnight with primary antibodies, and developed with a horseradish peroxidase-conjugated secondary antibody (Kangchen Biotech, Shanghai, China) diluted at 1:1000. Primary antibody (Zymed Laboratories, South San Francisco, CA, USA) was diluted as follows: claudin-4 at 1:100, claudin-5 at 1:100, and occludin at 1:300. The immune complexes were then visualized on X-ray film using chemiluminecent HRP substrate (Millipore, Boston, MA, USA). Additional immunoblots were performed using GAPDH antibody (Abcam, OFW, UK) as the primary antibody to evaluate equal loading.

Immunohistological analysis

Lung tissue sections (4 μm) were dewaxed in graded alcohols, and washed with tap water. Endogenous peroxidase activity was blocked with 3% (v/v) H_2O_2, and antigen was retrieved with microwave in 0.01 mol/L citrate buffer. The sections were then washed with phosphate-buffered saline (PBS, 0.1 mol/L). Mouse anti-rat claudin-4 and claudin-5, and rabbit anti-rat occludin polyclonal antibodies (Zymed Laboratories, South San Francisco, CA, USA) were diluted at 1:100 and incubated overnight at 4℃. The sections were washed 4 times with PBS, 5 min once. Power vision two-step histostaining reagent (ImmunoVision Technologies, Norwell, MA, USA) was used for detection of claudin and occludin expression. All sections were developed using diaminobenzidine and counterstained with hematoxylin.

Quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA was extracted with a TRIzol kit (Invitrogen Carlsbad, CA, USA) and converted to cDNA with a first strand cDNA synthesis kit (Fermentas, Burlington, Canada). Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using SYBR Green SuperMix-UDG (Invitrogen Carlsbad, CA, USA). The primer sequences used for PCR are as follows: claudin-4 (forward 5'-CCTTTCCCATACGGTCTTGCT-3', reverse 5'-CCCGTACCTTCCACAGACTG-3'), claudin-5 (forward 5'-TACTCAGCACCAAGGCGAACCAC-3', reverse 5'-GCGGCTTCCCACATCGGTC-3'), occludin (forward 5'-AGTACATGGCTGCTGCTGATG-3', reverse 5'-CCCACCATCCTCTTGATGTGT-3'), GAPDH (forward 5'-CAGTGCCAGCCTCGTCTCATA-3', reverse 5'-TGCCGTGGGTAGAGTCATA-3'). Amplification was performed at 50℃ for 2 min (UDG incubation), at 95℃ for 2 min, followed by 40 cycles of denaturing at 95℃ for 15 s and annealing at 60℃ for 30 s. All reactions were performed in triplicate. Melting curve analysis was performed to ensure the specificity of quantitative PCR. Data analysis was performed as previously described[20], with GAPDH used as a reference gene.

Statistical analysis

Data were expressed as mean ± SD. ANOVA was used to analyze differences between experimental and control groups. Student-Newman-Kleus method was used for multiple pair-wise comparisons. All statistical analyses were carried out using the SPSS version 11.5 for Windows (Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Emodin attenuated pulmonary edema and inflammation in rats with acute pancreatitis

The appropriate induction of pancreatitis-associated lung injury was demonstrated by histology and elevated serum amylase activity (Figure 1 and Table 1). Lung injury was characterized by pulmonary edema, leukocyte infiltration, and alveolar collapse. Pulmonary pathological scores and serum amylase activity were significantly lower after treatment with emodin.

Pulmonary edema was evaluated by measuring the water content in lung tissue samples and expressed as wet/dry ratio, which was significantly decreased after treatment with emodin (Table 1).

In the present study, the effect of emodin on pulmonary inflammation and MPO activity was evaluated. The TNF-α and IL-6 levels and MPO activity were decreased after treatment with emodin (Table 2).

Emodin promoted expression of claudin-4, claudin-5 and occludin in rats with acute pancreatitis

The expression levels of claudin-4, claudin-5, and occludin were markedly lower in experimental group than in control group (data not shown). Immunolocalization of claudin-4, claudin-5 and occludin in lung tissue samples was investigated with immunohistochemical staining. Moderate immunostaining of claudin-4, claudin-5, and occludin was detected in control group, which was distributed in alveolar epithelium, vascular endothelium, and bronchial epithelium, respectively (Figure 2A, D and G). Immunostaining of claudin-4, claudin-5, and occludin was markedly decreased in experimental group (Figure 2B, E and H), and moderately elevated after treatment with emodin (Figure 2C, F and I).

RT-PCR analysis showed that emodin could increase the expression levels of claudin-4, claudin-5, and occludin mRNA in rats with acute pancreatitis (Figure 3A).

Western blotting analysis showed that the expression levels of claudin-4, claudin-5, and occludin were significantly higher in emodin treatment group than in pancreatitis group (Figure 3B and C).

Emodin enhanced alveolar epithelial barrier function in rats with acute pancreatitis

The pulmonary dye extravasation, as a marker of local paracellular permeability, was significantly reduced in rats with acute pancreatitis after treatment with emodin, indicating that emodin can augment alveolar epithelial barrier function (Figure 4).

Table 1 Serum amylase level and pulmonary pathological scores in different groups (mean ± SD)

Group	Amylase (U/mL)	Edema (wet/dry)	Pathological score
Sham	3.5 ± 0.4	1.7 ± 0.2	0.3 ± 0.1
Pancreatitis	13.8 ± 1.6[a]	6.4 ± 0.5[a]	7.4 ± 0.8[a]
Emodin	8.2 ± 1.1[c]	4.1 ± 0.6[c]	4.8 ± 0.7[c]

Six rats were studied in each experimental group. [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* pancreatitis group.

Table 2 Pulmonary cytokines and MPO in different groups (mean ± SD)

Group	TNF-α (pg/mg protein)	IL-6 (pg/mg protein)	MPO (U/mg protein)
Sham	2.8 ± 0.3	25.8 ± 2.9	3.1 ± 0.3
Pancreatitis	34.1 ± 3.5[a]	114.4 ± 12.6[a]	25.8 ± 2.9[a]
Emodin	25.4 ± 2.4[c]	93.0 ± 9.8[c]	20.3 ± 1.9[c]

Six rats were studied in each experimental group. [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* pancreatitis group. MPO: Myeloperoxidase; TNF: Tumor necrosis factor.

DISCUSSION

In the present study, we identified the down-regulation of claudin-4, claudin-5, and occludin in rats with acute pancreatitis induced by sodium taurocholate. Intravenous administration of emodin promoted the down-regulation of tight junctions, enhanced alveolar epithelial barrier function, attenuated pulmonary edema and inflammatory infiltration in rats with acute pancreatitis.

Among the systemic complications of severe acute pancreatitis, pulmonary complication, also known as pancreatitis-associated lung injury, is the most frequent and serious[6]. Pancreatitis-associated lung injury is characterized by significant pulmonary edema, hyperemia and inflammatory infiltration in alveoli[5]. Increased interstitial edema cuts down the transport of carbon dioxide through the alveolar barrier, causing respiratory distress syndrome. It has been recently reported that claudins, the key components of tight junctions, restrict paracellular movement of water, proteins, and solutes across cellular barriers including pulmonary vascular endothelium and alveolar epithelium[8]. Disruption of claudins impairs barrier function and increases paracellular permeability, which may allow noxious contents to enter pulmonary interstitium and alveoli, further aggravating pulmonary edema and inflammation[5-9].

Recently, several studies have demonstrated the localization and function of claudin-4 in pulmonary cellular barriers[9-11]. In human airway epithelia, elevated claudin-4 level is associated with increased transepithelial electrical resistance, indicating that claudin-4 plays a role in alveolar epithelial barrier function[10]. Although increased claudin-4 expression has been found in a mice model of acute lung injury, inhibition of claudin-4 can lead to pulmonary edema in mice by decreasing transepithelial electrical

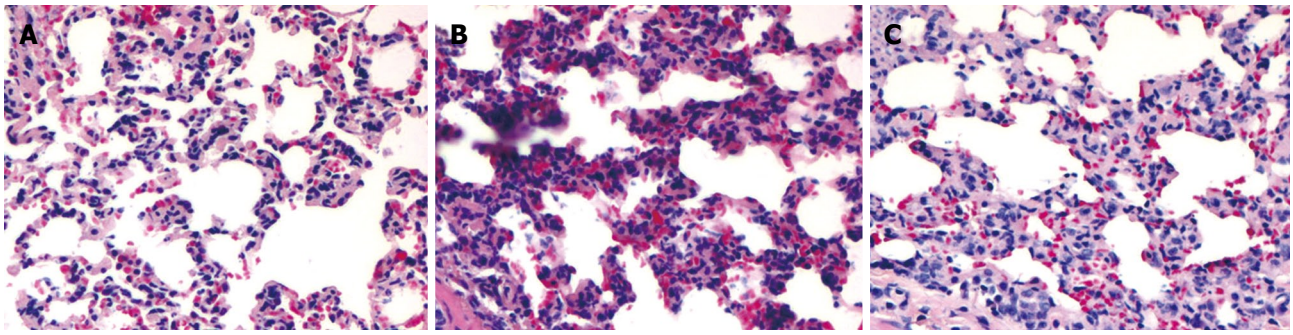

Figure 1 Effects of emodin on pancreatitis-associated lung injury in sham operation group (A), pancreatitis group (B), and emodin group (C) (original magnification, × 200).

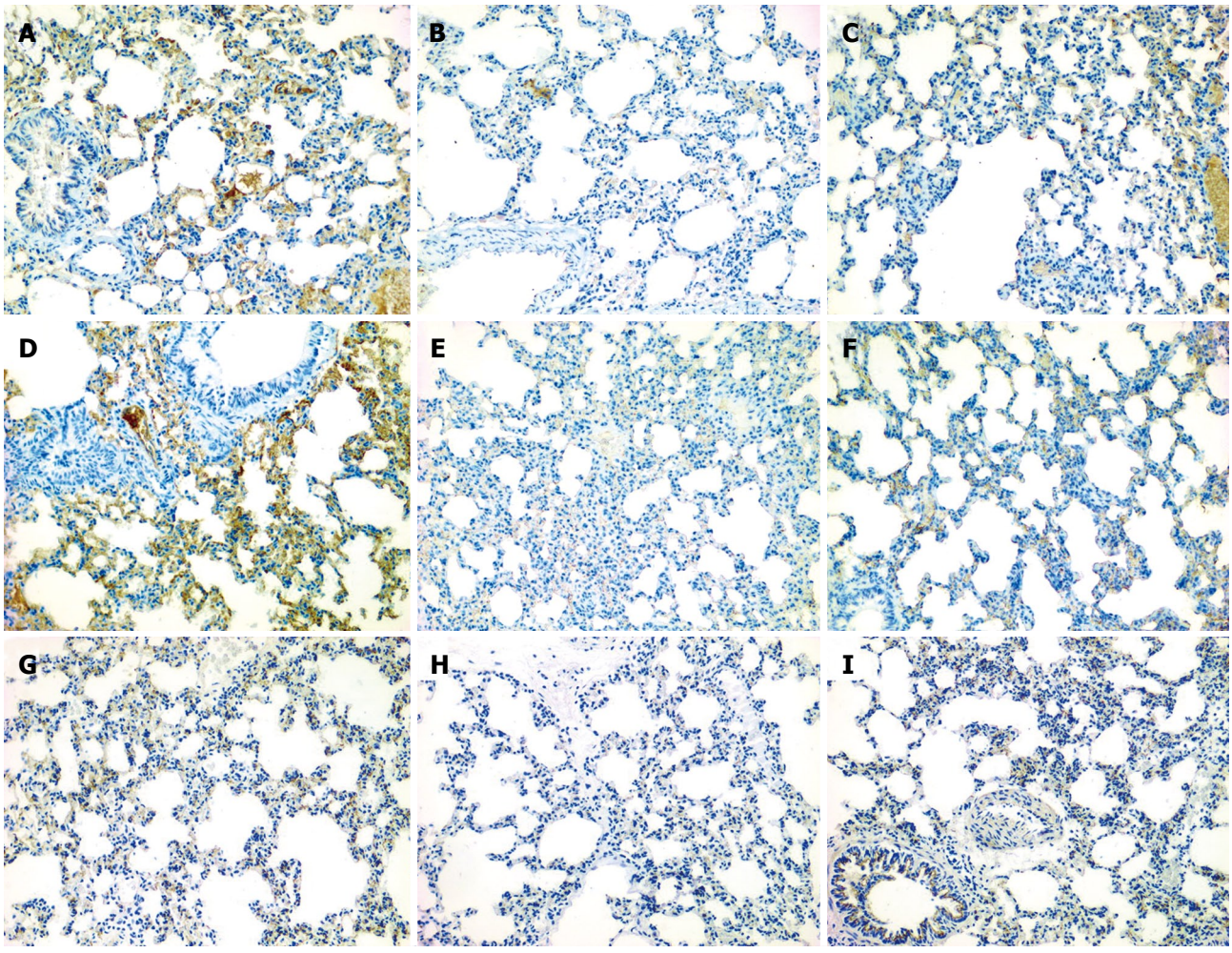

Figure 2 Immunohistochemical staining of claudin-4 (A-C), claudin-5 (D-F) and occludin (G-I) in sham operation group (A, D, G), pancreatitis group (B, E, H), and emodin group (C, F, I) (original magnification, × 200).

resistance and air space fluid clearance, suggesting that claudin-4 plays an important role in alveolar epithelial barrier function, and early increased claudin-4 expression may represent a mechanism by which pulmonary edema is limited[9]. Similar to claudin-4, claudin-5 also plays a role in cellular barrier function. Recombinant claudin-5 protects brain microvascular endothelial cell cultures against increased paracellular permeability induced by VEGF, showing that claudin-5 is a key determinant of blood-brain barrier function[21]. It has been recently reported that expression of pulmonary claudin-5 is decreased in models of carrageenan-induced acute lung inflammation, associated with the decreased pulmonary paracellular permeability, suggesting that claudin-5 may play role in alveolar epithelial barrier function[11].

Occludin shares a very similar membrane location with claudin. Based on the staining feature of claudins and occludin along the endothelial cell borders, Persid-

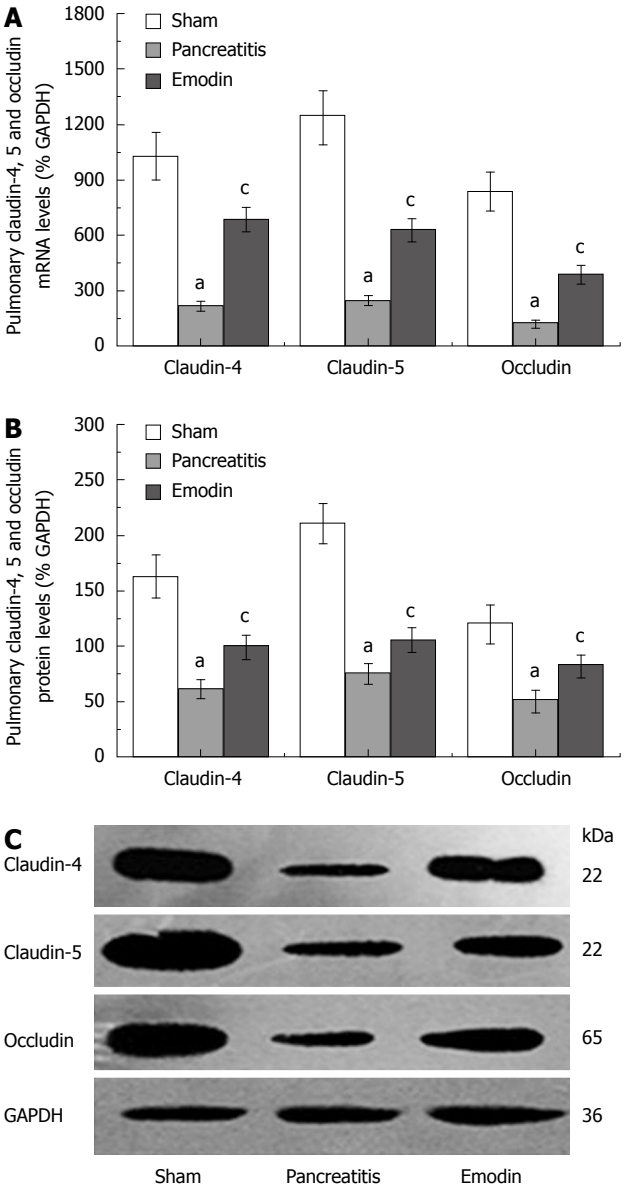

Figure 3 Effects of emodin on claudin-4 (A), claudin-5 (B), and occludin (C) mRNA transcription and protein synthesis in rats. Six rats were studied in each experimental group. Results are expressed as mean ± SD. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* pancreatitis group.

sky *et al*[22] speculated that claudins form the primary "makeup" of the tight junctions, and occludin further enhances tight junction tightness. In ethanol abused rats, which is decreased mRNA and protein expression of occludin has also been observed in lung tissues, associated with increased bronchoalveolar epithelial permeability[12]. Azithromycin-induced processing of occludin is accompanied by increased transepithelial electrical resistance[10], suggesting that occludin alteration may be related with alveolar barrier function.

In the present study, we identified the localization of claudin-4, claudin-5, and occludin in lung tissue samples from rats with acute pancreatitis, and found that claudin-4 and claudin-5 were uniformly and continuously distributed along the alveolar epithelium and vascular endothelium in normal lung tissue samples, which are consistent with the reported findings[9-11]. Furthermore, occludin was uniformly and continuously distributed along the alveolar epithelium, vascular endothelium, and bronchiolar epithelium, which is in line with the reported results[10,12]. In this study, RT-PCR and Western blotting showed that the expression of claudin-4, claudin-5 and occludin was down-regulated in lung tissue samples from rats with acute pancreatitis. Aggravated pulmonary edema and increased paracellular permeability (marked by extravasation of Evans blue) were in parallel with the down-regulation of claudin-4, claudin-5 and occludin expression, which is consistent with the findings in previous studies[9-12], suggesting that claudin-4, claudin-5 and occludin may play a role in alveolar barrier function.

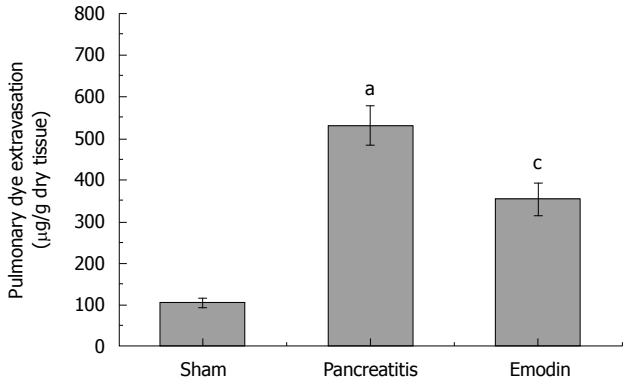

Figure 4 Effects of emodin on alveolar epithelial barrier in rats. Six rats were studied in each experimental group. Results are expressed as mean ± SD. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* pancreatitis group.

In the present study, emodin significantly promoted the expression of claudin-4, claudin-5 and occludin at mRNA transcription and protein synthesis level, and decreased pulmonary edema and paracellular permeability. Based on the previous and present studies, we speculate that emodin may contribute, in part at least, to the expression of claudin-4, claudin-5 and occludin by increasing the alveolar barrier function.

Emodin has long been used for anti-inflammatory purposes. Many studies have demonstrated that emodin intervention can significantly decrease TNF-α and IL-6 levels, or MPO activity in lung tissues[13,23,24], and the mechanism of emodin underlying cytokine inhibition is involved in NF-κB activity suppression[23-26]. Moreover, emodin also has antioxidant effects, promotes generation of ATP and antioxidant components, such as glutathione, α-tocopherol, and superoxide dismutase[27], and exhibits a promising free radical scavenging activity[28]. It has been shown that emodin markedly reduces serum amylase, TNF-α and IL-6 levels, attenuates lung damage in rats with acute pancreatitis[14,29,30], which is in line with the present study. Considering that MPO activity is a marker of local leukocyte sequestration[30], the results of our present study suggest that emodin ameliorates pancreatitis-associated lung injury by inhibiting the production of cytokines and the infiltration of leukocytes in lungs.

In conclusion, emodin can attenuate pulmonary edema and inflammation, enhance alveolar epithelial barrier function, and promote expression of claudin-4, claudin-5 and occludin in lung tissues.

COMMENTS

Background

The mortality associated with acute pancreatitis is attributed to inflammation-related complications such as pancreatitis-associated lung injury. Interventions with emodin are likely to reduce its morbidity and mortality of the disease.

Research frontiers

Claudins and occludin, the key components of tight junctions, restrict the paracellular movement of water, proteins, and solutes across cellular barriers including alveolar epithelium. Emodin, an anthraquinone derivative from the Chinese herb *Radix et Rhizoma Rhei*, has been used for anti-inflammatory purposes. Weather emodin has effects on pulmonary tight junction expression and alveolar epithelial barrier has not been defined.

Innovations and breakthroughs

A recent report has highlighted the importance of tight junction components in a model of ethanol-induced lung injury, or carrageenan-induced acute lung inflammation. This is the first study to report that down-regulation of pulmonary claudin-4, claudin-5 and occludin is parallel with pancreatitis-associated lung injury. Emodin can promote expression of claudin-4, claudin-5 and occluding in lungs, enhance alveolar epithelial barrier, and inhibit pulmonary inflammation.

Applications

The results of this study may improve our understanding of the pathogenesis of pancreatitis-associated lung injury. The present study also provides evidence for emodin in treatment of lung injury.

Peer review

This is a largely observational study representing an incremental advance in treatment of acute pancreatitis with emodin.

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ORIGINAL ARTICLE

Sulforaphane protects liver injury induced by intestinal ischemia reperfusion through Nrf2-ARE pathway

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Abstract

AIM: To investigate the effect of sulforaphane (SFN) on regulation of NF-E2-related factor-2 (Nrf2)-antioxidant response element (ARE) pathway in liver injury induced by intestinal ischemia/reperfusion (I/R).

METHODS: Rats were divided randomly into four experimental groups: control, SFN control, intestinal I/R and SFN pretreatment groups (n = 8 in each group). The intestinal I/R model was established by clamping the superior mesenteric artery for 1 h and 2 h reperfusion. In the SFN pretreatment group, surgery was performed as in the intestinal I/R group, with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation. Intestine and liver histology was investigated. Serum levels of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured. Liver tissue superoxide dismutase (SOD), myeloperoxidase (MPO), glutathione (GSH) and glutathione peroxidase (GSH-Px) activity were assayed. The liver transcription factor Nrf2 and heme oxygenase-1 (HO-1) were determined by immunohistochemical analysis and Western blotting analysis.

RESULTS: Intestinal I/R induced intestinal and liver injury, characterized by histological changes as well as a significant increase in serum AST and ALT levels (AST: 260.13 ± 40.17 U/L *vs* 186.00 ± 24.21 U/L, $P < 0.01$; ALT: 139.63 ± 11.35 U/L *vs* 48.38 ± 10.73 U/L, $P < 0.01$), all of which were reduced by pretreatment with SFN, respectively (AST: 260.13 ± 40.17 U/L *vs* 216.63 ± 22.65 U/L, $P < 0.05$; ALT: 139.63 ± 11.35 U/L *vs* 97.63 ± 15.56 U/L, $P < 0.01$). The activity of SOD in the liver tissue decreased after intestinal I/R ($P < 0.01$), which was enhanced by SFN pretreatment ($P < 0.05$). In addition, compared with the control group, SFN markedly reduced liver tissue MPO activity ($P < 0.05$) and elevated liver tissue GSH and GSH-Px activity ($P < 0.05$, $P < 0.05$), which was in parallel with the increased level of liver Nrf2 and HO-1 expression.

CONCLUSION: SFN pretreatment attenuates liver injury induced by intestinal I/R in rats, attributable to the antioxidant effect through Nrf2-ARE pathway.

Key words: Sulforaphane; Liver injury; Intestinal ischemia reperfusion; NF-E2-related factor-2; Antioxidant response element

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INTRODUCTION

Intestinal ischemia/reperfusion (I/R) is considered to be a grave and triggering event in development of local and distant organ dysfunction[1-4], which occurs in many clinical settings including acute mesenteric ischemia, hemorrhagic, traumatic or septic shock, severe burns, resuscitation, small bowel transplantation and thoracoabdominal aortic aneurysm repair[5,6]. The mechanism of liver injury induced by intestinal I/R is complicated, and it has not been fully elucidated although many studies have been done to mimic the pathophysiologic process by both cell culture and animal models in the past a few years[7-10].

Traditionally, decreased basement membrane integrity and barrier function of the intestine, which facilitate bacterial translocation and local production of inflammatory cytokines[11], promotes the systemic inflammatory response syndrome and multiple organ dysfunction syndrome[12,13]. Recent studies showed that reactive oxygen species (ROS) generated during tissue reperfusion, play an important role in intestinal I/R injury[14], which can initiate lipid peroxidation, oxidize proteins to inactive states and cause DNA strand breaks, and initiation of apoptotic and necrotic cascades[15]. Additionally, ROS also has a function as a second messenger to modulate the secretion of pro-inflammation cytokines and chemokines that can destroy the intestinal barrier, which is a critical junction point to amplify the inflammation reaction described above[11-13]. The administration of antioxidants has been shown to exert beneficial effects in the prevention of ischemia-reperfusion injury[16].

Sulforaphane (SFN) is a natural product derived from isothiocyanate which is present in cruciferous vegetables such as broccoli that has been used as a chemopreventive compound[17]. The cytoprotective effect of this compound is mediated by transcription factor NF-E2-related factor-2 (Nrf2), which binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes, including heme oxygenase-1 (HO-1), NAD(P)H: quinoine oxidoreductase 1, glutathione reductase, and glutathione peroxidase (GSH-Px)[18-20]. Phase 2 enzymes play a major role in the detoxification of ROS during ischemia/reperfusion[21,22]. Nrf2 is held in the cytoplasm by a cytoskeletal-associated specific inhibitory protein (Kelch-like ECH associated protein 1, Keap1) under circumstances of normal cellular quiescent state. Upon stimulation of oxidative stress, cysteine residues within the hinge region of Keap1 can be modified and cause a conformational change in KEAP1 with the loss of Nrf2 binding, then Nrf2 translocated into the nucleus, where it heterodimerizes with members of the maf protein family, and coordinates upregulation of cytoprotective genes[20]. SFN can disrupt the Nrf2-Keap1 complex, and permit Nrf2 to translocate into the nucleus to activate the ARE-driven genes[23,24]. Studies suggest that Nrf2 activation by SFN results in effective protection from cancers[25] and renal I/R[26] by upregulating ARE-related detoxification enzymes. These studies have focused on the chemoprevention by SFN, and to our knowledge, no one has evaluated SFN to determine if it can protect liver injury induced by intestinal I/R.

In this study, we investigated the effect of SFN on liver injury following intestinal I/R and explored the mechanism of its protective action through Nrf2-ARE pathway.

MATERIALS AND METHODS

Animal and experimental design

Male Sprague-Dawley rats weighing 190-220 g (from the Animal center of Dalian Medical University, Dalian, China) were used in this study, which was approved by the Institutional Ethics Committee. All rats were provided with standard laboratory chow and water and were housed in accordance with institutional animal care policies.

The rats were divided into four experimental groups randomly: control (A), SFN control (B), intestinal I/R (C) and SFN pretreatment group (D) (n = 8 in each group). The rats in the control group underwent surgical preparation including isolation of the superior mesenteric artery (SMA) without occlusion; the rats in the SFN control group underwent surgery as in the control group with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation (SFN was purchased from Sigma Chemical Company and was dissolved in 10% dimethylsulfoxide before administration); the rats in intestinal I/R group were subjected to 1 h intestinal ischemia and 2 h reperfusion after the SMA was isolated and occluded[27]; the rats in the SFN pretreatment group underwent surgery as in the intestinal I/R group with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation.

The dose of SFN administration was determined according to the previous studies with modification from preliminary experiments. The rats in the control and intestinal I/R groups were treated with an equal volume of 10% dimethylsulfoxide. Two hours after reperfusion, blood, intestine and liver tissue samples were obtained for further analysis.

Intestine and liver morphological analysis

The isolated intestine and liver tissues were harvested and fixed in 10% formalin. After being embedded in paraffin, the tissues were stained with hematoxylin and eosin for light microscopy.

Measurement of serum aspartate aminotransferase and alanine aminotransferase

The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with an OLYMPUS AU1000 automatic analyzer (AusBio Laboratories Co., Ltd. Beijing, China).

Liver superoxide dismutase, myeloperoxidase, GSH and GSH-Px activity assay

The liver tissues were harvested and homogenized im-

mediately on ice in 5 volumes of normal saline. The homogenates were centrifuged at 1200 r/min for 10 min to remove debris. The superoxide dismutase (SOD), myeloperoxidase (MPO), GSH and GSH-Px activity in the supernatant were determined using an assay kit (Nanjing Jiancheng Corp., China), following the manufacturer's recommendations.

The SOD activity was determined by hydroxylamine assay developed from xanthine oxidase assay. One unit of SOD sample inhibited the reaction by approximately 50% of the initially measured xanthine oxidase reaction at 37℃. SOD activity in the liver tissues was expressed as units per milligram protein (U/mgprot).

One unit of MPO activity is defined as degrading 1 μmol of hydrogen peroxide at 37℃ and MPO activity of tissue was expressed as units per gram (U/g).

Concentrations of GSH were determined by the 5,5' dithio-bis 2-nitrobenzoic acid (DTNB)-GSSG reductase-recycling assay. The amount of GSH was expressed as milligrams per gram protein (mg/gprot).

GSH-Px was measured by the enzymatic reaction which was initiated by addition of H_2O_2 to the reaction mixture containing reduced glutathione, NADPH and glutathione reductase, and the change in the absorbance at 340 nm was monitored by spectrophotometer. Activity was given in units per milligram protein (U/mgprot).

Liver Nrf2 and HO-1 immunohistochemical analysis

Paraffin-embedded tissue sections, 4 μm in thickness, were stained with SP immunohistochemical technique for Nrf2 and HO-1. The immunohistochemical experiments were performed according to the manufacturer's recommendations. After being dewaxed or washed in PBS, tissue sections were cultured in 3% hydrogen peroxide to eliminate intrinsic peroxidase, and quenched in normal goat serum for 30 min. The sections were incubated overnight at 4℃ with polyclonal rabbit anti-rat Nrf2 antibody (Santa Cruz Corp., Ltd.), followed by the addition of the anti-rabbit immunoglobulin and streptavidin conjugated to horseradish peroxides. Finally, 3,3'-diaminobenzidine was used for color development, and hematoxylin was used for counter staining. The brown or dark brown staining in cytoplasm and/or nucleus was considered to be positive. The results were evaluated semi-quantitatively according to percentage of positive cells in five fields at a 400 multiple signal magnification. The protein expression in tissue sections was graded as 0: less than 5%; 1: from 6% to 25%; 2: from 26% to 50%; 3: from 51% to 75%; and 4: more than 75%[28].

Western blotting analysis of liver Nrf2 and HO-1

Nrf2 is a nuclear transcriptional factor that binds to the ARE in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes such as HO-1 and GSH-Px. The level of Nrf2 in the nucleus was examined by Western blotting to assess Nrf2 activation.

Cellular plasma and nuclear protein were extracted from frozen liver tissues with a protein extraction kit (Pierce, Meridian Road, Rockford, IL, USA) for HO-1 and Nrf2 measurement separately. The protein was separated by 10% SDS-PAGE gel electrophoresis. The protein was electroblotted onto NC membranes (Millipore, Bedford, MA, USA) at 9 V for 30 min. The transferred membranes were then incubated overnight at 4℃ with rabbit polyclonal antibody HO-1, Nrf2, GAPDH and PCNA (Santa Cruz Corp., Ltd.) against rat in TBS-T (10 mmol/L Tris-HC1, pH 7.5, 150 mmol/L NaC1, 0.1% Tween-20) containing 5% skim milk. After washing three times in TBS-T, the membranes were incubated for 1 h at 37℃ with an anti-rabbit IgG conjugated with horseradish peroxidase. The signals were visualized using the DAB assay kit (Fuzhou Maixin Biological Technology Co., Ltd, Fuzhou, China) and documented with a gel imaging system (UVP Bioimaging System). The signals were analyzed with software Gel-Pro Analyzer 4.0.

Statistical analysis

All data, which expressed as the mean ± SD, were compared using the paired Student's *t* test with the SigmaStat 3.5 statistical software package. One-way analysis of variance (ANOVA) was used to determine significant differences in antioxidant enzyme activities between the groups. A value of $P < 0.05$ was considered significant.

RESULTS

Changes of liver histology and serum AST and ALT levels

Intestinal I/R induced apparent intestine and liver injury at 2 h after reperfusion, manifested as histological changes in the intestine and liver with edema, hemorrhage and neutrophil infiltration (Figure 1), as well as a significant increase in serum AST and ALT level (AST: 260.13 ± 40.17 U/L *vs* 186.00 ± 24.21 U/L, $P < 0.01$; ALT: 139.63 ± 11.35 U/L *vs* 48.38 ± 10.73 U/L, $P < 0.01$, Figure 2) when compared with the control group. Compared with the intestinal I/R group, the intestinal and liver pathological damage was improved in the SFN pretreatment group. In addition, there was a significant difference in the liver function between the intestinal I/R and SFN pretreatment group in serum AST and ALT levels (AST: 260.13 ± 40.17 U/L *vs* 216.63 ± 22.65 U/L, $P < 0.05$; ALT: 139.63 ± 11.35 U/L *vs* 97.63 ± 15.56 U/L, $P < 0.01$, Figure 2), which indicates that SFN significantly attenuated the intestinal I/R-induced liver injury. There was no significant difference between the SFN control group and the control group in liver pathological damage (Figure 1) and serum AST (AST: 193.38 ± 34.63 U/L *vs* 186.00 ± 24.21 U/L, $P > 0.05$, Figure 2) and ALT (ALT: 48.00 ± 8.52 U/L *vs* 48.38 ± 10.73 U/L, $P > 0.05$, Figure 2).

Changes of liver SOD and MPO activity

SOD is the major enzyme for scavenging ROS, and its activity can reflect its functional status. The liver homogenate SOD activity in the intestinal I/R group decreased significantly at 2 h of reperfusion in comparison with the control group ($P < 0.01$, Figure 3). SOD activity was elevated markedly after SFN pretreatment compared

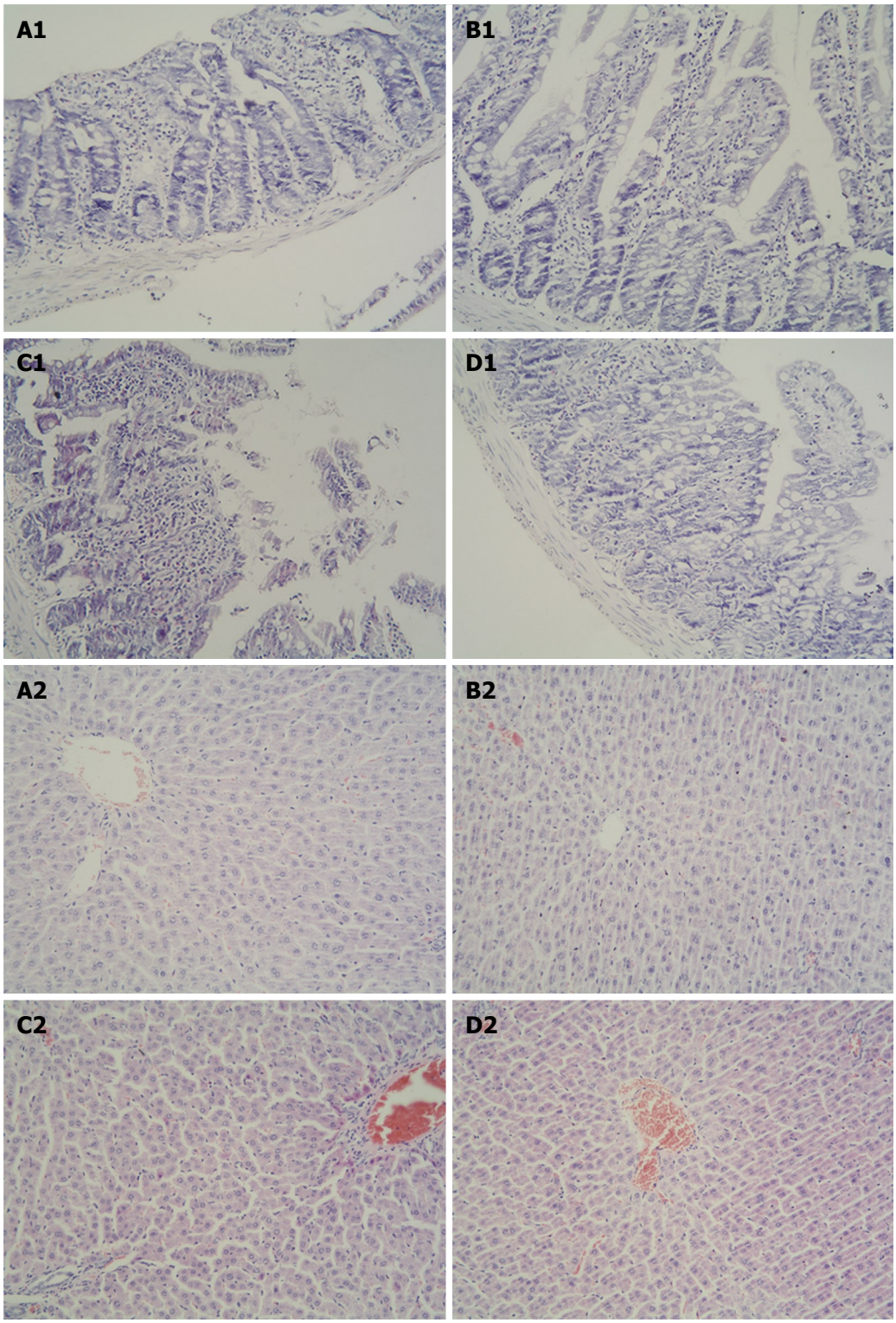

Figure 1 Changes in histology of intestine (A1-D1) and liver (A2-D2) tissues (HE stain, × 200) in the control (A), sulforaphane (SFN) control (B), intestinal ischemia/reperfusion (I/R) (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. A, B: Normal structure of intestine and liver; C: Edema, hemorrhage and neutrophil infiltration were observed in intestinal mucosa and liver tissue; D: Relatively normal histology of intestine and liver with less inflammatory cell infiltration.

with the intestinal I/R group ($P < 0.05$, Figure 3).

MPO activity in the liver homogenate was measured for estimating the leukocyte recruitment to liver tissues. The liver tissue MPO activity increased significantly after intestinal I/R at 2 h of reperfusion compared with the control group ($P < 0.05$, Figure 3). The administration of SFN reduced the MPO activity in liver tissues significantly in comparison with the intestinal I/R group ($P < 0.05$, Figure 3), thus indicating that SFN alleviated the leukocyte recruitment to liver tissues.

GSH is a kind of antioxidant which can scavenge ROS. Phase 2 enzyme GSH-Px present in the cell can

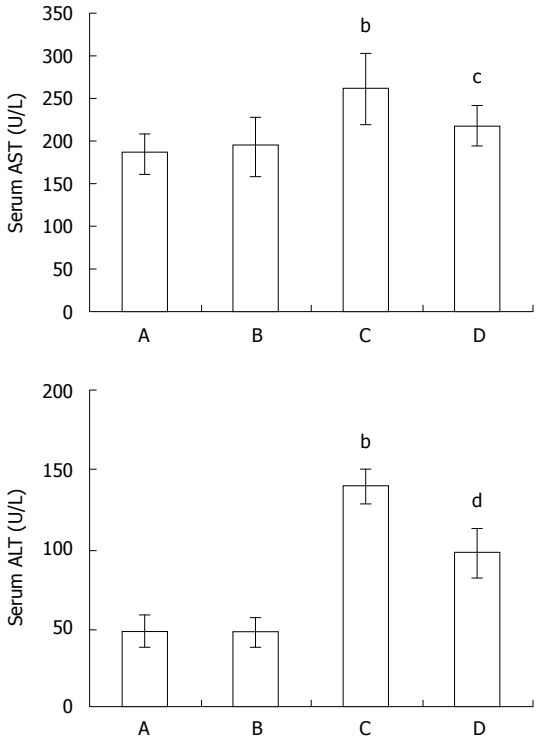

Figure 2 Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (mean ± SD, U/L) levels in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* intestinal I/R group.

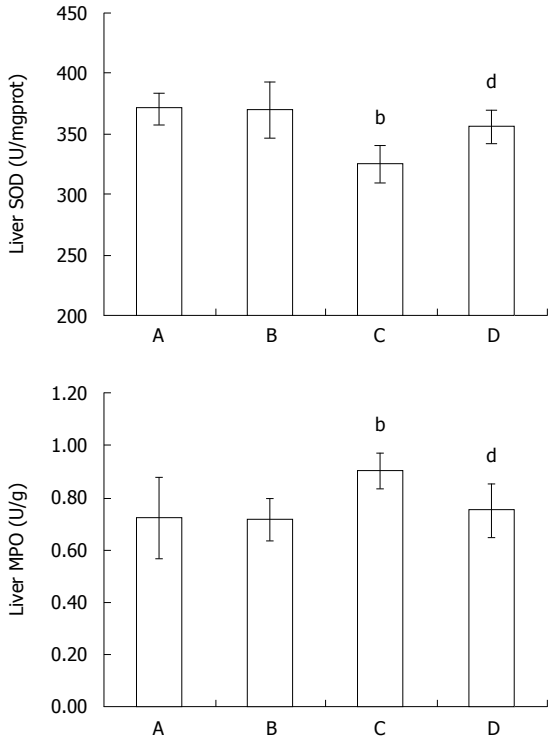

Figure 3 Liver tissue superoxide dismutase (SOD) (mean ± SD, U/mgprot) and myeloperoxidase (MPO) (mean ± SD, U/g) level in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

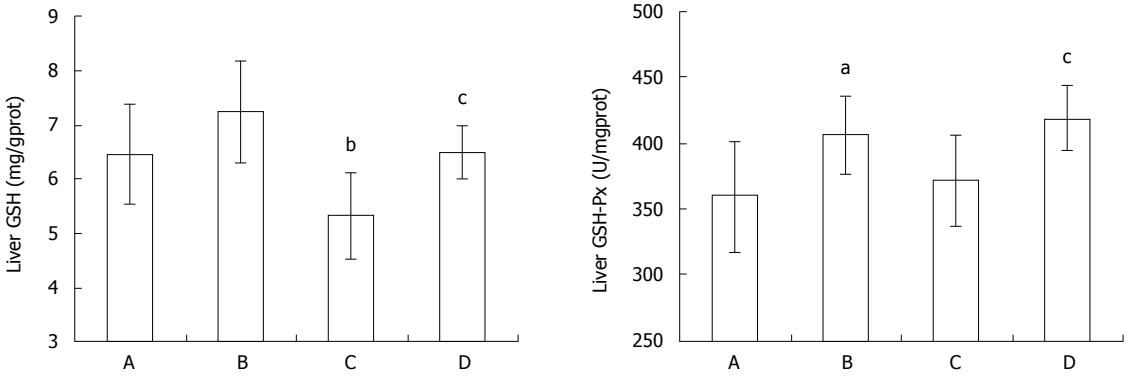

Figure 4 Liver tissue glutathione (GSH) and glutathione peroxidase (GSH-Px) (mean ± SD, U/gprot) level in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* intestinal I/R group.

catalyze this reaction. The liver homogenate GSH activity in the intestinal I/R group decreased significantly at 2h of reperfusion as compared with the control group ($P < 0.01$, Figure 4). GSH activity was elevated markedly after SFN pretreatment in comparison with the intestinal I/R group ($P < 0.05$, Figure 4).

The increased activity of the liver tissue GSH-Px was significant after SFN pretreatment as against the control group ($P < 0.05$, Figure 4). GSH-Px activity was elevated markedly after SFN pretreatment in comparison with the intestinal I/R group ($P < 0.05$, Figure 4), thus supporting the hepatoprotective effect of SFN.

Immunohistochemical expression of liver Nrf2 and HO-1

The expression of Nrf2 in control group showed light brown immunostaining in cytoplasm and no staining in the nuclei. The significantly positive expressions of Nrf2 as strong brown staining in cytoplasm and nuclei were observed in the intestinal I/R group and SFN control groups ($P < 0.01$, $P < 0.01$, Figures 5 and 6). Compared with the intestinal I/R group, the positive rates of Nrf2 expression increased significantly in SFN pretreatment group ($P < 0.01$, Figures 5 and 6).

The expression of HO-1 in the control group showed little brown immunostaining in cytoplasm while signifi-

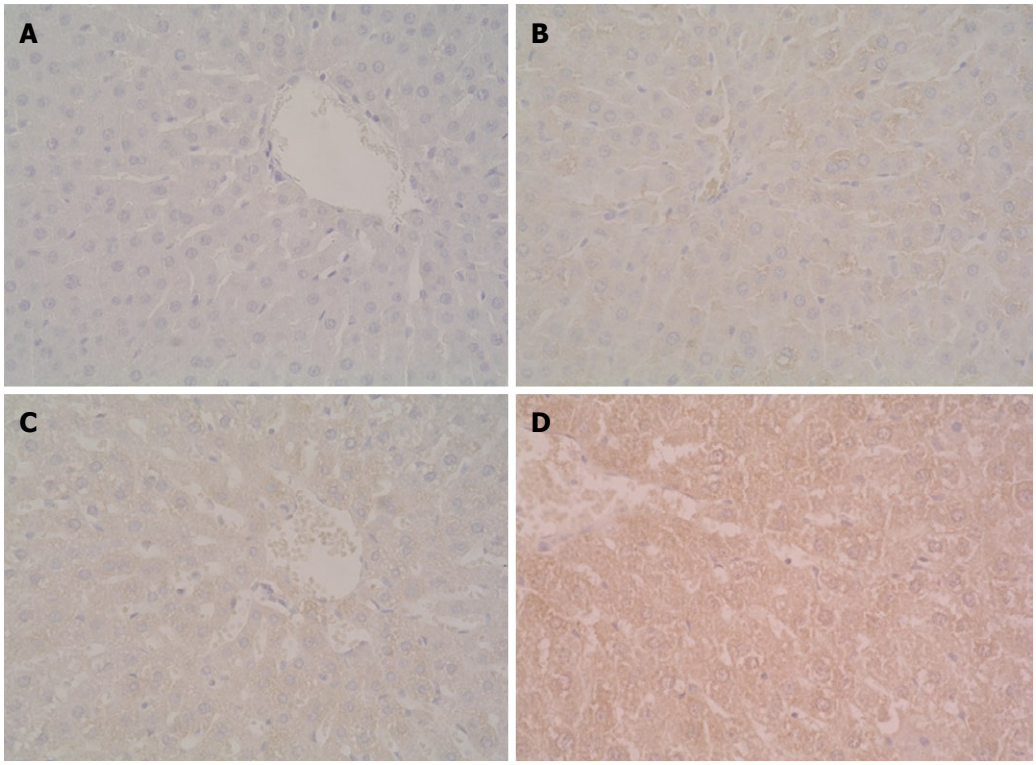

Figure 5 Immunohistochemical staining of liver NF-E2-related factor-2 (Nrf2) expression (original magnification × 400) in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups.

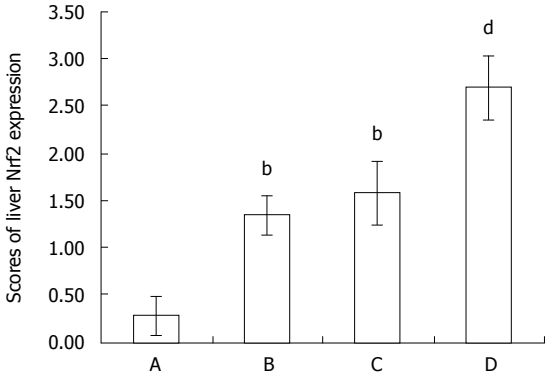

Figure 6 Immunohistochemical results (semi-quantitative analysis) of liver Nrf2 expression in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. Data are presented as mean ± SD of eight animals. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

cant positive expression of HO-1 as brown staining in cytoplasm was observed in the intestinal I/R group and SFN control groups ($P < 0.01$, $P < 0.01$, Figures 7 and 8). Compared with the intestinal I/R group, the positive rates of HO-1 expression increased significantly in cytoplasm in SFN pretreatment group ($P < 0.01$, Figures 7 and 8).

Western blotting analysis of liver Nrf2 and HO-1 expression

The expression of nuclear Nrf2 of the liver tissues in animals with 2-h reperfusion is shown in Figure 9. The results showed a weak Nrf2 positive signal in the liver of the control group. However, a strong Nrf2 protein signal was found in the intestinal I/R group ($P < 0.01$, Figure 9). The signal enhanced significantly in the SFN pretreatment group in comparison with the intestinal I/R group ($P < 0.01$, Figure 9), thereby indicating that SFN could increase the Nrf2 activation in liver tissues.

Western blotting showed weak positive staining for HO-1 in the liver in the control group. However, a significant HO-1 signal was observed in the intestinal I/R group. Compared with the control group, IOD level of HO-1 increased markedly ($P < 0.01$, Figure 9). The signal enhanced significantly in the SFN pretreatment group in comparison with the intestinal I/R group ($P < 0.01$, Figure 9), hinting that HO-1 was activated in liver tissues caused by SFN.

DISCUSSION

Intestinal I/R is not only necessary to the intestine itself, but involves severe distant organ dysfunction. The liver is the first distant organ involved in the severe attack from intestinal I/R[8] due to its vasculature being coupled in series with that of the intestine[29]. During the reperfusion, ROS formed as participants in the I/R-induced leukocyte-mediated liver inflammation responses[30-32], and many clinical and experimental studies showed that ROS plays a crucial role in the pathogenesis of I/R[7,33].

ROS can be restrained by endogenous free radical scavengers such as SOD, catalase, the glutathione peroxidase/glutathione/glutathione reductase system and the thioredoxin peroxidase/thioredoxin/thioredoxin reduc-

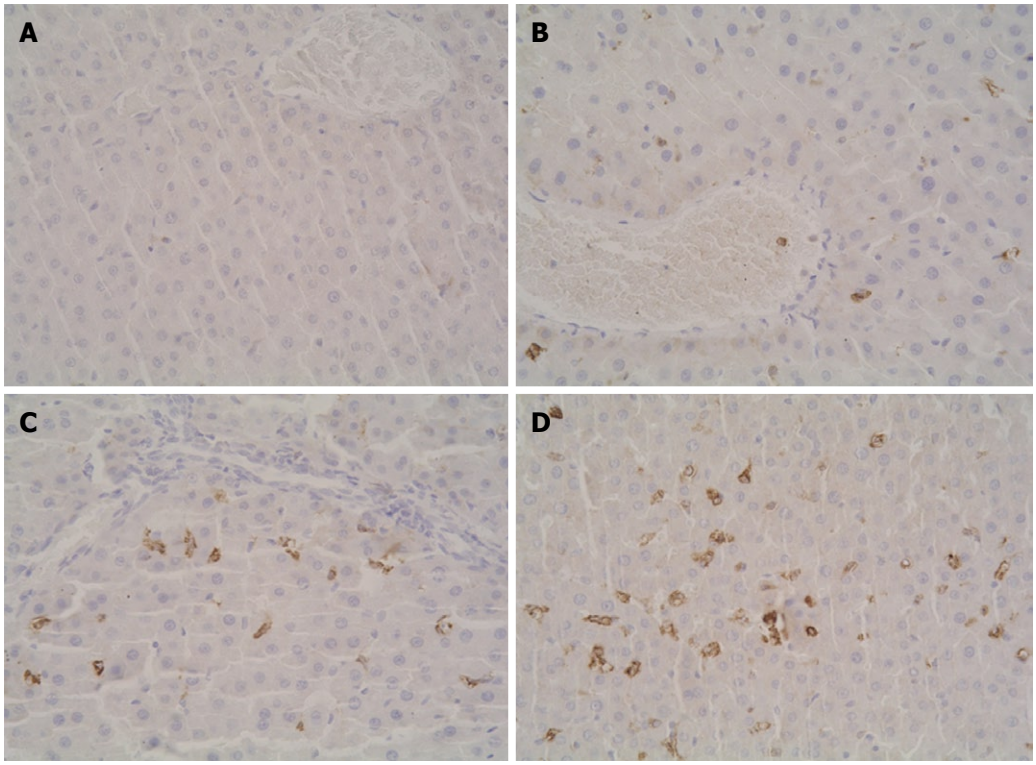

Figure 7 Immunohistochemical staining of liver heme oxygenase-1 (HO-1) expression (original magnification × 400) in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups.

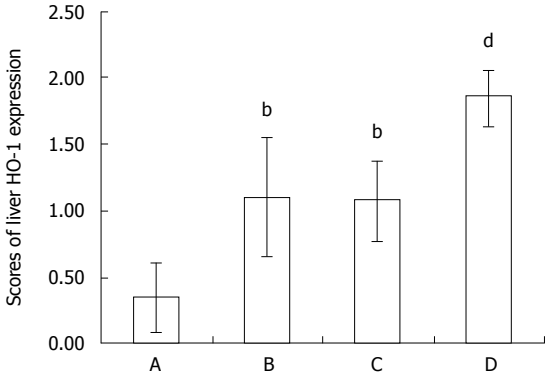

Figure 8 Immunohistochemical results (semi-quantitative analysis) of liver HO-1 expression in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. Data are presented as mean ± SD of eight animals. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

tase system[34]. SOD catalyses the dismutation of the superoxide anion ($O_2^{\cdot}$) into H_2O_2, which can be transformed into H_2O and O_2 by catalase (CAT). GSH as a kind of endogenous free radical scavenger can be regulated by GSH-Px. Ros also oxidizes proteins, induces lipid peroxidation and initiates DNA stand breaks, which can be blocked by the above-mentioned antioxidative agents.

ARE is an enhancer element that initiates the transcription of a battery of genes encoding phase 2 enzymes[35]. Activation of gene transcription through the ARE is mediated primarily by Nrf2[36]. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, and upon exposure of cells to inducers such as oxidative stress and certain chemopreventive agents, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to ARE, and transactivates phase 2 detoxifying and antioxidant genes[37]. Recent studies have implied that the activation of Nrf2/ARE pathway attenuate ischemia/reperfusion injury of the heart, brain and kidney[26,38,39]. And SFN induces Nrf2-driven phase 2 enzyme expression by modulating the activation in kidney ischemia reperfusion injury[40].

In this study, we evaluated the effect of SFN on regulating Nrf2/ARE in liver injury of the animal intestinal I/R model. We found that Nrf2 activation by sulforaphane pretreatment protected liver against injury induced by intestinal I/R, which was characterized by improved alternation in liver tissue pathology and liver function, and an enhanced antioxidant capacity, being consistent with the Nrf2 expression and content changes.

GSH-Px and HO-1 are two kinds of phase 2 enzymes. HO-1 is a ubiquitous heat shock protein (HSP32) that is highly induced by diverse stress-related conditions[41]. It is upregulated in response to oxidative stress in many tissues, providing generalized endogenous protection against oxidative stress[42]. Our results indicated that GSH-Px and HO-1 can be upregulated by SFN pretreatment through Nrf2-ARE pathway. Therefore, development of a kind of strategy to reduce oxidative stress by inducing endogenous phase 2 enzymes is attractive, which can be regulated by Nrf2. In order to clarify the mechanism comprehensively, further researches should determine the indexes of cell death such as apoptosis and inflammation in this pathophysiology process.

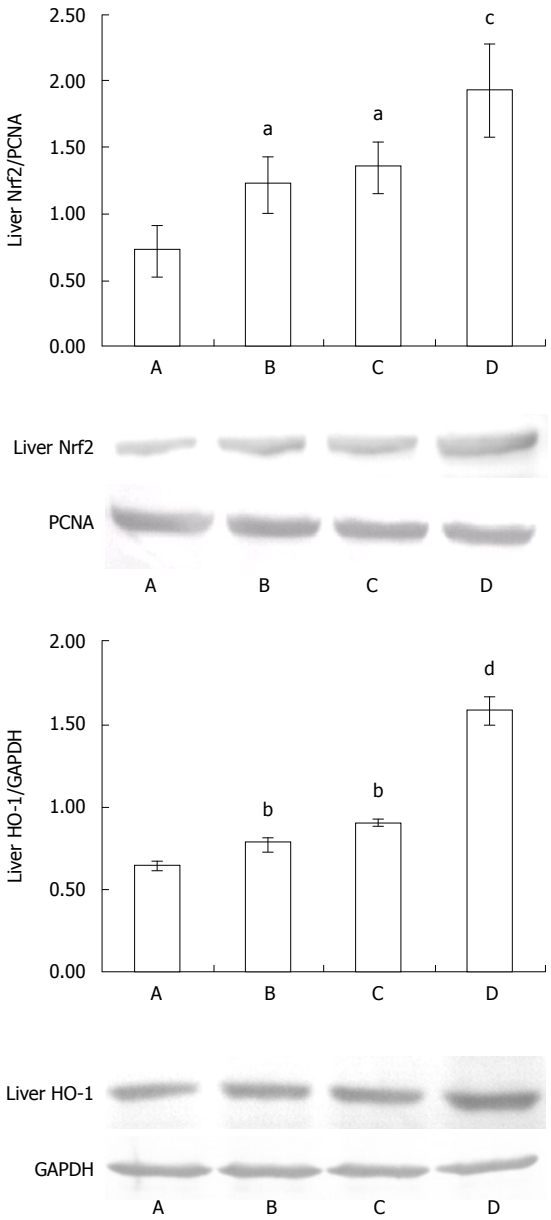

Figure 9 Western blotting analysis of expression of liver Nrf2 and HO-1 in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. PCNA and GAPDH were as the internal control respectively. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* intestinal I/R group.

In conclusion, ROS plays an important role in the pathogenesis of liver injury induced by intestinal I/R. SFN exerted the protective effect on the liver injury induced by intestinal I/R, attributable to the antioxidant effect through Nrf2-ARE pathway.

ACKNOWLEDGMENTS

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COMMENTS

Background

Sulforaphane (SFN) is a natural product derived from isothiocyanate which is present in cruciferous vegetables such as broccoli that has been used as a chemopreventive compound. The cytoprotective effect exerted by this compound is mediated by transcription factor NF-E2-related factor-2 (Nrf2), which binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes. SFN can disrupt the Nrf2-Keap1 complex, and permit Nrf2 to translocate into the nucleus to activate the ARE-driven genes.

Research frontiers

Studies suggest that Nrf2 activation by SFN results in effective protection from cancers and renal ischemia/reperfusion (I/R), but no one has evaluated SFN to determine if it can protect liver injury induced by intestinal I/R.

Innovations and breakthroughs

The study for the fist time showed that SFN exerted the protective effect on the liver injury induced by intestinal I/R, attributable to the antioxidant effect through Nrf2-ARE pathway.

Applications

This study has indicated that SFN pretreatment attenuates liver injury induced by intestinal I/R in rats, attributable to the antioxidant effect through Nrf2-ARE pathway.

Terminology

Nrf2 is sequestered in the cytoplasm by Keap1, and upon exposure of cells to inducers such as oxidative stress and certain chemopreventive agents, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to ARE, and transactivates phase 2 detoxifying and antioxidant genes. The activation of Nrf2/ARE pathway attenuates ischemia/reperfusion injury.

Peer review

The paper is well-written and data are convincing. All data confirm the hypothesis and adequately discussed.

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BRIEF ARTICLE

Management of recto-vaginal fistulas after prosthetic reinforcement treatment for pelvic organ prolapse

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Abstract

AIM: To communicate our findings on successful treatment of recto-vaginal fistulas (RVFs) after prosthetic reinforcement surgery of pelvic organ prolapse (POP).

METHODS: A retrospective single center study between 1998 and 2008 was performed. A total of 80 patients with RVF were identified, of which five patients (6%), with a mean age of 65 years (range: 52-73), had undergone previous surgery for POP with prosthetic reinforcement.

RESULTS: All patients complained about ongoing vaginal infections and febrile episodes. These symptoms were reported after a mean period of 18 mo after POP repair. As a first intervention, three patients underwent ablation of the prosthetic material (PM). As a second intervention, open proctectomy with a primary anastomosis, an omental patch, and a protective ileostomy were performed in two patients. One patient required a terminal colostomy due to complete destruction of the anal sphincters. In two other patients, ablation of the PM and proctectomy was performed as a one-step procedure. The postoperative course in all patients was uneventful, with a mean length of hospitalization of 20 d (range: 15-30). Closure of the ileostomy was achieved in all four patients within four months. After a mean period of 35 mo (range: 4-60) of follow-up, no recurrence was observed with normal continence in four patients.

CONCLUSION: In our experience, the definitive treatment of high RVFs after PM repair for POP necessitates ablation of the PM, proctectomy with a primary colorectal anastomosis, an omental patch interposition, and a temporary ileostomy.

Key words: Pelvic organ prolapse; Recto-vaginal fistula; Prosthetic treatment

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INTRODUCTION

Pelvic organ prolapse (POP) is a significant health issue in women worldwide. Approximately 250 000 procedures are performed annually in the United States alone[1,2]. The search for a permanent cure for POP has been ongoing for more than a century and remains a challenge today. A number of different approaches have been described for POP repair, including abdominal (open, laparoscopic, and robotic) and vaginal techniques. Recently, the use of prosthetic material (PM) in pelvic floor surgery has become increasingly popular due to the high incidence of recurrence with primary repairs and no surrogate material. Nevertheless, our knowledge about the possible benefit of PM reinforcement for POP repair remains limited. Currently, randomized trials support the superiority of techniques using PM for cystocele repair, whereas the results for colpo- and rectocele repair remain unclear or controversial[3,4].

With the use of PM, new and specific complications occur; these include PM infection, PM erosion or shrinkage, and visceral extrusion. In a systematic review of 2653 patients who underwent surgery for apical vaginal prolapse, Feiner *et al*[5] reported mesh erosion as the single most frequent complication in 4.6% to 10.7%, depending on the different PMs used. In a retrospective study of monofilament mesh placement in the anterior and posterior compartment for POP repair, Dwyer *et al*[6] reported a patient that had developed an recto-vaginal fistula (RVF). Two years later, Hilger *et al*[7] published a case study of RVF formation three months after a posterior intravaginal slingplasty and mesh augmented rectocele repair was performed, which ended in a permanent colostomy after two unsuccessful attempts to correct the fistula. Our knowledge on the incidence of RVF formation after prosthetic material repair for POP is still limited. In addition, uncertainty over the best management of this complication remains. This is in part due to the small number of case reports present in the medical literature. Here, we report our experience with POP management; to the best of our knowledge, this is the largest case series in the literature of RVFs after previous reinforcement surgery for POP.

MATERIALS AND METHODS

This was a retrospective single center study between 1998 and 2008. During that period, a total of 80 patients with RVF were treated at the Department of Visceral- and Oncological Surgery, Hôpital Timone, Marseille, France. Five female patients, with a mean age of 65 years (range: 52-73), were identified that had undergone previous surgery for POP with PM reinforcement. All patients had POP surgery done elsewhere. Details on previous POP repair were as follows: one Prolift® procedure (Ethicon Women's Health and Urology, Somerville, NJ) for urinary stress incontinence, one vaginal wall sling procedure (Bologna technique) and a laparoscopic repair with two multifilamentous polypropylene meshes implanted in both cases for a colpocystocele. Two other patients had open transabdominal implantation of a multifilamentous polypropylene mesh for a colpocysto- and a colporectocele, respectively, with the mesh fixed to the sacrospinous ligaments. Three patients were referred to our department after the PM had been removed transperinealy or transabdominaly, with creation of a temporary colostomy in one case.

At our department, all patients underwent a Gadolinium enhanced magnetic resonance imaging (MRI) of the pelvis using T1 and T2 weighted sequences, such as ano-rectoscopy or endorectal ultrasound (EUS), to evaluate the anal sphincter and a full gynecological work-up. Finally, in all cases, a complete colonoscopy to search for other underlying pathologies that could provoke RVF was routinely performed.

All surgical interventions were performed under general anesthesia and antibiotic cover. The definitive surgical therapy at our department consisted of an open proctectomy, colo-rectal anastomosis, an omental patch, and a protective ileostomy. One patient underwent a Hartmann procedure. A closed suction pelvic drainage was used in all patients.

For postoperative follow-up, all patients in our department underwent an initial clinical control (including Wexner score[8]), ano-rectoscopy, gynecological examination, and an MRI of the pelvis six months after final surgery had been performed.

RESULTS

Patient characteristics, details of surgery and follow-up are summarized in Table 1. The mean time interval between POP repair and the onset of ailments was 18 mo (range: 2-48). All five patients complained about vaginal discharge, dyspareunia, repetitive vaginal infections, and febrile episodes. However, in one patient (No. 3) the leading clinical symptom was complete stool incontinence. Patients No. 1, 2, and 3 underwent ablation of the PM before they had been referred to our department. Patient No. 1 had undergone open transabdominal ablation of the PM with creation of a temporary colostomy. Ablation of the PM in patients No. 2 and 3 was done *via* a transperineal or laparoscopic approach, respectively.

Pelvic MRI at our department provided evidence of a high RVF in all five patients. In patient No. 3 a complex low RVF with a destruction of the internal and external anal sphincter complex was additionally diagnosed (Figure 1).

EUS examination was normal in three patients; however, in patients No. 1 and 3, the anal sphincters were found with an inflammatory inhibition or destruction at a circumference of 20° and 180°, respectively. Nevertheless, in all patients, except for one (No. 3, Wexner score 19[8]), the preoperative Wexner score was normal. Diagnostic colonoscopy (including routine biopsies) was normal except for patient No. 5, where the mesh had completely eroded and migrated into the rectum (Figure 2).

The intra- and postoperative course was uneventful in all patients. Ileostomy closure was achieved in all four patients within four months after final surgery. The

Table 1 Patient characteristics and details of surgery and follow-up

Patients No.	Age (yr)	Primary pathology POP surgery/type of PM	Onset of symptoms (mo)	Primary surgery	Delay to secondary surgery (mo)	Secondary surgery	Follow-up (mo)/ Wexner score
1	73	Colpo-cystocele laparoscopic approach/two polypropylene meshes	24	Temporary colostomy + ablation of PM	6	Open proctectomy, omental patch + PI	36/3
2	65	Colpo-cystocele Bologna procedure/ two polypropylene meshes	12	Laparoscopic ablation of PM	12	Open proctectomy, omental patch + PI	36/2
3	60	SUI Prolift® procedure/two polypropylene meshes	6	Transperineal ablation of PM	6	Hartmann	36/NA
4	52	Colpo-rectocele transabdominal approach/one polypropylene mesh	2	-	-	Open proctectomy, omental patch + PI	60/0
5	73	Cysto-colpocele transabdominal approach/one polypropylene mesh	48	-	-	Open proctectomy, omental patch + PI	6/0

POP: Pelvic organ prolapse; NA: Not applicable; PM: Prosthetic material; SUI: Stress urinary incontinence; PI: Protective ileostomy.

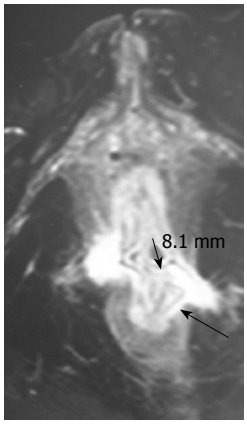

Figure 1 Pelvic magnetic resonance imaging (MRI) showing a transverse section of patient No. 3 with a complex recto-vaginal fistula (RVF) and destruction of the anal sphincter complex. The black arrows show a collection of 8.1 mm with complete destruction of the anal sphincter.

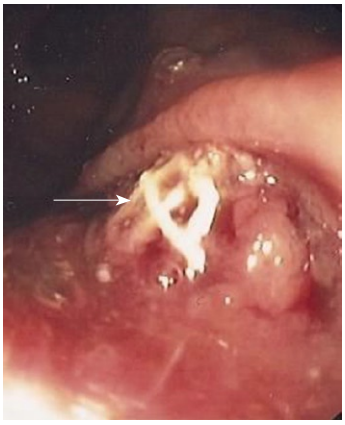

Figure 2 Colonoscopy. Recto-vaginal fistula (arrow) due to polypropylene mesh erosion and migration into the rectum.

mean hospitalization (including closure of the ileostomy) was 20 d (range: 15-40).

After a mean postoperative follow-up period of 34 mo (range: 6-60), no fistula recurrences were observed. In all patients where colo-rectal continuity could have been preserved, only patients No. 1 and 2 suffered from mild postoperative stool incontinence, with Wexner scores of 3 and 2, respectively. Stool continence in patients No. 4 and 5 was found to be completely normal (Wexner 0).

DISCUSSION

POP is a significant health problem in women worldwide, with an estimated 250 000 annual procedures performed in the United States alone[1,2]. These figures are predicted to rise by 45% over the next 30 years due to the increased life expectancy of women in the Western world, and an increasing prevalence of pelvic floor dysfunction with age[9].

Hundreds of techniques have been reported in POP surgery, whether vaginal, abdominal, or laparoscopic, but there is little or no real consensus on which procedure is most appropriate. This is in part due to the lack of high-quality, randomized controlled trials assessing the long-term anatomical and functional outcomes using validated tools and procedures[10].

As traditional surgical techniques for POP utilizing the patient's own tissue do not restore normal anatomy and have a very high failure rate, the use of PM has recently become more frequent. As far as the treatment for cystocele is concerned, better results are reported compared to the traditional surgical approach without use of PM[3,4].

However, with the more liberal use of different types of PM, new adverse effects and complications in patients who underwent POP surgery have been observed. In a public health notification published in October 2008 by the U.S Food and Drug Administration (FDA) it is stated, that over a period of three years, nine different mesh manufacturers reported about 1000 complications associated with transvaginal placement of surgical mesh in repair of POP and SUI. Furthermore, it stated that specific characteristics of patients at increased risk for complications have not been determined. However, contributing factors might include the overall health of the patient, the mesh material, the size and shape of the mesh, the surgical technique used, concomitant procedures undertaken (e.g. hysterectomy), and possibly estrogen status[11].

In our current series of five patients with high RVF after mesh repair of POP, all patients were in good overall health at the time of POP surgery. None of them had previous POP surgery or were under a concomitant medication or medical treatment that could have promoted RVF formation.

The type of mesh implanted has been found to be critical (reported to be pivotal). Mesh erosion through the vaginal epithelium is the most frequently observed mesh-related complication. However, this complication

differs between the different types of meshes used. A high rate of mesh erosion has been reported for non-resorbable synthetic meshes such as Gore-Tex®, Marlex®, and Mersilene®[12].

In our case series, RVF formation was related to multifilamentous polypropylene mesh implantation in all five cases, although the use of polypropylene mesh is currently promoted due to good biocompatability characteristics with a somewhat lower incidence of mesh related complications compared to other mesh types reported in the literature[12,13].

Information concerning RVF formation after mesh surgery for POP is rare in the medical literature. In a retrospective study of monofilament mesh placement in the anterior and posterior compartment for POP repair in 97 patients, Dwyer *et al*[6] observed one patient (1%) who had developed an RVF. In a case report, Hilger *et al*[7] reported an RVF that developed three months after a posterior intravaginal slingplasty and mesh augmented rectocele repair was performed. In another retrospective multicenter study of 687 patients who underwent a vaginal cure with the Prolift® procedure, one patient with an RVF (0.15%) in the postoperative follow-up period was found. However, in the same series, an intraoperative rectal erosion (0.15%), a rectal lesion (0.15%), and a recto-vesical fistula (0.15%) were also reported[14]. The exact incidence of this serious complication remains unknown, and is currently between 0.15% and 1%, as reported in the literature. However, the true incidence of RVF is probably somewhat higher, as the formation of RVFs is very likely the sequel of mesh infection and/or mesh extrusion, which are quite common.

Mesh extrusion after POP is generally observed between four and sixteen months postoperatively[15]. In our study, patients started to complain of vaginal discharge, repetitive vaginal infections, and febrile episodes after a mean period of 18 mo (range: 2-48) after POP repair. Therefore, the symptoms outlined above, even several years after POP surgery, should prompt clinicians to search for an RVF. In all five of our patients, MRI of the pelvis revealed a high RVF and furthermore helped to estimate the extent of pelvic inflammation and tissue destruction. Three patients underwent ablation of the PM before they were referred to our department. In one patient, a temporary colostomy had also been performed at the time of PM ablation. In cases of mesh infection, ablation of the mesh by a minimal invasive procedure, if possible, is currently recommended as a first treatment option[16]. This manoeuvre is generally facilitated by a bacterial biofilm, which isolates the PM from the surrounding tissue[13,17]. The definitive surgical treatment should then be performed after a delay of three to six months after pelvic inflammation has minimized[15]. In cases of a simple RVF, it is thought that once the infected PM has been removed, a spontaneous cicatrisation of the initially inflamed tissue will lead to spontaneous RVF closure[18].

However, in the three patients who underwent ablation of the PM in a primary surgical intervention outside our department, pelvic inflammation did not substantially minimize within 6 to 12 mo. Even protective colostomy did not resolve pelvic inflammation and closure of the RVF. This might be explained, in some part, by the finding that in all three patients some parts of PM were still found in the pelvis during final surgery, which might have promoted the ongoing pelvic inflammatory process. Therefore, it is unlikely that simple ablation of the PM, with or without the creation of a temporary colostomy, will completely resolve pelvic inflammation and lead to a permanent cure. In those two patients who were directly transferred to our department, ablation of the PM, proctectomy, colo-rectal anastomosis, an omental patch, and temporary ileostomy was performed as a one-step procedure. In cases of severe sphincter destruction, a definitive colostomy is the procedure of choice.

RVF after mesh surgery for POP is a rare, but severe, complication. The exact incidence of this complication depends on the type of PM implanted and the type of previous surgeries performed, and is currently about 0.15% to 1%, as reported in the medical literature. However, it is likely that the true incidence is somewhat higher. Ablation of the PM in a first surgical procedure is unlikely to remove all PM and there is a risk that parts of the infected mesh are left in place, which probably promotes an ongoing inflammation of the pelvis. If the general condition of the patient allows, we recommend a radical one-step surgical procedure with open PM ablation, proctectomy, an omental patch, colo-rectal anastomosis, and creation of a temporary ileostomy.

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COMMENTS

Background

An estimated 11% of women will require surgery for pelvic floor dysfunction, with 29% requiring at least a second surgery. To improve upon the results obtained with the use of native tissue, many surgeons have turned to different graft materials, especially synthetic mesh. Polypropylene mesh has been widely used and studied in such procedures, as abdominal sacral colpopexies and numerous mid-urethral slings. Over the last several years, the use of polypropylene mesh has been extended to augment vaginal repair of the anterior and/or posterior vaginal wall. With the use of prosthetic material (PM), new and specific complications have occurred. These include PM infection, PM erosion or shrinkage, and visceral extrusion. In a systematic review of 2 653 patients that underwent surgery for apical vaginal prolapse, Feiner *et al* reported mesh erosion as the single most frequent complication in 4.6% to 10.7%, depending on the different PMs used.

Research frontiers

Knowledge on the incidence of rectovaginal fistula (RVF) formation after prosthetic material repair for pelvic organ prolapse (POP) is still limited. In addition, uncertainly on the best management of this complication remains. This is in part due to the small number of case reports present in the medical literature. Here, the authors report on their experience with POP management, and to the best of their knowledge this is the largest case series the literature of RVFs after previous reinforcement surgery for POP.

Innovations and breakthroughs

In case of a simple RVF, it is thought that once the infected PM has been removed, a spontaneous cicatrisation of the initially inflamed tissue will lead to

spontaneous RVF closure. However, in three patients who underwent ablation of the PM in a primary surgical intervention outside our department, pelvic inflammation did not substantially minimize within 6 to 12 mo. Even protective colostomy did not resolve pelvic inflammation and closure of the RVF. This might be explained, in some part, by the finding that, in all three patients, some parts of the PM were still found in the pelvis during final surgery, which might have promoted the ongoing pelvic inflammatory process. Therefore, it is unlikely that simple ablation of the PM, with or without the creation of a temporary colostomy, will completely resolve pelvic inflammation and lead to a permanent cure.

Applications

RVF after mesh surgery for POP is a rare, but severe and complication. The exact incidence of this complication depends on the type of PM implanted and the type of previous surgeries performed, and is currently about 0.15% to 1%, as reported in the medical literature. However, it is likely that the true incidence is somewhat higher. Ablation of the PM in a first surgical procedure is unlikely to remove all PM and there is a risk that parts of the infected mesh are left in place, which probably promotes an ongoing inflammation of the pelvis. If the general condition of the patient allows, the authors recommend a radical one-step surgical procedure with open PM ablation, proctectomy, an omental patch, colo-rectal anastomosis, and creation of a temporary ileostomy.

Peer review

This is a good report of uncommon problem with clear-cut guidelines for this problem.

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BRIEF ARTICLE

Vitamin D receptor gene polymorphisms and hepatocellular carcinoma in alcoholic cirrhosis

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Abstract

AIM: To assess the relationship between vitamin D receptor (*VDR*) gene polymorphisms and the presence of hepatocellular carcinoma (HCC).

METHODS: Two-hundred forty patients who underwent liver transplantation were studied. The etiologies of liver disease were hepatitis C (100 patients), hepatitis B (37) and alcoholic liver disease (103). A group of 236 healthy subjects served as controls. HCC in the explanted liver was detected in 80 patients. The following single nucleotide gene polymorphisms of the VDR were investigated by polymerase chain reaction and restriction fragment length polymorphism: FokI C>T (*F*/*f*), BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) (BAT).

RESULTS: The frequencies of genotypes in patients without and with HCC were for FokI *F*/*F* = 69, *F*/*f* = 73, *f*/*f* = 18 and *F*/*F* = 36, *F*/*f* = 36, *f*/*f* = 8; BsmI *b*/*b* = 45, *B*/*b* = 87, *B*/*B* = 28 and *b*/*b* = 33, *B*/*b* = 35, *B*/*B* = 12; for ApaI *A*/*A* = 53, *A*/*a* = 85, *a*/*a* = 22 and *A*/*A* = 27, *A*/*a* = 38, a/a = 15; for TaqI *T*/*T* = 44, *T*/*t* = 88, *t*/*t* = 28 and *T*/*T* = 32, *T*/*t* = 38, *t*/*t* = 10. Carriage of the *b*/*b* genotype of BsmI and the *T*/*T* genotype of TaqI was significantly associated with HCC (45/160 *vs* 33/80, $P < 0.05$ and 44/160 *vs* 32/80, $P < 0.05$, respectively). The absence of the A-T-C protective allele of BAT was significantly associated with the presence of HCC (46/80 *vs* 68/160, $P < 0.05$). A strong association was observed between carriage of the *BAT* A-T-C and G-T-T haplotypes and HCC only in alcoholic liver disease (7/46 *vs* 12/36 *vs* 11/21, $P < 0.002$, respectively).

CONCLUSION: *VDR* genetic polymorphisms are significantly associated with the occurrence of HCC in patients with liver cirrhosis. This relationship is more specific for patients with an alcoholic etiology.

Key words: Alcohol; Hepatocellular carcinoma; Liver cirrhosis; Vitamin D receptor polymorphisms

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INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most common cause of cancer and the third leading cause of cancer-related death worldwide[1], accounts for 85% to 90% of primary liver cancers. It is characterized by dynamic temporal trends and marked demographic, geographic and ethnic variations[1]. In western countries, HCC is most often superimposed on cirrhosis, its major risk factor[2,3]. Alcohol, hepatitis B virus (HBV) and hepatitis C virus (HCV) are the main etiologic agents of cirrhosis and HCC worldwide. HBV and HCV supposedly exert a direct carcinogenic effect, accounting for the high prevalence of liver cancer among infected patients[2,3]; the same might not be true for alcohol[1]. Overall, it is prudent to affirm that differences in the incidence rates and the strong gender distribution in HCC are not entirely due to differences in the exposure to the three causative agents mentioned above. Genetic factors could also contribute, particularly gene polymorphisms of inflammatory cytokines and growth factor ligands and receptors[4].

The vitamin D receptor (VDR) is a member of the nuclear receptor super-family of ligand-inducible transcription factors which are involved in many physiological processes, including cell growth and differentiation, embryonic development and metabolic homeostasis. The transcriptional activity of this receptor is modulated by ligands, such as steroids, retinoids and other lipid-soluble compounds, and by nuclear proteins acting as co-activators and co-repressors[5,6]. The liganded VDR heterodimerizes with the retinoid X receptor and binds to vitamin D response elements in the promoter of target genes, thereby affecting their transcription. The genomic organization of the *VDR* at locus 12q13.1 shows that the *VDR* gene itself is quite large (over 100 kb) and has an extensive promoter region capable of generating multiple tissue-specific transcripts[7].

Several single nucleotide restriction fragment length polymorphisms have been described in the *VDR* gene in association with neoplastic and non-neoplastic diseases. In particular, *VDR* polymorphisms have been related to, although with conflicting observations, cancers of the breast, prostate, skin, colon-rectum, bladder and kidney[8-10]. Furthermore, *VDR* polymorphisms were found to influence the prognosis of prostate and breast cancer, renal cell carcinoma and malignant melanoma[11]. *VDR* polymorphisms have been investigated in the context of some chronic liver diseases, such as primary biliary cirrhosis and auto-immune hepatitis[12-16]. Surprisingly, however, there are no data in the literature on the possible association between *VDR* polymorphisms and the occurrence of HCC.

The aims of the present paper were (a) to investigate the possible relationship between *VDR* polymorphisms and the occurrence of HCC in patients with liver cirrhosis and (b) to assess whether the etiology of their liver disease exerts a role in modulating such a relationship.

MATERIALS AND METHODS

Patients

The study included 240 consecutive patients who underwent liver transplantation (LT) for end-stage liver disease due to hepatitis B (n = 37, 15.4%), hepatitis C (n = 100, 41.7%) and alcoholic liver disease (n = 103, 42.9%). The main demographic and clinical characteristics are reported in Table 1. Two-hundred thirty-six healthy community blood donors served as controls. They were 164 males (69.5%) and 72 females (30.5%); the median age was 48 years with a range of 18-77 years. The male gender distribution in the patients and controls (74% *vs* 70%) was similar, whereas these two groups differed in age: 54 ± 8 years *vs* 46 ± 13 years (mean ± SD, $P < 0.0001$). Control subjects did not have any clinical and/or laboratory evidence of liver disease or other major pathological conditions, such as diabetes mellitus. All patients and controls were Caucasian. Informed consent to participate in the study was obtained from each subject in accordance with the Declaration of Helsinki and following the guidelines of our ethical committee. All study participants approved the storage of their frozen DNA specimens for research purposes in our laboratory.

Histology

All total hepatectomy specimens were sectioned at intervals of approximately one cm to search for suspicious focal hepatic lesions. Standard histological staining techniques were applied to confirm the presence of HCC and to evaluate the characteristics of the identified tumors, such as pathologic tumor grade (Edmondson grade) and macro- or micro-vascular invasion.

Vitamin D assay

In 113 patients (47.1%), serum samples, which were collected the day before the transplant operation and were separated and stored at -80℃ until analysis, were available to assay the pre-LT serum vitamin D concentration. Circulating 25-hydroxyvitamin D levels were measured using a chemo-luminescent immunoassay implemented on a Liaison automatic analyzer (DiaSorin Inc, Stillwater, MN, USA). Data were expressed in ng/mL. Reference values of serum vitamin D adopted in this study were in accordance with those proposed by the Scientific Advisory Committee of Nutrition[17], which considers serum vitamin D levels < 10-15 ng/mL to be inadequate for bone and overall health in healthy individuals.

Molecular biology

Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Four diallelic polymorphisms of the *VDR* were genotyped: FokI C>T

Table 1 Main demographic and clinical characteristics of liver transplant recipients (*n* = 240) according to the presence (*n* = 80, 33.3%) or absence (*n* = 160, 66.7%) of HCC

		HCC present (*n* = 80)	HCC absent (*n* = 160)	*P* value
Male gender	178 (74.2)	70	108	< 0.0010
Age[1] (yr)	55 (22-68)	57.2 ± 5.9	52.0 ± 8.6	< 0.0001
Body mass index[1] (kg/m^2)	25.2 (14.8-48.5)	26.4 ± 4.2	24.7 ± 3.3	< 0.0010
Etiology				
Viral (*n* = 137)		50	87	NS
HBV	37 (15.4)			
HCV	100 (41.7)			
Alcoholic	103 (42.9)			
Child-Pugh score[1]	8 (5-14)	7 (5-14)	8 (5-13)	< 0.0001
Child-Pugh score[1] > 8 (*n* = 87)		22	65	< 0.0500
Diabetes mellitus	67 (27.9)	29	38	< 0.0500

The continuous variables are reported as means (standard deviation), whereas the categorical variables are reported as frequencies (%). Statistical analysis was performed using the Student's *t*-test for continuous variables and the Pearson χ^2 test for categorical variables. [1]At transplant operation. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NS: Not significant.

(rs10735810) and TaqI T>C (rs10735810) polymorphic sites on the coding sequence, BsmI A>G (rs1544410) and ApaI G>T (rs7975232) on the last intron. For the detection of the *VDR* polymorphisms, the polymerase chain reaction (PCR) technique was applied and followed by restriction fragment length polymorphism assays. The PCR amplifications were carried out in a total volume of 10 μL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.01% Tween-20, 0.2 mmol/L deoxyribonucleotides, 2-4 pmol of each primer, 2.0 mmol/L $MgCl_2$ and 0.5 U hot-start *Taq* DNA polymerase (RighTaq, Euroclone, Milan, Italy). The sequences of primers used for FokI were (*f*) 5'-TGCAGCCTTCACAGGTCATA-3', (*r*) 5'-GGCCTGCTTGCTGTTCTTAC-3'; for TaqI and ApaI were (*f*) 5'-ACGTCTGCAGTGTGTTGGAC-3', (*r*) 5'-TCACCGGTCAGCAGTCATAG-3'; for BsmI were (*f*) 5'-CAGTTCACGCAAGAGCAGAG-3', (*r*) 5'-ACCTGAAGGGAGACGTAGCA-3'. All the primers were newly designed with the aid of the NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The cycling conditions for all the *VDR* polymorphisms were set as 40 cycles at 95℃ for 30 s, 61℃ for 30 s and 72℃ for 1 min. In a total volume of 20 μL, amplified DNA (10 μL) was digested overnight with 2 U of restriction endonucleases using the buffers and temperatures recommended by the manufacturers. The presence of restriction sites for the FokI, TaqI, BsmI and ApaI enzymes were coded as '*f*', '*t*', '*b*' and '*a*' and the absence of restriction sites as '*F*', '*T*', '*B*' and '*A*', respectively. The FokI C>T (*F*/*f*) polymorphism was analyzed by digestion of a 157-bp PCR product with FokI (New England Biolabs, Hitchin, UK), which resulted in two fragments of 121 and 36 bp in the presence of the '*f*' allele and in an uncut fragment in the presence of the '*F*' allele. The TaqI T>C (*T*/*t*) and ApaI T>G (*A*/*a*) polymorphisms were analyzed by digestion of a 211-bp PCR product with TaqI (New England Biolabs, Hitchin, UK), which resulted in two fragments of 172 and 39 bp in the presence of the '*t*' allele. These same polymorphisms were also analyzed by digestion with ApaI (New England Biolabs, Hitchin, UK), which resulted in two fragments of 121 and 90 bp for the '*a*' allele. The BsmI A>G (*B*/*b*) polymorphism was analyzed by digestion of a 236-bp PCR product with BsmI (New England Biolabs, Hitchin, UK), which resulted in two fragments of 197 and 39 bp in the presence of the '*b*' allele. All PCR reactions were carried out in a Techne TC-412 thermal cycle, and PCR products were sized by electrophoresis on a 3% agarose gel stained with ethidium bromide.

Statistical analysis

The statistical analysis of data was performed using the BMDP Dynamic Statistical Software Package 7.0 (Statistical Solutions, Cork, Ireland). Continuous variables were presented as median (range) or mean ± SD, whereas categorical variables were expressed as frequencies (%). Differences between continuous variables were assessed by the Student's *t*-test, whereas differences between categorical variables were evaluated using the Pearson χ^2 test. The χ^2 *G* test "Goodness of Fit" was employed to verify whether the proportions of the four polymorphisms were distributed in controls and in patients in accordance with the Hardy-Weinberg equation. Differences in the allelic and genotypic frequencies between different groups were assessed by means of the Pearson χ^2 test and calculation of odds ratios with 95% confidence intervals (CI). Haplotype reconstruction from population genotype data and inferred phased diplotype calculation for each control subject or patient with liver cirrhosis were performed by means of the ARLEQUIN integrated software package for population genetics, version 3.1[18]. Analysis of molecular variance (AMOVA) with a global and a pair-wise approach was performed to assess whether haplotype allelic content differed among groups. Locus-by-locus AMOVA was utilized to assess the statistical contribution of each polymorphism. Pairwise differences in haplotype frequencies were assessed using the exact test for sample differentiation. Linkage disequilibrium between the four analyzed VDR poly-

Table 2 Allelic and genotypic frequencies of the VDR FokI C>T (*F*/*f*), BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) polymorphisms in controls and liver cirrhosis patients *n* (%)

VDR	Control subjects (*n* = 236)	Liver cirrhosis (*n* = 240)	OR	95% CI	*P*[1]
	F = 0.646	*F* = 0.665	1	Ref.	
FokI	*f* = 0.354	*f* = 0.335	1.085	0.831-1.417	NS
	B = 0.392	*B* = 0.421	1	Ref.	
BsmI	*b* = 0.608	*b* = 0.579	1.127	0.870-1.460	NS
	A = 0.570	*A* = 0.590	1	Ref.	
ApaI	*a* = 0.430	*a* = 0.410	1.084	0.838-1.402	NS
	T = 0.608	*T* = 0.579	1	Ref.	
TaqI	*t* = 0.392	*t* = 0.421	0.887	0.685-1.149	NS
	F/*F* = 104 (44.1)	*F*/*F* = 105 (43.8)	1	Ref.	
FokI	*F*/*f* = 97 (41.1)	*F*/*f* = 109 (45.4)	1.113	0.758-1.635	NS
	f/*f* = 35 (14.8)	*f*/*f* = 26 (10.8)	0.736	0.416-1.303	NS
	B/*B* = 41 (17.4)	*B*/*B* = 40 (16.7)	1	Ref.	
BsmI	*B*/*b* = 103 (43.6)	*B*/*b* = 122 (50.8)	1.214	0.732-2.014	NS
	b/*b* = 92 (39.0)	*b*/*b* = 78 (32.5)	0.869	0.513-1.473	NS
	A/*A* = 76 (32.2)	*A*/*A* = 80 (33.3)	1	Ref.	
ApaI	*A*/*a* = 117 (49.6)	*A*/*a* = 123 (51.3)	0.999	0.668-1.494	NS
	a/*a* = 43 (18.2)	*a*/*a* = 37 (15.4)	0.817	0.477-1.400	NS
	T/*T* = 89 (37.7)	*T*/*T* = 76 (31.7)	1	Ref.	
TaqI	*T*/*t* = 109 (46.2)	*T*/*t* = 126 (52.5)	1.354	0.909-2.017	NS
	t/*t* = 38 (16.1)	*t*/*t* = 38 (15.8)	1.171	0.681-2.013	NS

The odds ratios were constructed with the wild type for each polymorphism as the reference. The statistical analysis was carried out using the Pearson χ^2 test. [1]Pearson χ^2 test; OR: Odds ratio; CI: Confidence interval; VDR: Vitamin D receptor.

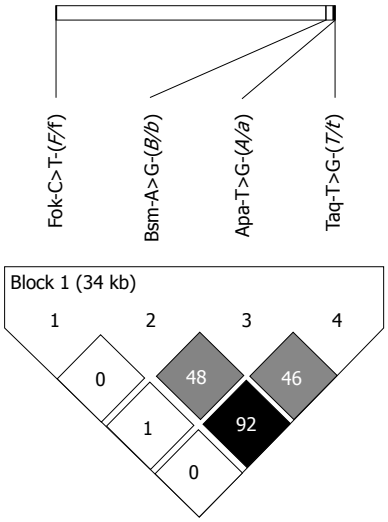

Figure 1 Schematic representation of linkage disequilibrium in the studied population (*n* = 476) between the four VDR polymorphisms: FokI C>T (*F*/*f*), BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*). R^2 for linkage disequilibrium between each marker is reported. Shades of gray are proportional to the R^2 value, expressing the strength of the linkage disequilibrium.

morphisms in the studied population was determined by means of the Haploview software[19]. Stepwise logistic regression analysis with a forward approach was used to verify whether the absence or presence of specific VDR haplotypes was an independent predictor of HCC.

RESULTS

Liver histology

HCC foci were detected in the native livers from 80 (33.3%) of the patients with end-stage liver disease; 33 patients (41.2%) were HCV positive, 17 (21.2%) HBV positive, and 30 (29.1%) had alcoholic liver disease. Table 1 illustrates the distribution of the values of the main demographic and clinical variables of cirrhotic patients according to the presence of HCC. Sixteen patients (20.0%) had an Edmondson grade of one, forty-eight patients (60.0%) had a grade of two, fifteen patients (18.8%) had a grade of three and one patient (1.2%) had a grade of four. Macro- and micro-vascular invasion was only observed in five cases (6.2%).

VDR polymorphisms in liver cirrhosis and controls

FokI C>T (*F*/*f*), BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) allele and genotype frequencies are reported in Table 2. No departure from the Hardy-Weinberg equilibrium equation was observed for each polymorphism in patients or controls. No significant difference was detected between patients and controls in allele or genotype frequencies. A strong linkage disequilibrium was detected between BsmI and TaqI (R^2 = 0.92); linkage disequilibrium was also detected between ApaI and TaqI (R^2 = 0.46) and between BsmI and ApaI (R^2 = 0.48) (Figure 1).

VDR polymorphisms in patients and controls in relationship to the etiology of liver disease

AMOVA was performed by grouping the liver cirrhosis patients according to the etiology of their liver disease: patients with cirrhosis of viral origin (*n* = 137) and patients with cirrhosis of alcoholic origin (*n* = 103); a third group consisted of control subjects (*n* = 236). A significant difference was detected among these three populations ($P < 0.05$) by global AMOVA; the comparison of

Table 3 Estimated VDR haplotype frequencies [FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms] in control subjects and in patients with liver cirrhosis of viral and alcoholic origin

Control subjects (n = 236)			Alcoholic cirrhosis (n = 103)			Viral cirrhosis (n = 137)		
Haplotypes	n	Frequencies	Haplotypes	n	Frequencies	Haplotypes	n	Frequencies
C-A-T-C	139	0.295	C-A-T-C	59	0.286	C-A-T-C	103	0.376
T-G-G-T	102	0.216	T-G-G-T	46	0.223	T-G-G-T	56	0.204
C-G-G-T	99	0.210	C-G-G-T	50	0.243	C-G-G-T	43	0.158
C-G-T-T	58	0.123	C-G-T-T	29	0.141	C-G-T-T	25	0.091
T-A-T-C	41	0.087	T-A-T-C	11	0.053	T-A-T-C	23	0.084
T-G-T-T	23	0.049	T-G-T-T	6	0.029	T-G-T-T	19	0.069
C-A-T-T	4	0.008	C-A-T-T	1	0.005	C-A-T-T	3	0.011
C-G-T-C	3	0.006	C-G-T-C	2	0.010	C-G-T-C	2	0.007
C-G-G-C	2	0.004	C-A-G-C	2	0.010			
T-A-T-T	1	0.002						

The statistical analysis was performed by means of the exact test for sample differentiation based on haplotype frequencies. Alcoholic *vs* viral liver cirrhosis $P < 0.05$.

pairs of population samples demonstrated a significant difference between patients with cirrhosis of viral and alcoholic origin ($P < 0.01$). Locus-by-locus AMOVA showed the following significant differences for the single nucleotide gene polymorphisms tested: FokI C>T (*F/f*) $P = 0.720$, BsmI A>G (*B/b*) $P = 0.047$, ApaI T>G (*A/a*) $P = 0.052$ and TaqI T>C (*T/t*) $P = 0.065$. Estimated VDR haplotype frequencies of FokI C>T, BsmI A>G, ApaI T>G and TaqI T>C polymorphisms are reported in Table 3. A significant difference in haplotype frequencies was detected between patients with cirrhosis of viral and alcoholic origin.

VDR polymorphisms in liver cirrhosis with and without HCC

Table 4 illustrates the genotype frequencies of FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms in patients with liver cirrhosis grouped according to the presence (n = 80) or absence (n = 160) of HCC. Patients with HCC were more likely to carry the *b/b* genotype compared with the *B/B* + *B/b* genotypes of the BsmI A>G (*B/b*) polymorphism and the *T/T* genotype compared with the *T/t* + *t/t* genotypes of the TaqI T>C (*T/t*) polymorphism. Considering the BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) (*BAT*) polymorphisms, carriage of the A-T-C haplotype was associated with the absence of HCC and carriage of the G-T-T haplotype with the presence of HCC. We then grouped the patients as follows: group (a) carriers of the *BAT* A-T-C haplotype (n = 126), group (b) carriers of both *BAT* A-T-C and G-T-T haplotypes or of none of the two haplotypes (n = 75) and group (c) carriers of the *BAT* G-T-T haplotype (n = 39). A significantly linear trend for increasing frequencies of HCC was detected starting with group (a) (34/126, 27.0%) to group (b) (29/75, 38.7%) to group (c) (17/39, 43.6%, $P < 0.05$). Stepwise logistic regression analysis was performed to verify whether carriage of the *BAT* A-T-C and G-T-T haplotypes was a predictor of HCC independent of gender, age (≤/> 50 years), body mass index (</≥ 25 kg/m^2), Child-Pugh score (≤/> 8) at the transplant operation, viral etiology of liver disease and presence of diabetes mellitus. The analysis confirmed that carriage of the *BAT* A-T-C and G-T-T haplotypes was a predictor of HCC occurrence (improvement of χ^2 $P < 0.05$, OR, 1.95, 95% CI: 1.06-3.57) independent of age > 50 years (improvement of χ^2 $P < 0.001$, OR 3.81, 95% CI: 1.75-8.29), male gender (improvement of χ^2 $P = 0.001$, OR 4.01, 95% CI: 1.80-8.96), viral etiology of liver disease (improvement of χ^2 $P < 0.05$, OR 2.19, 95% CI: 1.18-4.05) and body mass index ≥ 25 kg/m^2 (improvement of χ^2 $P < 0.05$, OR 1.82, 95% CI: 0.99-3.35).

VDR polymorphisms in liver cirrhosis with and without HCC in relationship to the etiology of liver disease

AMOVA was performed by grouping the patients with liver cirrhosis according to the etiology of their liver disease (viral origin n = 137 and alcoholic origin n = 103) and the presence (n = 80) or absence (n = 160) of HCC. A significant difference was detected among these four populations ($P < 0.002$); the comparison of pairs of population samples demonstrated significant differences between patients with liver cirrhosis of alcoholic origin with HCC *vs* (a) patients with liver cirrhosis of viral origin with HCC ($P < 0.0001$), (b) patients with liver cirrhosis of viral origin without HCC ($P < 0.0001$) and (c) patients with liver cirrhosis of alcoholic origin without HCC ($P < 0.01$). Locus-by-locus AMOVA showed the following significant differences for the single polymorphisms: FokI C>T (*F/f*) $P = 0.923$, BsmI A>G (*B/b*) $P = 0.000$, ApaI T>G (*A/a*) $P = 0.033$ and TaqI T>C (*T/t*) $P = 0.000$. Estimated VDR haplotype frequencies of FokI C>T, BsmI A>G, ApaI T>G and TaqI T>C polymorphisms are reported in Table 5. A significant difference in haplotype frequencies was detected between patients with alcoholic liver cirrhosis with HCC *vs* patients with viral liver cirrhosis without HCC ($P < 0.01$) and patients with viral liver cirrhosis with HCC ($P < 0.05$). Although no relationship was detected between carriage of the *BAT* A-T-C and G-T-T haplotypes and the pres-

Table 4 Genotypic frequencies of the VDR FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms in liver cirrhosis patients grouped according to the presence or absence of HCC

VDR	HCC absent (*n* = 160)	HCC present (*n* = 80)	OR	95% CI	*P*[1]
	F/F = 69 (43.1)	*F/F* = 36 (45.0)	1	Ref.	
FokI	*F/f* = 73 (45.6)	*F/f* = 36 (45.0)	0.945	0.537-1.663	NS
	f/f = 18 (11.3)	*f/f* = 8 (10.0)	0.852	0.345-2.113	NS
	B/B = 28 (17.5)	*B/B* = 12 (15.0)	1	Ref.	[2]
BsmI	*B/b* = 87 (54.4)	*B/b* = 35 (43.8)	0.939	0.433-2.028	NS
	b/b = 45 (28.1)	*b/b* = 33 (41.2)	1.711	0.766-3.813	NS
	A/A = 53 (33.1)	*A/A* = 27 (33.8)	1	Ref.	
ApaI	*A/a* = 85 (53.1)	*A/a* = 38 (47.5)	0.878	0.483-1.595	NS
	a/a = 22 (13.8)	*a/a* = 15 (18.7)	1.338	0.605-2.968	NS
	T/T = 44 (27.5)	*T/T* = 32 (40.0)	1	Ref.	[3]
TaqI	*T/t* = 88 (55.0)	*T/t* = 38 (47.5)	0.594	0.329-1.072	0.08
	t/t = 28 (17.5)	*t/t* = 10 (12.5)	0.491	0.212-1.141	0.09

The odds ratios were constructed with the wild type for each polymorphism as the reference. The statistical analysis was carried out using the Pearson χ^2 test. [1]Pearson χ^2 test; [2]*B/B* + *B/b* genotypes *vs* *b/b* genotype: $P < 0.05$; [3]*T/t* + *t/t* genotypes *vs* *T/T* genotype: $P < 0.05$.

Table 5 Estimated VDR haplotype frequencies [FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms] in patients with liver cirrhosis of viral and alcoholic origin according to the presence of HCC

Alcoholic cirrhosis (*n* = 103)						Viral cirrhosis (*n* = 137)					
HCC present (*n* = 30)			HCC absent (*n* = 73)			HCC present (*n* = 50)			HCC absent (*n* = 87)		
Haplotypes	*n*	Frequencies	Haplotypes	*n*	Frequencies	Haplotypes	*n*	Frequencies	Haplotypes	*n*	Frequencies
C-A-T-C	9	0.150	C-A-T-C	50	0.343	C-A-T-C	40	0.40	C-A-T-C	64	0.368
T-G-G-T	16	0.267	T-G-G-T	30	0.205	T-G-G-T	15	0.15	T-G-G-T	38	0.218
C-G-G-T	18	0.300	C-G-G-T	32	0.219	C-G-G-T	19	0.19	C-G-G-T	27	0.155
C-G-T-T	14	0.233	C-G-T-T	15	0.103	C-G-T-T	5	0.05	C-G-T-T	17	0.098
T-A-T-C	1	0.017	T-A-T-C	10	0.068	T-A-T-C	7	0.07	T-A-T-C	15	0.086
T-G-T-T	2	0.033	T-G-T-T	4	0.027	T-G-T-T	11	0.11	T-G-T-T	11	0.063
			C-A-T-T	1	0.007	C-A-T-T	2	0.02	C-G-T-C	1	0.006
			C-G-T-C	2	0.014	C-G-T-C	1	0.01	T-A-T-T	1	0.006
			C-G-G-C	2	0.014						

The statistical analysis was performed by means of the exact test for sample differentiation based on haplotype frequencies. Non-viral liver cirrhosis with HCC *vs* (a) viral liver cirrhosis without HCC, $P < 0.01$, (b) viral liver cirrhosis with HCC, $P < 0.05$.

ence of HCC in patients with viral liver disease, a strong association was observed between carriage of the *BAT* A-T-C and G-T-T haplotypes and HCC in alcoholic liver disease (27/80 *vs* 17/39 *vs* 6/18, P = NS) (7/46 *vs* 12/36 *vs* 11/21, $P < 0.002$).

Vitamin D levels and VDR haplotypes in relationship to HCC occurrence

A significant linear trend was detected when patients were grouped according to gender and vitamin D serum levels (cut-off level 15 ng/mL) in relation to HCC occurrence. Group A and B comprised female patients with vitamin D serum levels ≤ 15 ng/mL and > 15 ng/mL, respectively; group C and D comprised male patients with serum levels of vitamin D ≤ 15 ng/mL and > 15 ng/mL, respectively. HCC was detected with increasing frequency starting with group A (0/12, 0.0%) to group B (2/12, 16.7%) to group C (16/38, 42.1%) to group D (26/51, 51.0%, $P < 0.0005$). A synergistic effect was found between vitamin D serum levels > 15 ng/mL and carriage of the *BAT* ATC haplotype; in these patients, HCC occurred in 5/26 (19.2%) cases compared with 39/87 (44.8%) of the remaining cases ($P < 0.02$).

DISCUSSION

In the present study, patients with liver cirrhosis were found to present allele and genotype frequencies of FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms close to those observed in control subjects. Associations between specific polymorphisms and auto-immune hepatitis and primary biliary cirrhosis have been observed in several studies, although with conflicting results. In fact, the presence of the *B* allele of the BsmI A>G (*B/b*) polymorphism was found to be associated with the occurrence of primary biliary cirrhosis in three studies involving Caucasian and Japanese populations[12-14], whereas in two other studies performed in Caucasian and Chinese populations[15,16], primary biliary cirrhosis was related to the presence of the *b* allele. Similarly, Vogel *et al*[15] found an association between chronic auto-immune hepatitis and the *F* allele of the FokI C>T

(*F*/*f*) polymorphism, whereas the opposite results were observed by Fan *et al*[16]. Our study of patients affected by liver cirrhosis of viral (HBV or HCV) and alcoholic origin did not demonstrate any differences in the allele and genotype frequencies of the *VDR* polymorphisms between the patients and controls. The data agree with the only report in the literature on *VDR* polymorphisms in patients with chronic liver disease of a non-auto-immune origin. In fact, Suneetha *et al*[20] did not demonstrate differences in *VDR* polymorphism allele frequencies between controls and patients with chronic viral hepatitis due to HBV.

Some novel findings were provided by the present investigation. The first is represented by the observation of differences in the *VDR* polymorphisms in patients with liver cirrhosis in relationship to the etiology of their liver disease. The analysis of molecular variance found a significant difference in allele frequencies between patients with viral (HBV and HCV) and alcoholic liver cirrhosis; this was accounted for by the significant differences of the BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) (*BAT*) polymorphisms for which a strong linkage disequilibrium was detected. On the contrary, the FokI C>T (*F*/*f*) polymorphism was non-significantly distributed between these two groups. Consequently, haplotype analysis highlighted a different distribution between the two groups with liver cirrhosis; the *BAT* A-T-C haplotype was more represented in patients with viral cirrhosis (0.460%) than in those with alcoholic cirrhosis (0.339%). We are unable to provide a clear explanation of this previously unreported finding especially because the BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) (*BAT*) polymorphisms are located in a *VDR* gene region of unknown function. Two possible explanations are hypothesized: (a) the real genetic risk for liver cirrhosis might be in linkage disequilibrium with the observed haplotypes of these polymorphisms and/or (b) gene-environment interactions have a causative role.

The second novel finding of this paper is the significant association between *VDR* polymorphisms and the presence of HCC in patients with liver cirrhosis. HCC was found to be associated with the *b* allele of the BsmI A>G (*B*/*b*) polymorphism and with the *T* allele of the TaqI T>C (*T*/*t*) polymorphism. The *BAT* A-T-C haplotype was inversely related to the occurrence of HCC, whereas the *BAT* G-T-T haplotype was directly associated with this cancer; these associations were independent of the main demographic and clinical variables known to be strong predictors of the occurrence of HCC. *VDR* polymorphisms have been explored in cancers of epithelial origin, such as breast, ovarian, prostate, lung and skin cancers[20,21]. Even though some studies did not detect associations between the *VDR* polymorphisms and these diseases, e.g. Gsur *et al*[23] in prostate cancer and Dunning *et al*[24] in breast cancer, the majority of the authors found a significant association between the *VDR* polymorphisms and cancer[8-10,20]. In particular, carriage of the *B* BsmI A>G (*B*/*b*) allele and of the *t* TaqI T>C (*T*/*t*) allele has been described to exert a protective effect in prostate cancer[24], malignant melanoma[25,26] and breast cancer[27]; these results support our findings in the present series on HCC in liver cirrhosis.

The third observation that may be derived from these data concerns the strong interaction between the presence of HCC and the etiology of liver cirrhosis in relationship to *VDR* polymorphisms. Both the analysis of molecular variance and the estimated haplotype frequencies highlighted the different behaviors that were detected in the *VDR* polymorphisms in alcoholic patients with HCC, patients with alcoholic cirrhosis without HCC and patients with liver cirrhosis of viral origin complicated or uncomplicated by the presence of HCC. Locus-by-locus analysis demonstrated that the major contribution was provided by the BsmI A>G (*B*/*b*) and TaqI T>C (*T*/*t*) polymorphisms; the *BAT* A-T-C haplotype was strongly protective, whereas the G-T-T haplotype was associated with HCC in patients with alcoholic cirrhosis but not in patients with liver cirrhosis of viral origin.

Besides the classic action involving calcium and phosphate homeostasis, vitamin D possesses non-classic actions also known to be mediated through the VDR[28]. First, vitamin D exerts immune modulation activity either by stimulating innate immune function[29] and/or by inhibiting hyper-activity of adaptive immunity[30]. More closely related to the subject of the present study, vitamin D has been extensively evaluated for its potential anticancer activity in animal and cell studies. It has been suggested that the anticancer activity of vitamin D is due to its anti-proliferative and pro-differentiating action in most cell types[31]. From an epidemiological point of view, there is some evidence that low vitamin levels are associated with a higher incidence of cancer[32] even though there is no known beneficial effect of treating cancers with vitamin D[33].

Patients with liver cirrhosis are known to be at high risk for vitamin D deficiency in direct proportion to the severity of their chronic liver disease[34,35]. This observation applies particularly to cirrhotic patients with end-stage liver cirrhosis who are subjected to liver transplantation[36], such as those investigated in the present study. In contrast, chronic alcohol abuse is a factor that can interfere in multiple ways with vitamin D metabolism either through malnutrition with an assumption of reduced nutrient intake and/or reduced exposure to sunlight. Reduced vitamin D levels have been detected in the majority of alcoholic patients without chronic liver disease[37]. It is conceivable, therefore, that in patients with liver cirrhosis, the synergistic action of severely reduced serum vitamin D levels and of a specific *VDR* haplotype facilitates HCC development. In fact, supporting this hypothesis, we found vitamin D insufficiency ($\leqslant$ 15 ng/mL) in a large proportion (55.8%) of our patients with end-stage chronic liver disease; moreover, a gender-adjusted association between vitamin D insufficiency and the occurrence of HCC was also detected. Finally, HCC foci were observed less frequently in the native livers of patients carrying the protective *BAT* A-T-C haplotype and simultaneously possessing serum vitamin D levels > 15 ng/mL.

In conclusion, *VDR* genetic polymorphisms are signif-

icantly associated with the occurrence of HCC in patients with liver cirrhosis. This relationship is more specific for patients with an alcoholic etiology.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide. Liver cirrhosis due to alcohol consumption or to chronic infection by hepatitis C virus (HCV) and hepatitis B virus (HBV) are considered the main etiologic agents for the development of HCC. The differences in the incidence rates and the strong gender distribution of HCC are probably not entirely due to differences in exposure to the three causative agents mentioned above. Recently, several genetic factors regarding gene polymorphisms of inflammatory cytokines and growth factors have been considered.

Research frontiers

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily of ligand- inducible transcription factors which are involved in cell growth and differentiation. Several single nucleotide restriction length polymorphisms have been described in the *VDR* gene in association with cancers of the breast, prostate, colon, bladder and kidney. There are no data in the literature concerning the prevalence of *VDR* polymorphisms in HCC of different etiologies.

Innovations and breakthroughs

This is the first study to examine the potential role of *VDR* genetic polymorphisms in the occurrence of HCC in humans. In this study, the authors investigate the *VDR* single nucleotide gene polymorphisms FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) (BAT) in a large series of patients who underwent liver transplantation due to liver cirrhosis with or without HCC. They demonstrate that carriage of the *b/b* genotype of BsmI and the *T/T* genotype of TaqI was significantly associated with HCC, whereas carriage of the *BAT* A-T-C and G-T-T haplotypes was significantly more prevalent in patients with alcoholic liver disease and HCC.

Applications

The characterization of *VDR* genetic polymorphisms in patients with liver cirrhosis could help to identify those who are at high risk of developing HCC. This observation could be used to modify the strategy of periodical ultrasound surveillance in this category of patients.

Peer review

In this paper, the relationships between *VDR* gene polymorphisms and the presence of HCC have been evaluated in patients with viral or alcoholic cirrhosis. The most important result of this study is the observation that some *VDR* genetic polymorphisms are associated with the occurrence of HCC in patients with liver cirrhosis. The results are convincing, well presented and of some interest.

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BRIEF ARTICLE

Glycated hemoglobin and antidiabetic strategies as risk factors for hepatocellular carcinoma

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Abstract

AIM: To evaluate the relationship between glycemic control [assessed by glycated hemoglobin (HbA1c)], antidiabetic therapies and the risk of hepatocellular carcinoma (HCC).

METHODS: We recruited 465 patients with HCC, 618 cases with liver cirrhosis and 490 controls with no liver disease. Among subjects with type 2 diabetes mellitus (DM2), the associations between the antidiabetic strategies and HbA1c level with HCC were determined through 2 series of multivariate logistic regression models using cirrhotic patients and controls as comparison groups.

RESULTS: DM2 prevalence was 31.2% in patients with HCC, 23.2% in cirrhotic patients and 12.6% in controls ($P < 0.0001$). In 86% of study subjects, DM2 had been diagnosed for more than 1 year before the HCC diagnosis. HCC patients with DM2 had a 1.5-2.5-fold increased risk of liver cancer. The HbA1c mean levels were significantly higher in DM2 patients with HCC than in cirrhotic and control DM2 patients. Antidiabetic treatment with metformin was more common among cirrhotic and control DM2 subjects than among cases with HCC. In both series of multivariate analyses, treatment with metformin significantly reduced the risk of HCC by more than 80% compared with sulphonylureas and insulin therapy. No significant differences were seen between sulphonylureas and insulin treatment. Elevated HbA1c levels were positively related to the risk for HCC in diabetic patients, with a 26%-50% increase in risk for each 1% increase in HbA1c values.

CONCLUSION: In patients with preexisting DM2, the risk of HCC is positively associated with poor chronic glycemic control and significantly decreased by metformin therapy.

Key words: Hepatocellular carcinoma; Type 2 diabetes mellitus; Glycemic control; Metformin therapy; HbA1c level

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INTRODUCTION

The incidence and mortality rates of hepatocellular carci-

noma (HCC) have significantly increased in recent years, particularly in Western countries[1,2]. The main known risk factors for HCC are hepatitis C and B virus (HCV, HBV) infections and chronic alcohol abuse, but at least 25% of HCC cases do not have any recognized etiology. Diabetes mellitus has been proposed to be associated with HCC development[3], and several investigations in recent years clearly indicated that type 2 diabetes mellitus (DM2) is a risk factor for HCC[1,4-9]. During the past 2 decades, the prevalence of diabetes mellitus, and in particular of DM2, has dramatically increased in many countries, including Italy[10]. Sedentary lifestyles, excessive food consumption and obesity appear to be the main causes of the current diabetes mellitus epidemic[11].

It is unclear how DM2 influences hepatocarcinogenesis. Insulin resistance (IR) is a basic feature of DM2, a disorder characterized by hyperglycemia associated with IR and consequent hyperinsulinemia. Several reports have suggested that the mechanism underlying the effects of DM2 and antidiabetic therapies on hepatic carcinogenesis could be related to IR and hyperinsulinemia[12-16]. In DM2 patients, insulin plasma levels are chronically increased by therapies based on both exogenous insulin or insulin secretagogues, such as sulphonylureas. An excess of insulin plasma levels is the proposed mechanism underlying the well established association of DM2[16-18] and obesity[19] (the so called diabesity) with several types of solid tumors[20,21]. In a previous study, we found that DM2 in our population was an independent risk factor for HCC, and that it precedes HCC development[12]. In addition, in male chronic liver disease (CLD) patients with DM2, the risk of HCC is increased by insulin or sulphonylurea treatment. We also reported that the association between IR and CLD began in the early stages of liver fibrosis, and that DM2 significantly increased IR in HCC patients. Therefore, IR together with the consequent hyperinsulinemia, seems to play a major role in the link between DM2 and hepatocarcinoma[13].

Recent reports have shown that liver carcinogenesis is increased not only in HCV infected patients with previously diagnosed and/or treated DM2[22,23], but also in HCV infected subjects with no pre-existing history of DM2 and in the early stages of glucose intolerance, as diagnosed by a 75 g oral glucose tolerance test[24]. In addition, , a positive association between high dietary glycemic load and risk of HCC in patients with chronic HBV and HCV infection has been reported[25]; the pathogenesis and outcome of cryptogenic HCC seems to be more closely associated with the risk factors for metabolic syndrome than with HBV and HCV[26].

Despite extensive literature linking glucose intolerance with hepatocarcinogenesis, it remains unclear whether blood glucose control may influence the malignant potential that DM2 exerts on the liver. Only one prospective study reported that hyperglycemia was associated with increased total cancer risk in women and men, independently of obesity[27]. As far as we know, the effect of glycemic control on liver carcinogenesis and the role of treatment strategies to achieve the metabolic control in DM2 patients with CLD have not been investigated yet.

Therefore, the aims of our study were to explore the association between HCC and DM2 in CLD subjects, and to assess the relationships between glycemic control, as determined by glycated hemoglobin (HbA1c) measurement, antidiabetic strategies and the risk of HCC in DM2 patients with CLD.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. All of the studies were performed retrospectively by collecting and analyzing data from the patient records. This study had been approved by the Institutional Review Board of our hospital.

Design and case selection

For this retrospective, hospital based case-control study, subjects were recruited from patients attending the 3rd Internal Medicine of Pordenone General Hospital (Pordenone, Italy). The 3rd Internal Medicine is a tertiary referral center for liver diseases and diabetes mellitus. The case group consisted of patients with incident HCC admitted to the hospital. We then recruited two comparison groups: the first consisted of patients admitted for liver cirrhosis (LC); the second consisted of control individuals admitted for a wide spectrum of acute conditions other than diabetes mellitus and liver disease as primary diagnoses.

HCC group

The HCC group consisted of 465 (364 male, 101 female) consecutive patients with newly diagnosed HCC attending the 3rd Internal Medicine from January 1994 to June 2006. Of these, 398 (85.6%) were diagnosed by cytological or histological examination of hepatic focal lesions; the remaining 67 (14.4%) were diagnosed according to the following acknowledged criteria[28]: ultrasound examination (also by using micro-bubbles of sulfur hexafluoride as contrast dye in the last 3 years), α-fetoprotein (AFP) > 400 ng/mL, computerized tomography liver scan and/or magnetic resonance imaging. Based on the type of presentation, the HCC patients were subdivided into two groups: (1) 305 subjects from our surveillance program of HCC in cirrhotic patients (follow-up group: FU); and (2) 160 subjects presenting with symptomatic and advanced neoplastic disease of the liver (clinically overt: CO). The FU group included patients with a small single hepatic tumor who received the diagnosis within the HCC surveillance program in cirrhotic patients based on ultrasound examinations and AFP determinations every 3-6 mo; the CO group included cases with advanced, large size and symptomatic HCC at diagnosis. Clinical data, biochemical parameters and the antidiabetic treatment of patients were considered at the time of HCC diagnosis during the first admission to our Hospital.

In order to gain complete and accurate information on the time interval between DM2 onset and the diagnosis of HCC and to determine the levels of HbA1c testing before the HCC diagnosis, we reviewed, starting from October 1985, all medical documentation stored at the Diabetes Clinic.

Liver cirrhosis group

We enrolled 618 patients (450 male, 168 female) with LC, by selecting from 3560 cirrhotic patients treated at our Department subjects frequency-matched with the HCC cases, according to age (± 5 years), gender, body mass index (BMI), transaminases, serologic markers of HBV and HCV infections, alcohol consumption, time of hospital admission. The diagnosis of cirrhosis was performed either by hepatic biopsy or by ultrasound examination in the fasting state, showing the presence of splenomegaly, hypertrophy of the left or caudal lobes and surface irregularity or by transient elastography using the Fibroscan (Echosens, Paris) with a liver stiffness > 12.5 kPa[29]. LC patients were admitted to our Hospital for diagnosis, staging or therapy of LC. Clinical data, biochemical parameters and antidiabetic treatment were considered at the time of the first admission to our Hospital. According to Child's classification of cirrhosis, patients were classified as follows: class A: 55.5%; B: 24.3% and C: 20.2%. In cirrhotic patients, the presence of HCC was ruled out through ultrasound examinations, computed tomography or magnetic resonance imaging of the upper abdomen and AFP determination.

Control group

We enrolled 490 control subjects (385 male, 105 female) as follows: from 28740 inpatients of our hospital, we generated a pool of 7610 subjects with available tests as possible controls. Then we selected from them a frequency-matched subject for each HCC or LC patient, according to age (± 5 years), gender, BMI, time of hospital admission. Clinical data, biochemical parameters and therapeutic schedule of control subjects were considered at the time of the first admission to our Hospital. Subjects admitted for malignancies, alcohol-related disease, liver disease and DM2 as primary diagnoses were excluded from the study. The primary diagnoses of admission were: chronic heart failure (34.9%), hypertension (21.4%), chronic obstructive broncho-pneumopathies or pneumonia (16.5%), atrial fibrillation (7.8%), deep venous thrombosis (6.5%), fever of unknown origin (5.3%), benign tumors (4.1%), gastritis (3.5%). These subjects were assumed to represent our Region's general population as regards the prevalence of HCV, HBV infection, alcohol consumption and DM2. The prevalence of these parameters[30-33] in the free living population of the Pordenone area with an age range of 60-75 years and a similar age sub-sample of our control group is reported in Table 1.

Table 1 Mean prevalence of HCV infection, HBV infection, alcohol abuse and DM2 in the free living population of the Pordenone area and controls in this study

	General population (%)		Control group of the study (%)
	Global	60-75 yr age interval	
HCV positive	3.2	5.0	5.3
HBV positive	1.2	1.7	1.2
Alcohol abuse	4.5	5.0	4.7
DM2	4.8	12.4	12.6

HCV: Hepatitis C virus; HBV: Hepatitis B virus; DM2: Type 2 diabetes mellitus.

Methods

DM2 was diagnosed using the American Diabetes Association criteria[34]. Biochemical parameters were determined at the Pordenone Hospital central laboratory, using standardized and validated methods. Venous blood samples were taken in the morning after 12 h overnight fasting. Blood samples were available for 460 HCC cases and for all LC patients and controls. HbA1c was measured by high performance liquid chromatography (A1cHA-8160 Menarini, Italy)[35] for all diabetic subjects in our study. The nondiabetic range for our method of HbA1c testing was 4.0%-6.0% using a DCCT (Diabetes Control Complications Trial)-based assay[36]. For each subject we used the mean of 2 samples, taken on 2 consecutive days at the time of enrollment.

To assess the chronic diabetes controls in HCC patients with pre-existing DM2, we evaluated the individual HbA1c values tracked in the time before liver cancer diagnosis. Based on the records of the Diabetes Clinic, we determined the mean HbA1c levels of at least 3 tests/patient carried out approximately every 2 years before HCC clinical occurrence.

HBV surface antigen (HBsAg), anti-HBV surface antigen (anti-HBs), anti-HBV core antigen (anti-HBc), and hepatitis B e antigen (HBeAg) were determined using commercial assays (Abbott Diagnostic Division, Wiesbaden, Germany). Sera were also screened for antibodies against HCV (anti-HCV) using a third-generation microparticle enzyme immunoassay (AxSYM HCV version 3.0, Abbott Diagnostic Division). Positive samples were tested for anti-HCV using a third-generation line immunoassay (Immunogenetics, Gent, Belgium) and for serum HCV-RNA using the Roche Amplicor version 2.0 (Roche Molecular System, Pleasenton, CA, USA).

Information on consumption of alcoholic drinks was collected through a structured questionnaire which was returned by 455 HCC patients and by all LC and control subjects. Average alcohol content was estimated as 5% for beer, 12% for wine and 40% for spirits[37]. Alcohol abuse was defined as a daily consumption of over 30 g for males and over 20 g for females.

Statistical analysis

All the data were collected in a computerized database. Normality of the distributions of all continuous variables was tested by the Shapiro-Wilk test. The Student *t*-test and ANOVA were used to assess the statistical significance of

Table 2 Characteristics of patients in the HCC, LC and control groups *n* (%)

	HCC *n* = 465	LC *n* = 618	Controls *n* = 490	*P*
Sex, male	364 (78.3)	450 (72.8)	385 (78.6)	0.1706
Alcohol abuse	233 (51.2)	312 (50.6)	23 (4.7)	< 0.0001
DM2	145 (31.2)	144 (23.2)	62 (12.6)	< 0.0001
ALT ⩾ 53 IU/L	113 (24.3)	150 (24.3)	N/A	0.9911
HBV positive	39 (8.4)	39 (6.3)	N/A	0.1908
HCV positive	268 (57.6)	285 (46.1)	N/A	0.0002

P-value of χ^2 test. HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; N/A: Not available.

Table 3 Association of DM2 with HCC: results of multivariate analyses using controls and LC subjects as comparison groups

	OR	95% CI	*P*
Control group			
Age ⩾ 65 yr	0.873	0.606-1.259	0.4675
BMI ⩾ 25 kg/m^2	1.048	0.769-1.426	0.7681
Alcohol abuse	22.069	13.749-35.425	< 0.0001
DM2	2.507	1.703-3.692	< 0.0001
LC group			
Age ⩾ 65 yr	2.457	1.855-3.252	< 0.0001
BMI ⩾ 25 kg/m^2	1.560	1.197-2.032	0.0010
HBV positive	2.372	1.363-4.127	0.0022
HCV positive	2.783	1.896-4.084	< 0.0001
Alcohol abuse	1.937	1.318-2.845	0.0008
ALT ⩾ 53 IU/L	0.919	0.672-1.256	0.5951
DM2	1.456	1.072-1.979	0.0162

BMI: Body mass index; OR: Odds ratio; 95% CI: 95% confidence interval; ALT: Alanine transaminase.

differences among groups for continuous variables with normal distribution; the Wilcoxon rank sum and Kruskal-Wallis tests were used for continuous variables with non-normal distribution. The χ^2 test was used to evaluate the significance of differences among groups for categorical variables. Multivariate logistic regression was used to assess the association between HCC and DM2, antidiabetic therapy, and glycemic control after adjusting for potentially confounding factors. Separate models were built considering LC patients and controls as the comparison groups. When assessing the association of DM2 with the risk of HCC using LC patients as the control group, we considered sex, age (⩾ 65 years *vs* < 65 years), BMI (⩾ 25 kg/m^2 *vs* < 25 kg/m^2), HBV and HCV infection, alcohol abuse, alanine transaminase (ALT) level (> 53 IU/L *vs* ⩽ 53 IU/L); when we used control subjects as the comparison group, potential confounders included only sex, age, BMI, and alcohol abuse. When evaluating which DM2 characteristics were associated with risk of HCC among diabetics, covariates included sex, age, BMI, HBV and HCV infection, alcohol abuse, ALT level, triglycerides, cholesterol, type of antidiabetic therapy (metformin, sulphonylureas, insulin), HbA1c levels (continuous and in categories using the following cut-offs: 6.5%, 7.5%, 8.5%), DM2 duration (continuous and using the following 3 cut-offs: 5, 10, 15 years) when we used LC patients as the comparison group and sex, age, BMI, HBV and HCV infection, alcohol abuse, ALT level, triglycerides, cholesterol, type of antidiabetic therapy, HbA1c levels, and DM2 duration when we used the controls. Among HCC cases, HbA1c levels at the time of cancer diagnosis were compared with an average of at least 3 tests carried out approximately every 2-3 years before the diagnosis of HCC. The Wilcoxon signed rank test and Spearman's correlation coefficient (*r*) were used to compare the paired differences and the kappa statistic to evaluate concordance of categories. Since at enrollment DM2 duration was either unknown or < 12 mo duration in 14% of diabetic subjects and 1-2 years duration in an additional 8%, in the study of the association between DM2 and HCC we performed a sensitivity analysis considering as non-diabetic subjects those whose diagnosis of DM2 occurred in the 12, 24, 60, and 120 mo before enrollment to allow for adequate induction time. For the same reason, we performed a sensitivity analysis excluding from the analyses regarding diabetes therapy and glycemic control, those patients with diagnosis of DM2 in the 12, 24, 60, and 120 mo before enrollment. All *P*-values < 0.05 were considered statistically significant. Analyses were conducted with SAS v9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Demographic characteristics of the three groups studied, and exposures relevant to liver diseases are illustrated in Table 2. The prevalence of DM2 was significantly higher ($P < 0.0001$) in HCC patients (31.2%) than in the LC group (23.2%) and in controls (12.6%). Using control subjects as a comparison group, after adjusting for age, BMI and alcohol abuse, DM2 significantly increased the odds ratio (OR) of HCC by 2.5 times (Table 3). Using LC patients, after adjusting for sex, age, BMI, alcohol abuse, HBV and HCV infection, and ALT level, DM2 significantly increased the OR of HCC by 1.5 times (Table 3). Alcohol abuse was the strongest predictor of HCC using controls subjects as comparison; it also increased the risk of HCC, as did HBV and HCV infections by comparing HCC with LC patients.

Table 4 illustrates the levels of BMI, cholesterol, triglycerides, and HbA1c, in HCC, LC and control subjects with DM2. BMI, cholesterol and triglycerides were not different between the three groups of subjects studied. The HbA1c levels evaluated at the time of enrollment in the study were significantly higher in HCC patients (7.5%, with 8.1% ± 1.8% in FU and 6.3% ± 1.1% in CO subgroups $P < 0.0001$) than in controls (6.8%) and in LC patients (6.6%). Among HCC diabetic cases, current HbA1c levels were on average only 0.2 percentage points lower than the average value of 3 previous values that we assumed to be representative of glycemic control in the past years. Current and average past HbA1c levels were highly correlated ($r = 0.77$, $P < 0.0001$) and agreement between categories (using 6.5%, 7.5%, and 8.5% as the cut-offs) was moderate to good, as expressed by the weighted kap-

Table 4 Biochemical characteristics of diabetic HCC, LC patients and controls, DM2 duration and therapy (mean ± SD)

	HCC (n = 145)	LC (n = 144)	Controls (n = 62)	P
BMI (kg/m^2) (median)	25.6 ± 2.9 (26.0)	25.8 ± 3.8 (25.0)	25.1 ± 2.4 (25.3)	0.3175[a]
Cholesterol (mg/dL) (median)	155.4 ± 47.4 (152)	155.9 ± 37.7 (155)	160.6 ± 45.4 (173.0)	0.6013[a]
Triglycerides (mg/dL) (median)	110.6 ± 60.7 (94.5)	108.0 ± 45.5 (101.5)	111.5 ± 67.9 (95.0)	0.8060[a]
Fasting plasma glucose (mg/dL) (median)	126.7 ± 47.3 (110.5)	109.3 ± 32.9 (100.0)	98.0 ± 20.6 (95.0)	< 0.0001[a]
Current HbA1c (%) (median)	7.5 ± 1.8 (7.3)	6.6 ± 1.5 (6.4)	6.8 ± 1.5 (6.5)	0.0001[a]
Average past HbA1c (%) (range)	7.7 ± 1.5 (4-13.5)	N/A	N/A	
DM2 duration (mo) (median)	141.6 ± 81.0 (146)	135.4 ± 98.3 (117)	124.9 ± 100.5 (119)	0.2496[a]
Therapy, n (%)				< 0.0001[b]
Not reported	0 (0)	12 (8.3)	2 (3.2)	
Metformin	13 (9.0)	39 (27.1)	15 (24.2)	
Sulphonylureas	68 (46.9)	33 (22.9)	32 (51.6)	
Insulin	64 (44.1)	60 (41.7)	13 (21.0)	

[a]P of Kruskal-Wallis test; [b]P of Fisher's exact test. HbA1c: Glycated hemoglobin A1c.

pa 0.5456. Mean HbA1c levels were not different among HCC patients with or without HBV, HCV and alcohol abuse (data not shown).

Table 4 also shows the time interval between DM2 diagnosis and enrolment in the study. Duration of diabetes was not significantly different among the three groups of subjects and, on average, it was approximately 12 years. In addition, Table 4 displays the distribution of antidiabetic treatments in the three study groups. Treatment with metformin was less frequent among HCC patients than among the others; sulphonylureas were the most frequently used drugs among controls, whereas insulin was the most common treatment among HCC and LC patients.

At enrolment, DM2 had been diagnosed for more than 1 year in 86% of study subjects. Based on the records of our Diabetic Clinic, the mean duration of insulin treatment in HCC insulin-treated patients was 5 years; before insulin therapy, patients were treated only with diet. The mean period of treatment with the 2 antidiabetic oral agents was 10 years and before oral antidiabetic therapy patients had only dietary therapy.

Among diabetic subjects, the association of HbA1c levels, antidiabetic therapy, and DM2 duration with the risk of HCC is reported in Table 5. Both when using controls and when using LC as the comparison groups, after adjusting for the potential confounders shown in Table 5, therapy with metformin was associated with a strong and statistically significant reduction (> 80%) of the risk of HCC as compared with the use of sulphonylureas or insulin. In our HCC diabetic patients we found a significant 26%-50% increase in the HCC risk for each 1% increase in HbA1c level. Results did not significantly change in the sensitivity analysis after removing or moving from the diabetic to the non-diabetic category subjects whose diagnosis of diabetes occurred < 12, 24, 60, or 120 mo previously (data not shown).

Table 5 Association of antidiabetic therapy, metabolic control and DM2 duration with HCC among diabetic patients: results of multivariate analyses using controls and LC subjects as comparison groups

	OR[1]	95% CI	P
Control group			
Sex, male	1.860	0.636-5.441	0.2574
Age ≥ 65 yr	0.225	0.071-0.715	0.0115
BMI ≥ 25 kg/m^2	0.888	0.364-2.166	0.7936
Alcohol abuse	16.155	4.798-54.389	< 0.0001
Triglycerides (mg/dL, continuous)	1.001	0.994-1.008	0.8097
Cholesterol (mg/dL, continuous)	1.003	0.994-1.013	0.4890
Metformin *vs* sulphonylureas	0.149	0.039-0.507	0.0054
Insulin *vs* sulphonylureas	1.243	0.459-3.366	0.6686
DM2 duration (mo, continuous)	1.001	0.996-1.005	0.6740
HbA1c % (continuous)	1.265	0.943-1.699	0.1172
LC group			
Sex, male	0.120	0.0051-0.278	< 0.0001
Age ≥ 65 yr	1.508	0.719-3.165	0.2774
BMI ≥ 25 kg/m^2	1.473	0.716-3.029	0.2928
HBV+	3.535	0.428-29.184	0.2410
HCV+	2.870	1.129-7.293	0.0267
Alcohol abuse	1.455	0.567-3.732	0.4351
ALT ≥ 53 IU/L	0.808	0.394-1.675	0.5606
Triglycerides (mg/dL, continuous)	1.002	0.995-1.008	0.6366
Cholesterol (mg/dL, continuous)	1.003	0.995-1.011	0.4012
Metformin *vs* sulphonylureas	0.163	0.057-0.462	0.0006
Insulin *vs* sulphonylureas	0.428	0.203-0.901	0.0255
DM2 duration (mo, continuous)	1.001	0.997-1.005	0.6723
HbA1c % (continuous)	1.508	1.197-1.899	0.0005

[1]Patients with unknown therapy not included in analysis.

DISCUSSION

The results of our study show that chronic poor glycemic control, evaluated by HbA1c testing, is positively associated with risk of HCC in diabetic patients. In addition, therapy with metformin was associated with a strong and statistically significant reduction in the risk of HCC as compared with the use of sulphonylureas and insulin therapy.

Several studies have reported the relationship between DM2 and carcinogenesis in the liver[4-9], but, as far as we know, there are no investigations on the relationship between glycemic control and HCC risk in DM2 patients. In our study, we found that the prevalence of DM2 patients was significantly higher in HCC patients whereas in the LC group it was intermediate between those of HCC cases and controls. After adjusting for potential con-

founders, in our HCC patients DM2 was associated with an increased risk of HCC. The HbA1c mean levels were significantly higher in diabetic HCC patients than in LC and controls but not different between HCC patients with or without HBV and HCV infection or alcohol abuse. In HCC patients (above all in the FU subgroup), the HbA1c values were significantly higher and the mean levels during previous years of diabetic life were similar to those recognized at HCC occurrence. Multivariate analysis showed that high levels of HbA1c among diabetic HCC patients were associated with a significantly increased risk of liver cancer.

Because of IR, patients with DM2 have a long-term exposure to increased circulating insulin levels. It is well known that insulin stimulates cellular mitosis by direct action[14], and indirectly by stimulating the insulin-like growth factor-1 intracellular pathway, a major mitogenic and anti-apoptotic effector in carcinogenesis[15]. Sulphonylurea treatment increases insulin secretion and its circulating levels: this effect is detrimental in terms of weight gain and insulin levels[38]. In contrast, metformin treatment can ameliorate IR and reduce weight gain. The ability of metformin to reduce the insulin plasma levels and to activate cellular AMP-activated protein kinase (AMPK) represents, respectively, its direct and indirect proposed anti-oncogenic mechanisms. In fact, metformin not only lowers blood glucose and insulin levels but, through AMPK activation, also attenuates the *in vitro* response of cancer cells to insulin[39,40].

Several *in vivo* studies showed that cancer risk was lower in patients exposed to metformin than in unexposed patients[41,42]. Metformin has also been shown to be potentially beneficial in patients with specific types of cancer. For example, DM2 patients receiving neoadjuvant chemotherapy for breast cancer as well as metformin were more likely to have a complete remission than patients not receiving metformin[43]. In 2 studies patients receiving metformin seemed to have a lower incidence of prostate and pancreas cancer[44,45]. Furthermore, in the ZODIAC study[46], metformin use was associated with lower cancer mortality when compared to non metformin use.

In diabetic patients, good glycemic control prevents the onset and progression of acute and long-term diabetes-related complications[47]; however, it is presently unknown if poor metabolic control of diabetes could enhance or accelerate liver carcinogenesis in patients with CLD and DM2. Furthermore, it is not known whether an optimized control of diabetes can reduce the risk or delay the development of liver cancer. In the European Prospective Investigation into Cancer Study, a 1% increase in HbA1c levels was associated with a 1.3-fold increase in the risk of colorectal cancer[48]; recently it was reported that poor glycemic control is a predictor of clinically aggressive course for the colorectal cancer[49]. HbA1c reflects average glycemia over 3 mo; in our HCC patients, particularly in FU cases, the mean levels of HbA1c were above the recommended target level of 7%; HbA1c was significantly higher in HCC than in controls and LC patients, in which we found mean levels below 7%. Nonetheless it must be emphasized that the HbA1c levels observed in the three groups of subjects in this study were consistent with those seen in the vast majority of Italian diabetic patients[10].

It is known that diabetes is associated with more advanced lesions and poor outcomes in patient with HCC[50] but the observation that DM2 precedes the diagnosis of HCC in the majority of our cases, suggests that glucose intolerance and glycemic control are an outstanding feature of the hepatic tumor that may not always be related to the liver cancer.

This was a retrospective, hospital-based study drawn from a clinical series and not from the community: therefore the results may not be representative of those of the general population. There is no evidence that successful treatment for glucose intolerance can reduce hepatic carcinogenesis: therefore only a prospective study is required to demonstrate that optimized glycemic control can modify the risk of HCC in DM2 patients with CLD. However, this is the first study on the relationship between different antidiabetic strategies and the presence of HCC. In our diabetic HCC patients, HbA1c test results are available for years before HCC diagnosis, so a reliable assessment of prior glycemic control was possible. Our study was a single center investigation and all patients of the three groups studied were directly diagnosed and followed-up by us; all biochemical parameters were determined in a single laboratory. The study population was large. The controls, even though recruited from inpatients, were representative of the general population of our Region as regards the prevalence of HCV and HBV infection, alcohol consumption and diabetes mellitus.

To distinguish the temporal relationship between exposure and outcomes, due to the complex and reciprocal relationships between DM2, LC and HCC, we conducted a study on a large group of HCC patients comparing them with both a control group with no liver diseases and a group with LC, that represents the majority of patients with the clinical underlying cause of HCC. To have complete and accurate information on the time interval between the onset of DM2 and the diagnosis of HCC, we reviewed all medical documentation kept, from a period of 9 years before the enrollment of the first patient in the study, at our Diabetes Clinic. All our diabetic patients were on monotherapy of antidiabetic drugs and the selection of the antidiabetic therapy was based on the physician's clinical choice. In particular, in HCC and LC groups, diabetic patients treated with different antidiabetic oral agents had similar basic features as regards alcohol consumption, and hepatic and renal function. To make sure that a temporal relationship existed between diabetes and HCC, a sensitivity analysis was conducted excluding or reclassifying patients with recent diabetes diagnosis, but the results did not change.

Our study confirms that DM2 is associated with an increased risk of HCC. It also shows that in diabetic HCC patients metformin treatment is associated with a strong and statistically significant reduction (> 80%) in the risk of HCC compared with the use of sulphonylureas or

insulin. In diabetic patients with CLD, chronic poor glycemic control, evaluated by HbA1c testing, significantly increases the risk of HCC by 26%-50% for each 1% increase in HbA1c level. It has to be emphasized that in diabetic patients with CLD, it is important not only to attain an optimized glycemic control in order to prevent HCC, but also antidiabetic strategies may have an important effect on the relationship between DM2 and solid tumors.

COMMENTS

Background

In recent years, several investigations clearly indicated that type 2 diabetes mellitus (DM2) is a risk factor for hepatocellular carcinoma (HCC). The aims of the present study were to explore this association and to assess the relationships of antidiabetic therapy and glycemic control with the risk of HCC in DM2 patients with chronic liver disease.

Research frontiers

At the present time the relationship between DM2 and solid cancers is under intensive investigation. It remains unknown if DM2 has a direct carcinogenic effect on the liver and on other parts of the human body.

Innovations and breakthroughs

This study found that DM2 precedes HCC in the majority of patients. The cancer risk seems to be increased by chronic poor glycemic control. Metformin treatment is associated with a strong and statistically significant reduction (> 80%) in the risk of HCC compared with the use of sulphonylureas or insulin.

Applications

The study indicated that in diabetic patients with chronic liver disease it is important not only to attain an optimized glycemic control in order to prevent HCC, but also that antidiabetic strategies may have an important effect on the association between DM2 and solid tumors.

Peer review

This is a potentially important paper that continues to evaluate the link between diabetes and the risk of HCC. Its strength is in its numbers despite being from one institution.

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BRIEF ARTICLE

Helicobacter pylori in dental plaque and stomach of patients from Northern Brazil

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Abstract

AIM: To establish whether virulence factor genes *vacA* and *cagA* are present in *Helicobacter pylori* (*H. pylori*) retrieved from gastric mucosa and dental plaque in patients with dyspepsia.

METHODS: Cumulative dental plaque specimens and gastric biopsies were submitted to histological examination, rapid urease test and polymerase chain reaction (PCR) assays to detect the presence of *cagA* and *vacA* polymorphisms.

RESULTS: Detection of *H. pylori* from dental plaque and gastric biopsy samples was greater by PCR compared to histological examination and the rapid urease test. DNA from *H. pylori* was detected in 96% of gastric mucosa samples and in 72% of dental plaque samples. Sixty-three (89%) of 71 dental plaque samples that were *H. pylori*-positive also exhibited identical *vacA* and *cagA* genotypes in gastric mucosa. The most common genotype was *vacAs1bm1* and *cagA* positive, either in dental plaque or gastric mucosa. These virulent *H. pylori* isolates were involved in the severity of clinical outcome.

CONCLUSION: These pathogenic strains were found simultaneously in dental plaque and gastric mucosa, which suggests that gastric infection is correlated with the presence of *H. pylori* in the mouth.

Key words: *Helicobacter pylori*; Gastric mucosa; Dental plaque; *cagA*; *vacA*

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INTRODUCTION

Helicobacter pylori (*H. pylori*) gastric infection is considered one of the most common human infections[1]. It occurs in half of the world's population[2,3] and is the most common cause of adenocarcinoma of the distal stomach[4].

The risk in developing gastric cancer is believed to be related to differences among *H. pylori* strains and the inflammatory responses mediated by host genetic factors[3]. Therefore, it is presumed that severe gastric diseases are seen more often in patients who have been chronically infected with *H. pylori* isolates that bear both the *cagA* and *vacA* s1/m1 genes[1,5,6].

H. pylori is known to be acquired early in life[7,8], and is likely to be transmitted from person to person. The transmission is supported by crowded living conditions, accompanied by poor hygiene and intra-familial clustering. Nevertheless, the exact manner of transmission is not completely understood[4,9,10].

The accepted evidence is that the *H. pylori* strains reach the stomach by ingestion through the mouth, and because of its non-invasive nature, the stomach is the definitive site for colonization[10]. Evidence of transmission *via* the oral route includes the high prevalence of *H. pylori* among African children whose mothers premasticate their food before offering it to them[11], and to the higher prevalence of the infection in people who share chopsticks[12].

The presence of *H. pylori* in the oral cavity was first reported in 1989 when the bacterium was cultured from dental plaque of a patient with gastric disease associated with *H. pylori* infection[13].

Dental plaque is a biofilm that is formed by a microbial community of multiple species and represents a strategy to allow survival and evolution of microbial co-aggregation in a dynamic equilibrium, in a favorable environment with selective advantages[14,15]. Hence, bacterial biofilms are considered as sanctuaries, due to being protected from host defense mechanisms and antibiotic therapy. Additionally, antibiotic resistance is increased due to horizontal gene transfer[14,15].

The purpose of this study was to evaluate the prevalence of *H. pylori* isolates, in relation to *vacA* and *cagA* genotypes, in dental plaque and gastric antral biopsy samples in dyspeptic patients from a North Brazilian population.

MATERIALS AND METHODS

Patients and sampling

We studied the presence of *H. pylori* in the stomach and dental plaque in 99 adult patients (69 women and 30 men; mean age: 37 years, age range: 17-59), who underwent upper gastrointestinal endoscopy due to gastric problems. Most of them were of lower socioeconomic status and lived in the state of Pará, Brazil.

None of the patients had received antimicrobial drugs, H2-receptor antagonists, acid pump inhibitors, non-steroidal anti-inflammatory drugs, or any medication for at least 60 d before sampling, to avoid interference with *H. pylori* detection methods. During endoscopy, three antral gastric biopsy sections were taken from the stomach of each patient. One biopsy was analyzed by molecular methods, and the other sections were also analyzed with histological methods and the rapid urease test. Before endoscopic examination, cumulative dental plaque samples were collected by scraping tooth surfaces with sterile curettes and transferred into tubes that contained physiological saline solution. Dental plaque samples were frozen immediately and stored at -20℃ until required for DNA extraction. This study was approved by the Ethics Committee of Hospital Universitário João de Barros Barreto, Belém, PA, Brazil. All patients gave their informed consent to participate in the study.

Histological evaluation

The biopsy specimens were fixed in 10% buffered formalin solution, embedded in paraffin, cut into sequential 0.4-μm sections, and stained with hematoxylin and eosin (H&E). The presence of *H. pylori* in the sections was determined by using a modified Gram staining protocol and taking into consideration its morphological characteristics: curved and spiral form and intense purple coloring. The histological parameters were graded (0-3) using the criteria described in the update Sydney classification system[16] for analysis of chronic inflammation, polymorphonuclear activity and intestinal metaplasia.

H. pylori detection by the rapid urease test in the stomach and dental plaque

Additional fresh biopsies as well as dental plaque samples were placed in the gel of the CLOtest as recommended by the manufacturer (Uretest; Renylab, Barbacena, MG, Brazil). After 1 h, the CLOtest was inspected for a change in color. Yellow was interpreted as negative, indicating absence of urease. Red or pink was interpreted as positive. For comparison, a similar reading of each biopsy was also performed after 3 and 24 h. Results of the rapid urease test were compared with polymerase chain reaction (PCR) results and histological examination.

DNA isolation

Total DNA was extracted from frozen gastric biopsy and dental plaque specimens using the following procedure[17]: 10 μL proteinase K (Amresco, Cleveland, OH, USA) and 300 μL lysis buffer (200 mmol/L Tris-HCl, 25 mmol/L EDTA, 300 mmol/L NaCl, 1.2% SDS) were added to the biopsy or dental plaque pellets specimens. The mixture was incubated at 55℃ for 12 h. The lysate was extracted with an equal volume of phenol-chloroform, precipitated with isopropanol, and washed with 70% ethanol. The DNA pellet of each sample was dried and resuspended in 200 mL sterile distilled water. DNA extracts were stored at -20℃. Extensive care was taken to avoid contamination during all steps of collecting and preparing the samples.

PCR amplification and detection of amplified DNA products

PCR amplification for detection of *H. pylori* DNA in dental plaque and gastric mucosa was performed as described previously[18]. Briefly, one set of primers (p1-F and p2-R) that amplifies a 26-kDa antigen gene (fragment of 298 bp) present in all strains of *H. pylori* was used to detect bacteria[18]. The previously described F1-F and B1-R primers[19] were used to detect *cagA* gene. The amplification of *vacA* gene was performed by PCR with oligonucleotide primers described by Atherton *et al*[1], *vacA* signal (*vacA s1a, s1b* or *s2,* primers SS1- F, SS3-F, SS2-F/VA1-R, respectively) sequences and middle regions (*vacA m1* or *m2,* primers VA3-F/VA3-R and VA4-F/VA4-R).

All PCR mixtures were prepared in a volume of 25 μL that contained 0.5 nmol/L each primer; 1 × PCR buffer; 1.5 nmol/L $MgCl_2$, sterilized water, 0.2 nmol/L deoxynucleoside, 1.25 μL *Taq* DNA polymerase Platinum (Invitrogen Life Technologies, São Paulo, Brazil), and 2 μL DNA sample. The mixtures were placed in a thermal cycler. PCR amplification was performed under the following conditions: initial denaturation at 95℃ for 2 min, followed by 35 cycles of denaturation at 95℃ for 1 min, annealing and extension for 1 min, and final extension at 72℃ for 10 min. Annealing temperatures were set at 58℃ for primers VA3-F/VA3-R, VA4-F/VA4-R, F1/B1, and at 63℃ for SS1- F, SS3-F, SS2-F/VA1-R. Negative (sterile water) and positive (positive strain) controls were used in all reactions. PCR products were visualized by electrophoresis in 2% agarose gel, stained with ethidium bromide, and examined under UV illumination.

Statistical analysis

Data were analyzed with Biostat 4.0 version software[20]. Mann-Whitney and G tests were used to assess the association between *H. pylori* isolates in dental plaque and stomach and the distribution of *vacA* and *cagA* genotypes in both these specimens. Differences were considered statistically significant for P values less than 0.05.

RESULTS

Identification by PCR of *H. pylori* DNA in gastric biopsies and dental plaque

H. pylori was found in gastric biopsies of 96% (95/99) of the patients and the detected frequency of this organism in dental plaque was 72% (71/99). Both were detected by PCR, but none of the four patients that lacked *H. pylori* in the stomach had this organism in dental plaque. This difference was statistically significant, which indicated that the detection frequency of *H. pylori* in dental plaque was lower than in the stomach ($P < 0.001$).

Relationship of *H. pylori* DNA in dental plaque and stomach with age and sex

In the relative distributions of age and sex of the patients studied, no significant difference was observed between the groups with or without positive DNA for *H. pylori* simultaneously in the gastric mucosa and dental plaque. Nine of 30 (30%) men and 16 of 69 (27.5%) women were *H. pylori*-negative in dental plaque. Among these, 19 of 62 individuals belonged to the 17-39 years age group and eight of 33 to the 40-59 years group. Furthermore, three individuals in the first group and one in the second were *H. pylori*-negative in dental plaque and gastric mucosa.

Relationship of *H. pylori* DNA in dental plaque and stomach with clinical and histopathological parameters

All patients presented an inflammatory process in the gastric mucosa, with a diagnosis of functional dyspepsia. Ulcers or premalignant lesions, such as intestinal metaplasia, which were considered as lesions of higher severity, occurred in 17% (17/99) of the patients. Among these, 82.4% of cases harbored *H. pylori* in the stomach and dental plaque.

The genotypic frequencies of *vacA* and *cagA* of the positive *H. pylori* samples in the gastric biopsies and dental plaque DNA samples in relation to the endoscopic clinical diagnosis and histopathological findings are shown in Table 1. The *vacAs1m1/cagA*-positive genotype was found in 51 of 95 *H. pylori* gastric biopsies, which comprised 44 gastritis specimens and seven peptic ulcer samples, with 46 patients being *H. pylori*-positive in dental plaque DNA samples.

The presence of *H. pylori* mixed strains with *vacA s1*, *s2*, *m1*, *m2* and *cagA*-positive multiple genotypes was detected in 12 of 95 gastric biopsies of patients with gastritis and their corresponding dental plaque DNA samples.

The *vacAs2m2/cagA*-positive genotype was observed in four of 95 *H. pylori* gastric biopsies, which included one patient with peptic ulcer and three with chronic gastritis. The *vacAs2m2/cagA*-negative genotype was found in 28 of 95 *H. pylori* isolates from 27 gastritis specimens and one peptic ulcer sample, with 13 patients being simultaneously *H. pylori* positive in dental plaque DNA samples.

When comparing all these genotypes, there was 66% (63/95) agreement between *H. pylori* biopsy isolates and their corresponding dental plaque DNA samples. In addition, seven of nine patients with peptic ulcer and eight of 86 with chronic gastritis and diagnosed with intestinal metaplasia or gastric atrophy expressed *H. pylori* isolated with *vacAs1m1*- and *cagA*-positive genotypes, which indicated more severe gastritis and peptic ulceration (Table 1). In the same way, in relation to the histopathological data of these patients, a significant association has been observed between the presence of a bacterial virulence genotype and the high levels of inflammation and the neutrophilic activity, as well as an increased risk of developing atrophy and intestinal metaplasia in the gastric mucosa.

Detection of *H. pylori* in dental plaque and gastric biopsies by different diagnostic tests

In addition to the evaluation of *H. pylori* by PCR assay, bacteria in the stomach were also detected by the histological examination and the rapid urease test. However, the analysis of urease test indicated gastric colonization by *H. pylori* in only 49% of the patients. Similarly, evidence of *H. pylori* was detected in 52% of dental plaque speci-

Table 1 Frequency of *vacA* and *cagA* genotypes in patients positive for *H. pylori* DNA in dental plaque and stomach

H. pylori genotypes					Histopathological parameters[a]						Endoscopic diagnosis[b]		Total
Stomach		Dental plaque			DI		NA		IM	*H. pylori*	PU	G (+H)	
vacA	*cagA*	C	D	N	1	2/3	1	2/3	+	+			
s1m1	+	42	4	5	21	30	26	25	8	22	7	34/(10)	51
s1m1, s2m2	+	12	-	-	5	7	6	6	3	5	-	10/(2)	12
s2m2	+	-	-	4	2	2	2	2	1	1	1	2/(1)	4
s2m2	-	9	4	15	25	3	22	6	-	11	1	22/(5)	28
Total		63	8	24	53	42	56	39	12	39	9	72/(14)	95

DI: Degree of inflammation; NA: Neutrophilic activity (histopathological scores: 1, light; 2, moderate; 3, intense); IM: Intestinal metaplasia; *H. pylori*: *Helicobacter pylori*; G: Gastritis; H: Hiatus hernia; PU: Peptic ulcer; N: Polymerase chain reaction (PCR) negative for *H. pylori* in dental plaque; C: Identical genotypes in the stomach and dental plaque; D: Distinct genotypes in the stomach and dental plaque. [a]G tests (Yates) = 16.83, $P < 0.001$ (DI: *s1m1 cagA* + *x s2m2 cagA*-); G tests (Yates) = 4.86, $P = 0.027$ (NA: *s1m1 cagA* + *x s2m2 cagA*-); [b]G tests (Yates) = 4.36, $P = 0.037$ (disease: *s1m1 cagA* + *x s2m2 cagA*-).

Table 2 Results of the different diagnostic tests for *H. pylori* detection in gastric biopsies and dental plaque samples *n* (%)

Techniques used in the specimens	*H. pylori*		Total
	Positive	Negative	
Dental plaque[a]			
PCR	71 (72)	28 (28)	99
Urease	48 (52)	45 (48)	93
Stomach[b]			
PCR	95 (96)	4 (4)	99
Histological[1]	39 (48)	43 (52)	82
Urease	47 (49)	49 (51)	96

[1]31 of 39 were also urease positive; [a]G tests (Yates) = 7.39, $P = 0.006$; [b]G tests (Williams) = 76.82, $P = 0.0001$ (PCR/Histological/Urease); G tests (Yates) = 57.14, $P = 0.0001$ (PCR/Histological); G tests (Yates) = 58.48, $P = 0.001$ (PCR/Urease); G tests (Yates) = 0.001, $P = 0.97$ (Urease/Histological).

Table 3 Comparison of *H. pylori* isolates in the stomach and dental plaque, by PCR assay *n* (%)

Genotypes		Stomach	Dental plaque
vacA	*cagA*		
s1am1	+	16 (17)	16 (22)
s1bm1	+	35 (37)	26 (37)
s2m2	+	4 (4)	-
s1bm1/s2m2 (mixed infection)	+	12 (13)	16 (22)
s2m2	-	28 (29)	9 (13)
s1bm2/s2m2 (mixed infection)	-	-	4 (6)
		95 (100)	71 (100)

Mann-Whitney test: $U = 15.00$, $Z(U) = 0.48$, $P = 0.63$.

mens, using the rapid urease test (Table 2). There were significant differences among *H. pylori* positivity detected by PCR and the other methods used ($P < 0.001$).

Comparison of *H. pylori* vacA and cagA genotypes in dental plaque and gastric mucosa samples

Overall, 63 (89%) of 71 dental plaque samples, which were positive for the presence of the bacteria, revealed identical *H pylori* virulence factors in relation to *vacA* and *cagA* genotypes in the gastric mucosa. *H. pylori* isolates found in 71% (67/95) of gastric mucosa specimens were *cagA*-positive and 82% (58/71) were also *cagA*-positive for dental plaque, with significant correlations among the prevalence of *H. pylori cagA*-positive isolates in the stomach and dental plaque samples (Table 3).

In relation to the *vacA* gene, the *s1m1* genotype had a higher frequency, with 54% (51/95) of gastric biopsies and 59% (42/71) of dental plaque samples However, the *vacAs2m2* subtype combination was found among the patients that harbored *H. pylori* in 34% (32/95) of the stomach and in 13% (9/71) of the dental plaque samples, whereas *vacAs1bm1/s2m2* genotypes were detected in 13% (12/95) of gastric biopsies and 22% (16/71) of dental plaque samples (Table 3). The most common genotype was *vacAs1bm1* and *cagA*-positive, either in dental plaque (37%) or gastric mucosa (37%).

DISCUSSION

H. pylori infections of the stomach are common worldwide. These bacteria are also found in dental plaque, where the presence of this organism may be very low and the numbers of microorganisms appear to vary from one site to another within the mouth[21].

The present study detected DNA from *H. pylori* in 95% of clinical gastric biopsy samples. Likewise, as described in previous studies[6,22,23], the occurrence is very high in this community. In fact, *H. pylori* infection is influenced by precarious sanitation and low socioeconomic level, so that the prevalence of *H. pylori* infected subjects in Brazil can reach over 80%[24,25]. Furthermore, 72% of subjects with stomach infection showed a concomitant presence of *H. pylori* in dental plaque by PCR assay.

The high detection rate for *H. pylori* DNA in the dental plaque is sufficiently significant to support the assumption that *H. pylori* reaches the oral cavity by reflux[21], assisted by the finding that no positive case was found in dental plaque that was negative in gastric mucosa. This result is especially important because, in our sample, only untreated patients were selected, since treatment would likely eradicate the bacteria from the stomach but rarely from dental plaque, due to known resistance to systemic therapy in dental plaque, as has been reported by Gurbuz *et al*[26].

Similar results have been reported by Banatvala *et al*[13], who have verified a 63% correlation between *H. pylori* in the stomach and in dental plaque samples in patients from London. Also, Song *et al*[27] have found *H. pylori* in 97% (41/42) of dental plaque samples obtained in a German population.

Other studies have suggested a positive association between dental plaque and gastric *H. pylori*[28-32], but some others have failed to find *H. pylori* in oral samples[33-35]. In support of the former studies, our results indicate that the frequency of *H. pylori* in dental plaque is related to the existence of gastric infection.

In the patients studied, no significant difference was observed in the rate of *H. pylori* DNA in dental plaque and gastric mucosa of the diverse age groups. These results are similar to the infection patterns in developing countries[7,36]. A similar result was found in relation to sex, which indicated that there was no relation between sex ratio and *H. pylori* infection in the stomach and dental plaque DNA samples. However, Berroteran *et al*[34] have detected a higher level of *H. pylori* in gastric samples from women than from men, but in dental plaque, this difference was not observed.

On the other hand, these data indicate that, in the strains that harbor *vacAs1m1, cagA* genotypes are involved in the severity of gastritis and the final outcome of *H. pylori* infection. In addition, *H. pylori* isolates with *cagA* and *vacAs1m1* genotype are associated with an increased risk of developing distal gastric adenocarcinoma[37].

In the present study, different methods were used for detecting *H. pylori* in dental plaque and gastric mucosa. In this regard, the PCR assay has been demonstrated to be more efficient for the detection of *H. pylori* in clinical specimens, compared to the rapid urease test in dental plaque and gastric biopsies, as well as to the histopathological examination of the gastric mucosa. This result is supported by other studies[27,30,34,38].

Dönmez-Altuntaş and Güven[39] have detected higher specificity and sensitivity of the PCR assay in relation to the urease test, with 93.8% *vs* 65.6% of DNA from *H. pylori* being positive in both tests respectively. Zsikla *et al*[40] have reported that *H. pylori* DNA could be detected by PCR in 20.8% of biopsies with chronic gastritis but without histologically detectable bacteria. These results agree directly with our findings. Despite this, one of the critical points of the studies using PCR is the absence of uniformity of results, due to the establishment of distinct laboratory procedures[27,41-43]. Controlled trials with robust methodology are required to prove if there is any clinical or epidemiological relevance to the colonization of this bacterium in the oral cavity.

The molecular characterization of the *H. pylori* virulence factors, *cagA* and *vacA*, has revealed the existence of indistinguishable genotypes from oral cavity and gastric mucosa samples of the same patient[44]. Our results showed that, in 89% of the 71 cases in which the dental plaque samples were positive for bacteria, the vacA and cagA genotypes were similar to those in the gastric mucosa (Table 3). Thus, these data suggest that similar types of *H. pylori* isolates, in relation to *vacA* and *cagA* genotypes, are present in the mouth and stomach.

We found that the most common genotype was positive for *vacAs1bm1* and *cagA*, either in dental plaque or gastric mucosa (Table 3). In addition, the *H. pylori* isolates that were *vacAs1m1*/*cagA*-positive were detected in dental plaque at a higher percentage than the *vacAs2m2*/*cagA*-negative genotype. It seems that *H. pylori* in dental plaque is related more to severe gastrointestinal diseases, such as gastroesophageal reflux, which could facilitate oral colonization by *H. pylori* with a *vacAs1m1*/*cagA*-positive genotype.

In this context, several investigators have reported that reinfection is caused by the strains in the oral cavity, because they have isolated identical strains of *H. pylori* in the oral cavity and stomach[32,45-49]. Therefore, it seems necessary to apply additional molecular tests in the dental plaque to assist the diagnosis and therapy of gastric infection.

Finally, based on our findings we cannot exclude the possibility that transient colonization of the mouth with *H. pylori* may occur by reflux activities within symptomatic patients, which leads to the spread these organisms by direct transmission from person to person. In this way, it is a risk factor for the occurrence of diseases in the oral cavity and the stomach.

COMMENTS

Background

Infection by *Helicobacter pylori* (*H. pylori*) remains one of the most prevalent chronic bacterial diseases worldwide, with a distribution related to the degree of economic development in each country. The organism is transmitted from person to person, and some researchers have suggested that the oral cavity is an important reservoir of *H. pylori* besides the stomach. Although the oral cavity may be a source of transmission, it is unknown whether the mouth could be a common source for reinfection of the stomach after treatment.

Research frontiers

The variability of clinical manifestations in the population could be related to an interaction between the bacterial virulence factors and genetic susceptibility of the host. *H. pylori* strains that have *cagA*-positive and *vacAs1/m1* genotypes are associated with an intense inflammatory response in the stomach, which leads to peptic ulcer and other severe gastric diseases. For this reason, there is an increasing interest in generating different non-invasive diagnostic tests that allow the epidemiological and clinical study of *H. pylori* infection.

Innovations and breakthroughs

In this study, dental plaque and gastric biopsies were submitted to rapid urease test and polymerase chain reaction (PCR) assays to detect *H. pylori* and the virulence factors, *cagA* and *vacA* genotypes, respectively. In addition, gastric biopsies were evaluated by histological techniques. It was found a high correlation between the most pathogenic strains (*vacAs1m1/cagA*-positive) in dental plaque and gastric mucosa. It seems that the severity of gastrointestinal diseases, such as gastroesophageal reflux, could be facilitated by oral colonization by *H. pylori*.

Applications

The simultaneous presence of the *H. pylori* isolates with similar *cagA* and *vacA* genotypes in dental plaque and gastric mucosa was detected in a high percentage in patients with chronic gastritis and peptic ulcer, which suggests the necessity to apply additional molecular tests to dental plaque to assist with the diagnosis and therapy of gastric infection. This will add to the available knowledge about the pathogenetic relevance of *H. pylori* in the oral cavity.

Terminology

Bacterial virulence factors are expressed by the vacuolating cytotoxin gene (*vacA*) and cytotoxin-associated gene (*cagA*).

Peer review

The authors studied by means of PCR whether *H. pylori*-positive subjects also had the bacterium in the dental plaque. They found that the vast majority of patients with stomach colonization by *H. pylori* also had oral bacterial infection, thus lending further support to the concept that oral to oral transmission of *H. pylori* is highly possible. The manuscript is well written.

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BRIEF ARTICLE

Predisposing factors and surgical outcome of complicated liver hydatid cysts

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Abstract

AIM: To evaluate the predisposing factors for peritoneal perforation and intrabiliary rupture and the effects of these complications on surgical outcome in liver hydatid disease.

METHODS: A total of 372 patients with liver hydatid cysts who had undergone surgical treatment were evaluated retrospectively. Twenty eight patients with peritoneal perforation, 93 patients with spontaneous intrabiliary perforation, and 251 patients with noncomplicated hydatid cysts were treated in our clinics.

RESULTS: When the predisposing factors for complications were evaluated, younger age, superficial position, and larger cyst dimensions ($P < 0.05$; range, 0.001-0.017) increased peritoneal perforation rates. It was shown that older age increased cyst dimensions, and presence of multiple and bilobar cysts increased intrabiliary rupture rates ($P < 0.05$; range, 0.001-0.028). Partial pericystectomy and drainage was the most frequent surgical procedure in all groups (71.6%). The incidence of postoperative complications in the peritoneal perforated group, in the intrabiliary ruptured group, and in the noncomplicated group was 25%, 16.1% and 5.5%, respectively. When compared, complication rates were significantly different ($P = 0.002$). When length of hospital stay was compared, there was no significant difference between the groups ($P > 0.05$). The overall recurrence rate was 3.8% (14 patients), but there was not any statistical difference among the patient groups ($P = 0.13$). The early postoperative mortality rate was 1.1%.

CONCLUSION: In peritoneally perforated and intrabiliary ruptured cases, the most important steps are irrigation of the peritoneal cavity and clearance of the cystic material from the biliary tree.

Key words: Complicated liver hydatid cysts; Predisposing factors; Surgical treatment; Surgical outcome

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INTRODUCTION

Echinococcosis is a zoonosis transmitted by dogs that

accidentally infects humans. Both sheep and humans are intermediate hosts. Developing countries with poor hygiene where sheep and cattle are raised are high-risk areas for acquiring cystic echinococcosis. Endemic regions of human cystic disease include South America; the Mediterranean region, including North Africa, Spain, Portugal, and Turkey; the Middle East; central Asia; and many regions in China[1,2]. The prevalence of the disease increased in Europe and North America in recent years. In the United States and Europe, most infections are seen in immigrants from endemic areas[3].

In humans, 50% to 75% of hydatid cysts occur in the liver, 25% are found in the lungs, and 5% to 10% are distributed along the arterial system[4]. The majority of the patients have single-organ involvement (87%) and a solitary cyst (72%) at the right part of the liver[5]. If the cyst ruptures, scolices can grow in the peritoneum, pleura, bronchial tree, and bile ducts[5-7]. Cyst sizes range from 1 to 30 cm, and cyst growth varies from 1 to 30 mm per year[6,8].

The clinical picture can be severe, and complications may occur, making the already difficult treatment even more so[9,10]. Complications are observed in one third of patients with liver hydatid cysts. In large series, incidences of peritoneal perforation were reported at between 10% and 16%[11,12], and communications between the cyst and the biliary tree are seen in 9% to 25% of cases[13-15]. Without treatment, cysts grow and eventually may form fistulas into the peritoneal cavity or intrabiliary rupture, requiring emergency surgery[2,9,10]. Operative treatments vary from complete resection to minimal invasive procedures, but the ideal treatment is still controversial[16,17]. The choice of therapy depends on several factors: number and localization of the cysts, surgeon expertise, and presence of complications.

Only a few studies have reported the management and outcome of patients with complicated liver hydatid disease[13,18]. Surgery is the mainstay of treatment, although there is no consensus on the respective advantages of conservative and radical methods. Besides, in the treatment of peritoneal perforated cases, profuse peritoneal lavage with hypertonic solutions appears to be more necessary; in the setting of intrabiliary rupture, T-tube drainage, sphincteroplasty, and choledochoduodenostomy are appropriate treatment strategies to reduce the pressure in the biliary tree. In this study, we aimed to evaluate the predisposing factors for peritoneal perforation and intrabiliary rupture and the effects of these complications on morbidity, length of hospital stay (LOS), mortality, and recurrence in hydatid disease.

MATERIALS AND METHODS

Patients

We retrospectively evaluated medical records of 372 patients with liver hydatid cysts who were admitted to the Department of General Surgery, Faculty of Medicine, Erciyes University, between June 1990 and January 2008. In this period, 28 patients with peritoneal perforation (group Ⅰ), 93 patients with spontaneous intrabiliary perforation (group Ⅱ), and 251 patients with noncomplicated hydatid cysts (group Ⅲ) were treated in our clinics. The ratios of intrabiliary rupture and peritoneal perforation cases to noncomplicated cases were 37% and 11.1%, respectively.

Diagnosis

Patient age and sex, initial complaints, physical findings, cyst characteristics, imaging results, surgical procedures, reasons for peritoneal perforation and intrabiliary rupture, morbidity, LOS, recurrence rates, and mortality were evaluated. The patients with extrahepatic organ involvement were excluded.

Preoperative evaluation

The preoperative evaluation included blood tests (complete blood count, blood type, liver function tests), chest radiography, abdominal ultrasonography (US), and computed tomography (CT). Chest and abdominal radiography and abdominal US were performed in all patients at admission. The most important indications for CT were a need for additional anatomical and cystic details, the presence of multiple hydatid cysts, findings of a solid appearance, and findings of cyst infection. If intrabiliary rupture was suspected, magnetic resonance imaging (MRI) cholangiography was performed. Intraoperative ultrasound (IOUS) was used for identification of intraparenchimal cyst localization and selection of the best approach sight.

Surgical procedure

In all cases of liver hydatid cysts, the area around the cyst was covered with packs soaked in 3% hypertonic saline solution to prevent the further spread of the parasite during evacuation of the cyst. Cyst contents were aspirated from a suitable surface with a needle, which opened a nearly 1-2 cm incision; this was examined carefully for bile content. Subsequently, an aspirator was inserted into the cystic cavity to empty the remaining fluid, and then the incision was widened. The germinative membrane and daughter cysts were removed with forceps or spoons. With the roof excision of the redundant part of the cyst, an excellent exposure was obtained. After evacuation of the cyst, the cyst cavity was irrigated with 3% hypertonic saline for 10-15 min. Any orifices of bile ducts observed on the inner surface of the cavity were sutured with nonabsorbable sutures. To prevent peritoneal spreading in peritoneally perforated cases, the abdominal cavity was irrigated with 3% hypertonic saline or with povidone-iodine. Next, a surgical procedure, such as partial pericystectomy (PP) and capitonnage, PP and omentoplasty, PP and drainage, or PP and intraflexion, was performed. For pericystectomy, the hepatic pedicle was prepared for the Pringle maneuver. The cyst was then dissected along its boundary with healthy liver tissue. Blood vessels and small biliary structures passing through the plane between normal liver tissue and the cyst were clamped and divided. If the patient was fit and the cysts were away from the hepatic veins, large bile ducts, or major branches of the por-

tal vein and hepatic artery, pericystectomy was performed. Liver resections were performed only if multiple cysts were localized in one lobe and peripherally.

If the preoperative evaluation suggested a probability of a connection between the cyst and the biliary duct, or this was suspected during surgery, choledochotomy, placement of a T-tube, and an intraoperative cholangiographic and/or choledochoscopic evaluation were performed. In the case of any doubt about free biliary drainage, biliodigestive anastomosis or sphincteroplasty was added.

Medical treatment

Since 1993, medical treatment for hydatidosis has consisted of albendazole 10 mg/kg per day (Andazol, Biofarma, Istanbul, Turkey), beginning after surgery and continuing for 3 mo. Until 5 years ago, the therapy was performed over 3 mo and included 20 d of drug administration and 10 d drug free intervals. Over the last 5 years, the drug has been administered continuously with monthly blood liver function tests and complete blood count controls.

Follow-up

During follow-up, US was performed twice a year for 2 years, and then annually. CT scans were performed as needed.

Statistical analysis

Differences among categorical variables were compared using χ^2 tests, and Kruskal-Wallis tests were used for age, diameter, and number of cysts and LOS. Multinominal logistic regression analysis was performed to analyze the effects of variables (age; sex; previous hydatid disease surgery; location, number, position, and presence of cyst infection) influencing peritoneal perforation or biliary rupture. Data were analyzed with the SPSS software package (version 13.0; SPSS, Inc., Chicago, IL). Significance was set at $P < 0.05$.

RESULTS

The mean age (mean ± SD) of peritoneal perforated and intrabiliary ruptured patients was 36.32 ± 11.86 and 50.26 ± 13.88, respectively. When calculated, it was 45.62 ± 16.01 in the noncomplicated group. The mean age factor in the peritoneal perforation group was significantly different from others ($P = 0.001$). The sex ratio of the patients was not statistically different among the groups ($P = 0.95$, Table 1).

Previous hydatid disease surgery, cyst localization, number of cysts, and presence of infection were not statistically different among groups ($P > 0.5$; range, 0.109-0.683), whereas the differences in cyst diameters and positions (superficial or deep) were significant ($P = 0.001$ and 0.017, respectively; Table 1). Macroscopic appearance of an hydatid cyst that originates in the right lobe of the liver was seen in Figure 1.

The most common complaint was abdominal pain in all groups (67%). Other common complaints were nausea, vomiting, abdominal distension, and allergenic reactions in peritoneally perforated cases, jaundice (21%) in intrabiliary ruptured cases, and abdominal swelling (41%) in noncomplicated hydatid cysts. The most common physical examination findings were abdominal sensitivity and guarding (100%), acute abdomen findings (39%), distension (22%) in peritoneally perforated cases, hepatomegaly (28%) and jaundice (21%) in intrabiliary ruptured cysts, and finally, hepatomegaly (27%) in noncomplicated patients. In 49 (13%) patients, diagnosis was made incidentally by abdominal US or CT during a medical checkup or after a trauma.

When the predisposing factors for complications

Table 1 Patient and cyst characteristics of the three study groups

Characteristic	Group Ⅰ (n = 28)	Group Ⅱ (n = 93)	Group Ⅲ (n = 251)	P
Age[1] (yr)				0.001[b]
mean ± SD	36.32 ± 11.86	50.26 ± 13.88	45.62 ± 16.01	
Median (range)	37.0 (17-76)	53.0 (23-76)	43.0 (18-79)	
Sex				0.950
Male	15 (53)	51 (55)	141 (56)	
Female	13 (47)	42 (45)	110 (44)	
Previous hydatid disease surgery				0.204
No	24 (86)	74 (80)	219 (87)	
Yes	4 (14)	19 (20)	32 (13)	
Localization				0.109
Right lobe	17 (61)	54 (58)	178 (71)	
Left lobe	5 (18)	25 (26)	48 (19)	
Bilateral	6 (21)	14 (16)	25 (10)	
No. of cysts[1]				0.284
1	22 (78)	61 (66)	193 (77)	
2	4 (14)	17 (18)	39 (16)	
3	1 (4)	7 (8)	16 (6)	
4	1 (4)	5 (5)	3 (1)	
5	-	3 (3)	-	
Cyst diameter[1] (cm)	9.5 (4-24)	10 (6-29)	7 (3-31)	0.001[b]
Position				0.017[b]
Superficial	24 (86)	52 (56)	155 (62)	
Deep	4 (14)	41 (44)	96 (38)	
Cyst infection				0.683
Negative	26 (93)	89 (95)	234 (93)	
Positive	2 (7)	4 (5)	17 (7)	

Values are mean ± SD, median (range), or n (%). [b]Values indicate a significant difference. [1]Kruskal-Wallis test.

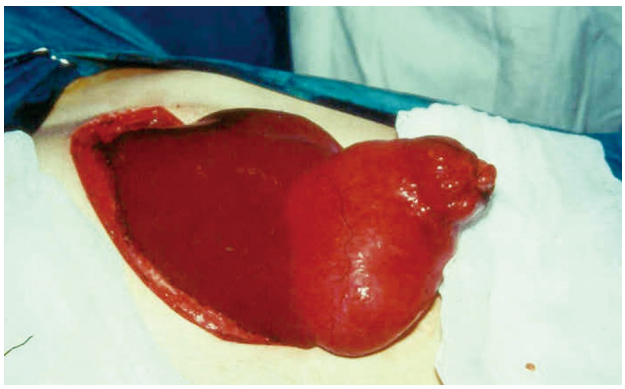

Figure 1 Macroscopic appearance of an hydatid cyst that originates in the right lobe of the liver.

Table 2 Multinominal logistic regression analyses of factors influencing peritoneal and biliary perforation of liver hydatid cysts

Factor	Group Ⅰ *vs* group Ⅲ		Group Ⅱ *vs* group Ⅲ	
	Odds ratio	95% CI	Odds ratio	95% CI
Sex				
Male	1.00		1.00	
Female	1.61	0.68-3.77	1.10	0.65-1.87
Age	0.59	0.41-0.84	1.30	1.07-1.57
Occurrence type				
Primary occurrence	1.00		1.00	
Recurrence	0.81	0.23-2.77	1.33	0.66-2.65
Localization				
Unilobar	1.00		1.00	
Bilateral	1.48	0.86-2.55	1.54	1.07-2.21
No. of cysts	1.21	0.65-2.23	1.80	1.30-2.50
Cyst diameter (cm)	1.11	1.04-1.19	1.12	1.07-1.57
Position				
Superficial	1.00		1.00	
Deep	0.27	0.09-0.84	1.40	0.18-4.33
Cyst infection				
Positive	1.00		1.00	
Negative	1.02	0.20-5.11	0.58	0.17-2.01

Group Ⅰ = 28, group Ⅱ = 93, group Ⅲ = 251 (reference value).

were evaluated, younger age, superficial position, and larger cyst dimensions increased peritoneal perforation rates. It has been seen that older age, increased cyst dimensions, and presence of multiple and bilobar cysts increases intrabiliary rupture rates. Sex, primary or recurrent disease, and cyst infection were not as effective variables in both logistic models (Table 2).

Abdominal US was done in 326 (87.6%) patients and demonstrated hepatic cysts with 96.7% sensitivity (Figure 2). CT was used as the second most frequent (n = 148, 46%) diagnostic modality, with 96.5% sensitivity. In 24 (6.5%) patients with intraparenchymal cysts, IOUS was performed for identification of cyst localization and selection of the best approach sight. MRI cholangiography was performed after 1996 to assess the spread of disease to the biliary tree but was not used routinely and was done in 37 patients (9.9%) (Figure 3). The indications for MRI cholangiography are the presence of jaundice seen during the physical examination, elevation of serum bilirubin and alkaline phosphatase levels, and other obstructive jaundice symptoms and demonstrated 98% sensitivity.

Surgical procedures used for the cysts of the 372 patients are shown in Table 3. Partial pericystectomy and drainage was the most frequent surgical procedure in all groups. Three of 4 jaundiced patients underwent cholecystectomy plus T-tube drainage, and the other patient underwent cholecystectomy plus sphincteroplasty in the peritoneal perforation group. In all intrabiliary rupture cases, cholecystectomy and common bile duct exploration were performed. Intraoperative cholangiography was performed in all patients, and choledochoscopy was also performed in 62 (66.6%) patients. Cyst remnants and daughter vesicles in the bile ducts were evacuated

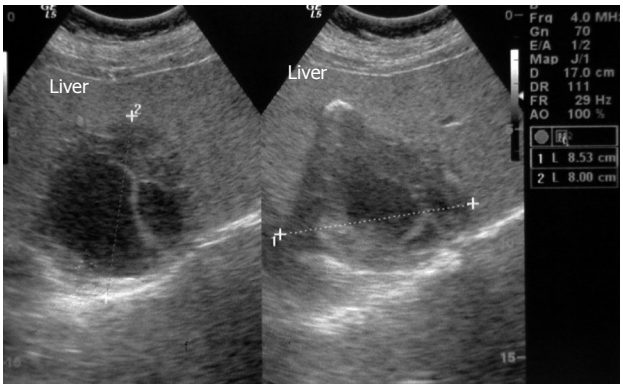

Figure 2 Ultrasonographic appearance of an hydatid cyst of the liver.

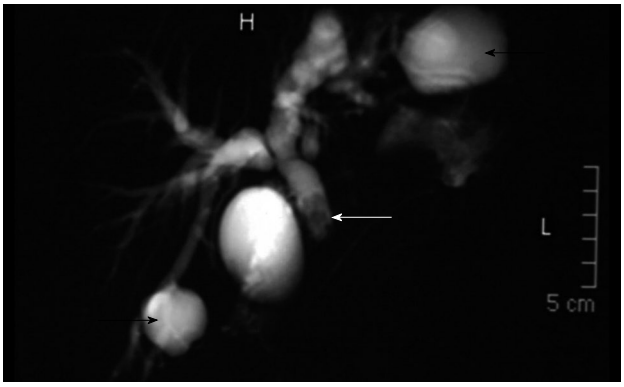

Figure 3 Magnetic resonance imaging cholangiography. Two biliary ruptured (right and left lobe) hydatid cysts (black arrows) and daughter vesicles in common bile duct (white arrow).

with Dormia forceps or a Fogarty catheter in 16 (17.2%) patients. A T-tube drainage was performed in 72 (77.4%) patients, choledochoduodenostomy was performed in 15 (16.1%) patients, and sphincteroplasty was performed in 6 (6.4%) patients.

The most frequent postoperative complications were wound infection (5.6%) and pulmonary complications (2.9%). In the peritoneal perforation group, 10 surgical complications occurred in 7 (25%) patients; in the intrabiliary rupture group, 18 complications occurred in 15 (16.1%) patients; and in the noncomplicated group, 16 complications occurred in 14 (5.5%) patients. When compared, complication rates were significantly different (P = 0.002, Table 4). When compared according to Dindo & Clavien Classification of Surgical Complications[19], the differences between grade Ⅰ, grade Ⅳa and grade Ⅳb surgical complications were not significant (P > 0.05, range, 0.08-0.57), however differences between grade Ⅱ, grade Ⅲa and grade Ⅲb complications were statistically significant (P < 0.05; range, 0.003-0.036).

Median postoperative LOS in the peritoneal perforation group, intrabiliary ruptured group, and noncomplicated group were 12 (range, 5-21), 13 (range, 5-37), and 9 (range, 4-19) d, respectively. When LOS was compared, there was no significant difference between the groups (P > 0.05). According to the additional surgical procedures,

Table 3 Surgical procedures used

Surgical procedure	No. of cysts (%)		
	Group Ⅰ (*n* = 28)	Group Ⅱ (*n* = 93)	Group Ⅲ (*n* = 251)
Main surgical procedure			
PP and drainage	26 (70.3)	101 (67.8)	231 (73.5)
PP and omentoplasty	2 (5.4)	13 (8.7)	26 (8.3)
PP and capitonnage	2 (5.4)	11 (7.4)	20 (6.4)
PP and intraflexion	-	3 (2.0)	8 (2.6)
Pericystectomy	5 (13.5)	14 (9.4)	19 (6.0)
Liver resection	2 (5.4)	7 (4.7)	10 (3.2)
Total No. of cysts	37	149	314
Additional surgical procedure			
Choledochotomy and T-tube	3 (10.7)	77 (82.8)	7 (2.7)
Choledochotomy and choledocho-duodenostomy or sphincteroplasty	1 (3.5)	16 (17.2)	2 (0.8)

PP: Partial pericystectomy.

Table 4 Morbidity, recurrence, and mortality rates of the three study groups *n* (%)

	Group Ⅰ (*n* = 28)	Group Ⅱ (*n* = 93)	Group Ⅲ (*n* = 251)	*P*
Medical complications[1]				0.163
Cardiac	1 (3.6)	1 (1.1)	3 (1.2)	
Respiratory	2 (7.2)	3 (3.2)	6 (2.4)	
Other	1 (3.6)	2 (2.2)	4 (1.6)	
Surgical complications				
Wound infection	6 (21.4)	6 (6.5)	9 (3.5)	0.001[b]
Biliary fistula	1 (3.6)	9 (9.7)	2 (0.8)	0.001[b]
Intra-abdominal abscess	-	1 (1.1)	2 (0.8)	0.855
Incisional hernia	2 (7.2)	2 (2.2)	3 (1.2)	0.087
Gastrocutaneous fistula	1 (3.6)	-	-	0.002[b]
Recurrence	3 (10.7)	3 (3.2)	8 (3.2)	0.132
Mortality	-	1 (1.1)	3 (1.2)	0.844

[b]Values indicate a significant difference. [1]Cardiac complications included heart failure and myocardial infarction; respiratory complications included atelectasis, pneumonia, adult respiratory distress syndrome, and pulmonary embolus; other complications included urinary tract infection and acute thromboembolic disease of the lower extremities.

the median LOS was 8 (range, 4-19) d in patients on which choledochotomy had not been performed, compared to 15 (range, 11-37) d in the choledochotomy with T-tube group and 10 (range, 6-13) d in the bilioenteric anastomosis group. There was a significant difference in LOS between the groups with choledochotomy with T-tube and without choledochotomy ($P < 0.05$). In contrast, no difference between the groups with biliodigestive anastomosis and without choledochotomy was seen ($P > 0.05$).

The follow-up data were complete for 28 (100%) of the peritoneal perforated group, 76 (82%) of the intrabiliary ruptured group, and 198 (79%) of the noncomplicated group. The mean follow-up periods were 37.3 ± 26.3 (range, 2-144) mo. The overall recurrence rate was 3.8% (14 patients), but there was not any statistical difference among the patient groups ($P = 0.13$, Table 4). The early postoperative mortality rate was 1.1% ($n = 4$). The causes of death were pulmonary embolism in one patient, myocardial infarct in one patient, and postoperative hemorrhage in two patients (Table 4). These causes were identified clinically, without necropsy.

DISCUSSION

Peritoneal perforation into the abdominal cavity and the spontaneous intrabiliary rupture of liver hydatid disease are not rare complications and cause serious problems. In our study, the ratios of peritoneal perforation and intrabiliary rupture cases to noncomplicated cases were 11.1% and 37%, respectively. The most common complication is rupture of the cyst, either internally or externally, followed by anaphylactic reaction and jaundice[5,9,10]. Systemic anaphylactic reactions have been reported in 1% to 12.5% of patients with intraperitoneal perforation, and these reactions may be life threatening[9,10]. Jaundice is the most important sign of intrabiliary rupture within a range of 9% to 30%[20,21]. In patients with peritoneal perforation and intrabiliary rupture, specific management has not been evaluated sufficiently. The presence of complications must be considered in the surgical treatment.

One third of 372 patients (32.5%) who underwent an operation for liver hydatid disease presented with complications. When compared, sex factor was not statistically different among groups ($P = 0.95$), but the mean age in the peritoneal perforated group was significantly different ($P = 0.001$, Table 1). Trauma is the most frequent etiological cause of perforation[9,22]. No patients in the current series experienced spontaneous peritoneal rupture. Hydatidosis is endemic in Turkey and the frequency of traffic accidents there is high[23]. Traffic accidents generally affect younger individuals, and the age risk factor found in our study may relate to this phenomenon. In superficial and bigger cysts, lack of normal liver tissue around the cyst to protect against trauma may be a cause of frequent rupture.

The differences in factors such as previous hydatid disease surgery, cyst localization, cyst number, and cyst infection were not significant among groups ($P > 0.05$, range, 0.109-0.683). Besides age, cyst diameter and cyst position (superficial or deep) were also significantly different factors ($P = 0.001$ and 0.017, respectively; Table 1). When compared to the same parameters with multinominal logistic regression analyses, young age, superficial position, and large diameter are significant predisposing factors for peritoneal perforation (Table 2). The hydatid cyst wall consists of an inner germinal layer and an outer laminated membrane. Surrounding the wall is the pericyst, which has an outer zone of compressed liver cells. A lack of presence of normal liver tissue around superficial cysts plays a role in relatively higher perforation rates. Similarly, an increase in cyst diameter may increase perforation risk either by becoming more superficial during growth or by increasing intracystic pressure.

Additionally, liver hydatid cysts at the dome of the liver grow progressively and have a tendency to erode

the diaphragm[24]. In our study, it has been shown that older age, bigger dimensions, multiplicity of the cysts, and bilobar cysts are predisposing factors for intrabiliary rupture. The relationship between older age and intrabiliary rupture probably depends on cyst age. Hydatid disease is a slowly growing disease, and in early times, most of the cysts produce no symptoms. In advanced cases, the intracystic pressure may cause tension on cyst walls and thinner wall formations. The communications between the cysts and the biliary tree usually involve small cholangioles, and direct extension into major bile ducts is rare[25,26]. The most probable theory may be the fenestration of the cyst and minor bile ducts, which have low pressure in the surrounding liver tissue. In the case of multiple and bilobar cysts, this communication rate may increase. Factors such as sex, primary or recurrent disease, or infection did not show effectiveness in both logistic models (Table 2).

Cyst dimension and age (younger age for peritoneal perforation and older age for intrabiliary rupture) have been determined as significant predisposing factors for the two complications. We do not believe a direct relationship exists between the two complications, but we can speculate that risk of peritoneal perforation risk may decrease in intrabiliary ruptured cysts because of the lower intracystic pressure probability. In our study the ratio of intrabiliary ruptured cysts was 97 out of 372 (26%), whereas intrabiliary rupture was 4 out of 28 (14.3%) peritoneally perforated cases, but this was not statistically significant.

The principles of hydatid surgery are (1) removal of all infective cyst parts; (2) inactivation of the cyst cavity; (3) avoidance of intra-abdominal spillage of cyst contents; and (4) management of the residual cavity. A lot of surgical techniques have been proposed for liver hydatid disease during the last three decades. Some authors suggest that obliteration procedures, such as capitonnage, intraflexion, or omentoplasty, are simple and safe methods[27]. In the past, obliteration procedures such as omentoplasty, capitonnage, and intraflexion were used for most patients in our clinic. However, in the last 10 years use of these management options decreased gradually and for the last 5 years we have not shown a preference for them. Recently, some authors have favoured the use of pericystectomy and liver resections because complete surgical resection is the ideal treatment for hydatid disease[18]. In our study, liver resections were performed only if multiple cysts were localized in one lobe, located peripherally, and were pedunculated, and pericystectomy was performed if the cysts were away from the major vascular and biliary structures of the liver. The most important advantages of these resections are that no dead space is left behind and that all the cyst wall is removed, which prevents liver regeneration. The need for sufficient technical infrastructure and surgical experience in the field of hepatic surgery limit these treatments in small centers, especially in developing countries. Relatively small-sized subcapsular cysts can be managed by nonanatomic resection of the cysts with a rim of healthy hepatic tissue but routine application of pericystectomy and liver resection may increase the operative complications, such as bleeding and postoperative morbidity and mortality.

For almost 15 years, our preferred surgical technique for treatment of liver hydatid cysts has been PP and drainage, which has been performed in 70% of the patients (Table 3). Leaving the cyst cavity open, the peritoneum has more support in recent years[13]. After controlling the relation between the biliary tree, it has been stated that this procedure does not increase recurrence or cause adhesion. The most important superior aspect of this technique is its applicability to every cyst at every localization. Other advantages of the procedure are the simplicity, no requirement of advanced experience, effectiveness, and a shorter operation time. The most important detail of this procedure in our experience is to perform the partial pericystectomy as widely as possible, and the drainage procedure should be added after the cyst contents are removed completely. After the cyst management, in peritoneal perforated cysts, the most important step is irrigation of the peritoneal cavity with a sufficient amount of scolicidal agents and removal of all cystic content, and in intrabiliary ruptured cases, clearance of the cystic material from the biliary tree is most important; also, the restoration of normal bile flow should be performed. The fistula was large enough to allow daughter cysts and debris to migrate into the biliary tree. For this reason introperative cholangiography was performed in all patients who presented with abnormal liver function tests preoperatively, bile-stained cyst fluid, and bile leak from the cyst wall. During the introperative cholangiography, visible bile fistulas must be sutured by using late absorbable suture materials.

After the intervention to the cyst, the most important step is irrigation of the cyst cavity with a sufficient amount of scolicidal agents and careful removal of all cystic content from the patient. Numerous solutions, such as hypertonic saline solutions (15%-30%), have been used as scolicidal agents for the purpose of inactivation[5,28]. The concentration of hypertonic saline solutions has diminished gradually. Now we use only 3% concentrations. Gargouri *et al*[29] used 3%-5% saline and stated that the application time is more important than the concentration. Although Derici *et al*[30] reported that hypertonic saline is not appropriate because it may damage the peritoneal surfaces and may cause hypernatremia, we have not encountered any significant complications with the use of this solution. Additionally, we believe that profuse peritoneal lavage with hypertonic sodium chloride is mandatory for preventing intra-abdominal recurrence of intraperitoneal ruptured hydatid disease.

The most effective surgical procedures following the cleaning of hydatid material from the biliary tree remain controversial. Some authors prefer T-tube[13-15]; in contrast, some reported that wide choledochoduodenostomy decreases morbidity and mortality[31]. In our study, complication rates in patients with bilioenteric anastomosis and T-tube were 21% and 27%, respectively.

In the literature, reported complication rates are be-

tween 6% and 47%; recurrence rates are 8% to 15%[32]. In this study, the overall postoperative complication and recurrence rates were 18% and 3.8%, respectively. Most often, surgical complications were wound infection (n = 21) and biliary fistulas (n = 12); the difference between groups was significant (P = 0.001, Table 4). The important causes of higher wound infection rates in the peritoneal perforation group were coinjuries, which were seen in 10 patients. There were small intestine and colon perforations in five cases; in addition, splenic, pancreatic, and renal injuries were each seen in one patient. Retroperitoneal hematoma was seen in the remaining two coinjured patients. External biliary fistulas did not develop in 68 of the T-tube patients and in none of the biliodigestive anastomosis or sphincteroplasty patients in the intrabiliary rupture group. T-tubes were removed after a control cholangiography, which was done after a mean period of 12 d postoperatively. External biliary fistulas developed in 9 (9.7%) patients. If the fistulas did not close with conservative follow-up, endoscopic sphincterotomy was performed after a mean period of 19 d. The causes of fistulas were remnant germinal vesicles and debris in the distal common bile duct in 5 patients and papillary stenosis in 4. In the peritoneally perforated and noncomplicated groups, biliary fistulas developed in 3 patients who had hepatic resections, and the problem was resolved with endoscopic sphincterotomy. Endoscopic procedures have been used not only for diagnostic purposes, but for treatment as well. Dumas *et al*[33] treated 7 of 28 patients with cholangitis due to cystic material with ERCP. Tekant *et al*[34] and Vignote *et al*[35] reported that endoscopic sphincterotomy achieved closure of fistulas within 5-7 d. In our series, fistulas closed in all external biliary fistula patients within a mean time of 7 (range, 3-17) d. At our center, intraoperative cholangiography with or without subsequent duct exploration remains the method of choice to prevent or decrease incidence of associated injuries and external biliary fistula. Several authors have advocated pre-operative or post-operative ERCP[33-35]. If performed in conjunction with a sphincterotomy, an ERCP may be used to clear the biliary tree as a planned procedure, thereby avoiding the need for an intraoperative cholangiography and bile duct exploration.

Recurrence occurred in 14 nonperforation cases (3.8%). When compared with the reported rates, the recurrence of our study is low. The cause of this may be the routine use of albendazol for 3 mo postoperatively. Recurrence rates were not significantly higher in peritoneal perforation and intrabiliary rupture cases when compared with noncomplicated cases (P = 0.132, Table 4). Recurrences of hydatid disease are usually due to inadequate cystic content removal, spillage of cystic liquid intraoperatively, undetected cysts, and satellite lesions and reinfestations. Readmissions in our series due to recurrent disease were generally after a long time and mostly from rural areas. Another clinical finding that suggests reinfestation is the presence of hydatid cyst at the same localisation which seen was 4 of 14 recurrent cases. For these reasons, the probability of reinfestation is high in endemic countries like Turkey. When compared with the literature, in our study the recurrence rate was low. Albendazole treatment may play an important role in this low rate. Although the efficacy of perioperative prophylaxis with albendazole has been controversial, recent studies suggest that medical treatment is effective in the prevention of recurrence after surgery[36,37]. To diminish recurrence and potential spread of the organism, we believe that medical treatment should apply as an adjuvant therapy to surgery when complete removal of the cyst is impossible or when the cyst contents threaten to disseminate due to peritoneal rupture. It is indicated in patients who are at high risk for surgery, and in the presence of bilateral multiple small cysts. Those with small cysts (< 4 cm) and cysts with thin walls are the best group for effective medical treatment. In addition, exogenous vesiculations have been pronounced in hydatid cyst recurrences[38]. The importance of medical treatment which is mainly effective on small cysts, may indicate the usefulness of the effect on recurrence.

Although there is insufficient published evidence to support a clear recommendation for the use of albendazole preoperatively[39], we highly recommend this treatment for one week before surgical exploration. Preoperative therapy may reduce the risk of intraperitoneal seeding of infection that develops *via* cyst rupture and spillage occurring spontaneously or during surgery. In our study no correlation was found between recurrence of cysts and albendazole use.

Serology detect specific serum antibodies or circulating antigen by a variety of immunodiagnostic methods. Of these, the enzyme immunosorbent assay (ELISA) employing hydatid fluid antigen for detection of echinococcal antibodies (IgG) in the serum is the most widely used. Serological tests are of little clinical use in monitoring patients after operation for cystic echinococcosis or patients on chemotherapy. The antibody titers rise following surgical treatment. The titers start falling at 3 mo and become negative in a period of 12-24 mo. False positive tests can occur in normal persons from endemic areas and in those with other parasitic infections[16]. In our study, 86 (58.9%) of 146 (42.4%) serologically evaluated patients of group Ⅱ and Ⅲ were positive, and 41 (28%) of these were also serologically positive 2 years after surgery. However, recurrence occurred in only 1 of the patients that was serologically positive for a long term. We think that the value of serological screening in areas endemic for cystic echinococcosis is enhanced by combining it with abdominal US.

Mortality rates of the study groups were not statistically different (P = 0.814, Table 4). Liver resections were performed in two mortality cases that were due to intraoperative and postoperative bleeding. One of the patients who had PP and drainage died because of myocardial infarct, and one patient had PP and omentoplasty cases of pulmonary thromboembolism. When compared, mortality rates were significantly different among groups (P < 0.001). This result requires discussion of the controversial radical methods in hydatid disease. Chautems *et al*[38] recommended complete resection of hepatic hydatid cysts. In contrast, Daali *et al*[40] preferred

conservative procedures. We think that radical methods should not be performed routinely for such a slowly growing benign pathology. It is wise to reserve radical surgery for peripheral, easily accessible cysts and for unilober multiple cysts.

LOS did not differ between the cases of peritoneal perforation and intrabiliary rupture when compared with noncomplicated cases ($P > 0.05$), whereas, according to the additional surgical procedures, there was a significant difference in LOS between choledochotomy with T-tube and procedures without choledochotomy ($P < 0.05$). An external biliary fistula formation and T-tube procedure, wherein the T-tube may be taken out after approximately 7-10 d, may affect LOS. In contrast, there was no difference between the groups with biliodigestive anastomosis and without choledochotomy ($P > 0.05$). Biliodigestive anastomosis may be a good alternative surgical procedure to T-tubes, with lower complication rates and shorter LOS.

According to this study's multinominal logistic regression analysis, peritoneal perforation risk increases with younger age, superficial localization, and larger dimensions of cysts, and older age, multiple and larger cysts, and bilobar localization are established as predisposing factors for intrabiliary rupture. Morbidity rates significantly increased in peritoneally perforated and intrabiliary ruptured cases, but there was no difference in recurrence rates and hospital stay times. Our preferred surgical technique for liver hydatid cysts is partial pericystectomy and drainage because the rate of postoperative complications and recurrence, and the duration of hospital stay are satisfactory. We believe that radical methods should not be performed routinely for such a benign pathology. The localization and number of cysts, relation to blood vessels and bile ducts, and general status of the patient are parameters influencing the choice of surgical procedure. In peritoneal perforated and intrabiliary ruptured cysts, the most important steps are irrigation of the peritoneal cavity with a sufficient amount of scolicidal agents, removal of all cystic contents from the peritoneum, and clearance of the cystic material from the biliary tree. However, randomized controlled studies are needed to establish the best surgical management for peritoneal perforated and intrabiliary ruptured liver hydatid cysts.

COMMENTS

Background

The development of hydatid disease associated with *Echinococcus granulosus* is still an important health problem. The clinical picture can be severe, and complications may occur, making the already difficult treatment even more so. Complications are observed in one third of patients with liver hydatid cysts. The most common complication is rupture of the cyst, either internally or externally.

Research frontiers

Only a few studies have reported the management and outcome of patients with complicated liver hydatid disease. Surgery is the mainstay of treatment, although there is no consensus on the respective advantages of conservative and radical methods. In this study, the authors evaluated the predisposing factors for peritoneal perforation and intrabiliary rupture and the effects of these complications on morbidity, length of hospital stay, mortality, and recurrence in hydatid disease.

Innovations and breakthroughs

According to this study's multinominal logistic regression analysis, peritoneal perforation risk increases with younger age, superficial localization, and larger dimensions of cysts, and older age, multiple and larger cysts, and bilobar localization are established as predisposing factors for intrabiliary rupture. Morbidity rates significantly increased in peritoneally perforated and intrabiliary ruptured cases, but there was no difference in recurrence rates and hospital stay times.

Applications

In peritoneal perforated and intrabiliary ruptured cysts, the most important steps are irrigation of the peritoneal cavity with a sufficient amount of scolicidal agents, removal of all cystic contents from the peritoneum, and clearance of the cystic material from the biliary tree. However, randomized controlled studies are needed to establish the best surgical management for peritoneal perforated and intrabiliary ruptured liver hydatid cysts.

Terminology

Echinococcus granulosus is a parasitic tapeworm that lives in the bowels of dogs. Humans contract the disease through enteral exposure and become accidental intermediate hosts.

Peer review

Alper Akcan *et al*, evaluate the predisposing factors for peritoneal perforation and intrabiliary rupture and the effects of these complications on surgical outcome in liver hydatid disease. This is one of the largest and well treated series of patients that had been publish. The structure and methodology followed by the authors is correct in my opinion and the techniques they applied for the treatment of these complex patients are modern and correct.

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BRIEF ARTICLE

Is inconsistency of α-fetoprotein level a good prognosticator for hepatocellular carcinoma recurrence?

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Abstract

AIM: To identify the clinical outcomes of hepatocellular carcinoma (HCC) patients with inconsistent α-fetoprotein (AFP) levels which were initially high and then low at recurrence.

METHODS: We retrospectively included 178 patients who underwent liver resection with high preoperative AFP levels (≥ 200 ng/dL). Sixty-nine HCC patients had recurrence during follow-up and were grouped by their AFP levels at recurrence: group Ⅰ, AFP ≤ 20 ng/dL (n = 16); group Ⅱ, AFP 20-200 ng/dL (n = 24); and group Ⅲ, AFP ≥ 200 ng/dL (n = 29). Their preoperative clinical characteristics, accumulated recurrence rate, and recurrence-to-death survival rate were compared. Three patients, one in each group, underwent liver resection twice for primary and recurrent HCC. AFP immunohistochemistry of primary and recurrent HCC specimens were examined.

RESULTS: In this study, 23% of patients demonstrated normal AFP levels at HCC recurrence. The AFP levels in these patients were initially high. There were no significant differences in clinical characteristics between the three groups except for the mean recurrence interval (21.8 ± 14.6, 12.3 ± 7.7, 8.3 ± 6.6 mo, respectively, P < 0.001) and survival time (40.2 ± 19.9, 36.1 ± 22.4, 21.9 ± 22.0 mo, respectively, P = 0.013). Tumor size > 5 cm, total bilirubin > 1.2 mg/dL, vessel invasion, Child classification B, group Ⅲ, and recurrence interval < 12 mo, were risk factors for survival rate. Cox regression analysis was performed and vessel invasion, group Ⅲ, and recurrence interval were independent risk factors. The recurrence interval was significant longer in group Ⅰ (P < 0.001). The recurrence-to-death survival rate was significantly better in group Ⅱ (P = 0.016). AFP staining was strong in the primary HCC specimens and was reduced at recurrence in group Ⅰ specimens.

CONCLUSION: Patients in group Ⅰ with inconsistent AFP levels had a longer recurrence interval and worse recurrence-to-death survival rate than those in group Ⅱ. This clinical presentation may be caused by a delay in the detection of HCC recurrence.

Key words: Hepatocellular carcinoma; Inconsistent α-fetoprotein; Outcome; Recurrence

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INTRODUCTION

Serum α-fetoprotein (AFP) level is widely used as a tumor marker for the diagnosis and detection of both occurrence and recurrence of hepatocellular carcinoma (HCC)[1,2]. For a patient with a history of liver disease, consensus for the diagnosis of HCC can be achieved with an elevated AFP level (≥ 200 ng/dL), along with a dynamic imaging study showing a hepatic tumor, with enhancement in the arterial phase and wash-out in the venous phase[3,4].

A high serum AFP level in HCC also indicates poor tumor differentiation, larger tumor burden, recurrence after tumor resection, and an unfavorable outcome[5-10]. In clinical practice, a dramatic decline in serum AFP level indicates that the tumor has been surgically removed[11]. The AFP level is broadly applicable both pre- and postoperatively.

AFP is secreted in only about 70% of HCC cases, so both false-negative and false-positive rates are high when using AFP as the serological marker for the detection of HCC[12]. In general, patients have an initially high serum AFP level when HCC is diagnosed. Serum AFP level is also considered an effective tumor marker in the surveillance of HCC recurrence[13,14]. Recurrent HCC is expected to have the same bioactivity as the primary tumor and to secrete high levels of AFP, making AFP a seemingly reliable choice. Regular checks of serum AFP levels in patients who initially had high AFP levels to identify HCC recurrence is suggested as a practice guideline by the National Comprehensive Cancer Network (NCCN) version 1, 2009[15].

However, inconsistent AFP levels have been found in patients diagnosed with HCC where initial AFP levels were high, but were normal when HCC recurred. A true relapse of HCC and a second primary tumor due to chromosomal aberration are possible mechanisms[16], and inconsistent AFP levels may differentiate them *via* AFP secretion bioactivity. If we depend on the AFP level to signal an HCC recurrence, we may miss or delay the diagnosis of recurrence. We may expect the clinical outcome of patients with inconsistent AFP levels to be similar to those with normal AFP levels. However, this clinical presentation has not been investigated before. Therefore, it is important to study the prognosis of this subgroup of HCC patients whose AFP levels are high initially and normal at recurrence. It is possible that the findings of this study could modify the surveillance principle for this subgroup of HCC patients and improve the quality of medical care.

MATERIALS AND METHODS

Patients

From 1996 to 2008, 642 patients newly diagnosed with HCC who underwent liver resection were entered into a computer database at the Tri-Service General Hospital, a tertiary referral medical center in Taipei, Taiwan. The diagnosis of HCC in all patients was established histologically from surgical specimens. HCC patients with an initial serum AFP level ≥ 200 ng/dL (n = 178) were selected and 82 had HCC recurrence during follow-up. Thirteen patients were excluded because of an American Joint Committee on Cancer (AJCC) stage Ⅳ diagnosis, lost to follow-up or missing data. Therefore, 69 patients were included in this study and grouped according to their serum AFP levels when HCC recurred (Figure 1).

Grouping by AFP level at recurrence

Sixteen patients with normal AFP levels (≤ 20 ng/dL) during HCC recurrence were assigned to group Ⅰ; 24 patients with AFP levels between 20 ng/dL and 200 ng/dL were assigned to group Ⅱ; and, 29 patients with AFP levels of 200 ng/dL or higher were assigned to group Ⅲ. In our hospital, the normal AFP level is ≤ 20 ng/dL measured by radioimmunoassay using an anti-AFP antibody (ELSA 2-AFP kit, CIS Bio International, Cedex, France).

Comparing the parameters of each group

Preoperative clinical characteristics were collected from the database and compared. These characteristics included age, gender, tumor size, serum alanine aminotransferase (ALT) level, total serum bilirubin, serum albumin level, platelet count, vessel invasion, tumor number, clinical stage (AJCC, Child-Pugh classification), hepatitis B virus, hepatitis C virus, presence of cirrhosis, mean recurrence interval, mean recurrence-to-death time, mean survival time and location of recurrence. Salvage treatments for recurrent HCC included repeat liver resection, liver transplantation, transarterial chemo-embolization, radiofrequency ablation, and thalidomide therapy. The mean AFP levels at operation (AFP op) and at recurrence (AFP recur) were calculated for these three groups. Intra-hepatic metastasis was defined as recurrent HCC located at the adjacent segment, section and same lobe. In contrast to intra-hepatic metastasis, multicentric recurrence was defined as recurrent HCC located at the contralateral liver lobe. The pathological grade was defined as: grade Ⅰ - well differentiated, grade Ⅱ - moderate, and grade Ⅲ - poorly differentiated, according to the report by the pathologist (Dr. Yu CP).

Endpoint

The mean recurrence interval was defined as the interval between the operation and HCC recurrence. The starting point of overall survival was the operation date, and recurrence-to-death was the date of recurrence diagnosis. The endpoint of accumulated recurrence rate was defined as the date of recurrence and the endpoint of recurrence-

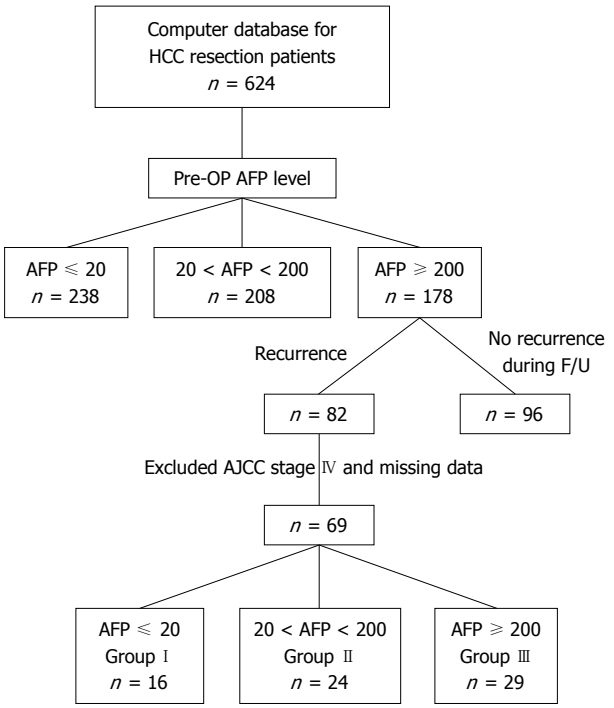

Figure 1 Flow chart for patient selection. HCC: Hepatocellular carcinoma; AFP: α-fetoprotein; AJCC: American Joint Committee on Cancer.

to-death survival was the date of death or the last follow-up date if the patients survived. The choice of salvage therapy was according to the willingness of the patient, the degree of tumor progression, and the criteria for treatment guidelines of the Cancer Committee at the Tri-Service General Hospital. The recurrence interval was defined as the day of the initial liver resection to the day of HCC recurrence diagnosis.

AFP immunohistochemistry

Three patients, one from each group, underwent a second liver resection for tumor recurrence. Surgical specimens from both operations in each of these three patients were reviewed by a pathologist (Dr. Yu CP). The specimens were examined by hematoxylin and eosin staining and immunohistochemistry for AFP levels and the differences were compared.

Follow-up

All patients were followed up regularly every month in the first year and every 3 mo thereafter. AFP level was monitored at each follow-up. Abdominal ultrasound imaging or computed tomography (CT) was performed every 3-6 mo in first two years, then annually, according to the NCCN practice guideline[15]. In some patients, magnetic resonance imaging, hepatic angiography, and percutaneous needle biopsy were considered if the CT images were not conclusive for a new hepatic lesion. All clinicopathological data were collected in the computer databases of the Cancer Board at the Tri-Service General Hospital, and the follow-up data were regularly updated. This study was approved by the Institutional Review Board of Tri-Service General Hospital. The follow-up period of this study ended on June 30, 2009.

Table 1 Clinical characteristics of groups Ⅰ, Ⅱ, and Ⅲ

	Group Ⅰ (*n* = 16)	Group Ⅱ (*n* = 24)	Group Ⅲ (*n* = 29)	*P*
Age (yr)	59.7 ± 12.5	57.1 ± 16.6	48.8 ± 16.3	0.055
Gender (M/F)	10/6	12/12	19/10	0.498
Tumor size (cm)	5.64 ± 3.07	4.80 ± 3.40	5.08 ± 3.11	0.696
ALT (U/L)	74.1 ± 50.9	77.2 ± 84.4	60.6 ± 44.1	0.615
T Bil (mg/dL)	0.79 ± 0.32	0.88 ± 0.49	0.96 ± 0.54	0.496
Albumin (g/dL)	3.74 ± 0.60	3.99 ± 0.50	3.74 ± 0.55	0.196
Plat (10^3/μL)	154.0 ± 65.8	134.9 ± 68.3	181.1 ± 94.5	0.155
Vessel invasion (+/-)	4/12	6/18	8/21	0.971
Tumor number (S/M)	9/7	16/8	15/14	0.541
AJCC Ⅰ/Ⅱ/Ⅲ	6/2/8	14/4/6	9/6/14	0.282
Child A/B	13/3	22/2	23/6	0.445
HBV (+/-)	12/4	15/9	25/4	0.241
HCV (+/-)	5/11	7/14	6/23	0.678
Cirrhosis (+/-)	10/6	14/10	20/9	0.720
AFP (op) (ng/dL)	1582 ± 2645	3049 ± 7215	2776 ± 6696	0.751
AFP (recur) (ng/dL)	7.5 ± 5.2	86.8 ± 57.9	8162.3 ± 15250.7	0.006
Recur interval (mo)	21.8 ± 14.6	12.3 ± 7.7	8.3 ± 6.6	< 0.001
Recur-to-death (mo)	18.4 ± 19.5	24.1 ± 19.8	13.6 ± 19.6	0.162
Survival time (mo)	40.2 ± 19.9	36.1 ± 22.4	21.9 ± 22.0	0.013
Intra-hepatic recur	2/14	8/16	17/8	0.001

ALT: Alanine aminotransferase; T Bil: Total bilirubin; Plat: Platelet; Child: Child-Pugh classification; AJCC: American Joint Committee on Cancer; HBV (+): Hepatitis B virus infection; HCV (+): Hepatitis C virus infection; Cirrhosis (+): Cirrhotic change by pathologic finding; AFP (op): α-fetoprotein level preoperatively; AFP (recur): α-fetoprotein level at tumor recurrence; Recur interval: The interval from operation to tumor recurrence; Recur-to-death: The interval from tumor recurrence to patient's death; Survival time: The time from operation to last follow-up date or death date; Intra-hepatic recur: Hepatocellular carcinoma (HCC) recurrence located at the adjacent segment, section and same lobe/HCC recurrence located at contralateral liver lobe.

Statistical analysis

Continuous clinical data were analyzed using the Student's *t* test and ANOVA, and the categorical data were analyzed using Fisher's exact test or the χ^2 test. Kaplan-Meier and Cox regression analyses were performed to determine the survival curves, and the survival relative ratio between the three groups of HCC patients. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using statistical software (SPSS version 15.0 for Windows, Chicago, IL, USA).

RESULTS

Of the 69 HCC patients with initially high AFP levels who underwent liver resection, 16 had normal serum AFP levels (⩽ 20 ng/dL) at recurrence. There were no significant differences between the three groups in terms of clinical characteristics such as age, gender, tumor stage, virology status, tumor pattern, biochemical examination results, and cirrhotic status (Table 1). Significant differences between the three groups were observed for recurrence intervals, survival times and location of recurrence ($P < 0.001$, $P = 0.013$, $P = 0.001$, respectively).

Recurrence interval among the groups

The accumulated recurrence rates in the three groups were

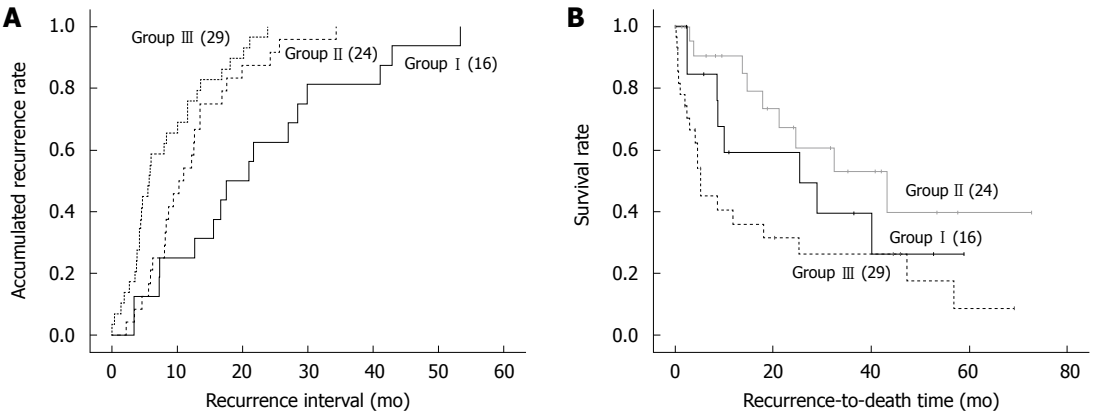

Figure 2 Accumulated recurrence rates and recurrence-to-death survival rates. A: Accumulated recurrence rates were calculated from the first liver resection date to recurrence date and compared between groups Ⅰ, Ⅱ, and Ⅲ ($P < 0.001$); B: Recurrence-to-death survival rates were calculated from the recurrence date to last follow-up date and compared between groups Ⅱ, Ⅰ, and Ⅲ ($P = 0.016$).

Table 2 Cox regression analysis of outcome for clinical parameters of HCC patients after curative liver resection

	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P*	RR	95% CI	*P*
Age (yr) > 50/≤ 50	1.023	0.532-1.968	0.95			
Gender (M/F)	0.640	0.325-1.262	0.20			
Tumor size (cm) > 5/≤ 5	0.444	0.230-0.856	0.02	0.836	0.404-1.729	0.63
ALT (U/L) > 40/≤ 40	0.929	0.471-1.831	0.83			
T Bil (mg/dL) > 1.2/≤ 1.2	0.357	0.175-0.730	0.005	0.430	0.159-1.168	0.10
Albumin (g/dL) > 3.5/≤ 3.5	1.674	0.867-3.232	0.13			
Plat (10^3/μL) > 10/≤ 10	1.600	0.808-3.169	0.18			
Vessel invasion (-/+)	0.470	0.228-0.969	0.04	0.251	0.096-0.658	0.005
Tumor number (S/M)	1.073	0.556-2.070	0.83			
Child B/A	0.372	0.182-0.759	0.007	0.452	0.153-1.339	0.15
HBV (-/+)	0.598	0.262-1.363	0.22			
HCV (-/+)	1.514	0.688-3.334	0.30			
Cirrhosis (-/+)	0.885	0.452-1.734	0.72			
Group Ⅰ reference			0.005			0.04
Ⅱ/Ⅰ	0.371	0.162-0.846	0.02	2.142	0.569-7.000	0.02
Ⅲ/Ⅰ	0.318	0.144-0.700	0.004	0.582	0.254-3.033	0.12
AFP (op) (ng/dL) > 200/≤ 200	0.048	0.000-14716.4	0.64			
AFP (recur) (ng/dL) > 200/≤ 200	0.341	0.177-0.653	0.001	0.851	0.301-1.389	0.45
Recur interval (mo) > 12/≤ 12	2.550	1.265-5.145	0.009	2.438	1.112-5.348	0.03
Path grade Ⅰ reference			0.42			
Ⅱ/Ⅰ	0.821	0.376-1.795	0.62			
Ⅲ/Ⅰ	0.504	0.197-1.291	0.15			

Path grade Ⅰ, Ⅱ, Ⅲ: Histopathologic grade of HCC. Ⅰ: Well differentiated; Ⅱ: Moderately differentiated; and Ⅲ: Poor or undifferentiated.

calculated using the Kaplan-Meier method (Figure 2A). Accumulated recurrence rates were calculated from the first liver resection date to the recurrence date. Recurrence took place in all patients and was compared among groups Ⅰ, Ⅱ, and Ⅲ ($P < 0.001$). The relative ratios [95% confidence interval (CI)] of groups Ⅱ and Ⅲ compared with group Ⅰ patients were 0.244 (0.119-0.498) and 0.591 (0.340-1.027), respectively, with group Ⅰ as the reference.

Independent factors affecting survival among the groups

The risk factors for clinical outcome related to survival are shown in Table 2. The six factors affecting clinical outcome by Cox proportion hazard analysis were tumor size (> 5 cm), total bilirubin (> 1.2 mg/dL), vessel invasion (+), recurrence interval (< 12 mo), Child-Pugh classification (B), and group Ⅰ and Ⅲ. When Cox regression analysis was performed, vessel invasion, group Ⅲ, and recurrence interval were identified as independent risk factors.

Recurrence-to-death survival rates among the groups

The recurrence-to-death survival rates were calculated from the recurrence date to the last follow-up date and compared among groups Ⅰ, Ⅱ, and Ⅲ as shown in Figure 2B ($P = 0.016$). Group Ⅱ had a significantly better survival rate than the other two groups. The relative ratios (95% CI) of groups Ⅱ and Ⅲ compared with group Ⅰ were 0.550 (0.241-1.253) and 0.337 (0.153-0.743), respectively.

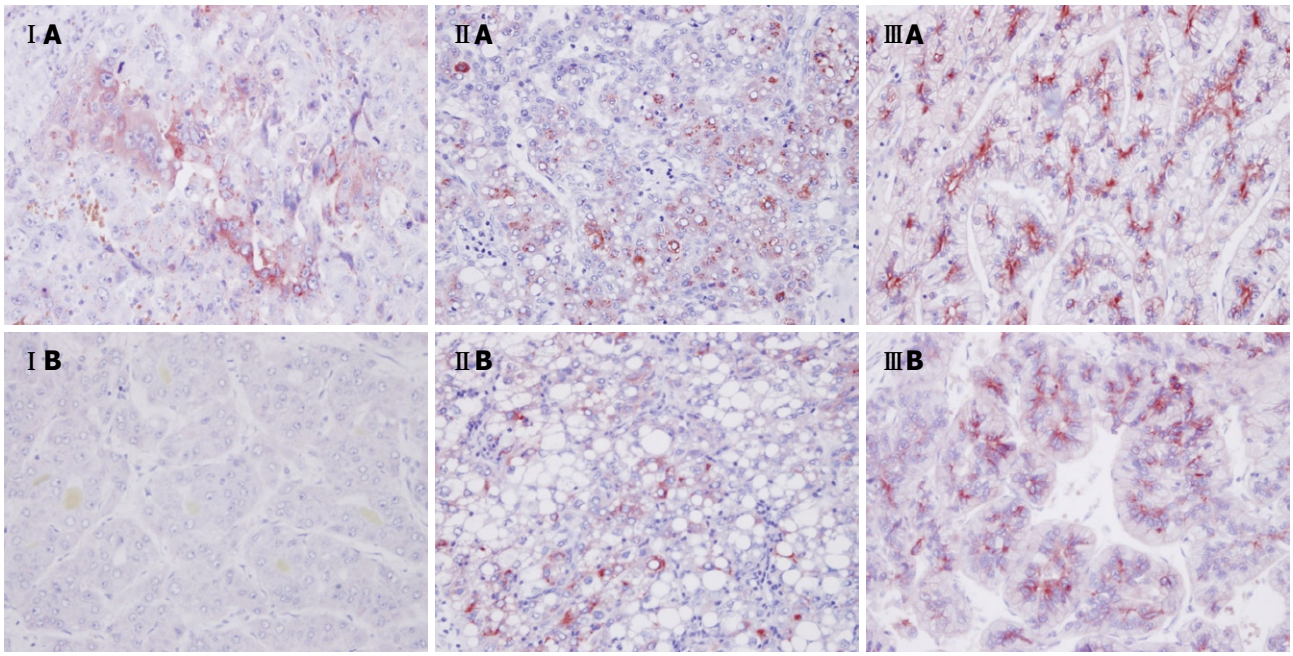

Figure 3 Immunohistochemical staining of AFP in representative specimens from the primary (A) and recurrent (B) HCC resections from each group (Ⅰ, Ⅱ, Ⅲ) × 400.

AFP immunohistochemistry in groups Ⅰ, Ⅱ, and Ⅲ

We compared the AFP immunohistochemistry results for a pair of specimens from each group. All groups revealed strong AFP staining in the primary HCC liver resection specimens. In the liver resection specimens from recurrent HCC, groups Ⅱ and Ⅲ showed strong AFP staining, but the specimen from group Ⅰ showed reduced staining (Figure 3).

DISCUSSION

Twenty-three percent of the patients in our study had inconsistent serum AFP levels between the time of initial HCC diagnosis and its recurrence. These patients had a longer recurrence interval. However, they did not have a better recurrence-to-death survival than patients with higher AFP levels at recurrence. We propose the following possible explanations: First, there was *de novo* HCC after the first resection, and the behavior or biological activity of the recurrent tumor was more malignant than the first. Second, a delay in the detection of the recurrent tumor as a result of normal AFP levels decreased the recurrence-to-death survival. This meant that these patients seemed to have a longer recurrence interval due to the delayed diagnosis caused by normal AFP levels. Once HCC recurrence was diagnosed *via* clinical symptoms, the recurrent tumor was advanced, which then shortened the time from recurrence to death.

In general, the principle we follow in HCC patients after resection is according to the NCCN guideline. For patients with initially high AFP levels, such as the patients of our study, the AFP level is followed every 3 mo, and imaging studies are carried out every 3-6 mo in the first two years. After 2 years, the AFP level is followed every 3 mo and imaging studies are carried out annually. If the AFP level is elevated, suggesting HCC recurrence then further intervention is arranged. However, in the group with inconsistent AFP levels, the imaging studies were carried out annually due to normal AFP levels. Once HCC recurrence based on the NCCN guideline is diagnosed, the recurrence will be detected late resulting in poor quality medical care and legal problems.

Histopathological change in a group Ⅰ patient and a possible mechanism

In our patients, some underwent resection twice for removal of HCC. The first and second specimens from liver resection did not show consistent histopathological findings in a patient from group Ⅰ, but did show similar histopathological findings in patients from groups Ⅱ and Ⅲ. Although these paired pathological findings are not conclusive, they do hint at an inconsistent pathological presentation. In a previous study, the authors proposed that the chromosomal changes and clonality relationship between primary and recurrent HCC could be proved using a comparative genomic hybridization technique[16]. In that study, 35% of HCC patients were considered to have a second primary HCC. In our study, the mechanism of HCC recurrence in group Ⅰ patients may be due to *de novo* HCC with low secretion of AFP. This mechanism in groups Ⅱ and Ⅲ may be due to intra-hepatic metastasis from the primary HCC. Many possible reasons for this phenomenon have been reported, including *de novo* HCC[16,17], a biologic tumor changed by dedifferentiation[18], or a multicentric origin[19].

Possible explanations for the clinical outcome

In our study, we used Cox regression analysis of the risk

factors for accumulated recurrence rate and no confounders were identified. Patients with inconsistent AFP levels had a longer accumulated recurrence rate than those in groups Ⅱ and Ⅲ. The second primary HCC might have needed a longer time to develop, resulting in a longer recurrence period. In groups Ⅱ and Ⅲ, the recurrent tumor was probably from an intra-hepatic metastasis or residual HCC resulting in a shorter recurrence interval than that suggested by other articles[20,21]. The recurrence-to-death survival rate of patients with inconsistent AFP levels was worse than that in group Ⅱ where the AFP level was consistent.

Dissociation of the tumor marker means inconsistent AFP, the clinical ramifications

Elevated serum AFP is useful for the diagnosis of HCC, and also implies a large tumor burden at presentation[6]. A low serum AFP level at recurrence in patients with an initially high AFP level at HCC diagnosis may be a good prognostic factor of survival[6]. However, this was not supported by our results. This poor outcome may be caused by a biological change in the malignancy or delayed diagnosis. Physicians need to diagnose recurrence as early as possible by regular surveillance to improve patient outcome. In the NCCN practice guidelines, version 1, 2009[15], surveillance for HCC recurrence in patients with initially elevated AFP levels includes checking AFP levels every 3 mo and performing imaging studies annually for 2 years after resection[15]. When physicians follow the NCCN guidelines, they should bear in mind that AFP level is not as sensitive as generally believed for tumor recurrence in this subgroup of HCC patients, similar to group I patients in our study. The results of our study showed that regular checks of AFP levels should be conducted every 1-3 mo, followed by imaging studies every 3 mo for patients with an initially elevated AFP level. Regular imaging studies should not be omitted even when the serum AFP level is normal. The results of our study are important for reminding practicing physicians that regular imaging is still the benchmark in the surveillance of HCC patients.

Limitations of this study

There were some limitations in this study. First, these preliminary data were from one medical center and the conclusion needs to be validated by a meta-analysis of multicentric data. Second, the diagnosis of HCC recurrence was not proven by pathologic findings. We diagnosed HCC recurrence by dynamic imaging studies, hepatic angiography, and clinical disease progression. In a further study, tumor biopsy should be performed before salvage therapy if possible. The results of histochemical staining in the recurrent tumor could explain the exact mechanism of this clinical presentation in patients with inconsistent AFP levels.

In conclusion, 23% of our selected patients demonstrated an inconsistency in serum AFP level between initial HCC diagnosis before resection and HCC recurrence diagnosis. To improve medical care and avoid missed or delayed diagnoses, regular checks of AFP level and imaging studies every three months are strongly recommended during follow-up, even for patients with normal serum AFP levels.

COMMENTS

Background

Serum α-fetoprotein (AFP) level is widely used as a tumor marker for the diagnosis and detection of both occurrence and recurrence of hepatocellular carcinoma (HCC). A high serum AFP level in HCC also indicates a poor outcome. However, inconsistent AFP levels have been found, where patients' AFP levels were initially high when HCC was diagnosed, but were normal when HCC recurred. There is no research on the prognosis of this subgroup of HCC patients.

Research frontiers

When clinical physicians follow the National Comprehensive Cancer Network guideline, they should bear in mind that AFP level is not as sensitive as generally believed for tumor recurrence in this subgroup of HCC patients. Regular checks of AFP level and imaging studies every three months are strongly recommended during follow-up, even for patients with normal serum AFP levels.

Innovations and breakthroughs

This is the first article to investigate the clinical outcomes of patients with inconsistent AFP levels. The clinical outcomes in this subgroup of HCC patients were not as good as those with normal AFP levels. To improve medical care and avoid missed or delayed diagnoses, clinical physicians should bear these findings in mind.

Applications

The authors modified the surveillance principle for this subgroup of HCC patients following the results of this study to improve the quality of medical care.

Peer review

This is a retrospective analysis of a cohort of patients with HCC, it maybe interesting for the readers.

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BRIEF ARTICLE

Combined resection and radiofrequency ablation for multifocal hepatocellular carcinoma: Prognosis and outcomes

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Abstract

AIM: To analyze the combined treatment of resection and intraoperative radiofrequency ablation (RFA) for multifocal hepatocellular carcinoma in terms of prognosis and surgical outcomes.

METHODS: This study was a retrospective case comparison study using prospectively collected data. The study covered the period from April 2001 to December 2006. The data of 200 patients with histologically confirmed hepatocellular carcinoma were reviewed. Nineteen patients (17 men and 2 women) having received resection in combination with RFA were chosen as subjects of the study (the combination group). Fifty-four patients (43 men and 11 women) having received resection alone were selected for comparison (the resection group). The two groups matched tumor number and tumor size, and all the patients in the two groups displayed no tumor rupture, major vascular involvement and distant metastasis. Their demographics, preoperative assessment, disease recurrence patterns, overall survival and disease-free survival were compared.

RESULTS: In the combination group, the median age was 65 years (range, 34-77 years), the median tumor number was 3 (range, 2-9), and the median tumor size was 6 cm (range, 1.2-14 cm). In the resection group, the median age was 51.5 years (range, 27-80 years, P = 0.003), the median tumor number was 3 (range, 2-9, P = 0.574), and the median tumor size was 6 cm (range, 1-14 cm, P = 0.782). The two groups were similar in characteristics of tumors and comorbidities, and had comparable results in preoperative liver function tests. All patients had Child-Pugh class A status. Bilobar involvement occurred in 14 patients (73.6%) in the combination group and 3 patients (5.5%) in the resection group (P = 0.04). Six patients (32%) in the combination group and 35 patients (65%) in the resection group underwent major hepatectomy. Thirteen patients (68%) in the combination group and 19 patients (35%) in the resection group underwent minor hepatectomy (P = 0.012). The combination group had fewer major resections (32% *vs* 65%, P = 0.012), less blood loss (400 *vs* 657 mL, P = 0.007), shorter operation time (270 *vs* 400 min, P = 0.001), and shorter hospital stay (7 *vs* 8.5 d, P = 0.042). The two groups displayed no major differences in surgical complications (15.8% *vs* 31.5%, P = 0.24), disease recurrence (63.2% *vs* 50%, P = 0.673), hospital mortality (5.3% *vs* 5.6%, P = 1), and overall survival (53 *vs* 44.5 mo, P = 0.496).

CONCLUSION: Safe and effective for selected patients with multifocal hepatocellular carcinoma, the combination of resection and intraoperative RFA widens the applicability of surgical intervention for the disease.

Key words: Hepatocellular carcinoma; Radiofrequency ablation; Combined resection; Resection; Cirrhosis

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INTRODUCTION

Management of hepatocellular carcinoma (HCC) in patients with cirrhosis is always a great challenge for clinicians. Prognosis of most HCC patients is poor due to the low rate of tumor resectability (20%-37%)[1,2]. Although resection is regarded as the gold-standard treatment for HCC, many patients are not suitable for it because of unfavorable anatomical location, major vessel involvement, multifocal involvement, distant metastasis, or poor liver function[3-7]. In the hope of improving the survival of patients having unresectable HCC, locoregional treatment aiming at local tumor control has been developed.

Radiofrequency ablation (RFA) is a locoregional treatment which has gained recognition in the management of liver diseases including HCC. During RFA treatment, heat energy generated by high-frequency alternating currents (460-480 kHz) targeted at the living tissues causes protein denaturation at a temperature of 60℃ through ionic vibration; coagulative necrosis of the target lesion then follows. RFA outperforms other locoregional treatments by its effective tumor ablation, minimal liver damage, low morbidity, and low mortality[3,8-12].

Lately, there have been detailed studies on RFA treating small HCC as well as isolated reports on the combined employment of resection and RFA for the treatment of liver metastasis of HCC[13-15]. Curley *et al*[16] reported a series of RFA treatment on 123 patients bearing unresectable primary (n = 48) or metastatic (n = 75) liver tumors. A total of 169 tumors were ablated with a clearance rate of 100%. There was no operative mortality. However, the efficacy of RFA on large tumors is still questionable. Although several reports showed that RFA with multiple processes and overlap of ablation zones was feasible for treating large liver tumors, the high recurrence rates did not favor it as a primary treatment tool for them[17-22]. Resection is still considered the first treatment option for large HCC.

Patients with multiple HCCs can opt for multiple resections, but multiple resections entail a higher risk of liver failure for patients with cirrhosis. Hence, in the management of multifocal HCC, combined employment of resection (for large tumors) and RFA (for small tumors) sounds like a reasonable measure for the achievement of complete tumor clearance.

Both resection and RFA are good treatment options for HCC while at the same time they both have their own limitations. Resection is limited by the patient's liver function and the location of tumor, and RFA is limited to small tumors only. In general, around one-fourth of patients with HCC are amenable to surgical intervention, and among them, around one-fourth develop multiple tumors on presentation. The combination of resection and RFA can subject more patients to potentially curative surgical intervention.

This study compared the combined treatment of resection and RFA with conventional hepatectomy alone in terms of prognosis and surgical outcomes in treating multifocal HCC.

MATERIALS AND METHODS

This study was a retrospective case comparison study using prospectively collected data. In our hospital, the data of all patients of liver surgery are input into a detailed computerized database by the surgeon-in-charge after patient discharge. Data collected include demographics, preoperative investigation results, operative details, and postoperative investigation results. This study covered the period from April 2001 to December 2006. The data of 200 patients with histologically confirmed HCC were reviewed. Nineteen patients having received resection in combination with RFA were chosen as subjects of the study (the combination group). Fifty-four patients having received resection alone were selected for comparison (the resection group). The two groups matched in terms of tumor number (range, 2-9) and tumor size (within 14 cm), and all patients in the two groups displayed no tumor rupture, major vascular involvement and distant metastasis. Their demographics, preoperative assessment, disease recurrence patterns, overall survival and disease-free survival were compared.

All the 73 patients had their medical history reviewed and were fully assessed with physical examination. They received chest X-ray (CXR) and computed tomography (CT) scan of the abdomen. They also received serum laboratory tests which yielded information on complete blood count, platelet count, coagulation profile, hepatitis B and hepatitis C serology, total serum bilirubin, serum albumin, serum alanine aminotransferase, serum aspartate aminotransferase, serum gamma-glutamyl transferase, serum alkaline phosphatase, serum α-fetoprotein (AFP), electrolytes, renal function, and indocyanine green (ICG) clearance rate. Resectability of tumor was confirmed upon three criteria: first, there was no distant metastasis according to the results of CXR and CT scan; second, the ICG rate was less than 14.4% at 15 min; and third, the estimated mass volume of the residual liver after resection was more than 30% of the original liver.

Surgical approaches

Each patient in the combination group underwent resection and RFA in one single operation intended to cure. The operation was performed with the open approach, starting with an initial exploration of the abdomen and pelvis to confirm the absence of extrahepatic lesion.

Intraoperative ultrasound was used to identify tumor location and number as well as the relation between the tumors and the vasculature of the liver. Anatomical resection was performed for the largest tumors or the largest groups of tumors which were deemed surgically resectable with clear margins. The extent of resection was determined by lesion location, the relation between the lesion and the surrounding vasculature, and biliary involvement. Parenchymal transection was performed with a Cavitron ultrasonic surgical aspirator. Segmental pedicles and other major vasculatures were divided with vascular staplers or ligatures. Pringle's maneuver was not applied.

Lesions not treated with resection were then subjected to RFA with standard treatment algorithm. Intraoperative ultrasound was used to guide the placement of the RFA needle at the target lesion. Single needle or needle cluster was used according to the size of the target tumor. For tumors larger than 3 cm, cluster probe was used. A margin of 1 cm was included in ablation. The Cool-tip™ system which consisted of a generator that supplied power up to 200 W was used. The electrode was optimally positioned to achieve complete tumor destruction and a normal parenchymal ablation zone of at least 1 cm.

All the patients were originally enlisted for resection only, but due to various reasons, the operative surgeon adopted RFA as an additional treatment modality with curative intention for complete control of tumors. The reasons for the adoption of a combination therapy are listed in Table 1.

Table 1 Reasons for adopting combination therapy *n* (%)

	Combination group (*n* = 19)
Bilobar disease	14 (73.6)
Proximity to major vessel or bile duct	5 (26.3)
Dense adhesion	3 (15.8)
Large resection required for small tumors	5 (26.3)
ICG rate at 15 min > 14.4%	5 (26.3)
Low platelet count (< 100 × 10^9/L)	3 (15.8)
Severe cirrhosis	9 (47.4)

ICG: Indocyanine green.

Table 2 Patient demographics

	Combination group (*n* = 19)	Resection group (*n* = 54)	*P* value
Age (yr)	65 (34-77)	52 (27-80)	0.003
Gender (M:F)	17:2	43:11	0.492
Comorbidities	10 (53%)	20 (37%)	0.235
Renal impairment	0 (0%)	2 (3.7%)	1.000
Diabetes mellitus	5 (26.3%)	10 (18.5%)	0.469
Chest infection	1 (5.3%)	3 (5.6%)	1.000
Coronary complications	6 (31.6%)	14 (25.9%)	0.635
Hepatitis B infection	16 (84.2%)	42 (77.8%)	0.745
Hepatitis C infection	2 (10.5%)	2 (3.7%)	0.478
Child-Pugh class A	19	54	1.000
Platelet count (× 10^9/L)	165 (91-64.5)	177 (86-458)	0.396
ICG (% at 15 min)	11.8 (3-25.7)	9 (3.7-18.2)	0.083
AFP level (ng/mL)	248 (6-38 040)	133 (2-530 600)	0.915
Tumor size (cm)	6 (1.2-14)	6 (1-12.5)	0.782
Tumor number	3 (2-9)	3 (2-9)	0.574

AFP: α-fetoprotein.

Postoperative follow-up

Monitoring of complete blood picture, coagulation profile, blood gas, liver function and renal function was carried out on day 1 and day 7 as part of the standard protocol. ICG clearance test was done on day 7 if possible. CXR, CT scan of the abdomen and serum AFP investigation were performed at one month, then quarterly in the first two years, and half-yearly afterwards.

Statistical analysis

All clinical data were analyzed with SPSS version 11.5 under the Window 98 operating system. Categorical variables were compared by a χ^2 test or Fisher's exact test. Continuous variables were compared by the Mann-Whitney *U* test. Survival rates were calculated by the Kaplan-Meier method, and differences in survival were analyzed by the log rank test. *P* value < 0.05 was considered statistically significant. The primary outcome measurement was overall survival and the secondary outcome measurement was disease-free survival. Immediate postoperative performance was also analyzed.

RESULTS

In the combination group, 17 men and 2 women, the median age was 65 years (range, 34-77 years). The median tumor number was 3 (range, 2-9) and the median tumor size was 6 cm (range, 1.2-14 cm). In the resection group, 43 men and 11 women, the median age was 51.5 years (range, 27-80 years, $P = 0.003$). The median tumor number was 3 (range, 2-9, $P = 0.574$) and the median tumor size was 6 cm (range, 1-14 cm, $P = 0.782$). The two groups were similar in characteristics of tumors and comorbidities, and had comparable results in preoperative liver function tests on serum bilirubin, serum albumin, prothrombin time, platelet count and ICG clearance. All patients in the two groups had Child-Pugh class A status (Table 2). Bilobar involvement occurred in 14 patients (73.6%) in the combination group and 3 patients (5.5%) in the resection group ($P = 0.04$).

Six patients (32%) in the combination group and 35 patients (65%) in the resection group received major hepatectomy. Thirteen patients (68%) in the combination group and 19 patients (35%) in the resection group received minor hepatectomy ($P = 0.012$). The types of resection performed are listed in Table 3. In the combination group, 5 patients had small tumor but received a large volume of resection for clearance, and 5 patients had tumors located close to major vessel, bile duct, or junction of hepatic veins. A total of 31 tumors were ablated, and the median size of the tumors was 1 cm (range, 1-3.8 cm). Ten patients had 1 tumor ablated, 6 patients 2, and 3 patients 3. Biopsies of the ablation sites were

Table 3 Types of resection performed according to Brisbane terminology (2005) of liver resection *n* (%)

	Combination group (*n* = 19)	Resection group (*n* = 54)
Right hepatectomy	1 (5.3)	17 (31.5)
Extended right hepatectomy	0 (0)	6 (11.1)
Right trisectionectomy	0 (0)	2 (3.7)
Left hepatectomy	3 (15.8)	3 (5.6)
Extended left hepatectomy	2 (10.5)	4 (7.4)
Left trisectionectomy	0 (0)	3 (5.6)
Left lateral sectionectomy	3 (15.8)	1 (1.9)
Segmentectomy	1 (5.3)	11 (20.4)
Wedge resection of liver	9 (47.4)	7 (13)

taken and all proven to be positive for HCC. The ablated sites were monitored with CT scan with contrast at one month, and no incomplete ablation was noted.

The combination group demonstrated certain advantages over the resection group in terms of surgical outcomes: the former had median blood loss of 400 mL (range, 20-1650 mL) whereas the latter had 657 mL (range, 50-3750 mL) (P = 0.007), the former had median operation time of 270 min (range, 150-465 min) whereas the latter had 400 min (range, 165-773 min) (P = 0.001), and the former had median hospital stay of 7 d (range, 1-20 d) and the latter had 8.5 d (range, 4-69 d) (P = 0.042). There was no difference in overall complications (15.8% *vs* 31.5%, P = 0.241) and hospital mortality (5.3% *vs* 5.6%, P = 1) between the two groups. Only one patient from the resection group needed blood transfusion. The median serum bilirubin level at day 1 was 21 μmol/L (range, 10-37 μmol/L) in the combination group and 25 μmol/L (range, 9-110 μmol/L) in the resection group (P = 0.012). The median serum albumin level at day 1 was 31.5 g/L (range, 10-37 g/L) in the combination group and 32 g/L (range, 17-39 g/L) in the resection group (P = 0.215). The median serum bilirubin level at day 7 was 18 μmol/L (range, 11-27 μmol/L) in the combination group and 21 μmol/L (range, 3-190 μmol/L) in the resection group (P = 0.075). The median serum albumin level at day 7 was 30 g/L (range, 24-39 g/L) in the combination group and 32 g/L (range, 26-41 g/L) in the resection group (P = 0.036).

Histopathology

With regard to the pathology of tumors, the combination group and the resection group shared similar characteristics. Margin involvement happened on none of the patients in the former group while on 4 patients (7.4%) in the latter (P = 0.567). Vascular permeation happened on 7 patients (36.8%) in the former group and 31 patients (57.4%) in the latter (P = 0.123). Microsatellite lesions appeared in 3 patients (15.8%) of the former group and 14 patients (25.9%) of the latter (P = 0.53). Poorly differentiated cell types by Edmondson classification appeared in 2 patients (10.5%) of the former group and 8 patients (14.8%) of the latter (P = 0.857). There was no statistical difference in histopathology between the two groups (Table 4).

Table 4 Histopathological results of tumors *n* (%)

	Combination group (*n* = 19)	Resection group (*n* = 54)	*P* value
Margin involvement	0 (0)	4 (7.4)	0.567
Vascular permeation	7 (36.8)	31 (57.4)	0.123
Microsatellite lesion	3 (15.8)	14 (25.9)	0.530
Poorly differentiated cell type	2 (10.5)	8 (14.8)	0.857
Complete ablation	19 (100)	-	
Histological proof of HCC at ablation sites	19 (100)	-	

HCC: Hepatocellular carcinoma.

Recurrence

Twelve patients (63.2%) in the combination group were found developing recurrence at the end of the study. Intrahepatic recurrence occurred in 8 patients (42.1%), and among them 4 patients (20.5%) had ablation site local recurrence, which happened at a median time of 3.6 mo (range, 3-24 mo). Extrahepatic recurrence occurred in 1 patient (5.3%) and intrahepatic and extrahepatic recurrence in 3 (15.8%). In the resection group, 27 patients (50%) were found developing recurrence. Among them, 15 patients (27.8%) had intrahepatic recurrence, 4 patients (7.4%) extrahepatic recurrence, and 8 patients (14.8%) intrahepatic and extrahepatic recurrence (P = 0.673).

Survival

The median follow-up period was 18.4 mo for the entire study period, 21 mo (range, 0.03-53 mo) for the combination group and 15.1 mo (range, 0.37-68.4 mo) for the resection group. No patient was lost to follow-up. The rates of overall survival at 1 year and 3 years were 88.8% and 62.6%, respectively for the former group (median 53 mo), and 88.9% and 51.8%, respectively for the latter (median 44.5 mo) (P = 0.496) (Figure 1A). The median disease-free survival of the former was 8.8 mo and that of the latter was 9.8 mo (P = 0.64) (Figure 1B). The two groups showed no statistically significant differences in survival.

DISCUSSION

A variety of treatment options are available for HCC. Liver transplantation offers good hope of cure to HCC patients, even those with poor liver function. However, donated livers are scarce, and patients having tumors exceeding the size limit for liver transplant or having low score in Model for End-stage Liver Disease are not suitable for transplantation. So, the first-line option is still, if technically feasible, hepatectomy[1,4,23,24]. Hepatectomy with clear margin can provide long postoperative survival to patients. Unfortunately, poor liver function poses extra challenge to hepatectomy because hepatectomy will diminish the already limited liver function reserve, thus risking liver failure. In Southeast Asia including Hong Kong, HCC patients usually have poor liver function because their HCC often coexists with hepatitis B cirrhosis.

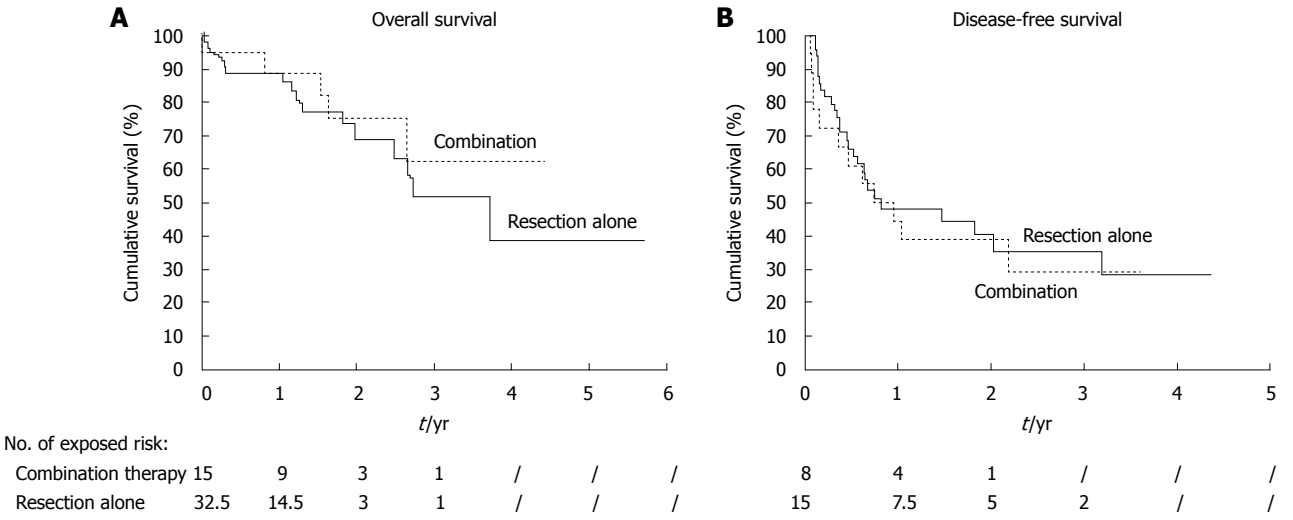

Figure 1 Survival curves showing overall survival and disease-free survival of the two groups of patients. A: Overall survival of patients; B: Disease-free survival of patients.

Striking a balance between achieving adequate resection and preventing liver failure is not easy at all. In patients with multifocal HCC, equilibrium between complete tumor clearance and minimal hepatectomy volume is even more difficult to grasp. Multiple partial resections, on the one hand, can achieve complete tumor clearance but, on the other hand, carry a higher risk of liver failure with doubtful long-term oncological outcomes.

RFA is another treatment option for HCC. Several clinical trials showed that RFA was as effective as hepatectomy for HCC smaller than 5 cm with a morbidity rate of less than 15%[22,25,26]. Hence, RFA has been gaining recognition in treating liver cancer including advanced HCC. Nonetheless, the efficacy of RFA on larger HCC is still questionable. In a study using a porcine model, RFA of 30%-35% of liver and hepatectomy of the same volume were compared, and it was found that the former resulted in significantly adverse systemic inflammatory response syndrome[27-29]. Hoshida *et al*[30] showed in another study that large parenchymal RFA volume was an independent poor prognostic factor for HCC patients. With recent advances in the design of radiofrequency electrodes and refinements of ablation techniques, favorable outcomes of RFA treatment on large tumors have been reported. However, extra caution should always be taken in treating large tumors with RFA since ablation of large tumors is associated with a high incidence of local recurrence, and the large amount of necrotic tissue left behind can cause serious problems for the patients. To ensure better outcome of RFA, careful patient selection, meticulous techniques and close monitoring of hepatic and renal functions after the procedure are essential.

Combined employment of hepatectomy and RFA for metastatic liver cancer has been documented. This combination of treatments can improve the tumor resectability rate for patients having multiple tumors. In this comparatively novel treatment, large tumors are resected and smaller ones at difficult locations are ablated. Currently, data on its performance in the management of HCC are very limited[14-16,22,25,26,31]. In our present study, the surgical outcomes of the combined treatment of hepatectomy and RFA on patients with multifocal HCC were investigated, and it was shown that patients having bilobar HCC could be treated safely with it. In the study, considerably less major hepatectomies were performed for the combination group, yet complete tumor clearance was still achieved. This is of crucial importance as hepatectomy in a lesser extent means better preservation of liver reserve, less liver failure, and quicker recovery[32,33].

For managing multifocal HCC, one may argue that multiple anatomical resections can achieve similar results as the combination approach. But in our study, we found that there are situations in which the combination approach is easier, if not better, than resection alone. First of all, if a tumor is small but requires a large resection volume for tumor clearance, RFA comes in as a safer option in terms of liver function preservation. Secondly, if a tumor is close to an important anatomical structure such as major vessels or bile ducts, RFA together with bile duct cooling can offer complete tumor clearance, obviating major hepatectomy. Thirdly, in the case of reoperation at a site where there is dense adhesion, RFA is a less traumatic treatment option, as mobilization for resection may lead to bleeding problem, especially in patients with thrombocytopenia and coagulopathy.

In this study, the median age of the combination group was 65 years, which was 13 years older than the 52-year median of the resection group. Choice of treatment was up to individual surgeons. It turned out that more elderly patients received the combined treatment. This was probably because the combined treatment would require less operation time and induce seemingly less surgical trauma. The resultant shorter operation time, less blood loss and shorter hospital stay have vindicated the postulation.

An ablation rate of 100% was achieved as confirmed by CT scan of the abdomen performed one month after

RFA treatment. The two groups displayed similar recurrence patterns. A margin of 1 cm was sought in all resections and ablations since a 1-cm margin is usually effective in preventing microscopic involvement by the major tumor. However, a clear margin alone is not enough for preventing tumor recurrence, as apart from margin involvement, there are other factors affecting recurrence such as poorly differentiated cell type, lymphovascular permeation, and microsatellite lesion, among which the latter two are the most significant independent risk factors. Unfortunately, we cannot alter these factors even by providing generous margins[24].

The combination group had more bilobar cases than the resection group (73.6% *vs* 7.4%). Theoretically, patients with stage IVa HCC would fare worse[34,35]. The predominance of bilobar disease in the combination group also explains why the group had more, though not significantly, intrahepatic and extrahepatic recurrences. A more advanced stage of HCC probably entails a poorer prognosis in general. Sub-group analysis of overall survival of these patients in the two groups was performed, and the median survival was 53 mo in the combination group and 44.5 mo in the resection group ($P = 0.496$). No significant difference was noted because of the small sample number in the resection group ($n = 3$). In our hospital, the median survival of patients having stage IVa HCC is 10 mo if they undergo no treatment. In the past, bilobar liver disease was considered a contraindication to hepatectomy, but with new tools and techniques, the whole concept of resectability is changing. Liu *et al*[36] pointed out that resection offered better survival outcomes than non-resectional treatments did to HCC patients with stage IVa bilobar disease. For this category of patients, resection in combination with RFA is an attractive choice of treatment. As to survival in our present study, the combination group and the resection group displayed comparable rates of overall survival and disease-free survival.

This study has clearly shown that when complete resection by major hepatectomy is dangerous because of marginal liver function or difficult tumor location, selective use of RFA is helpful. The integration of RFA into resectional surgery contributes to complete removal of tumors with adequate margin, diminishes the extent of parenchymal resection, and improves the resectability rate for patients with stage IVa liver disease. Safe and quick, it is especially handy when unexpected tumor is discovered during laparotomy, allowing complete tumor clearance in an unplanned situation during surgical operation without altering the original plan of treatment procedure too much; this is particularly important to patients having marginal hepatic function.

Fresh hope emerges as tumors previously considered unresectable due to multifocal involvement or poor liver function reserve can now be removed by the combined treatment, a new option in treating HCC. At present, a randomized controlled trial comparing it with surgical resection alone in patients with multifocal HCC is much desirable.

COMMENTS

Background

Management of hepatocellular carcinoma (HCC) in patients with cirrhosis is always a great challenge for clinicians. Prognosis of these patients is poor because many of their tumors are unresectable (with a resectability rate of 20%-37% only). Although resection is regarded as the gold-standard treatment for HCC, it is not applicable to cases with unfavorable anatomical location, major vessel involvement, multifocal involvement, distant metastasis, or poor liver function. On the other hand, radiofrequency ablation (RFA) is a treatment which has been gaining more and more recognition in the management of liver diseases including HCC. Both resection and RFA are good treatment options for HCC. However, they have their own limitations. Resection is hampered by patient's liver function as well as the location of tumor, whilst RFA is limited to smaller tumors only. In reality, only around one-fourth of HCC patients are amenable to surgical intervention, and in this sub-group, around one-fourth develop multiple tumors on presentation. There is no doubt that the combination of resection and RFA can subject more patients to potentially curative surgical intervention. The present study compared the combined treatment of resection and RFA with the treatment of sole resection and investigated the surgical outcomes of the combined treatment in patients with multifocal HCC.

Research frontiers

There is no detailed report on the combined use of resection and RFA in the management of HCC. However, this is an important topic as treating HCC with RFA is getting popular, and combining RFA with resection can definitely extend the operability of surgical intervention to patients with multifocal HCC.

Innovations and breakthroughs

In the management of multifocal HCC, one may argue that multiple resections can achieve what the combined treatment achieves, and thus there is no need to call in RFA. However, in the present study, it has been found that in some situations the combined treatment is easier than resection alone. First of all, since RFA is less traumatic and hence better for liver function preservation, it is preferable to resection when the tumor is small but still requires a large volume of resection for tumor clearance. Secondly, when the tumor is close to an important anatomical structure such as major vessel or bile duct, RFA together with bile duct cooling can achieve complete tumor clearance with lower risk. Thirdly, if dense adhesion is encountered, which is not uncommon in re-operation, RFA is safer as mobilization of the liver for resection may lead to bleeding problem especially in patients with thrombocytopenia and coagulopathy.

Applications

This study has clearly shown that when complete resection by major hepatectomy is dangerous because of marginal liver function or difficult tumor location, selective use of RFA provides the benefit of increased tumor resectability. The integration of RFA into resectional surgery contributes to complete removal of tumors with adequate margin, diminishes the extent of parenchymal resection, and improves the resectability rate for patients with stage IVa liver disease. Being safe and quick, it is especially handy when unexpected tumor is encountered during laparotomy, allowing achievement of complete tumor clearance in an unplanned situation during surgical operation without altering the original plan of treatment procedure too much; this is particularly important to patients with marginal liver function.

Terminology

RFA is a locoregional treatment. RFA treats tumors by inducing coagulative necrosis of the target lesion. During RFA, heat energy generated by high-frequency alternating currents (460-480 kHz) targeted at the lesion through ionic vibration causes protein denature at a temperature of 60℃, which causes coagulative necrosis of tissues.

Peer review

The Hong Kong group describes their experience with surgical treatment of HCC by performing a retrospective analysis of resected-only patients versus resected + RFA patients with HCC. This is important data to present and very clinically relevant as centers all over the world are taking care of rising numbers of patients with HCC.

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BRIEF ARTICLE

Evidence-based appraisal in laparoscopic Nissen and Toupet fundoplications for gastroesophageal reflux disease

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Abstract

AIM: To demonstrate the optimal surgical procedure for gastroesophageal reflux disease.

METHODS: The electronic databases of Medline, Elsevier, Springerlink and Embase over the last 16 years were searched. All clinical trials involved in the outcomes of laparoscopic Nissen fundoplication (LNF) and laparoscopic Toupet fundoplication (LTF) were identified. The data of assessment in benefits and adverse results of LNF and LTF were extracted and compared using meta-analysis.

RESULTS: We ultimately identified a total of 32 references reporting nine randomized controlled trials, eight prospective cohort trials and 15 retrospective trials. These studies reported a total of 6236 patients, of whom 4252 (68.18%) underwent LNF and 1984 (31.82%) underwent LTF. There were no differences between LNF and LTF in patients' satisfaction, perioperative complications, postoperative heartburn, reflux recurrence and re-operation. Both LNF and LTF enhanced the function of lower esophageal sphincter and improved esophagitis. The postoperative dysphagia, gas-bloating syndrome, inability to belch and the need for dilatation after LNF were more common than after LTF. Subgroup analyses showed that dysphagia after LNF and LTF was similar in patients with normal esophageal peristalsis (EP), but occurred more frequently in patients with weak EP after LNF than after LTF. Furthermore, patients with normal EP after LNF still had a higher risk of developing dysphagia than did patients with abnormal EP after LTF.

CONCLUSION: Compared with LNF, LTF offers equivalent symptom relief and reduces adverse results.

Key words: Laparoscopic fundoplication; Nissen, Toupet; Gastroesophageal reflux disease; Anti-reflux surgery; Esophageal peristalsis; Meta-analysis

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a chronic acid-peptic disorder characterized by the spontaneous and involuntary retrograde flow of stomach contents into esophagus mainly due to functional defect of the lower esophageal sphincter (LES)[1]. Acid reflux into the esophagus may cause esophagitis, tracheobronchitis,

stricture, Barrett's esophagus and even esophageal cancer, all of which affect a sizeable portion of patients, particularly in the USA and Europe[2-5]. Symptomatic GERD has fundamental effects on the quality-of-life (QOL) of patients. However, the complex pathophysiology of GERD[4] and limitation of the medicine[6] always make it extremely difficult to get a satisfactory efficacy under individual anti-acid medication in the long term.

Surgery is discussed as a very effective treatment for GERD which imparts a mechanical solution[6]. Surgical treatment was initially introduced by Rudolph Nissen[7] in 1956 and subsequently was developed as a safe and effective procedure by Dallemagne in 1991 through a minimal invasive approach[8]. Laparoscopic fundoplication has been established as the "gold standard" in the surgical treatment for GERD because of its immense success. There are two major anti-reflux procedures: 360º total (Nissen) fundoplication and 270º partial (Toupet) fundoplication. The superiority of one over the other is a matter of debate. The supporters of partial fundoplication argue that partial and total wrap construction offer equally effective forms of therapy for GERD[9-11]. To avoid the major postoperative complication, dysphagia, preoperative esophageal motility should be considered, and partial funduplication has the advantage of reducing this complication[12,13]. The proponents of total fundoplication propose that: (1) the 270º wrap provides a weaker anti-reflux barrier, and is therefore insufficient for reflux control[14,15]; (2) the prevalence of postoperative dysphagia after Nissen fundoplication is overestimated; and (3) motility disorders are not correlated with postoperative dysphagia[16], suggesting that the so-called "tailored procedure" should be abandoned[17,18]. Many crucial issues related to the mechanism of GERD, such as whether esophageal dysmotility is a consequence of GERD or just another component (along with LES dysfunction) of a complex foregut motor disease, have not been elucidated[19,20], so the controversy regarding the optimal surgical technique continues[21,22].

Meta-analysis is a powerful tool with an attempt to overcome the problem of reduced statistical power in studies with small sample sizes and to control variations between studies. The goal of surgical treatment for GERD is to provide optimal reflux control while minimizing adverse results. We focused this current systemic review on comparing which procedure was more effective in improving the QOL of patients while producing less adverse results. We attempted to depict a more comprehensive picture of the feature of laparoscopic anti-reflux operation, thus offering guidance for clinical practice.

MATERIALS AND METHODS

Search strategy

The electronic databases of Medline, ScienceDirect (Elsevier), Springerlink and Embase over the last 16 years from January 1994 to November 2009 were searched by two authors (Shan CX and Zhang W) to identify all clinical trials comparing laparoscopic Nissen fundoplication (LNF) with laparoscopic Toupet fundoplication (LTF). A search strategy using disease-specific search terms (e.g. gastroesophageal reflux disease), management-specific terms (e.g. laparoscopic anti-reflux fundoplication) and terms related to surgical procedures (e.g. Nissen, Toupet, partial and total) was adopted. All photocopied abstracts and citations were reviewed. The related articles were used to broaden the searching scope, and references of the articles acquired were also searched.

Selection criteria

Abstracts or full-text articles were initially screened, and then selected or rejected by the two reviewers (Shan CX and Zhang W) on the basis of the inclusion and exclusion criteria described below. A flow chart representing selection of clinical trials is shown in Figure 1.

Inclusion criteria: (1) Comparative clinical trials, including randomized and non-randomized controlled trials, involved in the efficacy and adverse results of different types of laparoscopic anti-reflux surgery (LARS); and (2) The exact data of dichotomous-type information and continuous-type information as well as standard deviation should be provided so as to integrate each single weight in each study.

Exclusion criteria: (1) Comparative trials between total and non-posterior partial fundoplication (e.g. total *vs* anterior partial fundoplication); (2) Fundoplications were carried out with laparotomy; (3) Trials involving children or patients younger than 16 years; and (4) Studies published repeatedly by different journals.

Data extraction

The two reviewers independently extracted details from selected studies which comprised (1) information and quality of the research: first author, year of publication, comparative design, sample capacity, follow-up duration; and (2) outcome analysis, including beneficial and adverse results. The specific rules of data extraction were listed below.

First, the assessment data of repeated trials published in different journals at different phases was extracted based on the latest article. For example, the results in the studies of Strate *et al*[16], Fibbe *et al*[23] and Zornig *et al*[24] were highly homologous, containing identical study objects and design protocol. Thus, the source of data extraction was focused on the latest citation of Zornig *et al*[24].

Second, the assessment data of trials containing multiple groups were initially divided into single groups, and then extracted individually. For example, the research of Mickevicius *et al*[22] included two subgroups of LARS with a 1.5-cm wrap fundoplication and 3.0-cm wrap. We split it into two independent trials according to the wrap length.

And third, the evaluation indices of surgical efficacy and incidence of postoperative morbidities were subject to the final updates based on long-term follow-up results. For example, Chrysos *et al*[25] reported the 3- and 12-mo incidences of postoperative dysphagia in their

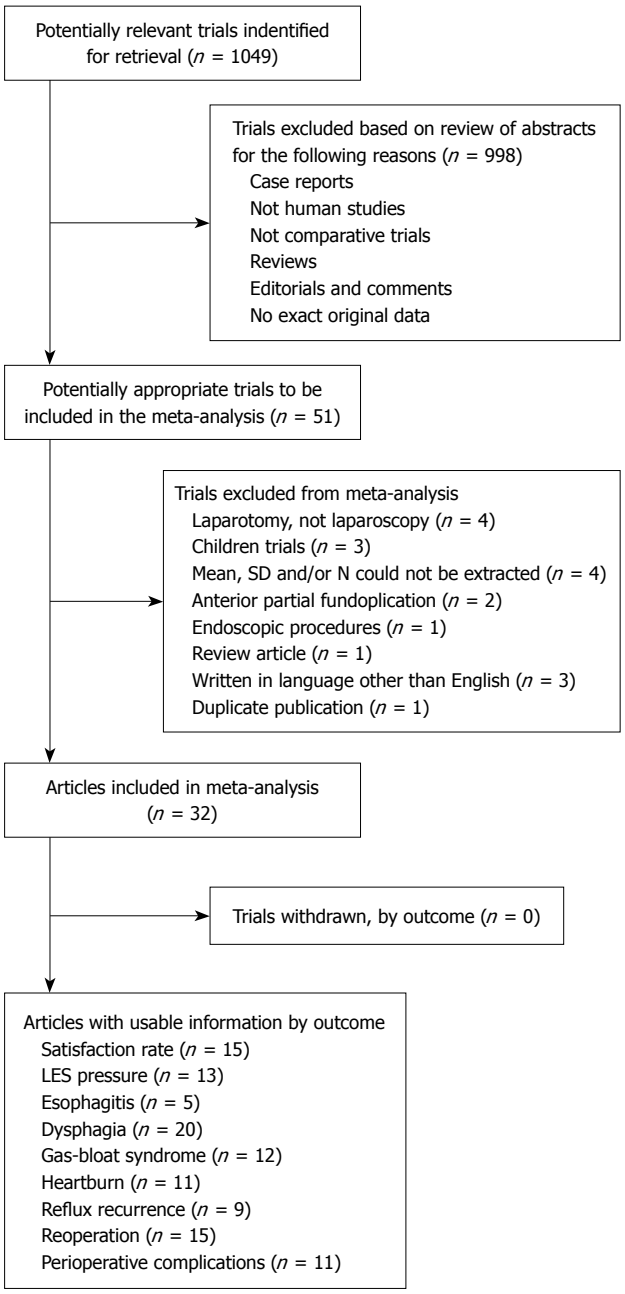


Figure 1 A flow chart showing the progress of trials through the review. LES: Lower esophageal sphincter.

article. We ultimately extracted the information on the 12-mo incidence of dysphagia after LARS and integrated it into the total meta-analysis.

Statistical analysis

All individual outcomes were integrated with the meta-analysis software: Review Manager Software 5.0 (Cochrane Collaborative, Oxford, England). Results were analyzed with the random-effect method if significant heterogeneity ($P < 0.05$ was used to define statistically significant heterogeneity) was detected among studies. Otherwise, a fixed-effect model was adopted. The odds ratio (OR) and the weighted mean difference were calculated for dichotomous data and continuous data, respectively. In addition, subgroup analyses were done to estimate if the results of postoperative dysphagia would change after LARS with respect to preoperative esophageal motility (EM). Thus, three subgroups were established. Subgroup 1: all patients underwent LNF or LTF and had normal esophageal peristalsis (EP); subgroup 2: all patients underwent LNF or LTF and had abnormal EP; and subgroup 3: LNF for patients with normal EP *vs* LTF for patients with abnormal EP.

RESULTS

Identification and characteristics of studies and patients

We ultimately identified 32 references reporting 29 independent controlled studies published between January 1994 and November 2009. Nine randomized controlled trials[10,16,21-27], eight prospective cohort trials[11,17,28-33] and 15 retrospective trials[9,12,20,33-45] met the selection criteria reporting 6236 patients, of whom 4252 (68.18%) underwent LNF and 1984 (31.82%) underwent LTF. Twenty studies provided the sex and 11 trials provided the age of included patients. The percentage of males varied from 39.39% to 70.13% in the LNF group, and from 39.53% to 66.67% in the LTF group. The mean age ranged from 45.2 to 59.2 years in the LNF group and from 44.2 to 61.7 years in the LTF group. The details of these studies are shown in Table 1.

Subjective assessment: Satisfaction rate

Fifteen studies assessed patient satisfaction as the major efficacy of LARS. In addition, patient satisfaction with the outcome of surgery was always expressed by Visick scores evaluating QOL. After follow-up, each single research suggested that > 90% patients were satisfied with postoperative outcomes after LNF or LTF, and 91.78% (1674/1824) of patients in the LNF group and 91.33% (938/1027) in the LTF group reported excellent or good results after LARS. There was no statistically significant difference between the two groups (OR 0.96, 95% CI: 0.72-1.28, $P = 0.77$).

Objective evaluation

LES pressure: The change in LES pressure after LARS was investigated in 13 trials. The mean preoperative LES pressure was 3.1-12.8 mmHg in the LNF group, and about 2.3-13.6 mmHg in the LTF group, and increased significantly to 10.3-26 mmHg after LNF and 11-18 mmHg after LTF, respectively. Moreover, 360° total fundoplication could form a relatively stronger anti-reflux barrier than 270° partial funduplication because the amplitude of LES pressure increase was significantly higher after LNF than after LTF (OR 2.76, 95% CI: 1.57-3.95, $P < 0.05$).

Esophagitis: Five authors outlined the improvement of preoperative esophagitis after LARS by endoscopy. Esophagitis severity was graded according to the Savary-Miller classification in most studies. Remission of moderate-to-severe esophagitis was observed by endoscopic re-examination after anti-reflux procedures; there was a reduction in case number from 87/210 to 12/191 in the

Table 1 LNF and LTF in treatment of patients with gastroesophageal reflux disease

Data source	Design	PF (°)	Sample capacity (LNF/LTF)	Group or subg. depend on EM	Follow-up (mo)	Level of evidence
Mickevicius *et al*[22], 2008 Fibbe *et al*[23], 2001	RCT	200-270	76/77	No detail	12	1b Level A
Zornig *et al*[24], 2002 Strate *et al*[16], 2008	RCT	270	100/100	More subgroups	24	1b Level A
Chrysos *et al*[25], 2003	RCT	270	14/19	Both abnormal	12	2b Level B
Guérin *et al*[26], 2007	RCT	270	77/63	Normal-Nissen/Abnormal-Toupet	36	1b Level A
Laws *et al*[10], 1997	RCT	200	23/16	Both normal	27.2	2b Level B
Shaw *et al*[27], 2010	RCT	270	50/50	No detail	> 55	1b Level A
Booth *et al*[21], 2008	RCT	270	64/63	No detail	12	1b Level A
Coster *et al*[28], 1997	PCT	No detail	125/101	No detail	12	2b Level B
Granderath *et al*[29], 2007	PCT	270	28/28	Both normal	3	3b Level B
Wykypiel *et al*[30], 2008	PCT	300	20/20	Normal-Nissen/Abnormal-Toupet	6	3b Level B
Hunter *et al*[31], 1996	PCT	180-300	101/83	Both normal	3	3b Level B
Bessell *et al*[17], 2000	PCT	200-270	761/85	More subgroups	12	2b Level B
Radajewski *et al*[32], 2009	PCT	No detail	51/43	No detail	12	2b Level B
Kamolz *et al*[33], 2000 Kamolz *et al*[11], 2002	PCT	No detail	107/68	No detail	60	2b Level B
Sgromo *et al*[34], 2008	RT	No detail	150/116	No detail	72	2b Level B
Erenoğlu *et al*[35], 2003	RT	270	118/26	Normal-Nissen/Abnormal-Toupet	27.5	2b Level B
Herbella *et al*[20], 2007	RT	240	55/16	More subgroups	16	3b Level B
Wetscher *et al*[12], 1997	RT	No detail	17/32	Normal-Nissen/Abnormal-Toupet	15	3b Level B
Fernando *et al*[36], 2002	RT	270	163/43	No detail	19.7	2b Level B
Wykypiel *et al*[37], 2005	RT	300	77/132	Normal-Nissen/Abnormal-Toupet	52	2b Level B
McKernan *et al*[38], 1994	RT	180-200	14/14	No detail	> 3.4	3b Level B
Fein *et al*[45], 2008	RT	No detail	88/10	Normal-Nissen/Abnormal-Toupet	120	2b Level B
Patti *et al*[39], 2004	RT	240	216/141	More subgroups	> 23	2b Level B
Zügel *et al*[9], 2002	RT	270	40/122	No detail	19	2b Level B
Pessaux *et al*[40], 2000	RT	180	1078/392	No detail	24	2b Level B
Bell *et al*[41], 1996	RT	180	11/11	No detail	13	4 Level C
Lund *et al*[42], 1997	RT	270	16/46	Both abnormal	6	3b Level B
Farrell *et al*[43], 2000	RT	270	293/52	More subgroups	12	3b Level B
Heider *et al*[44], 2003	RT	270	15/4	Both abnormal	29.5	4 Level C

LNF: Laparoscopic Nissen fundoplication; LTF: Laparoscopic Toupet fundoplication; PF: Circumferential degrees of the partial wrap; Subg.: Subgroup; EM: Esophageal motility; RCT: Randomized controlled trial; PCT: Prospective controlled trial; RT: Retrospective trial.

Nissen group, and from 99/218 to 17/197 in the LTF group. There was no significant difference in remission rate between the two groups (6.28% *vs* 8.63%, OR 0.72, 95% CI: 0.59-1.33, $P = 0.42$).

Adverse results: Perioperative complications

Eleven studies reported the surgical complications in 1622 patients with LNF and 836 patients with LTF. Both techniques entailed a low (but definite) risk of surgical morbidity which occurred in 1.30%[26]-14.28%[25] of the population after Nissen fundoplication and in 2.46%[9]-15.63%[12] of the population after Toupet fundoplication. Lacerations of the gastric fundus and spleen, bleeding from the spleen or short gastric blood vessels and pneumothorax were the main disease categories. However, surgical mortality was also a natural sporadic event. Only two patients died: one on the 15th day after LNF due to secondary peritonitis from necrosis of the wrap[40], and the other died of esophageal perforation and mediastinitis[22]. The cumulative prevalence of perioperative complications after LNF and LTF was 4.01% and 5.14%, respectively, with no significant difference between the groups (4.01% *vs* 5.14%, OR 0.73, 95% CI: 0.47-1.12, $P = 0.15$).

Postoperative symptoms

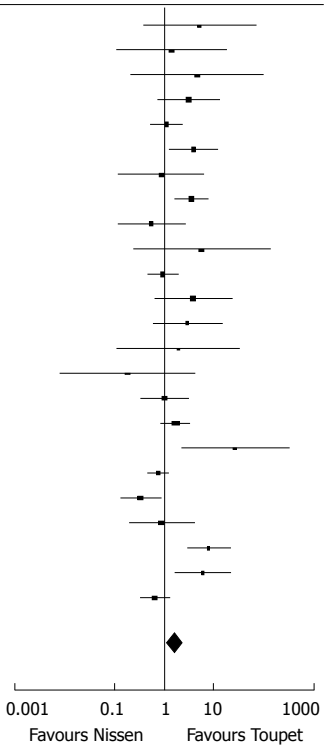
Dysphagia and need for bougie dilatation: Twenty-four studies assessed the outcome of dysphagia after LARS. Various questionnaires were used to define dysphagia severity, ranging from no symptoms to very severe episodes at the end of follow-up. The overall prevalence of dysphagia after LNF was significantly higher than after LTF (14.29% *vs* 11.00%, OR 1.68, 95% CI: 1.12-2.52, $P = 0.01$) (Figure 2A). A similar result was also obtained if only patients with moderate-to-severe dysphagia were included (12.30% *vs* 2.74%, OR 3.11, 95% CI: 1.94-5.00, $P < 0.01$). For patients with severe symptoms needing bougie dilatation, prevalence was still significantly higher after LNF than after LTF (7.91% *vs* 1.44%, OR 3.67, 95% CI: 1.90-7.09, $P < 0.01$). We thought it necessary to carry out subgroup analyses because preoperative EM was an important variable in the choice of surgical procedure and may have an effect on the analyses. Defining esophageal dysmotility was not consistent in the surgical literature. Most surgeons would agree that a distal esophageal amplitude > 40 mmHg and peristaltic contraction of the esophageal body of at least 70% indicate normal motility[18]. As described above, subgroup 1 analysis showed that the surgical procedure had no effect on the occurrence of

A

Study or subgroup	Nissen Events	Nissen Total	Toupet Events	Toupet Total	Weight (%)	Odds ratio M-H, random (95% CI)	Allocation concealment?
Shaw *et al*[27], 2010	3	7	1	8	1.9	5.25 (0.40-68.95)	Yes
Laws *et al*[10], 1997	2	23	1	16	2.0	1.43 (0.12-17.23)	Yes
Guérin *et al*[26], 2007	2	62	0	56	1.5	4.67 (0.22-99.38)	Yes
Mickevicius 3.0 *et al*[22], 2008	8	31	3	30	4.0	3.13 (0.74-13.20)	Yes
Patti *et al*[39], 2004	20	216	12	141	6.4	1.10 (0.52-2.32)	Yes
Booth *et al*[21], 2008	16	59	5	58	5.2	3.94 (1.34-11.63)	Yes
Chrysos *et al*[25], 2003	2	14	3	19	2.9	0.89 (0.13-6.18)	Yes
Strate *et al*[16], 2008	30	100	11	100	6.3	3.47 (1.62-7.40)	Yes
Mickevicius 1.5 *et al*[22], 2008	3	33	5	33	3.8	0.56 (0.12-2.56)	Yes
McKernan *et al*[38], 1994	2	14	0	14	1.4	5.80 (0.25-132.56)	No
Farrell *et al*[43], 2000	83	293	15	52	6.7	0.97 (0.51-1.87)	No
Wykypiel *et al*[30], 2008	6	20	2	20	3.3	3.86 (0.67-22.11)	No
Hunter *et al*[31], 1996	7	101	2	83	3.6	3.02 (0.61-14.93)	No
Wetscher *et al*[12], 1997	1	17	1	32	1.6	1.94 (0.11-33.05)	No
Granderath *et al*[29], 2007	0	28	2	28	1.4	0.19 (0.01-4.05)	No
Erenoğlu *et al*[35], 2003	23	118	5	26	5.2	1.02 (0.35-2.98)	No
Sgromo *et al*[34], 2008	48	99	22	62	6.7	1.71 (0.89-3.29)	No
Bell *et al*[41], 1996	10	11	3	11	2.1	26.67 (2.31-308.00)	No
Pessaux *et al*[40], 2000	57	1078	27	392	7.3	0.75 (0.47-1.21)	No
Fernando *et al*[36], 2002	17	113	10	29	5.7	0.34 (0.13-0.85)	No
Fein *et al*[45], 2008	23	74	3	9	3.9	0.90 (0.21-3.93)	No
Wykypiel *et al*[37], 2005	21	77	6	132	5.6	7.88 (3.01-20.57)	No
Lund *et al*[42], 1997	10	16	10	46	4.7	6.00 (1.75-20.55)	No
Bessell *et al*[17], 2000	87	761	14	85	6.9	0.65 (0.35-1.21)	No
Total (95% CI)		3365		1482	100	1.68 (1.12-2.52)	
Total events	481		163				

Heterogeneity: Tau^2 = 0.53; χ^2 = 65.84, *df* = 23 (*P* < 0.00001); I^2 = 65%

Test for overall effect: *Z* = 2.50 (*P* = 0.01)



B

Study or subgroup	Nissen Events	Nissen Total	Toupet Events	Toupet Total	Weight (%)	Odds ratio M-H, random (95% CI)	Allocation concealment?
2.2.1 Motility normal (both)							
Strate *et al*[16], 2008	16	50	3	50	16.7	7.37 (1.99-27.32)	Yes
Laws *et al*[10], 1997	2	23	1	16	7.8	1.43 (0.12-17.23)	Yes
Bessell *et al*[17], 2000	58	558	5	21	19.9	0.37 (0.13-1.05)	No
Hunter *et al*[31], 1996	7	101	2	83	13.7	3.02 (0.61-14.93)	No
Farrell *et al*[43], 2000	75	267	9	31	22.7	0.95 (0.42-2.17)	No
Fernando *et al*[36], 2002	14	88	4	7	13.7	0.14 (0.03-0.70)	No
Granderath *et al*[29], 2007	0	28	2	28	5.6	0.19 (0.01-4.05)	No
Subtotal (95% CI)		1115		236	100	0.93 (0.32-2.64)	
Total events	172		26				

Heterogeneity: Tau^2 = 1.31; χ^2 = 21.66, *df* = 6 (*P* = 0.001); I^2 = 72%

Test for overall effect: *Z* = 0.14 (*P* = 0.89)

2.2.2 Motility abnormal (both)							
Patti *et al*[39], 2004	9	55	12	141	19.3	2.10 (0.83-5.32)	Yes
Strate *et al*[16], 2008	14	50	8	50	18.7	2.04 (0.77-5.42)	Yes
Chrysos *et al*[25], 2003	2	14	3	19	9.9	0.89 (0.13-6.18)	Yes
Farrell *et al*[43], 2000	8	26	6	21	15.6	1.11 (0.31-3.92)	No
Bessell *et al*[17], 2000	29	203	9	64	20.7	1.02 (0.45-2.28)	No
Lund *et al*[42], 1997	10	16	10	46	15.9	6.00 (1.75-20.55)	No
Subtotal (95% CI)		364		341	100	1.75 (1.04-2.94)	
Total events	72		48				

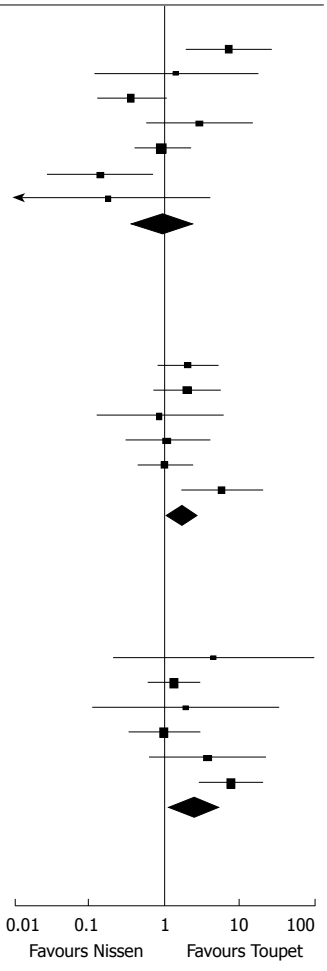
Heterogeneity: Tau^2 = 0.11; χ^2 = 6.78, *df* = 5 (*P* = 0.24); I^2 = 26%

Test for overall effect: *Z* = 2.11 (*P* = 0.04)

2.2.3 Motility normal (Nissen) *vs* motility abnormal (Toupet)							
Guérin *et al*[26], 2007	2	62	0	56	6.4	4.67 (0.22-99.38)	Yes
Patti *et al*[39], 2004	18	161	12	141	26.5	1.35 (0.63-2.92)	Yes
Wetscher *et al*[12], 1997	1	17	1	32	7.2	1.94 (0.11-33.05)	No
Erenoğlu *et al*[35], 2003	23	118	5	26	22.0	1.02 (0.35-2.98)	No
Wykypiel *et al*[30], 2008	6	20	2	20	14.1	3.86 (0.67-22.11)	No
Wykypiel *et al*[37], 2005	21	77	6	132	23.7	7.88 (3.01-20.57)	No
Subtotal (95% CI)		455		407	100	2.47 (1.08-5.68)	
Total events	71		26				

Heterogeneity: Tau^2 = 0.51; χ^2 = 10.94, *df* = 5 (*P* = 0.05); I^2 = 54%

Test for overall effect: *Z* = 2.14 (*P* = 0.03)



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Study or subgroup	Nissen Events	Nissen Total	Toupet Events	Toupet Total	Weight (%)	Odds ratio M-H, random (95% CI)	Allocation concealment?
3.3.1 Postoperative gas bloating							
Chrysos *et al*[25], 2003	3	14	3	19	6.7	1.45 (0.25-8.58)	Yes
Guérin *et al*[26], 2007	1	62	0	56	3.1	2.76 (0.11-69.04)	Yes
Booth *et al*[21], 2008	11	59	6	58	9.7	1.99 (0.68-5.79)	Yes
Wykypiel *et al*[37], 2005	18	77	6	132	10.1	6.41 (2.42-16.97)	No
Sgromo *et al*[34], 2008	33	99	23	62	11.5	0.85 (0.44-1.65)	No
Wykypiel *et al*[30], 2008	6	20	0	20	3.6	18.38 (0.96-352.57)	No
Granderath *et al*[29], 2007	17	28	7	28	9.3	4.64 (1.48-14.54)	No
Fernando *et al*[36], 2002	60	113	18	29	10.8	0.69 (0.30-1.60)	No
Bell *et al*[41], 1996	9	11	3	11	5.8	12.00 (1.58-91.08)	No
Fein *et al*[45], 2008	63	74	5	9	7.9	4.58 (1.06-19.78)	No
Coster *et al*[28], 1997	70	82	38	62	10.9	3.68 (1.66-8.18)	No
Radajewski *et al*[32], 2009	36	51	29	43	10.6	1.16 (0.48-2.79)	No
Subtotal (95% CI)		690		529	100	2.42 (1.37-4.26)	
Total events	327		138				
Heterogeneity: Tau^2 = 0.55; χ^2 = 30.48, *df* = 11 (*P* = 0.001); I^2 = 64%							
Test for overall effect: *Z* = 3.06 (*P* = 0.002)							
3.3.2 Postoperative inability to belch							
Guérin *et al*[26], 2007	0	62	2	56	4.9	0.17 (0.01-3.71)	Yes
Booth *et al*[21], 2008	34	59	24	58	16.0	1.93 (0.92-4.02)	Yes
Wykypiel *et al*[30], 2008	6	20	0	20	5.1	18.38 (0.96-352.57)	No
Sgromo *et al*[34], 2008	74	99	55	62	14.9	0.38 (0.15-0.93)	No
Granderath *et al*[29], 2007	26	28	7	28	10.1	39.00 (7.32-207.87)	No
Fernando *et al*[36], 2002	22	113	5	29	13.8	1.16 (0.40-3.38)	No
Wykypiel *et al*[37], 2005	26	77	9	132	15.4	6.97 (3.05-15.90)	No
Coster *et al*[28], 1997	12	82	0	62	5.4	22.16 (1.29-382.06)	No
Radajewski *et al*[32], 2009	20	51	7	43	14.4	3.32 (1.24-8.89)	No
Subtotal (95% CI)		591		490	100	3.02 (1.14-7.95)	
Total events	220		109				
Heterogeneity: Tau^2 = 1.54; χ^2 = 42.66, *df* = 8 (*P* < 0.00001); I^2 = 81%							
Test for overall effect: *Z* = 2.23 (*P* = 0.03)							

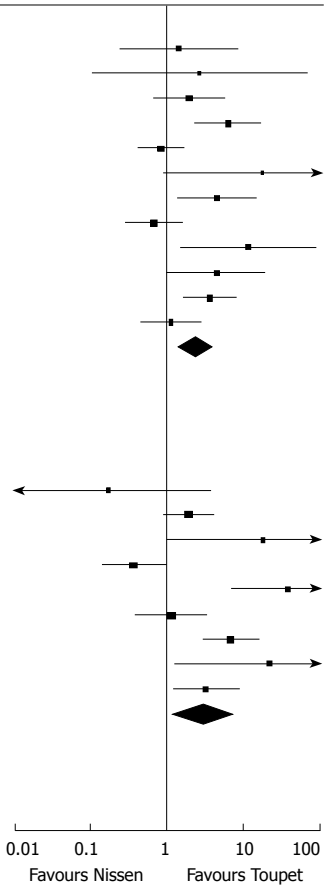

Figure 2 Pooled analysis using the Mantel-Haenszel method and a random-effect model. A: Overall rates of dysphagia after laparoscopic Nissen fundoplication (LNF) and laparoscopic Toupet fundoplication (LTF); B: Subgroup dysphagia depending on preoperative esophageal motility after LNF and LTF; C: Postoperative gas bloating and inability to belch after LNF and LTF.

postoperative dysphagia if preoperative EM was normal (*P* = 0.89). However, in subgroup 2, patients with abnormal EP, the prevalence of dysphagia after LNF was still more common than after LTF (*P* = 0.04). In subgroup 3, even though the surgical choice was in accordance with the so-called "tailored procedure", LNF also led to a significantly higher risk of developing postoperative dysphgia than LTF (*P* = 0.03) (Figure 2B).

Gas-bloat syndrome or gas-related symptoms: Gas-related symptoms ("gas-bloat syndrome") were described in 12 studies involving > 1200 patients. Gas-bloat syndrome included gas bloating, inability to belch, flatulence, postprandial fullness, and epigastric pain. The main objective evaluation of gas-related symptoms was done using a verbal rating scale using the items mentioned above. Overall, 47.39% (327/690) of patients after LNF and 26.09% (138/529) of patients after LTF suffered from gas bloating, whereas 37.22% (220/591) after LNF and 22.24% (109/490) after LTF were unable to belch. Gas bloating and inability to belch were more common in the LNF group and a significant difference between LNF and LTF was also further confirmed to reach a significant level (Figure 2C).

Recurrence and re-operation

Heartburn and reflux recurrence: Eleven studies investigated the incidence of postoperative heartburn (considered to be the cardinal symptom of GERD recurrence). Heartburn occurred in 6.45%[26]-60.29%[43] of the population after LNF, and in 5.26%[25]-55.10%[43] after LTF. No statistically significant difference was found between LNF and LTF concerning the cumulative prevalence of heartburn (32.97%, 331/1004 *vs* 31.09%, 157/505, OR 0.83, 95% CI: 0.52-1.33, *P* = 0.45, respectively).

Nine studies reported the postoperative recurrence of GERD. However, the standard of GERD recurrence was not uniform because some were judged according to symptoms such as reflux, chest pain, and heartburn, whereas others were based on the endoscopic characteristics and 24-h gastric monitoring of pH (e.g. persistent esophagitis, DeMeester score[46]). In our analysis, the cumulative prevalence of reflux recurrence was slightly higher after LTF than after LNF, but this difference was not significant (6.50%, 64/985 *vs* 9.42%, 84/892, OR 0.73, 95% CI: 0.35-1.53, *P* = 0.40).

Re-operation: Re-operation prevalence was described in 15 trials involving > 3800 patients. The main causes

of re-operation were persistent dysphagia, severe reflux symptoms, hiatus hernia recurrence, and other severe treatment failures. Although the re-operation rate after LNF was slightly higher than after Toupet fundoplication, 4.40% (115/2616) *vs* 3.68% (47/1276), the difference was not significant (OR 1.29, 95% CI: 0.73-2.29, $P = 0.38$). The morbidity associated with re-operation after LNF for GERD ranged from 1.56%[21] to 27.27%[41], and in the LTF group the rates varied from 1.00%[28] to 17.5%[22].

DISCUSSION

Lower morbidity and mortality, shorter hospitalization and faster convalescence made LARS so attractive to patients with GERD. Since Nissen procedure was first reported a decade ago, it has been adopted as a tremendously successful procedure for reflux control, and it is therefore more often performed than partial fundoplication. However, numerous recent researches witnessed a strong debate between Nissen and Toupet fundoplications[47], shifting the attention to postoperative failures due to mechanical problems (e.g. dysphagia), rather than worries about the recurrence of disease.

Our analysis is a comprehensive and detailed systematic literature review of 32 articles reporting the surgical outcomes of 6236 patients with GERD after LNF and LTF. It demonstrated that the latter is advantageous over the former, showing similar outcomes of satisfaction rate, endoscopic improvement, perioperative complication occurrence, reflux recurrence and re-operation, but a substantially reduced prevalence of postoperative symptoms (e.g. dysphagia, gas-bloat syndrome).

Patient satisfaction is a reasonable and accurate index for assessing the efficacy of surgical treatment for GERD[11,47]. In our meta-analysis, > 90% of the study populations reported excellent or good results in the LNF and LTF groups. Bearing in mind that the subjective description of patients was not always in accordance with objective findings[48], we further analyzed the alteration of LES pressure and remission of esophagitis after construction of a mechanical anti-reflux barrier. Elevating the resting pressure of the LES played a crucial role in controlling reflux symptoms, blocking the natural history of Barrett's esophagus and reducing the risk of malignancy. We also found that LNF and LTF could increase the LES pressure and improve preoperative esophagitis. Even though the amplitude of elevation of LES pressure was significantly lower after LTF than after LNF, it seemed that it remained sufficiently powerful to resist the reflux of gastric contents. These subjective assessments and objective evaluations supported the point of view that LTF can control reflux symptoms for GERD.

The prevalence of perioperative complications showed no significant difference between groups, indicating that neither procedure was more technically difficult or more demanding of surgical skills. However, the prevalence of postoperative dysphagia and gas-related symptoms was much higher after LNF than after LTF. Even though the mechanism of gas-related symptoms is unclear[29], these results suggest that wrap type and alteration in LES pressure were the underlying causes.

Dysphagia was still the complication of greatest concern and presented in three scenarios: acute total dysphagia immediately after surgery; mild dysphagia within the first postoperative 6-8 wk; and persistent chronic dysphagia after the postoperative 6-8 wk[18,49-51]. Some authors thought that dysphagia converges and the difference normalizes within 12 mo[52-55]. This study analyzed the prevalence of chronic dysphagia at the end of follow-up, and the mean duration of follow-up of most included studies was > 12 mo. A subgroup analysis was also executed to incorporate the variable of EM. It seemed that in normal motility patients (subgroup 1), extent of anti-reflux barrier did not affect the possibility of dysphagia. But in patients with abnormal motility (subgroup 2), the higher dysphagia rate after LNF supported that the tailored approach should be preserved. Even using the tailored approach (subgroup 3), the prevalence of dysphagia after LTF was still significantly lower than after LNF. These results indicated that, irrespective of EM status, LTF would be a safer choice in reducing this complication.

Concerns regarding GERD recurrence made surgeons select the Nissen technique rather than the Toupet technique for a long time. The similar prevalence of reflux recurrence between LNF and LTF groups in our analyses appeared to confirm the effectiveness of the Toupet procedure from another viewpoint. However, the value of this index may be slightly inferior. A DeMeester score > 14.7 was a generally accepted criterion, but the standards of GERD recurrence of selected researches were not uniform. Some were judged based on symptoms, some on endoscopic manometry. Furthermore, even though specific pH changes in the gastric environment were related to symptoms to some extent, inconsistency of poor correlation between postoperative reflux symptoms and reflux abnormal DeMeester scores were noted in many instances[27,45,48].

For re-operation, we did not divide subgroups according to causes such as recurrence of reflux or complications. The reason was that they were a miserable experience for the patients and also indicated the failure of primary surgery. The re-operation prevalence between the two groups was comparable. A remarkable phenomenon discovered in some studies[16,23,24] was that a large proportion of patients needing re-operation after LNF possessed a intact wrap during surgery with herniation of the wraps into the mediastinum. Nevertheless, it was rarely detected and presented after LTF. The technical issue of a suture between the posterior wall of the wrap and the two crura of the diaphragm, or a suture between the upper portion of the wrap and the anterior edge of the hiatus, has been proposed to maintain the wrap in an adequate abdominal position and to avoid its rotation and migration.

In conclusion, the results of the present meta-analysis suggested that LTF might be the current procedure of choice to treat GERD. It should be advocated as a more physiologic alternative for Nissen repair, allowing a reduced morbidity rate with similar efficacy in reflux control

and recurrence. The surgical patterns might be a prior factor to preoperative esophageal motility in affecting the postoperative dysphagia after LARS. More multicenter, randomized controlled trials including objective outcome assessment are required to further confirm the value of LNF and LTF.

COMMENTS

Background

The laparoscopic Nissen fundoplication and the laparoscopic Toupet fundoplication are the two major anti-reflux surgical procedures for gastroesophageal reflux disease (GERD). There has been much debate about the superiority of these two techniques.

Research frontiers

A decade ago, Nissen procedure was first reported, then adopted as a tremendous successful procedure for reflux control, and was therefore more often performed than posterior partial fundoplication. However, numerous recent researches witnessed a strong debate about Nissen and Toupet fundoplication, shifting the attention to postoperative failures due to mechanical problems (e.g. dysphagia), rather than worries about the recurrence of disease.

Innovations and breakthroughs

To the best of the authors' knowledge, the present study was the most comprehensive and detailed systematic literature review to demonstrate the optimal surgical procedure for GERD.

Applications

The research showed that laparoscopic Toupet fundoplication might be the current procedure of choice to treat GERD. It should be advocated as a more physiologic alternative for Nissen repair, allowing a reduced morbidity rate with similar efficacy in reflux control and recurrence.

Peer review

This article is important and interesting.

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BRIEF ARTICLE

Simultaneous detection of different serum pepsinogens and its primary application

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Abstract

AIM: To develop the simple, rapid and sensitive dual-label time-resolved fluoroimmunoassay for pepsinogens in human serum.

METHODS: Based on two-site sandwich protocol, monoclonal antibodies (McAbs) against pepsinogen Ⅰ (PG Ⅰ) and PG Ⅱ were co-coated in 96 microtitration wells, and tracer McAbs against PG Ⅰ and PG Ⅱ were labeled with europium (Eu) and samarium (Sm) chelate, respectively. Diluted serum samples of Eu^{3+}- and Sm^{3+}-McAbs were added into microtitration wells simultaneously. After washing, fluorescence of bound Sm^{3+} and Eu^{3+} tracers was detected.

RESULTS: The detection limit was 0.2 μg/L for PG Ⅰ and 0.05 μg/L for PG Ⅱ. The assay range was 5.0-320.0 μg/L for PG Ⅰ and 1.0-55.0 μg/L for PG Ⅱ. The average recovery rate was 102.7% for PG Ⅰ and 98.8% for PG Ⅱ. Sera from healthy controls and patients with gastric disease were analyzed. The PG detected by dual-label assay was in good agreement with that detected by single-label assay or by enzyme-linked immunosorbent assay.

CONCLUSION: Dual-label assay can provide high-throughput serological screening for gastric diseases.

Key words: Serum pepsinogen; Simultaneous detection; Time-resolved fluoroimmunoassay

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INTRODUCTION

There is considerable interest in developing methods to simultaneously quantify two or more analyses of one sample in a single assay[1,2], which is advantageous to the confidence level of results, especially in cases when the ratio of compounds gives important information[3,4]. Dual-label has potential applications in various fields such as microbiology, molecular biology, drug analysis, and clinical research. Fluorescence immunoassay, like other immunoassays involving non-isotopic labeling, has been well accepted as a stable, inexpensive, rapid, and sensitive

method. However, conventional fluorescent labeling has a limited success in assay of multiple analytes because of its high background, short decay time and broad spectrum, which make it difficult to be distinguished between its emission bands[5,6]. Up to now, fluorescent lanthanide is a favorable choice owing to its narrow emission peak at different wavelengths. Its lifetime ranges 50-1000 μs (over four decades longer than the average background duration) depending on the temperature and the solvent presented[7]. These features can be utilized for optimization of the measurement conditions to get the maximal sensitivity and to minimize the signal spillover. The europium ion (Eu^{3+}), is the lanthanide mainly used in time-resolved fluoroimmunoassay (TRFIA)[8]. Eu^{3+} and terbium ion (Tb^{3+}) form the most efficient fluorescent chelates, but Tb^{3+} requires fluorinated aliphatic β-diketone for simultaneous detection[9], rather than β-naphthoyltrifluoroacetone (β-NTA) used in the enhancement solution optimized for Eu^{3+} detection. β-NTA is also applicable to samarium ion (Sm^{3+}) excitation, which has thus been suggested that Sm^{3+} can be used as a counterpart to Eu^{3+} with the same enhancement formulation (enhancement solution or DELFIA inducer) in a dual-label system[10].

Human pepsinogens originating from gastric mucosa can be classified into two immunochemically distinct groups: pepsinogen I (PG I) and PG Ⅱ[11], which are mostly secreted into the gastric lumen and nearly 1% of them are leaked into the blood circulation. Serum PG levels reflect the morphological and functional status of gastric mucosa. Human pepsinogens have a diagnostic value for various gastroduodenal disorders, especially for peptic ulcer and atrophic gastritis, which have been widely discussed[12,13]. The PG I /PG Ⅱ ratio can provide even better information on the extent of chronic gastritis than gastric intubation[14].

Since PG I and PG Ⅱ serve as useful predictors in early diagnosis of gastric cancer and in mass screening of populations at a high risk of gastric cancer[15,16], a reliable and sensitive method is needed to detect PG I and PG Ⅱ in human sera. In our previous study[17], a fast and highly sensitive TRFIA was developed to measure serum PG I and PG Ⅱ. The present study was to evaluate the dual-label TRFIA for simultaneous detection of PG I and PG Ⅱ in human serum.

MATERIALS AND METHODS

Chemicals and instruments

Diethylenetriaminepentaacetate (DTPA), bovine serum albumin (BSA), Tris and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). PD-10 column and sepharoseCL-6B column were from the Pharmacia Company (Chalfont St Giles, UK). Q2 anion exchange chromatography, DEAE-52 chromatography, and gel filtration HPLC were purchased from Bio-Rad Company (Hercules, USA). Pure water was produced by Barnstead Equipment (Dubuque, Iowa, USA). Ninety six -well polystyrene microtitre plates were obtained from Nunc International (Roskilde, Denmark). Eu-labeling reagent 1244-302 and Sm-labeling reagent 1244-303, both including N'-[*p*-isothiocyanatobenzyl]-diethylenetriamine-N^1, N^2, N^3, N^4-tetraacetic acid, were purchased from Perkin-Elmer (Waltham, Massachusetts, USA). β-NTA was synthesized in our laboratory. Two counterparts of monoclonal antibodies (McAbs) to human PG I and PG Ⅱ respectively for capture and detection were obtained from Chinese Institute of Cancer with a purity of over 95% (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for detection of PG I and PG Ⅱ were from Biohit Plc(Helsinki, Finland). AutoDELFIA1235, from Perkin-Elmer (Waltham, Massachusetts, USA), was used to measure Eu^{3+} and Sm^{3+} fluorescence in microtiter wells. All other reagents used were of analytical grade.

Reagent solutions

Labeling buffer contained 50 mmol/L Na_2CO_3-$NaHCO_3$ (pH 8.5), and 0.155 mol/L NaCl. Elution buffer contained 50 mmol/L Tris-HC1 (pH 7.8), 0.9% NaC1, and 0.05% NaN_3. Assay buffer contained 50 mmol/L Tris-HCl (pH 7.8), 0.9% NaC1, 0.2% BSA, 0.05% NaN_3, 20 μmol/L DTPA, and 0.1% Tween-20. Washing solution was a Tris-HCl buffered saline solution (pH 7.8) containing 0.9% NaCl, 0.2% Tween-20, and 0.05% NaN_3. Enhancement solution was a 0.1 mol/L acetate-phthalate buffer (pH 3.2) containing 0.1% triton X-100, 15 μmol /L βNTA, and 50 μmol/L tri-*n*-octylphosphine oxide.

Serum samples

Serum samples, collected from healthy volunteers who had no upper abdominal complaints and evidence of gastroduodenal disorder and liver diseases, were stored at -20℃. Blood samples were collected from patients at endoscopic and histological examinations. This study was conducted with the approval of the Ethics Committee of Jiangyuan Hospital Affiliated to Jiangsu Institute of Nuclear Medicine.

Immobilization of McAbs

Five micrograms of the capture McAbs to PG I and PG Ⅱ in 200 μL of 50 mmol of Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) was co-immobilized in each well and incubated overnight at room temperature. After washing, 200 μL of 1 g/L BSA in 50 mmol of Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) was added to block the coated surface for 2 h. After the blocking solution was removed, the plates were dried in a high vacuum, and then stored at -20℃ in a sealed plastic bag with desiccant.

Labeling of McAbs with Eu^{3+}- and Sm^{3+}-chelates

McAbs to human PG I (PG I McAbs) and PG Ⅱ (PG Ⅱ McAbs) were labeled with Sm^{3+}- and Eu^{3+}-chelates, respectively. The buffer for McAbs was replaced with the labeling buffer. Five hundred micrograms of PG I McAbs was gently mixed in 200 μL of labeling buffer with 250 μg of Sm^{3+}-chelates in 100 μL of the same buffer. After an 18-h incubation with continuous gentle shaking at room

temperature, free Sm^{3+}-chelates and aggregated McAbs were separated from Sm^{3+}-McAbs conjugates using a 1 cm × 40 cm column packed with sepharose CL-6B (lower 20 cm), eluted with a descending elution buffer, and collected with 1.0 mL per fraction. The concentration of Sm^{3+}-conjugates in collected fraction was measured with fluorescence, and diluted with an enhancement solution (1:1000). The fluorescence in microtitration wells (200 μL per well) was detected by comparing the signal of samples to that of stock standards diluted at 1:100 in an enhancement solution. The fractions from the first peak with the highest Sm^{3+} count were pooled and characterized. PG Ⅱ McAbs were labeled with Eu. The labeled McAbs were rapidly lyophilized under high vacuum after dilution with an elution buffer containing 0.2% BSA as a stabilizer, and stored at -20℃. No loss of immunoreactivity was observed during a 6-mo storage period.

Purification of PG and calibrators

Surgically resected stomach tissues were free from the invaded part. PG Ⅰ and PG Ⅱ were purified by DEAE-52 chromatography, gel filtration HPLC, and Q2 anion exchange chromatography, as previously described[18]. The purity of PG Ⅰ was over 98% and that of PG Ⅱ was over 95.0%. Calibrators were prepared by diluting them with the assay buffer containing 0, 5, 10, 50, 100, 300 μg/L of highly purified PG Ⅰ and 0, 5, 10, 20, 30, 50 μg/L of highly purified PG Ⅱ, respectively.

Assay procedure

Dual-label TRFIA was performed to detect PG Ⅰ and PG Ⅱ simultaneously in serum with a one-step "sandwich-type" protocol. In brief, 25 μL of calibrators (samples) and 200 μL of 50-fold diluted Eu^{3+} and Sm^{3+} tracer McAbs solution in assay buffer were pipetted into the coated microtitration wells. The plates were incubated with continuous shaking for 2 h at 25℃. After washed 6 times, 200 μL of enhancement solution was added into each well. The plates were shaken for 5 min before fluorescence reading. All procedures were automatically performed by $_{auto}$DELFIA$_{1235}$ with the software designed in our laboratory. Calibration curve was plotted and concentrations in unknown samples were measured using Multicalc software program, where a spline algorithm on logarithmically transformed data was employed. ELISA was performed with a kit following its instructions.

Statistical analysis

Data about PG Ⅰ or PG Ⅱ were expressed as mean ± SD. The limit of detection was defined by the concentration of PG Ⅰ or PG Ⅱ corresponding to the fluorescence of the zero calibrators plus two SD. The average intra- or inter-assay coefficient of variation (CV) was calculated for the precision of the assay. The recovery rate was evaluated by comparing the measured and theoretical values. Regression analysis was used to display the linearity and correlations. Differences in patients with gastric disease and healthy controls were analyzed using paired *t*-test. $P < 0.05$ was considered statistically significant. Analysis of data was performed using SPSS 13.0 (Chicago, IL, USA).

Table 1 Precision of dual-label assay for PG Ⅰ and PG Ⅱ in serum of controls[1]

	PG Ⅰ (μg/L)		PG Ⅱ (μg/L)	
	mean ± SD	CV (%)	mean ± SD	CV (%)
Serum pool 1				
Within-run (n = 10)	43.2 ± 1.36	3.2	5.23 ± 4.62	4.8
Between-run (n = 6)	42.8 ± 2.28	5.1	5.65 ± 6.45	6.7
Serum pool 2				
Within-run (n = 10)	105.0 ± 1.88	2.3	11.7 ± 3.66	3.9
Between-run (n = 6)	103.8 ± 2.73	3.6	10.5 ± 4.55	5.1
Serum pool 3				
Within-run (n = 10)	198.2 ± 5.31	6.3	22.0 ± 3.88	4.6
Between-run (n = 6)	186.7 ± 4.35	5.4	21.2 ± 5.71	8.3

[1]Served as controls. PG: Pepsinogen; CV: Coefficient of variation.

RESULTS

Kinetics, detection limits and precision

The calibrators covered a range of 10-300 μg/L of PG Ⅰ and 2-50 μg/L of PG Ⅱ. The serum samples from healthy controls and patients with chronic atrophic gastritis and peptic ulcer were incubated for different periods of time (60, 90, 120, 150 min) at different temperatures (25℃, 37℃). Both calibrators and serum samples reached a plateau value around 120 min at 25℃ and around 60 min at 37℃, respectively. In this study, the incubation time was 120 min and the temperature was 25℃ for the assay on $_{auto}$DELFIA$_{1235}$.

The calibration curves of PG Ⅰ and PG Ⅱ were linear over the concentration. The equation was y = 46.4x + 383.2 for the calibration curve of PG Ⅰ and y = 2198.2x + 5189.1 for the calibration curve of PG Ⅱ, where y indicates the response counts (cps), x indicates the concentration (μg/L). With 25 μL of serum samples, the measurement range of PG Ⅰ, ED_{20}, ED_{50}, and ED_{80} was 5.0-320.0, 19.87 ± 4.3, 64.32 ± 6.2, and 176.0 ± 12.9 μg/L, respectively, and the measurement range of PG Ⅱ, ED_{20}, ED_{50}, and ED_{80} was 1.0-55.0, 3.546 ± 2.2, 9.746 ± 4.7, and 23.79 ± 6.3 μg/L, respectively.

The limit of detection was 0.2 μg/L for PG Ⅰ and 0.05 μg/L for PG Ⅱ. The average intra-assay CV of the calibrators was 4.6% for PG Ⅰ and 5.3% for PG Ⅱ. The intra- and inter-assay CV of serum-based controls was summarized in Table 1. The results showed that the assay had a good precision not only for the calibrators but also for the clinical samples.

The cross-reactivity between anti-PG Ⅰ antibody to PG Ⅱ and anti-PG Ⅱ antibody to PG Ⅰ was detected. No interference between them was found. The result showed that the specificity of the assay was good.

Analysis of samples

The correlations between dual-label and single-label assay are shown in Figure 1. The correlation ratio between

Table 2 Serum PG level and PG Ⅰ/PG Ⅱ ratio in healthy controls and patients with duodenal ulcer, gastric ulcer, atrophic gastritis, superficial gastritis and gastric cancer (mean ± SD)

Diagnosis	*n*	Age (yr)	PG Ⅰ (μg/L)	PG Ⅱ (μg/L)	PG Ⅰ/PG Ⅱ ratio
Healthy controls	500	41.5 ± 18.8	150.3 ± 45.1	10.4 ± 8.4	14.5 ± 4.3
Patients with duodenal ulcer	112	43 ± 14.2	252.9 ± 84.5[d]	18.4 ± 16.0[d]	13.7 ± 8.0
Patients with gastric ulcer	44	37 ± 11.2	221.2 ± 91.7[d]	15.6 ± 12.4[b]	14.2 ± 7.1
Patients with atrophic gastritis	21	48 ± 9.7	89.5 ± 51.2[d]	12.9 ± 9.39	6.9 ± 6.2[b]
Patients with superficial gastritis	76	35 ± 7.4	175.3 ± 45.8[a]	12.7 ± 10.1	13.8 ± 5.3
Patients with gastric cancer	126	55 ± 12.2	157.1 ± 81.9	15.6 ± 14.4[b]	13.4 ± 7.8

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* healthy controls.

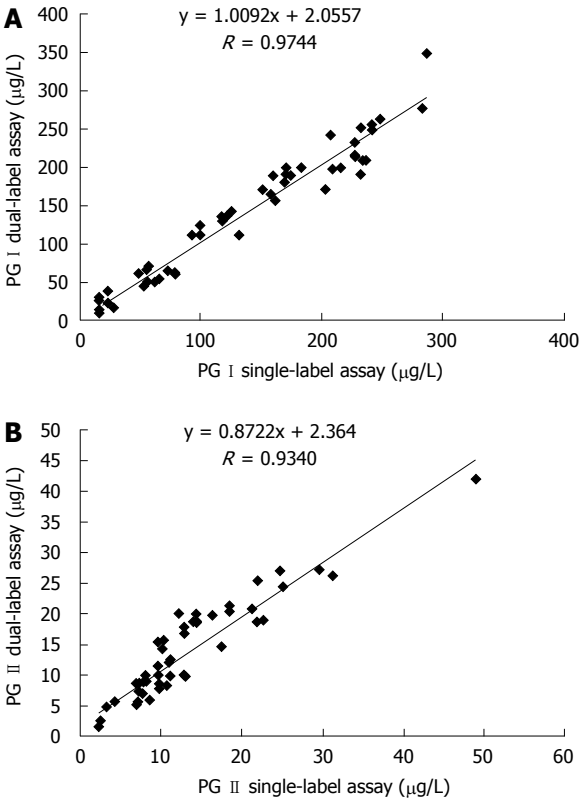

Figure 1 Correlation between single-label and dual-label assay of pepsinogen (PG) Ⅰ (A) and PG Ⅱ (B) in human sera (*n* = 50).

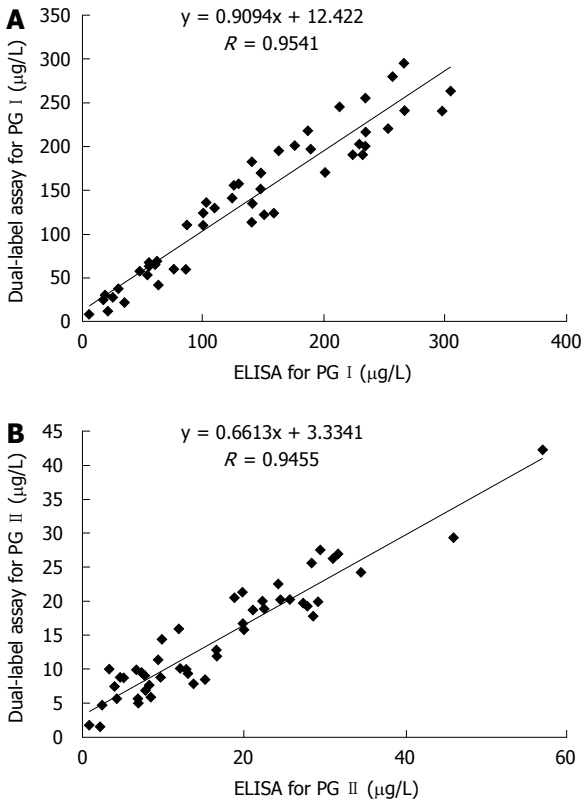

Figure 2 Correlation between enzyme-linked immunosorbent assay (ELISA) and dual-label assay of PG Ⅰ (A) and PG Ⅱ (B) in human sera (*n* = 50).

single-label and dual-label assay for PG Ⅰ and PG Ⅱ was 0.9744 and 0.9340, respectively. The correlations between dual-label assay and ELISA for PG Ⅰ and PG Ⅱ are shown in Figure 2. The correlation ratio between ELISA and dual-label for PG Ⅰ and PG Ⅱ was 0.9541 and 0.94550, respectively. The results indicate that the dual-label assay is in agreement with ELISA or with single-label assay.

The serum samples were analyzed by dual-label assay (Table 2). The normal range of serum PG Ⅰ in healthy controls was 60.1-240.3 μg/L. The range of serum PG Ⅱ was lower than 27.2 μg/L. The cut-off point was 5.9 for the PG Ⅰ/PG Ⅱ ratio.

The serum PG level was lower or higher than its normal range in dyspeptic patients including those with gastric cancer. The distributions of PG Ⅰ and PG Ⅱ value and PG Ⅰ/PG Ⅱ ratio in patients with gastric cancer and in those with duodenal ulcer are shown in Table 3. The difference in PG Ⅰ value was relatively small. The serum PG Ⅰ level was remarkably higher in peptic ulcer patients, especially in those with active duodenal ulcer than in healthy controls. The increased PG Ⅰ level would be a high risk factor for duodenal ulcer, and a remarkably low serum PG Ⅰ level could exclude the diagnosis of peptic ulcer[19].

DISCUSSION

Eu^{3+} chelate is the most commonly used label in time-resolved fluorometry-based analysis because of its higher fluorescence yield and lower background than other lanthanide complexes. Tb^{3+} chelate usually has a longer decay time and a higher fluorescence yield than Sm^{3+} chelate, and their fluorescence is less sensitive to aqueous quench-

Table 3 PG Ⅰ and PG Ⅱ values and PG Ⅰ/PG Ⅱ ratio in patients with gastric carcinoma and duodenal ulcer *n* (%)

	n	PG Ⅰ (μg/L)			PG Ⅱ (μg/L)		PG Ⅰ/PG Ⅱ ratio	
		< 60	60-240	> 240	≤ 27	> 27	≤ 6	> 6
Patients with gastric cancer	126	17 (13.5)	88 (69.8)	21 (16.7)	109 (86.5)	17 (13.5)	25 (19.8)	101 (80.2)
Patients with duodenal ulcer	112	0	69 (61.6)	43 (38.4)	100 (89.2)	12 (10.8)	3 (2.7)	109 (97.3)

ing. However, the relatively shorter emission wavelength of Tb^{3+} chelate (545 nm) makes it more prone to interference (e.g. phosphorescence) derived from plastic or glass materials. Additionally, it is required to use an aliphatic β-diketone to enhance the fluorescence of Tb^{3+} in immunoassay for DELFIA-type of multiple analytes[9]. Considering these factors, we selected Eu^{3+} and Sm^{3+} as labels in the present study.

As the Sm photoluminescence yield is lower than that of Eu, Sm^{3+} is usually used as a tracer in assays not requiring a great sensitivity. The detection limit for PG Ⅰ in dual-label assay is 0.2 μg/L, whereas that of single-label assay is 0.05 μg/L[17]. The sensitivity and precision for PG Ⅰ can be improved significantly by increasing Sm^{3+} label yield. The labeling reaction between PG Ⅰ McAbs and Sm labeling reagent can be prolonged with a suitable excess of the Sm labeling reagents, which may help to get a higher Sm^{3+} label yield. Despite this, the detection sensitivity for PG Ⅰ with a limit of 0.2 μg/L is more than adequate for measuring the PG Ⅰ concentration in clinical samples.

Direct passive absorption of two or more binders is still the routine method for multi-analyte immunoassay (MAIA)[20]. When only the sandwich-type configuration is employed in MAIA, it is necessary to prepare an activated surface binder (e.g. antibody) in order to get a high sensitivity. In this study, the anti-PG Ⅰ and anti-PG Ⅱ antibodies were co-coated simultaneously in a strip well, which was found to be beneficial and economical for a suitable fluorescence by adjusting the concentration of coated antibodies. The total concentration of the co-coated antibodies which can achieve favorable results was no more than 5 μg/mL (1000 ng per well) in this study.

Samples with a relatively high PG Ⅰ or PG Ⅱ were analyzed at various dilutions. The diluting buffer was identical to the calibrator buffer. The percentage of expected value was 96.3%-101.7% for PG Ⅰ and 98.1%-109.6% for PG Ⅱ. No hook effect of dual-label assay was found at a relatively high PG Ⅰ or PG Ⅱ concentration. Recovery was identified by supplementing PG Ⅰ calibrators at 20 μg/L and 100 μg/L, and PG Ⅱ calibrators at 5 μg/L and 50 μg/L. The average recovery rates for PG Ⅰ and PG Ⅱ were 102.7% and 98.8%, respectively, showing that the analytical accuracy is satisfactory for clinical use.

Different analytes (i.e. t-PSA/f-PSA, AFP/CEA) can be detected at present by dual-label TRFIA[3,21] and the corresponding instruments are commercially available. In this study, the human PG detected by dual-label TRFIA was similar to that detected by ELISA and single-label TRFIA. The sensitivity, measurement range and stability of dual-label TRFIA were substantially better than those of ELISA. Unlike radioimmunoassay or ELISA[22,23], dual-label TRFIA can measure the concentration of PG Ⅰ and PG Ⅱ, as well as the ratio of PG Ⅰ/Ⅱ, thus reducing the random handling errors and increasing the clinical confidence level of PG Ⅰ/PG Ⅱ ratio. Direct labeling of immunoreagents with lanthanide chelates and lack of overlapping between Eu^{3+} and Sm^{3+} chelates allow a rapid assay. In addition, 25 μL of samples is enough for the simultaneously detection of PG Ⅰ and PG Ⅱ.

In summary, dual-label TRFIA can serve as a high-throughput tool for the detection of serum PG and has good prospects of clinical application.

COMMENTS

Background

Non-invasive serum pepsinogen (PG) test provides much information on intestinal metaplasia, atrophic gastritis, as well as *Helicobacter pylori* infection and peptide ulcer, which has a significant clinical value for the mass screening of patients at a high risk of gastric cancer. A reliable and effective detection method covering a wide concentration range with good sensitivity for PG is required. Furthermore, simultaneous determination of PG Ⅰ and PG Ⅱ can improve the confidence level of the PG Ⅰ/PG Ⅱ ratio.

Research frontiers

In this study, the authors found that multi-analyte immunoassay could increase the throughput and reduce the overall cost per test. A simple, rapid and sensitive dual-label time-resolved fluoroimmunoassay (TRFIA) for pepsinogens in human serum was developed.

Innovations and breakthroughs

TRFIA is a sensitive technique used in analysis of trace substances. Compared with traditional methods, such as radioimmunoassay or enzyme-linked immunosorbent assay (ELISA), dual-label assay can reduce random handling errors and increase the confidence level of PG Ⅰ and PG Ⅱ (especially for the PG Ⅰ/PG Ⅱ ratio). Only 25 μL of serum samples is enough for each test, which is useful for mass screening.

Applications

The PG detected by dual-label TRFIA was in good agreement with that detected by single-label assay or by ELISA. The analytical accuracy, precision and stability are satisfactory for its use in clinical practice. Dual-label TRFIA may serve as a high-throughput tool for the detection of serum PG and has good prospects of clinical application.

Peer review

The analysis of pepsinogens in serum/plasma of patients is a well established method to identify subjects at a higher risk of developing gastric cancer. The described method for analyzing Pep-Ⅰ and Pep-Ⅱ simultaneously seems to have similar parameters in relation to sensitivity and specificity as EIA or ELISA. The manuscript is well written.

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BRIEF ARTICLE

Construction and characterization of calreticulin-HBsAg fusion gene recombinant adenovirus expression vector

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Abstract

AIM: To generate recombinant adenoviral vector containing calreticulin (CRT)-hepatitis B surface antigen (HBsAg) fusion gene for developing a safe, effective and HBsAg-specific therapeutic vaccine.

METHODS: CRT and HBsAg gene were fused using polymerase chain reaction (PCR), endonuclease digestion and ligation methods. The fusion gene was cloned into pENTR/D-TOPO transfer vector after the base pairs of DNA (CACC) sequence was added to the 5′ end. Adenoviral expression vector containing CRT-HBsAg fusion gene was constructed by homologous recombinantion. The human embryo kidney (HEK) 293A cells were transfected with linearized DNA plasmid of the recombinant adenoviral vector to package and amplify recombinant adenovirus. The recombinant adenovirus titer was characterized using the end-dilution assay. The expression of the CRT/HBsAg fusion protein in Ad-CRT/HBsAg infected 293A cells was detected by Western blotting.

RESULTS: The CRT-HBsAg fusion gene was characterized by PCR and sequencing and its length and sequence were confirmed to be accurate. The CRT-HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector was constructed. The recombinant adenoviral vector, Ad-CRT/HBsAg, was generated successfully. The titer of Ad-CRT/HBsAg was characterized as 3.9 × 10^{11} pfu/mL. The CRT-HBsAg fusion protein was expressed by HEK 293A cells correctly.

CONCLUSION: CRT/HBsAg fusion gene recombinant replication-defective adenovirus expression vector is constructed successfully and this study has provided an experimental basis for further studies of Hepatitis B virus gene therapy.

Key words: Calreticulin; Hepatitis B virus; Hepatitis B surface antigen; Adenovirus expression vector; Fusion protein; Therapeutic vaccine

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INTRODUCTION

Development of a safe, effective and therapeutic vaccine represents the best hope for treatment of hepatitis B virus (HBV) infection. Recent data have indicated that immunotherapeutic strategies stimulating both cellular and humoral immune responses to HBV antigens are es-

sential to cure chronic HBV infection[1]. In this regard, DNA-based vaccination appears to be a particularly pertinent approach for chronic hepatitis B treatment, since it has been well documented to elicit durable humoral and cell mediated immunity including cytotoxic T lymphocytes and cytokines in normal mice[2,3], ducks[4] and chimpanzees[5]. It is a good candidate for immunization of non-responders to recombinant hepatitis B surface antigen (HBsAg) vaccines and for therapeutic vaccination[2]. However, even at higher doses of DNA, human clinical trials of HBV DNA vaccine have yielded a much lower level of immune responses than that observed in small animals[6,7]. Therefore, the potency of DNA vaccines must be enhanced for successful human application. The use of calreticulin (CRT) represents an innovative and feasible approach for enhancing immune responses and generating an antiangiogenic effect[8]. CRT is an abundant 46 kDa Ca^{2+}-binding protein located in the endoplasmic reticulum (ER)[9]. CRT is considered to be related to the family of heat shock proteins[10,11]. The protein has been shown to associate with peptides delivered into the ER by transporters associated with antigen processing (TAP-1 and TAP-2)[12] and with major histocompatibility complex (MHC) class I -β2 microglobulin molecules to aid in antigen presentation[13].

It is well known that available recombinant HBsAg protein vaccine is effective for preventing HBV infection for healthy persons, but for the infected patients there have been no effective and therapeutic vaccines until now. In this study, CRT-HBsAg fusion gene recombinant adenovirus expression vector was generated to establish the basis for curing chronic HBV infection and HBsAg-positive liver cancer.

MARTERIALS AND METHODS

Plasmids, cells and reagents

ViraPower Adenoviral Expression System including pENTR/D-TOPO Cloning Vector, Adenoviral Expression Vector, HEK293A cells, TOP10 competent cells of *Escherichia coli (E. coli)* were all purchased from Invitrogen Corporation (USA). The pJW4303, plasmid was provided by professor Lu Shan (Umass, USA).

Taq DNA polymerase, *Pfu* DNA polymerase, antibodies marked by HRP, *Pac*I, plasmid DNA purification kit and Gel purification kit were purchased from Tianwei Shidai Corporation (China), New England Corporation (UK), Takara Corporation (China), and Promega Corporation (USA), respectively. Human anti-HBsAg antibody positive serum was collected by our laboratory. Primers were synthesized in Shanghai Invitrogen Corporation (China).

Construction and preparation of CRT/HBsAg fusion gene recombinant plasmid

For the generation of pJW43033-CRT, CRT was amplified with polymerase chain reaction (PCR) using rabbit CRT cDNA as the template[14] and a set of primers, 5'-CCGGAGACTCATGCTGCTCCCTGTGCCGCT-3' and 5'-CCGGGAATTCCAGCTCGTCCTTGGCCTGGC-3'. There is more than 90% homology between rabbit, human, mouse, and rat CRT[14]. The amplified product was then cloned into the *Sac*I/*Eco*RI sites of pJW4303 vector. For the generation of pJW4303-CRT/HBsAg, HBsAg gene was amplified with a set of primers, 5'-GTGGGGAATTCATGGAGAACACAACATCAGG-3' and 5'-GGGGTGGATCCAATTTACATATGGGTTTCTGT-3', and then cloned into the *Eco*RI/*Bam*HI sites of pJW4303-CRT to generate pJW4303-CRT/HBsAg. The accuracy of these constructs was confirmed by endonuclease digestion and DNA sequencing. The sequencing was finished by Shanghai Invitrogen Biological Engineering Corp (China).

Construction of recombinant pENTR/D-TOPO transfer vector containing CRT/HBsAg fusion gene

CRT/HBsAg fusion gene was amplified using pJW4303-CRT/HBsAg plasmid as the template and a set of primers, 5'-CACCATGCTGCTCCCTGTGCCGCT-3' and 5'-AATTTACATATGGGTTTCTGT-3'. In order to construct directional TOPO cloning transfer vector in the desired direction, the CACC sequence was incorporated into the PCR upstream primer at its 5' end. The final optimized conditions of PCR consisted of 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ for 1 min. After PCR, the PCR products of fusion gene were purified for generating the pENTR/D-TOPO transfer vector. The ligated products were transformed into TOP10 chemically competent *E. coli* and incubated overnight at 37℃. Subsequently, extracted and purified plasmids containing CRT/HBsAg fusion gene were characterized by PCR and sequencing, respectively. The sequencing was finished by Shanghai Invitrogen Biological Engineering Corp.

Construction and characterization of CRT/HBsAg fusion gene recombinant adenoviral expression vector, Ad-CRT/HBsAg

Using LR Clonase Mix™ (Invitrogen, US) as catalysis, the purified CRT/HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector was mixed with adenoviral expression vector DNA plasmid to generate the recombinant adenoviral expression vector. The recombinant products were transformed into TOP10 chemically competent *E. coli* and incubated on Luria-Bertani (LB) plates containing 100 μg/mL ampicillin at 37℃ overnight. Subsequently, we selected eight putative positive clones which were ampicillin-resistant to amplify, extract and purify for PCR amplification and electrophoresis detection, respectively. The reason why using LB plates containing 30 μg/mL chloramphenicol to select positive clones is because true expression clones would be ampicillin-resistant and chloramphenicol-sensitive.

Transfecting the HEK 293A cells line with Ad-CRT/HBsAg plasmid

Purified Ad-CRT/HBsAg DNA plasmid was digested with *Pac*I. The day before transfection, the 293A cells were

trysinized, counted, and plated at 5×10^5 cells per well in a 6-well plate. Two milliliter of normally growing Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal bovine serum (FBS) was plated into each well. Those 293A cells were transfected using Lipofectamine 2000™ (Invitrogen, USA) and Pac I-digested expression plasmid DNA complexes. Culture medium was replaced with refresh complete culture medium every 2-3 d until visible regions of cytopathic effect (CPE) were observed. When approximately 80% of CPE was observed, adenovirus-containing cells and media were harvested.

LacZ gene recombinant adenoviral expression vector plasmid (Ad-LacZ) provided by the manufacturer (Invitrogen, USA) was used as control.

Characterization and titering of Ad-CRT/HBsAg

The titer of Ad-CRT/HBsAg was characterized using the End-Point Dilution Assay. When the 293A cells prepared 24 h before incubated at 37℃ in a CO_2 incubator were at more than 80% confluency in 96-well plates, the recombinant adenovirus stock solution harvested above was diluted to a concentration of 10^{-1}-10^{-12} and 100 μL was added to each well. After cultured for 10 d, the number of wells with CPE was calculated according to the formula of titre (pfu/mL) = $10^{(x + 0.8)}$.

Western blotting analysis

After being cultured for 72 h, the Ad-CRT/HBsAg infected and normal control 293A cells were harvested and frozen to -80℃. Some of them were prepared for Western blotting analysis. Protein samples were fractionated on 5%-12% SDS-PAGE and transferred to PVDF membrane using protein transfer apparatus (Bio-Rad, USA). The membrane was blocked for 1 h with 5% skimmed milk in TBS buffer (50 mmol/L Tris base, 150 mmol/L NaCl, pH 7.5) containing 0.5% Triton X-100. The membrane was probed with human anti-HBsAg antibody positive serum. After washing the membrane, goat anti-human HRP-conjugated antibody was added and incubated. The results were finally revealed using the sensitive substrate of DAB kit (Wuhan Boster, China) for Western blotting detection.

RESULTS

Characterization of CRT/HBsAg fusion gene recombinant plasmid

The CRT/HBsAg fusion gene recombinant plasmid pJW4303-CRT/HBsAg was digested by *Sac*I and *Bam*HI endonucleases. An approximately 2000 bp (1944 bp = 1263 bp + 681 bp) long DNA band was observed using 1.5% agarose gel electrophoresis (Figure 1A).

Characterization of CRT/HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector

Kanamycin-resistant putative positive clones were prepared as template to amplify CRT/HBsAg fusion gene by specific PCR. Approximately 2000 bp long DNA bands were observed in those positive clones using 1.5% agarose gel electrophoresis (Figure 1B). The sequencing results of CRT and HBsAg gene were the same as CRT sequence of NM004343.3 and HBsAg sequence of AF013631 in GenBank.

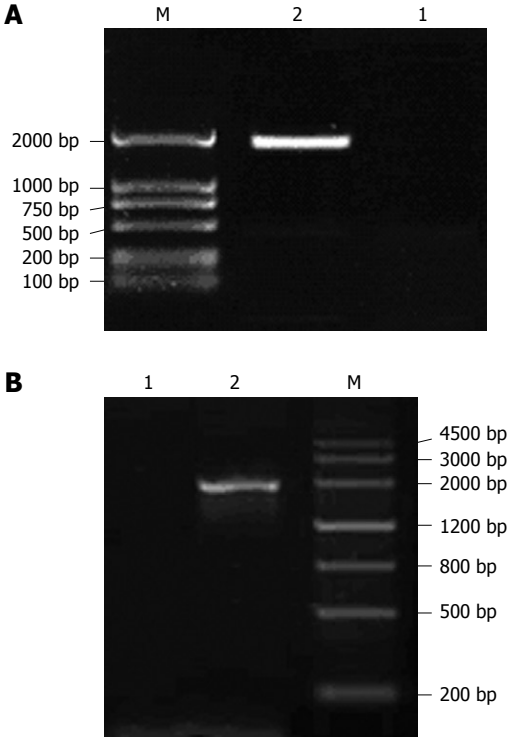

Figure 1 Characterization of polymerase chain reaction (PCR) amplification by electrophoresis. A: Amplification of calreticulin (CRT)/hepatitis B surface antigen (HBsAg) fusion gene by PCR. Lane 1: Negative control; Lane 2: CRT/HBsAg fusion gene recombinant pJW4303 plasmid; Lane M: DNA marker; B: Characterization of CRT/HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector by PCR. Lane 1: Negative control; Lane 2: CRT/HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector amplified by specific PCR; Lane M: DNA marker.

Characterization of CRT/HBsAg fusion gene recombinant adenoviral expression vector, Ad-CRT/HBsAg

All of the selected clones were sensitive for culturing in LB plates containing 30 μg/mL chloramphenicol because those true expression clones would be chloramphenicol-sensitive. After PCR amplification, a nearly 2000 bp long DNA band was observed using 1.5% agarose gel electrophoresis.

Harvest and detection of recombinant adenovirus, Ad-CRT/HBsAg

*Pac*I-digested Ad-CRT/HBsAg DNA plasmid was transfected into 293A cells by Lipofectamine 2000™. About 10 d after transfection, 80% of the cells rounded up and were floating or lightly attached to the tissue culture dish. This indicated that cells were loaded with adenovirus particles (Figure 2A). The shape of normal 293A cells are shown in Figure 2B. The adenovirus-containing cells were harvested and characterized by PCR amplification. A DNA band, which was approximately 2000 bp long, was found with 1.5% agarose gel electrophoresis.

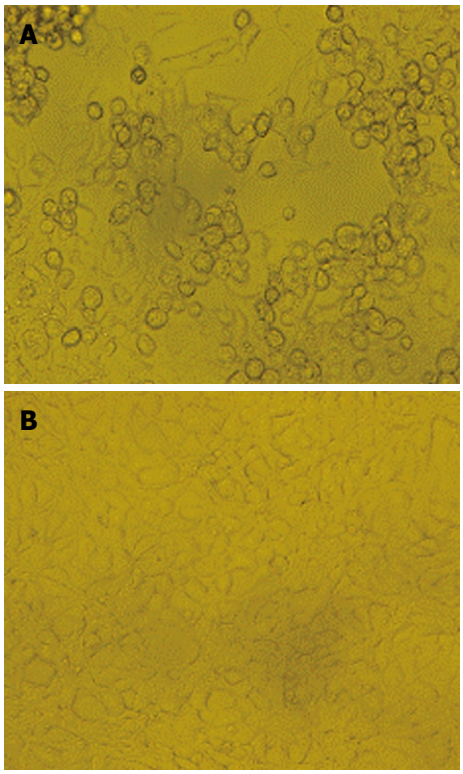

Figure 2 Culture of recombinant adenovirus in HEK293A cells. A: CPE was observed in recombinant adenovirus-containing HEK293A cells; B: Normal HEK293A cells cultured as control.

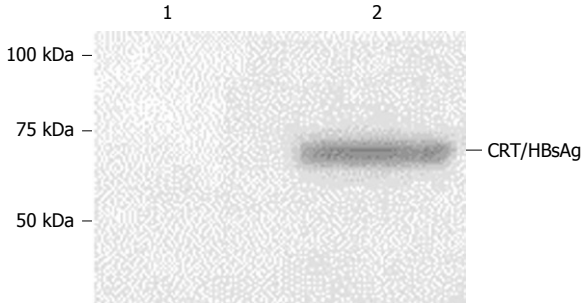

Figure 3 Characterization of CRT/HBsAg fusion protein expression by Western blotting. The expression of CRT/HBsAg fusion protein in 293A cells was determined in 293A cells transfected with Ad-CRT/HBsAg and Ad-LacZ by Western blotting analysis. Human anti-HBsAg positive serum was used at a 1:100 dilution for the detection of CRT/HBsAg fusion protein expression. Lane 1: Lysate from 293 cells transfected with Ad-LacZ; Lane 2: Lysate from 293 cells transfected with Ad-CRT/HBsAg.

Titering of the adenoviral stock, Ad-CRT/HBsAg

After having been amplified, the recombinant adenoviral stock was characterized by the End-Point Dilution Assay. Its titer was calculated as 3.9×10^{11} pfu/mL.

Characterization of CRT/HBsAg fusion protein expression

The expression of CRT/HBsAg fusion protein in Ad-CRT/HBsAg infected 293A cells was detected by Western blotting. Figure 3 shows the 71 kDa CRT/HBsAg fusion protein expression.

DISCUSSION

There are approximately four hundred million chronic hepatitis B patients in the world, and effective therapeutic vaccines are needed urgently. The purpose of this study is to facilitate the development of a new type of therapeutic HBV vaccine. The research hotspot in this field is how to develop an effective therapeutic vaccine by modifying the HBV antigens such as HBsAg, pres1Ag, pres2Ag and HBcAg to induce a high level of humoral and cellular immune responses for clearing the HBV infection. The HBV therapeutic vaccines include protein vaccine, DNA vaccine and virus vector vaccine, but none of them has been developed successfully.

Recently, investigators have performed a head-to-head comparison of linkage of antigen to CRT in a DNA vaccine, and achieved the greatest enhancement of the humoral and T-cell-mediated immune responses in vaccinated mice[15]. Previous studies have shown that adenoviral vectors expressing the fusion of CRT and E7 can effectively be used as a prophylactic and therapeutic vaccine against E7-expressing tumors[16]. In this study, the HBV surface gene was fused with CRT, which can improve immunogenicity by enhancing the MHC class I presentation of linked peptide/protein. CRT-HBsAg fusion gene recombinant adenovirus vector vaccine will be an ideal candidate for HBV therapeutic vaccine, because it can induce HBsAg specific high-level immune responses.

Members of adenovirus family infect a great variety of post-mitotic cells, even those associated with highly differentiated tissues such as skeletal muscle, lung, brain and heart. This characteristic, together with being relatively easy for preparation and purification, has led to their extensive use as gene vectors. Since they deliver their genome to the nucleus and can replicate with high efficiency, they are prime candidates for the expression and delivery of therapeutic genes[17]. The adenovirus vectors used in this study are to be utilized for delivering genes of CRT fused HBsAg to liver tissues to suppress and eliminate chronic inflammation and tumors caused by HBV infection. This is for the first time to construct the CRT-HBsAg gene recombinant adenovirus as a therapeutic vaccine. This study suggested that CRT-HBsAg fusion gene recombinant adenovirus vector may be a potent tool for treating chronic HBV carriers and liver cancer patients.

COMMENTS

Background

The hepatitis B virus (HBV) infection is one of the most widespread viral infections of human and causes acute and chronic hepatitis and hepatocellular carcinoma. Problem of HBV infection has necessitated the development of an effective therapeutic vaccine. Calreticulin (CRT) is a ubiquitously expressed Ca^{2+} binding protein in endoplasmic reticulum of eukaryotic cells. Previous studies have shown that CRT enhances the major histocompatibility complex (MHC) class I presentation of linked peptide/protein and may serve as an effective vaccination strategy for antigen-specific cancer treatment. In this study, CRT-hepatitis B surface antigen (HBsAg) fusion gene recombinant adenovirus

expression vector was generated to establish the basis for curing acute and chronic HBV infection and HBsAg-positive hepatocellular carcinoma.

Research frontiers

There are approximately four hundred million chronic hepatitis B patients in the world, and effective therapeutic vaccines are needed urgently. The research hotspot is how to develop an effective therapeutic vaccine by modifying the HBV antigens such as HBsAg, pres1Ag, pres2Ag and HBcAg to induce a high level of humoral and cellular immune responses for clearing the HBV infection. HBV therapeutic vaccines include protein vaccine, DNA vaccine and virus vector vaccine, but none of them has been developed successfully.

Innovations and breakthroughs

Most of the previous researches about HBV therapeutic vaccines focus on modifying HBsAg to enhance its immunogenicity. In this study, the HBV surface gene was fused with CRT, which can improve immunogenicity by enhancing the MHC class I presentation of linked peptide/protein. CRT-HBsAg fusion gene recombinant adenovirus vector vaccine will be an ideal candidate for HBV therapeutic vaccine, because it can induce HBsAg specific high-level immune responses. Adenovirus can infect a great variety of cell types and tissues in both dividing and non-dividing cells. This characteristic, together with being relatively easy for preparation and purification, has led to their extensive use as gene vectors. The adenovirus vectors used in this study are to be utilized for delivering genes of CRT fused HBsAg to liver tissues to suppress and eliminate tumors and inflammation caused by HBV infection. This is for the first time to construct the CRT-HBsAg gene recombinant adenovirus as a therapeutic vaccine.

Applications

CRT linked HBsAg gene recombinant adenovirus vectors were constructed in this study to develop a new sort of HBV therapeutic vaccine for the purpose of curing chronic hepatitis and hepatocellular carcinoma.

Peer review

It is a nice work to construct CRT/HBsAg fusion gene recombinant replication-defective adenovirus expression vector and it has provided a therapeutic vaccine and an experimental basis for further research of HBV gene therapy.

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CASE REPORT

Obstructing fungal cholangitis complicating metal biliary stent placement in pancreatic cancer

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Abstract

Biliary obstructions can lead to infections of the biliary system, particularly in patients with occluded biliary stents. Fungal organisms are frequently found in biliary aspirates of patients who have been on antibiotics and have stents; however, fungal masses, or "balls", that fully obstruct the biliary system are uncommon and exceedingly difficult to eradicate. We present 4 cases of obstructing fungal cholangitis in patients who had metal biliary stents placed for pancreatic malignancies, and subsequently required aggressive antifungal administration along with endoscopic and radiologic interventions. This report also reviews approaches previously undertaken to manage severe obstructing fungal cholangitis.

Key words: Obstructing fungal cholangitis; Biliary stents; Fungal balls; Pancreatic cancer; Biliary obstruction

Peer reviewers: Pauli Antero Puolakkainen, MD, PhD, Professor of Surgery, Chairman, Department of Surgery, Turku University Hospital, Turku, Finland; Jon Arne Søreide, Professor, MD, PhD, FACS, Department of Surgery, Stavanger University Hospital, N-4068 Stavanger, Norway; Ashok Kumar, MD, Department of Surgical Gastroenterology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, 226014, India

Story B, Gluck M. Obstructing fungal cholangitis complicating metal biliary stent placement in pancreatic cancer. *World J Gastroenterol* 2010; 16(24): 3083-3086 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i24/3083.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i24.3083

INTRODUCTION

Obstructing lesions of the biliary tree include benign and malignant strictures, bile duct stones, stenotic sphincters, and obstructed endoprostheses. Obstructions can lead to infections of the biliary system, particularly in patients with occluded biliary stents. Fungal organisms are frequently found in biliary aspirates of patients who have been on antibiotics and have stents; however, fungal masses, or "balls", that fully obstruct the biliary system are uncommon and exceedingly difficult to eradicate[1-3]. We present 4 cases of obstructing fungal cholangitis in patients who had metal biliary stents placed for pancreatic malignancies and subsequently required aggressive endoscopic and radiologic interventions.

CASE REPORT

Case 1

A 48-year-old male with locally advanced unresectable pancreatic cancer on gemcitabine and docetaxel chemotherapy, presented to our institution with fevers, rigors, jaundice, and epigastric pain 7 d after placement of a metal biliary stent at another institution. Antibiotics were started and endoscopic retrograde cholangiopancreatography (ERCP) showed multiple large filling defects resembling stones (Figure 1). The bile duct was cleared of all defects and the biliary stent was exchanged for a new longer metal biliary stent. Biliary aspirates grew *Candida albicans*. Despite starting 200 mg daily intravenous (iv) fluconazole, the biliary tree rapidly reoccluded within 3 d with large fungal balls. A repeat ERCP in which a plastic

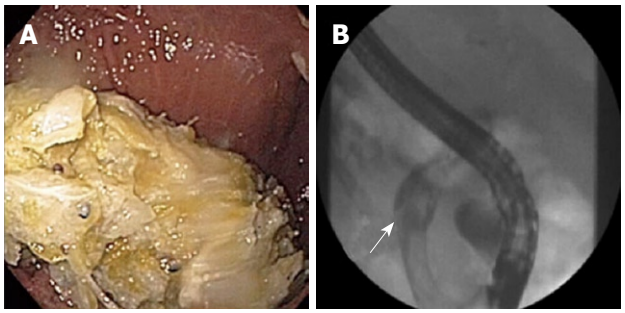

Figure 1 Endoscopic and cholangiographic views of biliary obstruction in patient 1. A: Biliary/fungal debris occluding metal biliary stent; B: "Fungal balls" (arrow) obstructing the bile ducts by cholangiography.

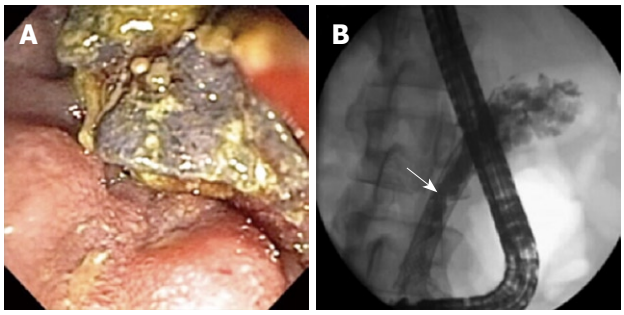

Figure 2 Endoscopic and cholangiographic views of biliary obstruction in patient 2. A: "Fungal ball" visible in the common bile duct; B: Cholangiogram showing fungal debris (arrow) around the metal biliary stent.

endoprosthesis was placed also occluded within 5 d with large fungal balls. A percutaneous transhepatic biliary drain (PTBD) was placed and twice daily 100 mg fluconazole flushes were initiated through the drain. The IV fluconazole was switched to 50 mg daily iv caspofungin, and this antifungal combination resulted in no further obstructions. The patient was discharged home on 200 mg/d oral fluconazole and twice daily 100 mg fluconazole flushes to be continued indefinitely. At 2 mo, repeat biliary cultures through the PTBD identified fluconazole-resistant *Candida*, prompting the patient's oral regimen to be switched to 200 mg twice daily oral voriconazole. The remainder of the patient's clinical course required occasional PTBD catheter exchanges, daily oral voriconazole, and daily intrabiliary flushing of fluconazole until he died 5 mo later secondary to his advanced pancreatic cancer.

Case 2

A 66-year-old female with metastatic pancreatic cancer on gemcitabine chemotherapy underwent metal biliary stent placement and percutaneous cholecystostomy tube placement for obstructive jaundice and acute cholecystitis. Her symptoms improved and 3 mo later, the gallbladder was removed. Shortly after surgery, she was readmitted for fevers, chills, and recurrent jaundice. Antibiotics for presumptive bacterial cholangitis were started and an ERCP was performed revealing caked debris on the proximal edge of her metal biliary stent and multiple soft filling defects within the common hepatic duct (Figure 2). Biliary aspirates grew *Candida glabrata* and *Candida albicans*. Despite clearing the duct of all debris and starting 200 mg twice daily oral voriconazole and 100 mg twice daily fluconazole flushes through the cholecystostomy tube, her biliary tree rapidly reoccluded in 10 d with large fungal balls. A nasobiliary drain was then placed to further facilitate fluconazole flushing into the biliary system. Eradication was finally achieved with daily oral voriconazole and daily fluconazole flushing through both the cholecystostomy tube and the nasobiliary tube. On this regimen indefinitely, the patient remained clear of further obstructions and died from her advanced pancreatic cancer 8 mo later.

Case 3

A 56-year-old male with a Merkel cell lymphoma of his leg and a large metastasis to the pancreatic head, presented to our institution with increasing fevers, abdominal pain, nausea, vomiting, and jaundice. He had been undergoing chemotherapy with irinotecan and carboplatin, and had required duodenal stent placement for tumor overgrowth as well as PTBD and percutaneous metal biliary stent placement 7 mo previously for biliary obstruction. At the time of admission, empiric antibiotic therapy was initiated for presumptive bacterial cholangitis, and an upper endoscopy was performed revealing gastric outlet obstruction with tumor overgrowth of the duodenal stent. A new duodenal stent was placed but the biliary stent could not be visualized, requiring repeat PTBD placement. A PTBD tube check 5 d later revealed multiple soft filling defects within the PTBD catheter consistent with fungal balls (Figure 3). The PTBD catheter was exchanged and after biliary aspirates grew *Candida* spp, 400 mg/d oral fluconazole and 100 mg fluconazole flushes twice daily were initiated through the new PTBD catheter. However, after fluconazole-resistant *Candida glabrata* and *Candida albicans* were isolated from culture, antifungals were switched to 50 mg iv caspofungin once daily and 100 mg amphotericin B flushes once daily through the PTBD catheter. The patient was subsequently discharged from the hospital on a 3-wk course of this antifungal regimen. One month after completion of antifungal therapy, there was no further recurrence of biliary obstruction.

Case 4

A 64-year-old male with metastatic pancreatic cancer on gemcitabine and docetaxel chemotherapy, with an indwelling metal biliary stent in place, presented to an outside facility with biliary obstruction and bacterial cholangitis. Symptoms resolved after a PTBD was placed and antibiotics were administered. However, 2 wk later, the patient re-presented to our institution with recurrent fevers and jaundice. Empiric antibiotic therapy was again initiated for presumptive bacterial cholangitis. Cholangiography performed during ERCP demonstrated obstruction due to migration of the metal biliary stent, tumor overgrowth and multiple soft filling defects consistent with fungal balls. Exchange of the PTBD catheter allowed for external drainage of biliary debris, and the aspirate grew *Candida albicans* in culture. Intravenous fluconazole

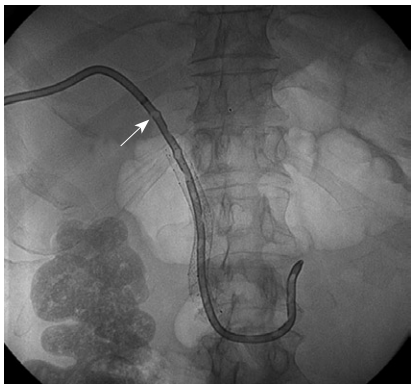

Figure 3 Cholangiographic view of fungal balls (ex. arrow) within the percutaneous transhepatic biliary drainage catheter in case 3.

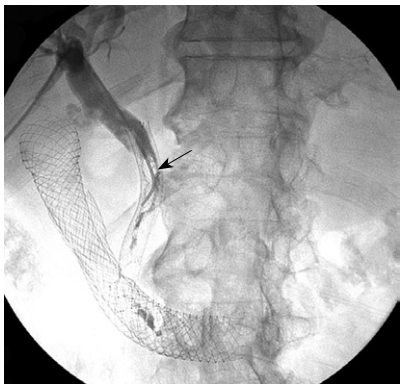

Figure 4 Cholangiographic view of fungal debris (arrow) within a metal biliary stent in case 4.

Table 1 Obstructing fungal cholangitis, summary of the literature

Author	Presentation	Treatment	Fungal eradication
Marcucci *et al*[1], 1978	Abdominal (Abd) pain, jaundice	Surgical removal of fungal balls/drainage. No chemotherapy	Yes
Magnussen *et al*[11], 1979	Acute myelogenous leukemia, elevated liver function tests (LFTs)	Surgical removal of fungal balls, amphotericin B	Yes
Carstensen *et al*[2], 1986	Abd pain, jaundice	Surgical removal, T-tube drainage, antifungals	Yes
Irani *et al*[7], 1986	Jaundice, s/p thoracic abdominal aortic aneurysm repair	Amphotericin B	No, died of cerebral infarct
Ho *et al*[3], 1988	Sickle cell disease, abd pain, jaundice, s/p cholecystecomy	Surgical removal, intrabiliary amphotericin B	Yes
Wig *et al*[4], 1998	Abd pain, jaundice	Nasobiliary drainage, ex-lap with T-tube placement; no chemotherapy	Yes
Reeves *et al*[8], 2000	Abd pain, jaundice	Endoscopic debridement, intrabiliary amphotericin B, oral fluconazole	Yes
Domagk *et al*[5], 2001 and	Elevated LFTs, dilated bile ducts	Sphincterotomy, debridement, systemic/intrabiliary antifungals	Yes
Domagk *et al*[6], 2006	Dilated bile ducts	Sphincterotomy, debridement, systemic/intrabiliary antifungals	Yes
	Abd pain, jaundice	Sphincterotomy, nasobiliary drainage, systemic/intrabiliary antifungals	Yes
	Abd pain, jaundice	Sphincterotomy, debridement, nasobiliary drainage, systemic/intrabiliary antifungals	Yes
	Elevated LFTs	Sphincterotomy, debridement, nasobiliary drainage, systemic/intrabiliary antifungals	Yes
	Elevated LFTs	Sphincterotomy, debridement, nasobiliary drainage, systemic/intrabiliary antifungals	No
	Abd pain, jaundice	Sphincterotomy, debridement, nasobiliary drainage, systemic/intrabiliary antifungals	No

200 mg/d was initiated for antifungal coverage. Over the next several days, biliary obstruction persisted due to stent malpositioning, which ultimately was relieved by severing the patient's migrated metal biliary stent. Despite these measures, within 5 d of PTBD catheter exchange and trimming of the metal biliary stent, the patient's bile duct reobstructed with fungal balls visualized on repeat tube check (Figure 4). The PTBD catheter was exchanged and the patient was initiated on an indefinite regimen of 200 mg oral voriconazole daily and 100 mg fluconazole flushes through the PTBD catheter twice daily. Two weeks later, the patient had no further evidence of obstruction.

DISCUSSION

The patients described in this case report highlight the difficulties in treatment of obstructing fungal cholangitis following the placement of metal biliary stents. Clinical improvement was not seen with endoscopic maneuvers alone or in combination with initial systemic antifungals. Ultimately, infection control was only achieved after flushing antifungals through percutaneous intrabiliary or nasobiliary drains and escalating systemic antifungal therapy from fluconazole to voriconazole or caspofungin. All patients remained free of *Candida* infection following optimization of their antifungal regimens.

While the presence of biliary stents likely played a role in the development of fungal cholangitis in our case report, there are several other factors that can contribute to the development of fungal infections. These include immunosuppression secondary to chemotherapy agents, malignant hematologic disease, broad-spectrum antibiotic administration, corticosteroid therapy, diabetes mellitus, improper sterilization of endoscopes or prostheses, previous surgery, or trauma[1-6]. The patients in our case study demonstrated a number of these risk factors; all were

treated with broad-spectrum antibiotics, all were immunocompromised secondary to systemic chemotherapy for their pancreatic malignancies, and all had been given intermittent corticosteroids for chemotherapy administration.

The data regarding optimal treatment strategies for management of obstructing fungal masses is limited. To our knowledge, only 13 cases of obstructing fungal cholangitis have been described in the literature, the majority of which underwent systemic and intrabiliary infusion of antifungals. Several studies support our treatment approach of initiating therapy with fluconazole, given its favorable efficacy toward *Candida albicans* and relatively few side effects[4,6]. The literature further supports the use of escalation of therapy to voriconazole or caspofungin[6] for infection control. Other case studies, prior to the availability of voriconazole, utilized amphotericin B or liposomal amphotericin[5-8]. While the use of systemic amphotericin has been shown to have numerous complications, several case reports demonstrated that local intrabiliary infusion could be effective[5,6,8]. Interestingly, 2 case studies[1,4] reported no further antifungal administration was necessary following bile duct drainage of fungal balls for complete fungal eradication. Further details are shown in Table 1.

Fungal biliary aspirates are frequently positive in patients who have stents and have been on antibiotics[9,10]. However, it is uncommon to find completely obstructed bile ducts caused by large fungal "balls," implying that growth of the organism was rapid in a previously contaminated system due to foreign bodies and antibiotics. The pathogenesis of common bile duct fungal balls is largely unknown; however, 2 possible explanations exist. Firstly, formation of microabscesses in the liver parenchyma from fungal sepsis may allow movement of fungal organisms into the extra-hepatic biliary system[11]. A second hypothesis involves direct invasion of *Candida* into the common bile duct from the duodenum, with "fungal ball" formation resulting from rapid growth of a superficial mat of fungal mycelia on the bile duct surface[7,11]. Fragmented pieces of fungi and mucoid debris become molded to bile duct walls. Subsequent invasion and proliferation of fungal hyphae may be further aided by the presence of metal biliary stents, acting as a scaffold for infection[7,11].

The treatment strategies described herein address both of the above hypotheses. Systemic antifungal administration targets fungal sepsis, preventing further aggregation of fungal organisms. The direct invasion hypothesis is supported by the fact that fungal balls are easily swept out of the biliary system by endoscopic maneuvers. As such, treating the fungal conglomerates directly by flushing antifungal medications through percutaneous or nasobiliary drains may be a more effective treatment strategy than systemic hematologic dissemination of antifungal therapies[8]. The results seen in the 4 cases described in this report further support this idea. While it seems intuitive that intrabiliary antifungal concentrations would be higher with direct flushing than with systemic antifungal administration, both approaches seem to be required to prevent reocclusion of the biliary system. Further studies are needed to expand on these findings.

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CASE REPORT

Stone extraction balloon-guided repeat self-expanding metal stent placement

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Abstract

Self-expanding metal stent (SEMS) placement offers safe and effective palliation in patients with upper gastrointestinal obstruction due to a malignancy. Well described complications of SEMS placement include tumor growth, obstruction, and stent migration. SEMS occlusions are treated by SEMS redeployment, argon plasma coagulation application, balloon dilation, and surgical bypass. At our center, we usually place the second SEMS into the first SEMS if there is complete occlusion by the tumor. We discovered an unusual complication during SEMS redeployment. The guidewire passed through the mesh of the first SEMS and caused the second SEMS to become entangled with the first SEMS. This led to the distortion and malfunction of the second SEMS, which worsened the gastric outlet obstruction. For lowering the risk of entanglement, we studied stone extraction balloon-guided repeat SEMS placement. This is the first report of a SEMS entangled by the mesh of the first SEMS and stone extraction balloon-guided repeat SEMS placement for lowering the risk of this complication.

Key words: Gastric outlet obstruction; Self-expanding metal stent

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INTRODUCTION

In patients presenting with gastric outlet obstruction, upper gastrointestinal malignancies are the source in up to 39% of cases[1]. Surgical resection is the standard of care in patients who lack significant comorbidities[2]. In unresectable patients, gastrojejunostomy remains the standard of care if a surgical intervention is to be undertaken. In those patients with significant comorbidities, the morbidity rate approaches 40%[3], encouraging alternatives to surgery. In patients who are not surgical candidates, enteral stenting offers an attractive option[4]. Self-expanding metal stent (SEMS) has proven itself to be a safe and relatively cost-effective alternative to surgical palliation allowing the patient to be discharged and start postoperative (PO) intake earlier[5]. Complications related to SEMS placement include stent migration, managed by removal of the original SEMS and replacement with a new SEMS, and obstructions due to local disease progression, treated respectively with repeat SEMS placement, argon plasma coagulation application, balloon dilation, and surgical bypass[2]. We usually perform repeat SEMS placement when there is an obstruction after first stenting. Overlapping the second SEMS into the first SEMS is a safe and easy way for resolving stent obstruction. However, repeat SEMS placement can cause a serious problem if a guidewire transverses the mesh of

the first SEMS and the second SMES is deployed in the mesh of the first SEMS. It must be extremely rare and happens in specific conditions; we experienced just one case of this serious complication. However, once it has happened, this causes irreversible SEMS entanglement leading to further obstruction. Therefore, it is necessary to lower the risk of SEMS entanglement during repeat SEMS placement. To prevent a guidewire from passing through the mesh of the first SEMS, we devised our stone extraction balloon-guided repeat SEMS placement and applied it to one case successfully.

CASE REPORT

A seventy-year-old woman received an 80 mm long SEMS (Niti-S™ Pyloric uncovered stent, Taewoong Medical, Ilsan, Korea) for relieving gastric outlet obstruction due to advanced gastric cancer. Six months later, she presented with vomiting and abdominal distention. Investigation revealed tumor overgrowth distal to the placed SEMS. We decided to deploy another 120 mm long uncovered SEMS (Niti-S™ Pyloric covered stent, Taewoong Medical, Ilsan, Korea), overlapping the previous one. An endoscopy did not pass the existing stent because the proximal part of the existing SEMS was narrow. Thus, we advanced a guidewire from the proximal part of the first SEMS. After performing repeat SEMS placement, we encountered an unusual complication. The newly deployed SEMS was trapped and unexpanded at the entangled site (Figure 1). The guidewire passed through the mesh of the existing stent and caused the second SEMS to be trapped by the mesh of the first one. Because of this complication the gastric outlet obstruction was not improved. This painful experience made us think of stone extraction balloon-guided repeat SEMS placement to avoid SEMS entanglement in specific conditions; the insertion of a guidewire from the proximal or middle part of the first SEMS guided only by fluoroscopy and the acute angulation of duodenum. Several months later, we faced a similar situation. An eighty-four-year-old woman with an 80 mm long SEMS (Niti-S™ Pyloric uncovered stent, Taewoong Medical, Ilsan, Korea) presented with gastric outlet obstruction symptoms. Investigation found that tumor ingrowth had caused SEMS occlusion. We decided to deploy a 100 mm long covered SEMS (Niti-S™ Pyloric covered stent, Taewoong Medical, Ilsan, Korea) into the existing one. Endoscopy was advanced to the middle part of the first SEMS where tumor ingrowth caused near total occlusion. We used a 15 mm stone extraction balloon (ESCORT II™, Willson Cook, Winston-Salem, USA) before advancing a guidewire. An inflated 15 mm stone extraction balloon was passed through from the middle to the distal part of the existing stent without falling into the mesh and led a guidewire to reach the target area safely (Figures 2 and 3). Repeat SEMS placement was successfully performed without the misguidance of a guidewire (Figure 4).

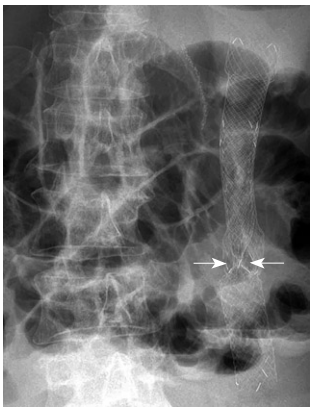

Figure 1 Plain film of the abdomen seven days after deploying the second self-expanding metal stent (SEMS). The unexpanded part of the second SEMS due to entanglement (arrows).

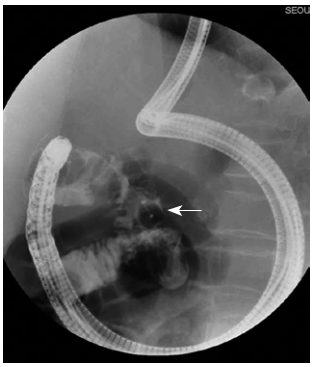

Figure 2 Fluoroscopy. The stone extraction balloon (arrow) was advanced distal to the existing SEMS.

DISCUSSION

The endoscopic placement of enteral SEMS relieves malignant gastroduodenal obstruction in the majority of patients, allowing discharge from hospital and the resumption of enteral nutrition[6]. A multi-center trial of patients with gastric outlet obstruction due to malignancy has concluded that enteral stent insertion allows a purely enteral diet to be maintained in up to 92% of patients, with 73% tolerating solid or semi-solid food[7]. Overall, if migration, fracture, tumor ingrowth, erosion, and perforation are taken into account, the global long-term patency rate obtained is 76%[2]. Stent migration occurs more often with covered stents since tumor ingrowth and subsequent incorporation of the stent into the surrounding tissue is less likely. In contrast, recurrent obstruction is more problematic for uncovered stents[8]. About 9% of total patients who received SEMS developed stent occlusion by local tumor growth with progression of the primary disease process[2]. These were treated, respectively, with repeat SEMS placement, argon plasma coagulation application, balloon dilation, and surgical bypass[2]. At our center, we usually perform repeat SEMS placement when there is a stent occlusion by tumor growth. We have performed repeat SEMS placement through endoscopic-fluoroscopic positioning. An endoscope with a working channel is placed at the distal portion of the first SEMS. Under fluoroscopic guidance, a guidewire pre-loaded through a catheter is used to gently cannulate the stenosis. Once the guidewire has passed through the stenosis, the catheter is advanced over the guidewire through the lesion. As soon as the catheter is passed through the stenosis, the guidewire is

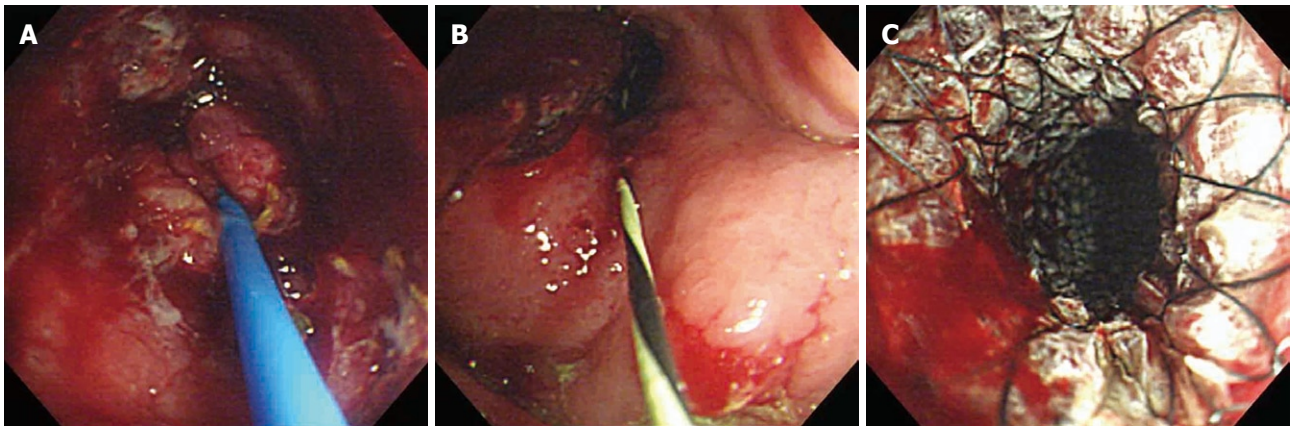

Figure 3 Duodenoscopy. A: The stone extraction balloon advancing over the region of tumor ingrowth; B: The guidewire advancing through the stone extraction balloon; C: The expanded covered SEMS.

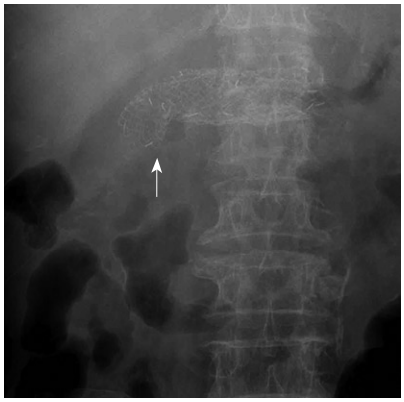

Figure 4 Plain film of the abdomen seven days after replacement of the SEMS. The fully expanded second SEMS overlapping the first SEMS without entanglement (arrow).

removed and water soluble contrast medium is injected to document the characteristics and length of the obstruction and to rule out the occurrence of perforation. At this point, it is very important to push and pull a guidewire through the catheter to find the right position. The stent apparatus is passed through the scope, advanced over the first SEMS under endoscopic and fluoroscopic guidance and deployed so that the middle of the stent covers the first SEMS with 2-2.5 cm extending beyond the proximal and distal margins of the obstructing neoplasm.

Repeat SEMS placement is generally safe and does not cause complications. However, in a specific condition, this procedure can result in irreversible SEMS entanglement. SEMS entanglement can happen by the combination of two conditions: acute angulation of the duodenum and stenosis of the first SEMS that prohibits an endoscope from passing through it. Stenosis of the first SEMS makes practice totally dependent on fluoroscopy, and acute angulation causes a guidewire to touch the duodenal wall. These two conditions make it possible for a guidewire to pass through the mesh of the first SEMS. It is definite that the possibility for a guidewire to transverse the mesh depends more on the angle of a guidewire direction to the mesh wall than on the catheter diameter. Because of the acute angulation of the SEMS in the duodenum, this situation can happen in duodenal obstruction treated by repeat SEMS placement, with frequent misinsertion of the guidewire. Although the acute angulation of an SEMS and the duodenum is the major factor responsible for SEMS entanglement, we thought that a thin guidewire was also responsible for this complication. Because the tip of the guidewire is smaller than the pores of the mesh, it can transverse the mesh and misguide the second SEMS under these specific conditions. When it does occur, the entanglement leads the second SEMS remaining unexpanded and causes further occlusion. For avoiding entanglement of two SEMSs, it is important to push forward and pull back a guidewire repeatedly to ensure the correct position of the guidewire, before starting the insertion of a second SEMS. It is also necessary for the deployment of the new stent to be slow and under careful radiologic control, because most stents can be recovered in their own sheath during the deployment maneuvers, when difficulties are encountered. Adding to these important skills, using a stone extraction balloon can be considered instead of using an ordinary guidewire. When inflated, this system is larger than the pores of the mesh, and therefore, the stone extraction balloon is less likely to pass through the mesh. We used three steps to perform stone extraction balloon-guided repeat SEMS placement in the specific conditions mentioned before. First, we advanced the stone extraction balloon distal to the first SEMS. Second, we inserted the guidewire through the stone extraction balloon so that it was placed at the target area. Finally, we placed the second SEMS within the first one following the guidewire. When there was an acute angulation, and an endoscope was not able to pass through the first SEMS, it seemed easier and safer to perform repeat SEMS placement by the guidance of a stone extraction balloon under careful radiologic control with repeat push and pull of a guidewire.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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Acknowledgments

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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 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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 DOI:10.1046/j.1526-4610.42.s2.7.x]

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 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and 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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Write as mean ± SD or mean ± SE.

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