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EDITORIAL

Antioxidant therapy in the management of acute, chronic and post-ERCP pancreatitis: A systematic review

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Abstract

We systematically reviewed the clinical trials which recruited antioxidants in the therapy of pancreatitis and evaluated whether antioxidants improve the outcome of patients with pancreatitis. Electronic bibliographic databases were searched for any studies which investigated the use of antioxidants in the management of acute pancreatitis (AP) or chronic pancreatitis (CP) and in the prevention of post-endoscopic retrograde cholangio-pancreatography (post-ERCP) pancreatitis (PEP) up to February 2009. Twenty-two randomized, placebo-controlled, clinical trials met our criteria and were included in the review. Except for a cocktail of antioxidants which showed improvement in outcomes in three different clinical trials, the results of the administration of other antioxidants in both AP and CP clinical trials were incongruent and heterogeneous. Furthermore, antioxidant therapy including allopurinol and N-acetylcysteine failed to prevent the onset of PEP in almost all trials. In conclusion, the present data do not support a benefit of antioxidant therapy alone or in combination with conventional therapy in the management of AP, CP or PEP. Further double blind, randomized, placebo-controlled clinical trials with large sample size need to be conducted.

Key words: Antioxidant; Post-endoscopic retrograde cholangio-pancreatography pancreatitis; Oxidative stress; Therapy; Acute pancreatitis; Chronic pancreatitis

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INTRODUCTION

Pancreatitis, both chronic and acute, contributes to thousands of annual hospital admissions and consecutive complications[1]. Acute pancreatitis (AP), an acute inflammatory condition, is thought to be due to activation of enzymes in the pancreatic acinar cells, with inflammation spreading into the surrounding tissues[2]. Patients with AP were either treated with strict bowel rest or given parenteral nutrition to allow the pancreas to rest until the serum enzyme levels returned to normal[3]. Chronic pancreatitis (CP) is a progressive inflammatory disorder that is characterized by recurrent episodes of severe abdominal pain. Affected patients typically suffer years of disabling pain, and conventional therapeutic interventions are often unable to offer satisfactory analgesia[4].

Oxidative stress caused by short lived intracellular reactive oxygen and nitrogen species, can oxidize lipids in the cell membrane, proteins, depolarize the mitochondrial

membrane, and induce DNA fragmentation. Active free radicals in the body can be produced during diseases or exposure to xenobiotics[5,6].

Basic and clinical evidence suggests that the pathogenesis of both AP and CP can be associated with oxidative stress seeming independent of the etiology of pancreatitis, because oxidative stress is observed in different experimental pancreatitis models[7,8]. Findings show that free radical activity and oxidative stress indices such as lipid peroxide levels are higher in the blood and duodenal juice of patients with AP or CP[9,10].

Based on the mentioned findings, the idea of using antioxidant regimens in the management of both AP and CP as a supplement and complementary in combination with its traditional therapy is rational and reasonable. As a result of this hypothesis, antioxidant therapy should improve the inflammatory process that is involved in pancreatitis and therefore ameliorate the recovery rate.

In addition, pancreatitis is the most common serious complication of endoscopic retrograde cholangiopancreatography (ERCP), occurring in 1%-7% of cases[11]. Although, the exact mechanisms involved in the pathophysiology of post-ERCP pancreatitis (PEP) are not clear, the role of oxidative stress cannot be neglected. Therefore, the use of antioxidants before, during or after this intervention has already been studied in a few clinical trials[12,13]. Although some clinical trials have proved the benefits of using various antioxidants in AP or CP, there are still controversies[14].

To our knowledge, there is no definite consensus on the benefits of antioxidant therapy in the management of AP or CP. Our objective was to systematically review and summarize the literature on antioxidant therapies for AP and CP as well as PEP, to provide recommendations for future research.

METHODS

PubMed, Scopus, Google Scholar, Cochrane library database, and Evidence based medicine reviews were searched for any relevant studies that investigated the use of antioxidants in the management of AP or CP and in the prevention of PEP up to February 2009. We also hand-searched references in key articles. The search terms were: AP or CP, pancreatic inflammation, antioxidant, vitamin, superoxide dismutase, manganese, glutamine, butylated hydroxyanisole, taurine, glutathione, curcumin, catalase, peroxidase, lutein, xanthophylls, zeaxanthin, selenium, riboflavin, zinc, carotenoid, cobalamin, retinol, alpha-tocopherol, ascorbic acid, beta-carotene, carotene and all MeSH terms for pharmacologically active antioxidants. Studies were limited to clinical trials and those written in the English language.

To assess the quality of clinical trials, we employed the Jadad score, a previously validated instrument that assesses trials based on appropriate randomization, blinding, and description of study withdrawals or dropouts[15]. The description of this score is as follows: (1) whether randomized (yes = 1 point, no = 0); (2) whether randomization was described appropriately (yes = 1 point, no = 0); (3) double-blind (yes = 1 point, no = 0); (4) was the double-blinding described appropriately (yes = 1 point, no = 0); (5) whether withdrawals and dropouts were described (yes = 1 point, no = 0). The quality score ranges from 0 to 5 points; a low-quality report score is $\leqslant 2$ and a high-quality report score is at least 3.

Data synthesis was conducted by three reviewers who read the title and abstract of the search results separately to eliminate duplicates, reviews, case studies, and uncontrolled trials. The inclusion criteria were that the studies should be clinical trials which used an antioxidant for the treatment or prevention of pancreatitis. Outcomes of the studies were not the point of selection and all studies that analyzed the effects of an antioxidant on pancreatitis, from pain reduction[16] to changes in plasma cytokines, were included.

Data from selected studies were extracted in the form of 2×2 tables. All included studies were weighted and pooled. The data were analyzed using Statsdirect (2.7.3). Relative risk (RR) and 95% confidence intervals (95% CI) were calculated using the Mantel-Haenszel and DerSimonian-Laird methods. The Cochran Q test was used to test heterogeneity. The event rate in the experimental (intervention) group against the event rate in the control group was calculated using L'Abbe plot as an aid to explore the heterogeneity of effect estimates. Funnel plot analysis was used as a publication bias indicator.

RESULTS AND DISCUSSION

A total of 211 potentially relevant papers were identified, of which 22 papers were eligible[4,16-36]. Amongst the 22 papers, 19 (86%) scored 3 and only three studies[17,25,31] scored 2 or lower according to the Jadad score. Table 1 presents controlled clinical trials of antioxidants in patients with AP or CP. Trials that used antioxidants to prevent PEP are summarized in Table 2. To perform a meta-analysis we included only four studies in which allopurinol was used in PEP.

Antioxidants in AP and CP

Glutamine: Glutamine is the most abundant amino acid both in plasma and in the intracellular free amino acid pool. It is essential for a wide variety of physiologic processes, in particular, the growth and function of immune cells including lymphocytes and macrophages[17]. Glutamine is normally synthesized *de novo* by a number of cells and therefore is not an essential amino acid. Although glutamine is an antioxidant, in conditions of excess glutamine utilization such as sepsis, trauma, major surgery or severe AP, endogenous glutamine production may not be adequate and glutamine depletion occurs[23].

In four studies[17,18,22,23] glutamine was supplemented to standard total parenteral nutrition (TPN) in AP patients. In one randomized controlled study (n = 28), glutamine was used in AP in combination with standard TPN and demonstrated a decrease in the duration of TPN therapy and hospitalization without a change in the total

Table 1 Controlled clinical trials of antioxidants in patients with acute or chronic pancreatitis

Study/ Ref.	Drug/supplements	Study design	Jadad score	Participants	Treatment (intervention)		Outcome (results)		Adverse effects/events
					Case	Control	Clinical	Laboratory	
Bhardwaj *et al*[16] 2009	Combined antioxidant (organic selenium, vitamin C, β-carotene, α-tocopherol and methionine)	Randomized; double blind; placebo-controlled	5	147 patients with CP	71 patients; combined antioxidants: 600 μg organic selenium, 0.54 g ascorbic acid, 9000 IU β-carotene, 270 IU α-tocopherol and 2 g methionine; per day; for 6 mo	76 patients; placebo	Number of painful days per month[2] Numbers of oral analgesic tablets and parenteral analgesic injections per month[2] Hospitalization[2] Percentage of patients become pain-free[2] Number of man-days lost per month[2]	Lipid peroxidation (TBARS)[2] Serum SOD[2] Total antioxidant capacity (FRAP)[1] Serum vitamin A[1] Serum vitamin C[1] Serum vitamin E[1] Erythrocyte superoxide dismutase[2]	Headache & constipation (all during the first month of treatment)
Xue *et al*[17] 2008	Glutamine	Randomized	1	80 patients with severe AP	38 patients; 100 mL/d of 20% AGD intravenous infusion; for 10 d; starting on the day 1 (Early treatment)	38 patients; 100 mL/d of 20% AGD intravenous infusion/for 10 d starting on the day 5 (Late treatment)	Infection rate[2] Operation rate[2] Mortality[2] Hospitalization[2] Duration of ARDS[2] Renal failure[2] Acute hepatitis[2] Encephalopathy[2] Enteroparalysis[2] Duration of shock[2] 15-d APACHE Ⅱ core[2]		-
Fuentes-Orozco *et al*[18] 2008	Glutamine	Randomized; double blind; controlled	4	44 patients with AP	22 patients; 0.4 g/kg per day of L-alanyl-L-Glutamine in standard TPN; 10 d	22 patients; standard TPN; 10 d	Infectious morbidity[2] Hospital stay day[3] Mortality[3]	Serum IL-10[1] Serum IL-6[2] CRP[2] Ig A[1] Protein[1] Albumin[1] Leucocyte[2] Total lymphocyte[1] Nitrogen balance was (+) in treated group *vs* (-) in control group	-
Siriwardena *et al*[19] 2007	Combined antioxidant (N-acetylcysteine, selenium, vitamin C)	Randomized; double blind; placebo-controlled	5	43 patients with severe AP	22 patients; N-acetylcysteine, selenium and vitamin C; for 7 d	21 patients; placebo	Organ dysfunction[3] APACHE-Ⅱ[3] Hospitalization[3] All case mortality[3]	Serum vitamin C[3] Serum selenium[3] GSH/GSSG ratio[3] CRP[3]	-
Kirk *et al*[4] 2006	Combined antioxidant (selenium, β-carotene, L-methionine, vitamins C and E)	Randomized; double-blind; placebo-controlled; crossover	4	36 patients with CP	36 patients; Antox tablet: 75 mg of selenium, 3 mg β-carotene, 47 mg vitamin E, 150 mg vitamin C, and 400 mg methionon; four times per day; for 10 wk	36 patients; placebo; four times per day; for 10 wk	Quality of life[1] Pain[2] Physical and social functioning[1] Health perception[1] Emotional functioning, energy, mental health[3]	Plasma selenium[1] Plasma vitamin C[1] Plasma vitamin E[1] Plasma β-carotene[1]	Two patients complained of nausea and one of an unpleasant taste during treatment with Antox
Durgaprasad *et al*[20] 2005	Curcumin	Randomized; single blind; placebo-controlled	3	20 patients of tropical pancreatitis (CP)	Eight patients; capsule: 500 mg curcumin (95%) with 5 mg of piperine; three times per day; for 6 wk	Seven patients; placebo (lactose)	Pain[3]	Erythrocyte MDA[2] GSH level[3]	-

Du *et al*[21] 2003	Vitamin C	Randomized; controlled	3	84 patients with AP	40 patients; IV vitamin C; 10 g/d; for 5 d	44 patients; IV vitamin C; 1 g/d; for 5 d	Hospitalization[2] Deterioration of disease[2] Improvement of disease[1] Cure rate[1]	Tnf-α[2] IL-1[2] IL-8[2] CRP[2] Serum interleukin-2 receptor[2] Plasma vitamin C[1] Plasma lipideroxide[1] Plasma vitamin E[1] Plasma β-carotene[1] Whole blood glutathione[1] Activity of erythrocyte superoxide dismutase[1] Erythrocyte catalase[1]	-
Ockenga *et al*[22] 2002	Glutamine	Randomized, double blind; controlled	4	28 patients with AP	Standard TPN which contains 0.3 g/kg per day L-alanine-L-glutamine; at least 1 wk	Standard TPN	Hospitalization[2] Duration of TPN[2] Cost of TPN[3]	Cholinesterase[1] Albumin[1] lymphocyte count[1] CRP[2]	-
de Beaux *et al*[23] 1998	Glutamine	Randomized; double-blind; controlled	5	14 patients with AP	Six patients; 0.22 g/kg per day of glycyl-glutamine in standard TPN; for 7 d	Seven patients; standard TPN	-	Lymphocytic proliferation (by DNA synthesis)[1] TNF[3] IL-6[3] IL-8[2]	-
Banks *et al*[24] 1997	Allopurinol	Randomized, double-blind, two-period crossover clinical trial	4	13 patients with CP	13 patients; 300 mg/d allopurinol; 4 wk	13 patients; placebo	Pain[3]	Uric acid level[2]	-
Sharer *et al*[25] 1995	Glutathione precursors (S-adenosyl methionine and N-acetylcysteine)	Randomized	-	79 patients with AP	SAMe 43 mg/kg and N-acetylcysteine 300 mg/kg	-	APACHE Ⅱ score reduction[3] Complication rate[3] Days in hospital[3] Mortality[3]	-	-
Bilton *et al*[26] 1994	S-adenosyl methionine (SAMe)	Randomized; double-blind; crossover; placebo-controlled	5	20 patients with AP or CP	20 patients; SAMe 2.4g/d; 10 wk	Placebo	Attack rate and background pain[3]	Free radical activity[2] Serum selenium[2] Serum β-carotene[2] Serum vitamin E[2,3] Serum vitamin C[2] Serum SAMe[1]	-
	Selenium and β-carotene + SAMe				20 patients; SAMe 2.4 g/d, Selenium 600 μg and β-carotene 9000 IU; 10 wk			Free radical activity[2] Serum selenium[2] Serum β-carotene[1] Serum vitamin E[1,3] Serum vitamin C[2] Serum SAMe[1]	
Salim[27] 1991	Allopurinol; dimethyl sulfoxide	Randomized; double-blind; placebo-controlled	4	78 patients with CP	25 patients; allopurinol; 50 mg four times per day, with analgesic regimen (IM pethidine hydrochloride; 50 mg every 4 h, and IM metoclopramide hydrochloride; 10 mg every 8 h)	27 patients; placebo with analgesic regimen	Pain[2] Hospitalization[2] Epigastric tenderness[2]	WBC count[2] Serum amylase[2] Serum LDH[2]	Allergies General malaise Headache Nausea Vomiting Dyspepsia Abdominal pain

Uden *et al*[28,29] 1992, 1990	Combined antioxidant (selenium, β-carotene, vitamin C, vitamin E, methionine)	Randomized; double-blind; crossover; placebo-controlled	5	28 patients with CP	26 patients; dimethyl sulfoxide; 500 mg four times per day; with analgesic regimen 23 patients; daily doses of 600 mg organic selenium, 9000 IU β-carotene, 0.54 g vitamin C, 270 IU vitamin E and 2 g methionine; 10 wk	23 patients; placebo	Pain[2]	Free radical activity[2] Serum selenium[1] Serum β-carotene[1] Serum vitamin E[1] Serum SAMe[2]	-

[1]Significant increase as compared with control; [2]Significant decrease as compared with control; [3]No significant difference between groups. TBARS: Thiobarbituric acid reactive substances; FRAP: Ferric reducing antioxidant power; SOD: Superoxide dismutase; AGD: Alanyl-glutamine dipeptide; CRP: C-reaction protein; MDA: Malondialdehyde; LDH: Lactate dehydrogenase; APACHE Ⅱ: Acute Physiology and Chronic Health Evaluation Ⅱ; GSH: Glutathione; TPN: Total parenteral nutrition; TNF-α: Tumor necrosis factor-α; IL: Interleukin.

Table 2 Controlled clinical trials of antioxidant therapy to prevent post-ERCP pancreatitis

Ref.	Drug/ supplements	Study design	Jadad score	*n*	Treatment (intervention)		Outcome (results)		Adverse effects/events	Other comments
					Case	Control	Primary	Other		
Romagnuolo *et al*[30] 2008	Allopurinol	Randomized; double blind; placebo-controlled	4	586	293 patients; 300 mg oral allopurinol 60 min before ERCP	293 patients; placebo	Rate of PEP[3] (5.5% *vs* 4.1%)	Disease-related adverse events[3] Procedure-related complications[3] Hospitalization[3]	-	In the non-high-risk group (n = 520), the crude PEP rates were 5.4% for allopurinol and 1.5% for placebo (P = 0.017), favoring placebo, indicating harm associated with allopurinol, whereas in the high-risk group (n = 66), the PEP rates were 6.3% for allopurinol and 23.5% for placebo (P = 0.050), favoring allopurinol
Milewski *et al*[31] 2006	N-acetylcysteine	Randomized; placebo-controlled	2	106	55 patients; 600 mg oral N-acetylcysteine 24 and 12 h before ERCP and 1200 mg IV for 2 d after the ERCP	51 patients; isotonic IV saline twice for 2 d after the ERCP	Rate of PEP[3] (7.3% *vs* 11.8%)	Urine amylase activity[3] Serum amylase activity[3]	-	-
Katsinelos *et al*[32] 2005	Allopurinol	Randomized; double blind; placebo-controlled	4	250	125 patients; 600 mg oral allopurinol 15 and 3 h before ERCP	118 patients; placebo	Rate of PEP[2] (3.2% *vs* 17.8%)	Hospitalization[2] Severity of pancreatitis[2]	-	-
Katsinelos *et al*[33] 2005	N-acetylcysteine	Randomized; double-blind; placebo-controlled	3	256	124 patients; 70 mg/kg 2 h before and 35 mg/kg at 4 h intervals for a total of 24 h after the procedure	125 patients; placebo (normal saline solution)	Rate of PEP[3] Hospitalization[3]	-	Nausea; skin rash; diarrhea; vomiting	-
Mosler *et al*[34] 2005	Allopurinol	Randomized; double blind; placebo- controlled	4	701	355 patients; 600 mg 4 h and 300 mg 1 h oral allopurinol before ERCP	346 patients; placebo	Rate of PEP[3] (13.0% *vs* 12.1%)	Severity of pancreatitis[3]	-	-
Lavy *et al*[35] 2004	Natural β-carotene	Randomized; double-blind; placebo-controlled	5	321	141 patients; 2 g oral β-carotene 12 h before ERCP	180 patients; placebo	Rate of PEP[3] (10% *vs* 9.4%)	Severe pancreatitis[2]	-	-
Budzyńska *et al*[36] 2001	Allopurinol	Randomized; placebo-controlled	3	300	99 patients; 200 mg oral allopurinol 15 and 3 h before ERCP	101 patients; placebo	Rate of PEP[3] (12.1% *vs* 7.9%)	Severity of pancreatitis[3]	-	-

[1]Significant increase as compared with control; [2]Significant decrease as compared with control; [3]No significant difference between groups. PEP: Post endoscopic pancreatitis.

cost of parenteral feeding[22]. Another similar study (n = 44) showed that even though TPN therapy containing glutamine reduces infectious morbidity, it has no significant effect on hospitalization and total mortality[18]. However, both studies showed laboratory improvement in AP after administration of glutamine such as an increase in serum albumin or decrease in C-reaction protein (CRP).

Proinflammatory cytokine release was assessed in another study with a small patient number (n = 14). Glutamine supplementation did not significantly influence tumor necrosis factor-α or interleukin (IL)-6 release, but, in contrast, median IL-8 release was reduced by day 7 in the glutamine group while it was increased in the conventional group[23]. Another non-blinded study examined the administration of glutamine in AP for 10 d starting either on the day of admission or 5 d after admission. Investigators reported an improvement in all clinical findings including hospitalization, infection, and mortality rate[17]. No adverse effects were reported in these trials.

Allopurinol: Allopurinol, a xanthine oxidase inhibitor that historically has been effective in preventing attacks of acute gouty arthritis, is an effective anti-oxidant with anti-apoptotic effects. It has been shown that allopurinol is a hydroxyl radical scavenger[37,38]. Two studies used allopurinol to reduce chronic pain in CP[24,27]. In one clinical trial (n = 78), CP patients with chronic pain were admitted to hospital and received an analgesic regimen of pethidine with or without allopurinol. Their results showed that allopurinol could reduce pain and gastric tenderness. Hospitalization was also decreased in allopurinol-treated patients[27]. Another clinical study (n = 13) showed that 4 wk of allopurinol administration did not reduce pain in CP when compared with placebo[24]. Allergy, general malaise, and gastrointestinal disturbances were adverse events of allopurinol.

Vitamin C: Ascorbic acid or vitamin C is a monosaccharide antioxidant. This water-soluble vitamin is a reducing agent and can neutralize oxygen species. Vitamin C is an important antioxidant which protects the body from damage caused by inflammation, and high-dose vitamin C can improve immune function[21]. Vitamin C alone was only investigated in one study and other studies used vitamin C in combination with other antioxidants which will be discussed later. In one randomized study (n = 83), 10 g/d of vitamin C was used intravenously compared to 1 g/d of vitamin C in the control group for 5 d in patients with AP. Their results indicated that 10 g vitamin C decreases hospitalization and duration of disease, and increases the cure rate. Proinflammatory cytokines and CRP were also diminished by vitamin C administration[21].

Combined antioxidants (selenium, β-carotene, vitamin C, vitamin E and methionine): A combination of various antioxidants including selenium, β-carotene, vitamin C, vitamin E, and methionine was studied in three controlled clinical trials[4,16,28]. In the first clinical trial, the efficacy of antioxidant therapy in the management of pancreatitis was determined using the above combination in CP patients (n = 28). Their results showed that this cocktail can reduce the pain which is experienced by patients[28]. Another study with a slightly larger sample size (n = 36) used the above combination at the same doses but with greater bioavailability in CP patients. In this trial, congruent with the previous trial, pain was reduced after 10 wk of the combined antioxidants. Indeed, quality of life, physical and social functioning, and health perception were also enhanced as a result of antioxidant therapy[4]. The latest published controlled clinical trial in the field of antioxidants and pancreatitis has also used this combination at the same doses as the previous studies. In this larger clinical trial (n = 147), the antioxidants were administered for 6 mo, and showed that, similar to the two preceding trials, pain and hospitalization were reduced[16]. All three studies showed that serum concentrations of the above-mentioned antioxidants were higher after a period of intake and laboratory indices of oxidative stress markers such as lipid peroxidation, free radical activity, and total antioxidant capacity improved after therapy. Another cohort study which is not presented in Table 1 examined this combination of antioxidants in 12 CP patients and showed that this combination reduces pain and hospitalization[39]. Headache, nausea, vomiting, and constipation were some of the adverse effects of this combination. A clinical trial studied the effect of selenium, vitamin C and N-acetylcysteine (NAC) combination for 7 d in 43 AP patients. All primary endpoints including hospitalization, Acute Physiology and Chronic Health Evaluation Ⅱ score, and organ dysfunction were statistically similar between the placebo and antioxidant-treated groups[19].

Curcumin: Curcumin is a polyphenolic compound commonly found in the dietary spice turmeric[40]. Curcumin is an inhibitor of nuclear factor-κB and has various biological activities such as anti-inflammatory, antioxidant, antiseptic, and anticancer activity[41]. In the one available pilot study (n = 20), patients with CP received 500 mg of curcumin with 5 mg of piperine or placebo for 6 wk. There was a significant reduction in erythrocyte malondialdehyde levels following curcumin therapy when compared with placebo. A significant increase in glutathione (GSH) levels was also observed. There was no corresponding improvement in pain and no adverse effects were reported[20].

Glutathione precursors [S-adenosyl methionine (SAMe)]: SAMe, a highly bioactive metabolite of methionine is a precursor of glutathione, which is the key defense against reactive species. Of the two clinical trials that examined SAMe in pancreatitis, in one, SAMe was administered to AP patients[25] and in the other, SAMe was administered to CP patients[26]. SAMe did not enhance the clinical outcomes in either AP or CP patients. However, laboratory indices such as free radical activity were better after 10 wk of SAMe administration

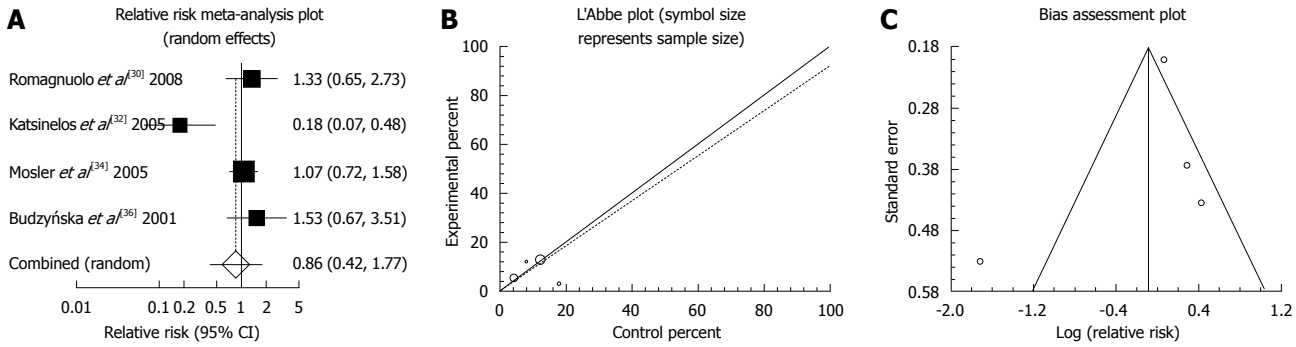

Figure 1 Individual and pooled relative risk (A), heterogeneity indicators (B), and publication bias indicators (C) for the outcome "prevention of all kinds of pancreatitis" in the studies considering allopurinol *vs* placebo therapy.

in CP patients. Methionine in combination with other antioxidants was discussed previously under the topic of combined antioxidants.

Antioxidants in PEP

NAC: NAC is a free radical scavenger capable of stimulating glutathione synthesis. NAC was used in two clinical trials. In one of these trial (n = 106), 600 mg NAC was given orally 24 h and 12 h before ERCP and 600 mg was given intravenously, twice a day for 2 d after ERCP. Their results showed that the rate of PEP was not significantly reduced. In addition, urine amylase activity, total bilirubin, alanine, aspartate aminotransferases and white blood cells showed no change[31].

In the other double-blind, placebo-controlled trial (n = 256), patients received intravenous NAC at a loading dose of 70 mg/kg 2 h before and 35 mg/kg at 4-h intervals for a total of 24 h after the procedure. Similar to the previous study, there were no statistical differences in the incidence or severity of PEP grades between the groups. The mean duration of hospitalization for pancreatitis was not different in the NAC group as compared to the placebo group[33]. The results of those studies showed the absence of any beneficial effect of NAC on the incidence and the severity of ERCP-induced pancreatitis.

Natural β-Carotene: β-carotene is a natural antioxidant which has been used as a supplement in various conditions. In a double-blind trial, 321 patients were given a single dose of natural β-carotene, 12 h prior to the procedure, and monitored for procedure complications, antioxidant levels, and plasma oxidation for 24 h post-procedure. The overall incidence of AP was not significantly different between the β-carotene and the placebo groups. The rate of severe pancreatitis was lower in the β-carotene-treated group. No reduction in the incidence of PEP was reported but there may be some protective effect of treatment with β-carotene regarding the severity of disease. Adverse events were not reported[35].

Allopurinol: There were four randomized clinical trials which used allopurinol orally before ERCP to prevent PEP (Table 3). These studies were meta-analyzed for their primary PEP outcome. The summary RR for "prevention of all kinds of pancreatitis" in the four trials[30,32,34,36] was 0.86 with a 95% CI of 0.42-1.77 and a non-significant RR (P = 0.6801, Figure 1A). The Cochrane Q test for heterogeneity indicated that the studies were heterogenous (P = 0.0062, Figure 1B) and could not be combined. Thus the random effect for individual and the summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among allopurinol *vs* placebo therapy was -1.961983 (95% CI: -14.671469 to 10.747502, P = 0.5749), and Kendall's test on standardized effect *vs* variance indicated tau = 0, P = 0.75 (Figure 1C). The summary RR for "prevention of mild pancreatitis" in three trials[30,32,36] was 1.08 with a 95% CI of 0.7-1.67, a non-significant RR (P = 0.7238, Figure 2A). The Cochrane Q test for heterogeneity indicated that the studies were homogenous (P = 0.2255, Figure 2B) and could be combined. Thus the fixed effect for individual and the summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among allopurinol *vs* placebo therapy could not be calculated because of too few strata. The summary RR for "prevention of moderate pancreatitis" in the three trials[30,32,36] was 0.655 with a 95% CI of 0.388-1.105 and a non-significant RR (P = 0.113, Figure 3A). The Cochrane Q test for heterogeneity indicated that the studies were homogenous (P = 0.0614, Figure 3B) and could be combined. Thus the random effect for individual and the summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among allopurinol *vs* placebo therapy could not be calculated because of too few strata.

Table 3 Studies evaluating post-ERCP pancreatitis after allopurinol administration

Study	Allopurinol	Placebo
Romagnuolo *et al*[30] 2008	16/293	12/293
Katsinelos *et al*[32] 2005	4/121	21/118
Mosler *et al*[34] 2005	46/355	42/346
Budzyńska *et al*[36] 2001	12/99	8/101

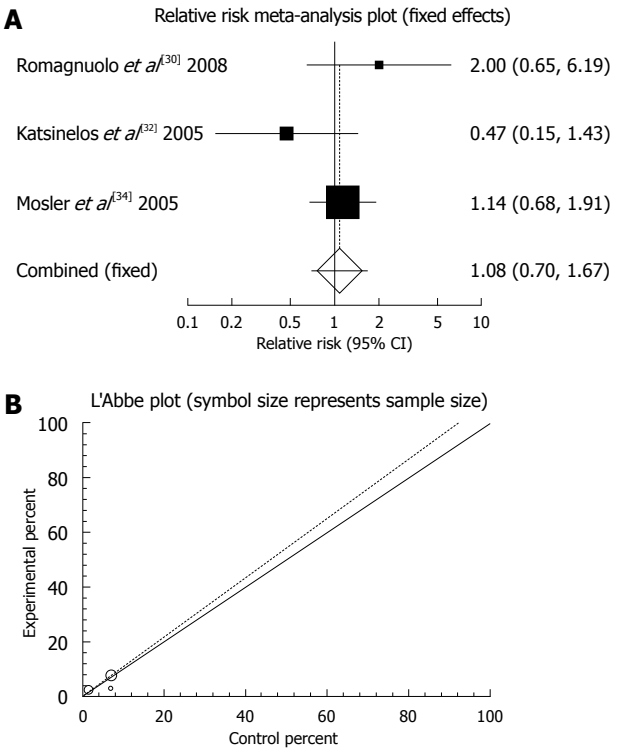

Figure 2 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome "prevention of all kinds of pancreatitis" in the studies considering allopurinol *vs* placebo therapy.

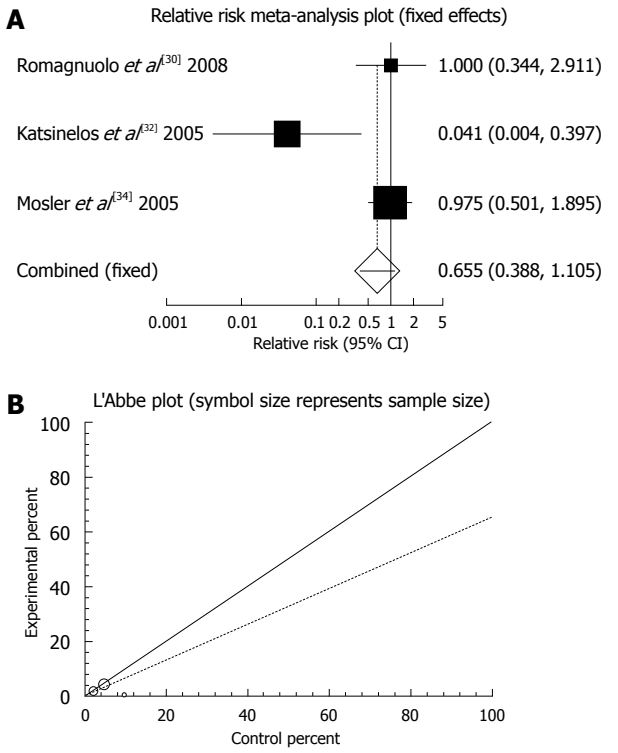

Figure 3 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome "prevention of moderate pancreatitis" in the studies considering allopurinol *vs* placebo therapy.

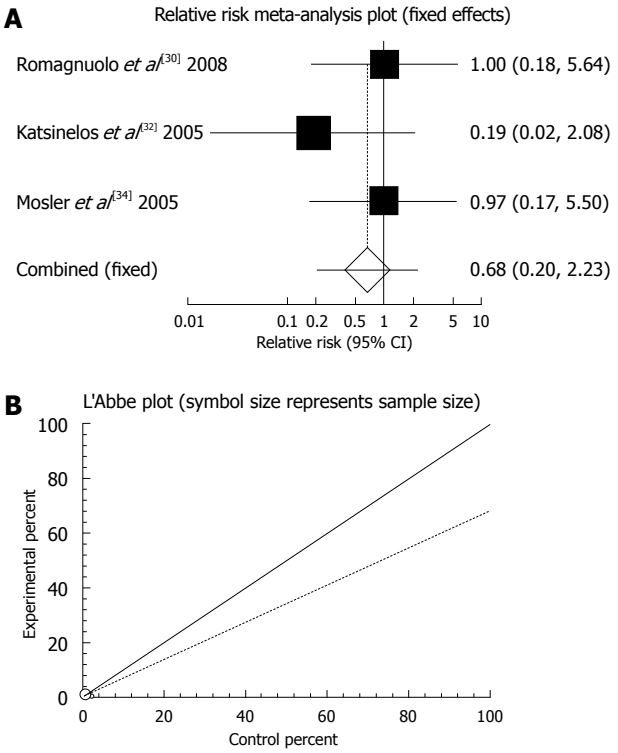

Figure 4 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome "prevention of severe pancreatitis" in the studies considering allopurinol *vs* placebo therapy.

The summary RR for "prevention of severe pancreatitis" of allopurinol *vs* placebo therapy among the three trials[30,32,36] was 0.68 with a 95% CI of 0.2-2.23, indicating a non-significant RR for allopurinol administration ($P = 0.5206$, Figure 4A). The Cochrane Q test for heterogeneity indicated that the studies were not significantly heterogeneous ($P = 0.6154$, Figure 4B) and the fixed effects for individual and the summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for any adverse events among allopurinol *vs* placebo therapy could not be calculated because of too few strata.

Oxygen radicals play an essential role in the development of inflammation in various conditions[42-50]. The involvement of free radicals in the pathogenesis of pancreatitis has been shown in both animal and human studies[51]. Oxidative stress expedites mechanisms which lead to cell damage. It can directly destruct the cell membrane, accelerate lipid peroxidation, deplete cell reserves of antioxidants, and change signaling pathways inside the cells[52,53].

Although the pathophysiology of pancreatitis has been studied before, there is no specific therapy for this disastrous disease yet. Enteral or parenteral nutrition, antibiotic therapy, surgical procedures such as removal of abscess and necrosis, and cholecystectomy have been developed to treat AP[14]. In CP, pain management and probably surgical resection of pseudocysts are the goals of treatment. Because these treatments do not target the main problem and are recommended for symptoms and complications, investigators are still looking for new effective approaches in combination with current treatment.

Clinical studies of the evaluation of typical antioxidants on AP and CP were performed firstly at Man-

chester Royal Infirmary by Braganza and her colleagues. Two placebo controlled clinical trials[28,29] examining combined antioxidant therapy on recurrent CP showed a significant decrease in pain and an elevation in serum antioxidant biomarkers; however, in one study in which SAMe was examined as an antioxidant, alone or in combination with selenium and β-carotene, the results showed that SAMe was ineffective in patients with recurrent pancreatitis. Another two recently published clinical trials[4,16], particularly the latter study with a larger number of subjects (147), which used the same cocktail of antioxidants also showed pain reduction after administration. Therefore, the results of these studies show that such a combination of antioxidants could have a positive effect in the treatment of CP. However, we were unable to meta-analyze these three studies for pain as the primary outcome because pain reduction was assessed in a different way in each study. Except for the mentioned antioxidant cocktail, results of the administration of other antioxidants in both AP and CP clinical trials were incongruent and heterogeneous; and we cannot draw a definite conclusion on the efficacy of such therapy in the management of pancreatitis. We also evaluated the effect of etiology of pancreatitis including alcoholic, gallstone or idiopathic on the results of pain reduction and other outcomes, however, there was no relation between the cause of pancreatitis and clinical outcomes.

Furthermore, antioxidant therapy failed to prevent the onset of PEP in almost all trials (Table 2). Only one clinical trial in which 600 mg of allopurinol was administered twice before ERCP showed a significant decrease in the rate of PEP. However, our meta-analysis revealed that the RR for "prevention of mild, moderate and severe pancreatitis" of allopurinol *vs* placebo therapy was non-significant for allopurinol administration.

However, the present review indicates that there is insufficient data to support using antioxidants alone or in combination with conventional therapy in the management of AP, CP or PEP. Further double blind, randomized, placebo-controlled clinical trials with a larger sample size need to be conducted.

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REVIEW

Adenosine: An immune modulator of inflammatory bowel diseases

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Abstract

Inflammatory bowel disease (IBD) is a common and lifelong disabling gastrointestinal disease. Emerging treatments are being developed to target inflammatory cytokines which initiate and perpetuate the immune response. Adenosine is an important modulator of inflammation and its anti-inflammatory effects have been well established in humans as well as in animal models. High extracellular adenosine suppresses and resolves chronic inflammation in IBD models. High extracellular adenosine levels could be achieved by enhanced adenosine absorption and increased *de novo* synthesis. Increased adenosine concentration leads to activation of the A2a receptor on the cell surface of immune and epithelial cells that would be a potential therapeutic target for chronic intestinal inflammation. Adenosine is transported *via* concentrative nucleoside transporter and equilibrative nucleoside transporter transporters that are localized in apical and basolateral membranes of intestinal epithelial cells, respectively. Increased extracellular adenosine levels activate the A2a receptor, which would reduce cytokines responsible for chronic inflammation.

Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Epithelial cells; Membrane transporters; Immuno-modulator

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INTRODUCTION

Adenosine is a purine molecule necessary for normal cell metabolism and growth. Recently, adenosine has been recognized as a potential anti-inflammatory molecule. In general, cellular adenosine is produced by both *de novo* synthesis and by absorption from the diet into the body through transporters in the gastrointestinal tract. It is thought that activation of adenosine receptors deactivates the synthesis of critical components necessary for activation of chronic inflammatory diseases, including inflammatory bowel disease (IBD). Many reviews have focused on the general aspects of adenosine activation of its receptors in inflamed tissues. This review focuses on the identification of the role of intestinal epithelial cell adenosine transporters during IBD.

CURRENT UNDERSTANDING OF INFLAMMATORY BOWEL DISEASES

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a common and lifelong disabling gastrointestinal disease[1,2]. It has highest incidence and prevalence in the developed countries. The worldwide incidence varies greatly with that of UC ranging from 0.5-24.5/100 000 and that of CD ranging from 0.1-16/100 000 in different populations. There are more than 2 million IBD patients in the United States[3]. The precise mechanism of IBD is still unknown. CD and UC differ in their histological presentation and cytokine profile. The accumulated data indicate that IBD results from a complex interplay of genetic, environmental, and immunologic factors. The presence of one or more genetically determined defects leads to an over-reaction of the host mucosal

immune system to normal constituents of the mucosal microflora. The genetically determined alterations of gut epithelial barrier function enhance exposure of the mucosal immune system to microflora components. The over-reaction causes either a Th1-type T cell-mediated inflammation (Crohn's disease) or a Th2-type T cell-mediated inflammation (ulcerative colitis). Multiple cytokines are released in the inflammatory process. The most important factors are tumor necrosis factor (TNF)-α, interleukin (IL)-1, interferon (INF)-γ, IL-6, 12, 13 and 17, monocyte chemotactic protein (MCP)-1 and IL-8[4]. These cytokines attract and activate neutrophils, eosinophils, mast/plasma cells and macrophages. These inflammatory cells produce large amounts of unstable chemical species such as reactive oxygen species (ROS) or oxyradicals (i.e. superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitrite), resulting in tissue injury[5,6].

Since the cause of IBD is still unknown, currently available treatments for the disease are non-specific and may cause side effects such as osteoporosis and suppression of the immune system. Many patients respond and maintain remission with existing therapy. Thus, at present, there is no cure for IBD. But for some patients, the available therapeutic options for IBD are still inadequate. The conventional treatments use corticosteroids, mesalamine, and immunosuppressants. These either nonspecifically block downstream inflammatory events, such as the secretion of cytokines and activation of immunocytes and neutrophils, or increase tissue adenosine levels, regardless of the nature of the underlying T cell response that generated these events. These agents have been used for treatment of mild and moderate IBD with some success for many years despite shortcomings and toxicities. The newer therapies using biologics, such as antibodies against TNF-α and α-integrin molecules, eliminate a specific major inflammatory cytokine or act by disrupting accumulation of cells at areas of inflammation. Both strategies have been successful in subsets of IBD patients but have also been associated with significant complications including fatal infections[7-12].

Emerging treatments are being developed to target the hierarchy of the inflammatory cytokine effect including IL-12/IL-23[13], IFN-γ[14], IL-6[15], and IL-10 levels[16]. Several antibodies currently on clinic trial include: anti-IL-12p40, an antibody against IL-12 and IL-23, the master cytokines underlying the Th1 response, for Crohn's disease[13]; anti-IL-23p19, a potentially useful treatment for patients with resistance to anti-TNF therapy which acts by targeting IL-23 and IL-17 rather than IL-12 and IFN-γ in experimental colitis. Other approaches to the treatment of IBD currently under investigation are leukocytapheresis to eliminate effector cells[17,18], administration of probiotics, use of GM-CSF to enhance innate immune function[19], administration of microbe-derived agents or intestinal parasites to activate the innate immune system by inducing counter-regulatory immune responses to quell established inflammation[20], administration of anti-CD3 antibodies[21], autologous hematopoietic stem cell transplant[22], extracorporeal photophoresis to restore immunoregulation[23], and adipose stem cell infusion[24].

ADENOSINE MODULATES CHRONIC INFLAMMATION IN IBD

Adenosine exerts broad biologic effects, including smooth muscle contraction, neurotransmission in the peripheral and central nervous systems, platelet aggregation, pain, exocrine and endocrine secretion, lipolysis, glycogenesis, immune system development and response (e.g. severe combined immunodeficiency is due to lack of adenosine-deaminase), cardiac conduction and contractility, and anti-inflammation[25]. It has long been reported that adenosine, a purine nucleoside that is released at injured and inflamed sites, plays a central role in the regulation of inflammatory responses and in limiting inflammatory tissue destruction[26]. Early after the injurious or infectious signal, high concentrations of extracellular adenosine favor a transition from neutrophil infiltration to macrophage recruitment, to facilitate a highly efficient specific immune response carried out by macrophages. At later stages of immune or inflammatory processes, adenosine contributes to the resolution of inflammation, both by down-regulating macrophage activation and by advancing Th2- *vs* Th1-cell response[26]. Some anti-inflammatory and immunomodulating drugs, such as salicylates, methotrexate and purine analogs like 6-MP and cyclosporine, exert their therapeutic actions in inflammatory diseases, at least in part, by decreasing intracellular adenosine 5'-triphosphate (ATP) concentrations and increasing extracellular adenosine levels[27].

There are numerous reports that have demonstrated the ability of adenosine to exert anti-inflammatory actions in a variety of animal models. The anti-inflammatory effects can be achieved by increasing intracellular or extracellular adenosine levels through the mechanism of either enhanced production or inhibition of adenosine catabolism. The majority of work has been focusing on inhibition of adenosine catabolism or direct activation of adenosine receptors. A recent article by Antonioli *et al*[28] reported that inhibition of adenosine deaminase can attenuate mucosal inflammation in experimental colitis through the mechanism of reducing mucosal myeloperoxidase activity, production of malondialdehyde and TNF-α levels as well as plasma TNF-α and interleukin-6 levels. Other studies have demonstrated that adenosine acting on the A2a receptor of T-lymphocytes can selectively suppress the expression of pro-inflammatory cytokines while sparing anti-inflammatory activity mediated by IL-10 and TGF-β[29]. The tissue injury and inflammation in mice with enteritis induced by *Clostridium difficile* toxin A can be alleviated by a new A2a receptor agonist, ATL 313, through the mechanism of inhibiting neutrophil infiltration, TNF-α production and adenosine deaminase activity[30]. Adenosine can down-regulate neutrophil functions by

decreasing their adhesion, degranulation, and oxidant activities[25]. An increase in endogenous adenosine levels by inhibition of adenosine kinase ameliorates colitis by suppression of IFN-γ in colonic tissue and CD69 expression in splenocytes, as well as maintaining tissue integrity by reducing energy demand, increasing nutrient availability, and modulating the immune system[31]. At a molecular level, adenosine has been demonstrated to be a negative regulator of NF-κB and MAPK signaling in human intestinal epithelial cells[32]. Based on these findings, the adenosine system can represent a very promising target for therapies of inflammatory bowel diseases.

PHYSIOLOGY OF ADENOSINE METABOLISM BY INTESTINAL EPITHELIUM CELLS

Intracellular adenosine level is maintained by constant synthesis and degradation, as well as by trans-membrane transport through nucleoside transporters. The intracellular adenosine is produced by *de novo* synthesis from amino acids, CO_2, carbon-1-tetrahydrofolate and ribose-5-phosphate, salvage of endogenous adenine, dephosphorylation of ATP, ADP and AMP, as well as by transportation of exogenous nucleobases and nucleosides through concentrative nucleoside transporters (CNTs) or equilibrative nucleoside transporters (ENTs). Adenosine is metabolized to inosine by adenosine-deaminase, to either an end product uric acid or phosphorylated to ATP by adenosine-kinase or diffused into the extracellular space *via* ENTs[33,34].

Most tissues and cells have *de novo* synthesis capacity to produce adenine endogenously from amino acids, CO_2 and carbon-1-tetrahydrofolate for their own use. However, some tissues and cells either lack or have very limited *de novo* synthesis capacity. These tissues and cells rely largely on exogenous nucleoside supply and salvage of endogenous nucleobases and nucleosides. Bone marrow, lymphocytes, leukocytes and intestinal epithelial cells are among them[35-39]. The liver is the major organ supplying the nucleobases to these tissue and cells. The dietary nucleobases and nucleosides are absorbed by intestinal villous epithelial cells and degraded to end products such as uric acid. The uric acid is brought to the liver by blood and is taken up by hepatocytes and transformed into nucleobases. The synthesized nucleobases are released into the blood stream and carried to the tissues.

The intestinal epithelial cells have a very limited capacity for *de novo* synthesis; the villous cells can directly use the absorbed dietary nucleosides but the cryptal cells depend on blood supply. The impact of lack or limitation of nucleoside supply to cryptal cells on epithelial repair and barrier function during IBD has not been fully investigated[40,41]. It is reasonable to speculate that poor absorption in the intestine or suppression of synthesis in the liver will ultimately result in disruption of epithelial barrier function in chronic bowel inflammatory diseases like IBD, which in turn will lead to over-exposure of the innate immune system to intraluminal bacterial antigens and cause persistent inflammation or exacerbation of the diseases.

In general, the extracellular adenosine is produced by dephosphorylation of ATP by an enzymatic cascade consisting of Ntpases and ecto-5'-nucleotidase (Ecto 5'NTase), and direct diffusion of intracellular adenosine through ENTs. It is removed by enzymatic degradation by adenosine deaminase to inosine or by adenosine kinase to AMP. It can also be transported back into cells by membranous transporters like CNTs/ENTs. The extracellular adenosine level is believed to be lower than 1 μmol/L in normal tissue but can be as high as 100 μmol/L in inflamed or ischemic tissues[25]. Only a high adenosine level can exert immunomodulatory and immunosuppressive effects. Luminal adenosine level is estimated to be 5 mmol/L in normal intestine, while it is 6 mmol/L in inflamed intestine due to ATP and adenosine secretion in inflammatory and other cell types[42].

It is not entirely clear which cell types are the most important producer of extracellular adenosine, but endothelial cells, neutrophils, nerve terminal and epithelial cells have been identified in the literature[43,44]. Extracellular adenosine binds to adenosine receptors (AR) 1, 2a, 2b and 3, all of which are expressed on the surface of immune cells. Low level expression of A1R is demonstrated in small intestine. A2bR is the only receptor expressed in epithelial cells of cecum and colon. A3R can be detected in jejunum and proximal colon[45,46]. Adenosine receptors are members of the G protein-coupled family of receptors[47]. A1 and A3 receptors are usually coupled with Gi proteins that inhibit adenylate cyclase, whereas the A2aR and A2bR receptors are coupled with Gs proteins that activate adenylate cyclase. Several studies have demonstrated that adenosine attenuates intestinal inflammation predominantly through the effects of the A2aR receptor of neutrophils and T-lymphocytes[29,30,48]. However, Yang *et al*[49] found that activation of the A2bR can also have anti-inflammatory effects, using a gene knock-out method to delete this gene in order to show a pro-inflammatory phenotype.

MOLECULAR MECHANISM OF ADENOSINE TRANSPORT

The significance of exogenous adenosine transport by intestinal epithelial cells in the treatment of IBD and its impact on epithelial cell barrier function has not been explored. Concentrative nucleoside transporters (CNTs) have been identified as the major transporters for absorption of exogenous nucleosides from the diet. Three distinct CNTs (CNT1, CNT2 and CNT3) that exhibit different substrate specificity have been cloned and characterized from humans, rats and mice[34]. CNT1 predominantly transports pyrimidines. CNT2 transports purine and uridine, while CNT3 transports purines and

pyrimidines. The expression of CNTs and their substrate specificity vary among species. CNT3 was not found in intestinal epithelial cells of human and rat[34,50,51]. CNTs belong to the solute carrier family 28 (SLC-28). CNTs are expressed in the apical membranes of intestinal epithelial cells, as well as in other cell types including hepatocytes, endothelial cells, neutrophils, lymphocytes and macrophages.

CNT2 has been cloned and characterized in humans, mice, rats and rabbits. The rat CNT2 cDNA is 2.9 Kb and encodes a 659 amino acid protein with molecular weight of 72 kDa[52]. The apparent molecular weight on western blot is usually around 60 kDa due to high hydrophobicity of membrane protein. Fourteen putative transmembrane domains were identified by hydropathy analysis. The presence of several consensus sequences for protein kinase-C (PKC) and protein kinase-A (PKA) phosphorylation sites on both N- and C-termini, and an ATP/GTP binding motif in N-terminus, suggest that CNT2 may be regulated by protein kinases and intracellular ATP and GTP. CNT2 may be a glycoprotein as there are five possible N-linked glycosylation sites. Na^+-adenosine cotransport in brush-border membranes from rabbit ileum was identified and partially characterized in one previous study[53]. *In vitro* expressed CNT2 in *Xenopus laevis* oocytes exhibited Na^+-dependent adenosine uptake with an apparent *Km* for adenosine of 6 μmol/L, with substrate selectivity to purine and uridine[54].

REGULATION OF ADENOSINE TRANSPORT AND CNT2 EXPRESSION IN GENERAL

The CNT2 expression and adenosine uptake are highly regulated processes and are species and tissue specific[55]. Sub-cellular trafficking (i.e. internalization of membrane transporters to sub-cellular storage vesicles) has been shown to be a regulatory mechanism for CNT2 in several cell lines including adrenal chromaffin cells, reticulocytes and cholangiocytes[56-58]. Although CNT1 has been shown to be up-regulated in intestine and down-regulated in hepatocytes of pyrimidine-free diet fed animals, the dietary effects of adenosine on CNT2 activity and molecular expressions are not known[59,60]. Several studies have shown that nucleoside transport functions and expressions are regulated by hormones[61-66]. Tyrosine and glucagon have been shown to stimulate adenosine transport and CNT2 expression in both *in vitro* and *in vivo* models[61-63]. Studies with insulin and glucose have yielded different results regarding adenosine transport and molecular expression. Insulin regulates adenosine transport through different signaling pathways that involve PI3K, MAPK, NO synthase, PKC and MAP kinase[64-66]. One study showed that adenosine transport is up-regulated by activation of the A1 adenosine receptor through ATP-sensitive K-channels in hepatocytes, suggesting some positive feedback regulation among adenosine receptors and the adenosine transporter[67].

The effects of proliferative (EGF and TGF-α) and differentiating (glucocorticoid) hormones were demonstrated in IEC-6 cells (a rat intestinal cryptal cell line). A four-fold increase of adenosine transport activity and CNT2 molecular expression after treatment with dexamethasone was observed while no significant impact was noted from treatment with proliferative hormones[68]. However, a more recent paper by the same research group found that TGF-β can transcriptionally up-regulate *CNT2* gene expression in rat hepatocytes[69]. Adenosine transport and CNT2 expression are altered during cell growth cycle and differentiation. Hepatocarcinogenesis is accompanied by loss of CNT2 expression and increased expression of ENTs. CNT2 mRNA and protein levels were increased right before the peak of incorporation of thymidine into DNA and during liver regeneration after partial hepatectomy[70,71].

CNT2 expression was up-regulated by lipopolysaccharide (LPS), NO, INF-α and TNF-α in macrophages and B-lymphocytes in several studies[72-74]. The LPS-induced increase of adenosine transport and molecular expression is TNF-α-dependent but not iNOs-dependent. cNOs is required for maintaining the basal transport activities of adenosine in activated B-cells. The CNT2 is recognized as an important regulator of extracellular adenosine concentrations. CNT2 expression is suppressed in inflamed tissue as a mechanism to maintain high extracellular adenosine concentration. The CNT2 expression is suppressed in neutrophils and macrophages during inflammation[75]. There is controversy between the *in vivo* and *in vitro* studies about the functional status of adenosine transport and CNT2 expression during inflammation and the effects of inflammatory mediators.

MECHANISM OF REGULATION OF ADENOSINE TRANSPORT AND CNT2 EXPRESSION IN INTESTINAL EPITHELIAL CELLS

A systematic study for the mechanism of regulation of adenosine transport and CNT2 expression in intestinal epithelial cells is not available. Adenosine functions as a nutrient for nucleic acid metabolism, an energy carrier molecule for cell energy metabolism, and a second messenger in autocrine and paracrine hormone regulation. Adenosine transport is regulated differently from other nutrient transporters such as glucose and amino acid transporters. Luminal adenosine and ATP have been reported to regulate glucose and bile acid transport in intestine, proximal renal tubule cells and cholangiocytes[42,76,77]. The role of the activation of purinergic receptors on adenosine uptake was also studied in vascular endothelial cells and chromaffin cells. ATP has been shown to up-regulate adenosine transport and protein expression in vascular endothelial cells and chromaffin cells[78-80]. However, the effect of ATP on

adenosine transport in normal and inflamed intestine remains to be elucidated.

MECHANISM OF REGULATION OF NA^+-CO-TRANSPORT IN INTESTINAL EPITHELIAL CELLS DURING CHRONIC INTESTINAL INFLAMMATION

The regulation of intestinal epithelial transporters during chronic inflammation is a very complex process, involving many cell types and immune-inflammatory mediators. The cells involved in chronic enteritis include all inflammatory cells (neutrophils, basophils, eosinophils, macrophages, lymphocytes), fibroblasts, vascular endothelial cells, nerve cells and epithelial cells. These cells produce numerous cytokines and immune-inflammatory mediators such as prostaglandins, leukotrienes, reactive oxidative metabolites (ROMs) and nitric oxide[1-6]. So far, there are no perfect animal models for IBD though several different models exist with chemically-induced and genetically-induced enteritis. It is very difficult to fully understand the regulation of nutrient and electrolyte transport in the chronically inflamed intestine.

The mast cell has been implicated as an important player in chronic intestinal inflammation. It releases multiple inflammatory mediators including histamine, serotonin, cytokines, prostaglandins, leukotrienes and ROMs. The released mediators are very important regulators of transporters in epithelial cells. It was consistently demonstrated that blocking mast cell degranulation prevented down-regulation of a number of transporters including Na^+-glucose and Na^+-amino acid cotransport as well as electrolyte transporters such as Cl^-/HCO_3^- exchange[81].

Nitric oxide has been identified as an important regulating factor in the intestinal tract. Constitutive nitric oxide synthase (cNOs) plays an important role in the regulation of transporters in normal intestine. It is essential for some transporters to function properly under physiologic conditions. Inducible nitric oxide synthase (iNOS) is activated during chronic inflammation, ischemia and tissue injury. iNOS produces large amounts of nitric oxide (NO) during chronic inflammation, which is generally considered as detrimental to tissue and cells because of formation of peroxynitrous acid, though controversy continues about its role in inflammation. The mechanism of cNOS and iNOS effects on nutrient co-transporters in epithelial cells is possibly through direct and indirect effects, by stimulating the production and/or release of other inflammatory mediators such as arachidonic acid metabolites (e.g. prostaglandins and leukotrienes)[82-84].

Prostaglandins have been shown to be very important inflammatory mediators in chronic intestinal inflammation. A large amount of prostaglandins are produced in the intestinal tissue during IBD. Prostaglandins inhibit electrolyte absorption and Na^-

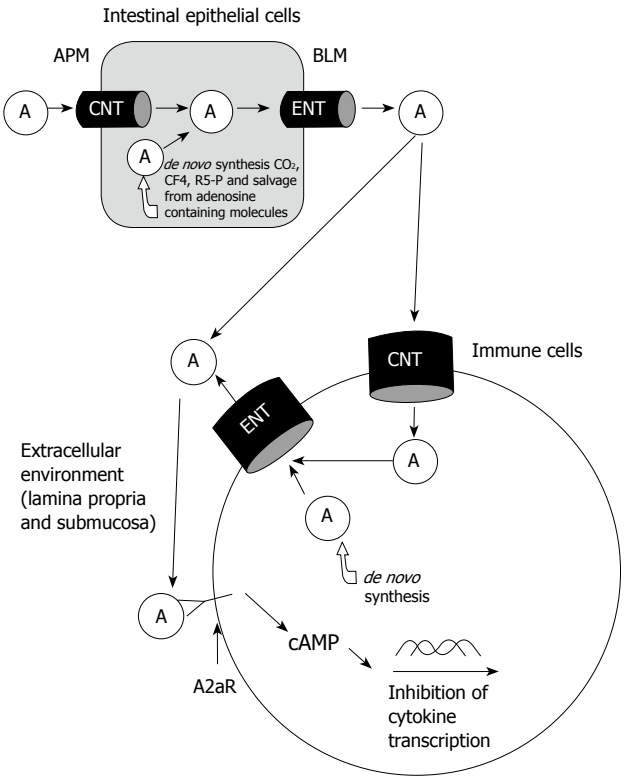

Figure 1 Pathways and roles of adenosine. Absorbed and *de novo* synthesized adenosine delivered to extracellular space. Adenosine binds to A2aR of immune cells and activates signaling pathways to inhibit the production of inflammatory mediators. A: Adenosine; APM: Apical membrane; BLM: Basolateral membrane; CNT: Concentrative nucleoside transporter; ENT: Equilibrative nucleoside transporter.

nutrient co-transporter functions[85-89]. In addition, they also promote mucous secretion and cytoprotection in the gastrointestinal tract during IBD[85,88]. Prostaglandin E2 (PGE2) was also reported to suppress glucose transport in the ovine intestine[90]. Leukotrienes have been demonstrated to inhibit electrolyte transport in a similar pattern to prostaglandins[91-98].

Corticosteroids are the most frequently used broad spectrum immunomodulators in IBD for blocking the production of most major inflammatory mediators. Corticosteroids prevent mast cell degranulation and block the phospholipase A2 (PLA2) enzyme pathway. They also down-regulate arachidonic acid release and production of prostaglandins and leukotrienes. In addition, corticosteroids suppress the production of iNOS and inducible cyclooxygenase (COX-2) during chronic inflammation[99].

It is possible that mast cells, nitric oxide, arachidonic acid metabolites and steroids are all involved in the regulation of adenosine transport and CNT2 expression during chronic enteritis. It should be noted that adenosine itself is an inflammatory modulator. Extracellular adenosine can suppress all these above-mentioned inflammatory cells and mediators. The interplay among adenosine, inflammatory cells and mediators can be very complex and is also very interesting for further study.

CONCLUSION

The pathways of adenosine metabolism and transport are fully illustrated in Figure 1. Luminal adenosine is absorbed by intestinal epithelial cells through CNT and ENT transporters and can also be synthesized *de novo*. The increase in intracellular levels of adenosine leads to extracellular transport along with the release of adenosine from damaged cells during inflammation. Using various therapies during IBD, it may be possible to eventually increase the extracellular levels of adenosine so that the appropriate receptors can be activated to inhibit and reduce the effects of chronic inflammation on the gut. IBD is a common and lifelong disabling gastrointestinal disease. At present, there is no cure for IBD. Emerging treatments are being developed to target cytokines that perpetuate the chronic inflammatory response. Adenosine is an important modulator of inflammation and its anti-inflammatory effects have been well established in humans as well as in animal models. Therapeutic targeting of receptors such as the A2a receptor could reduce cytokine levels and thus reduce the effects of chronic inflammation during IBD.

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ORIGINAL ARTICLES

Iron increases HMOX1 and decreases hepatitis C viral expression in HCV-expressing cells

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Abstract

AIM: To investigate effects of iron on oxidative stress, heme oxygenase-1 (HMOX1) and hepatitis C viral (HCV) expression in human hepatoma cells stably expressing HCV proteins.

METHODS: Effects of iron on oxidative stress, HMOX1, and HCV expression were assessed in CON1 cells. Measurements included mRNA by quantitative reverse transcription-polymerase chain reaction, and protein levels by Western blots.

RESULTS: Iron, in the form of ferric nitrilotriacetate, increased oxidative stress and up-regulated *HMOX1* gene expression. Iron did not affect mRNA or protein levels of Bach1, a repressor of HMOX1. Silencing the up-regulation of HMOX1 nuclear factor-erythroid 2-related factor 2 (Nrf2) by Nrf2-siRNA decreased FeNTA-mediated up-regulation of HMOX1 mRNA levels. These iron effects were completely blocked by deferoxamine (DFO). Iron also significantly decreased levels of HCV core mRNA and protein by 80%-90%, nonstructural 5A mRNA by 90% and protein by about 50% in the Con1 full length HCV replicon cells, whereas DFO increased them.

CONCLUSION: Excess iron up-regulates HMOX1 and down-regulates HCV gene expression in hepatoma cells. This probably mitigates liver injury caused by combined iron overload and HCV infection.

Key words: Deferoxamine; Core protein of hepatitis C virus; Hepatitis C; Iron; Heme oxygenase-1; Nuclear factor-erythroid 2-related factor 2; Bach1; Oxidative stress; Nonstructural 5A protein of hepatitis C virus

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INTRODUCTION

Iron overload is known to be toxic to many organs, particularly to the liver. The liver is the major site of storage of excess iron. The most common form of iron overload is that related to classic hereditary hemochromatosis, in which, due to mutations in the *HFE* gene, there is excessive uptake of iron into enterocytes[1-3]. In hemochromatosis, decreased hepatic production and secretion of hepcidin leads to increased ferroportin expression at the plasma membranes,

especially of enterocytes and macrophages. Ferroportin is the only known physiologic iron exporter from cells and its uncontrolled over expression leads to excess uptake of iron from the enterocytes into the portal blood and to increased release of iron from macrophages and other cells of the reticulo-endothelial system, including the Kupffer cells of the liver[4-6]. The excess iron in the portal blood and/or released by Kupffer cells within the liver is taken up by hepatocytes where it is stored, chiefly in the form of holo-ferritin. Iron in ferritin is relatively non-reactive and non-toxic. However, release of tissue ferritin from damaged or dying cells leads to activation of hepatic stellate cells and a cascade of pro-inflammatory and pro-fibrogenic events. This may eventuate in the development of hepatic fibrosis, cirrhosis, and hepatocellular carcinoma, as well as all of the usual complications of advanced chronic liver disease[7-9].

In recent years, it has become increasingly clear that only modest amounts of iron in the liver may play a role as a co-morbid factor in the development and progression of non-hemochromatotic liver diseases[10-15]. The link between iron and non-hemochromatotic liver diseases is particularly strong for steatohepatitis, both non-alcoholic and alcoholic[10,14,15] and viral hepatitis B and C[16-18].

Porphyria cutanea tarda, the most common form of porphyria, is known to be triggered or exacerbated by iron and is often associated with *HFE* gene mutations, chronic hepatitis C, and alcohol use[19-21]. The treatment of choice for porphyria cutanea tarda involves removal of iron, which leads to remission of the biochemical and clinical features of the disease. Blumberg and colleagues were among the first to stress the importance of iron status in influencing outcomes and progression of acute hepatitis B infection[22,23]. In the case of hepatitis C infection, a number of investigators from throughout the world have noted high prevalences (35%-50%) of elevations of serum ferritin and high, albeit somewhat lower, frequencies of elevations of serum transferrin saturation in patients with chronic hepatitis C[10,24-26]. Despite this, the occurrence of heavy iron overload in chronic hepatitis C is infrequent and is chiefly related to advanced liver disease. Increases in serum measures of iron and stainable iron in the liver have been directly correlated with more severe chronic hepatitis C and with lower likelihoods of response to currently available antiviral therapy, especially type 1 interferons[24,27,28]. In addition, it has been shown repeatedly that reduction of body iron by therapeutic phlebotomy improves the responsiveness of chronic hepatitis C infection to interferon therapy[29].

Heme oxygenase-1 (HMOX1) has emerged as a key cytoprotective gene and enzyme in numerous experimental and clinical contexts (For reviews, see[30-33]). The *HMOX1* gene is under complex regulation and can be up-regulated markedly by heme, the physiologic substrate for the HMOX1 protein, by iron and other transition metallic ions, and by oxidative and heat stress and other stressful perturbations. Regulation of *HMOX1* gene expression is related in part to alterations in levels of several transcription factors, including Bach1, and nuclear factor-erythroid 2-related factor 2 (Nrf2). Normally, Bach1 in nuclei represses *HMOX1* gene expression, whereas Nrf2, in concert with small Maf proteins, up-regulates its expression[34-36].

The study of hepatitis C viral (HCV) infection has been difficult because of the lack of a readily available, inexpensive animal model of acute or chronic hepatitis C infection. The recent development of human hepatoma cell lines, which stably express HCV proteins, and support the replication of HCV RNA or the formation of complete infectious virions of HCV[37,38], has facilitated studies on pathogenesis and the role of potential co-morbid factors, such as iron. We used such lines to investigate the effects of iron on oxidative stress, HMOX1 and HCV expression. Here we report that excess iron results in further increased oxidative stress and up-regulation of HMOX1 *via* Nrf2, and down-regulation of HCV protein expression in human hepatoma cells in culture (Huh-7) expressing HCV RNA and proteins. These effects are reversed by deferoxamine (DFO), the selective and potent iron chelator.

MATERIALS AND METHODS

Reagents and materials

Mouse anti-HCV nonstructural 5A (NS5A) protein was purchased from Virogen (Plantation, FL). Goat anti-human Bach1, goat anti-human GAPDH polyclonal antibodies, goat anti-mouse IgG, and donkey anti-goat IgG were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). ECL-Plus was purchased from Amersham Biosciences Corp (Piscataway, NJ). Dimethyl sulfoxide was purchased from FisherBiotech (Fair Lawn, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), zeocin, geneticin, trypsin and TRIzol were from Invitrogen Inc. (Carlsbad, CA). $FeCl_3$, Na_3NTA, H_2O_2, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and its oxidation-insensitive analog 2',7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (Allentown, PA). DFO mesylate was from Novartis (Cambridge, MA).

Cell cultures

The human hepatoma cell line, Huh-7, was purchased from the Japan Health Research Resources Bank (Osaka, Japan). 9-13 and CNS3 cell lines derived from Huh-7 cells, which stably express HCV proteins were gifts from Dr. R Bartenschlager (University of Heidelberg, Heidelberg, Germany). Human hepatoma Huh-7 cells were maintained in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) FBS. 9-13 and CNS3 replicon cells were cultured with additional antibiotics (1 mg/mL geneticin or 10 μg/mL zeocin), respectively. 9-13 replicon cells stably express HCV nonstructural proteins (NS3-5B), and CNS3 cells stably express subgenomic proteins from core to nonstructural protein 3 (core-NS3). The Con1 subgenomic genotype 1b HCV replicon cell line was

from Apath LLC (St, Louis, MO). The Con1 cell line is a Huh-7.5 cell population containing the full-length HCV genotype 1b replicon. The Con1 cells were maintained in DMEM supplemented with 10% (v/v) FBS and 0.1 mmol/L nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, and selection antibiotic 750 μg/mL geneticin. Cells were maintained in a humidified atmosphere of 95% room air and 5% CO_2 at 37℃.

siRNA transfection

A smart pool of siRNAs targeting four positions of the human Nrf2 mRNA, was purchased from Dharmacon (Lafayette, CO). Transfections of Nrf2-siRNA were performed with Lipofectamine 2000 from Invitrogen (Carlsbad, CA) as described previously[35]. Cells were transfected for 48 h with 20-100 nmol/L Nrf2-siRNA, or an irrelevant control, and subsequently were exposed for 4 h to indicated concentrations of ferric nitrilotriacetate (FeNTA). Cells were harvested and total RNA and proteins were extracted for measurements of mRNA or protein levels by quantitative RT-PCR or Western blots.

Quantitative RT-PCR

Total RNA from treated cells was extracted and cDNA was synthesized and real time quantitative RT-PCR was performed using a MyiQ™ Single Color Real-Time PCR Detection System (BIO-RAD) and iQ™ SYBR Green Supermix Real-Time PCR kit (BIO-RAD, Hercules, CA) as described previously[39,40]. Sequence-specific primers used for HMOX1, HCV core, NS5A and GAPDH were synthesized. We included samples without template and without reverse transcriptase as negative controls, which were expected to produce negligible signals (Ct values > 35). Standard curves of HMOX1, HCV core, NS5A and GAPDH were constructed with results of parallel PCR reactions performed on serial dilutions of a standard DNA (from one of the controls). Fold-change values were calculated by comparative Ct analysis after normalizing for the quantity of GAPDH in the same samples.

Western blotting

Protein preparations and Western blots were performed as described previously[39,40]. In brief, total proteins (30-50 μg) were separated on 4%-15% gradient SDS-PAGE gels (Bio-Rad). After electrophoretic transfer onto immunoblot PVDF membrane (Bio-Rad), membranes were blocked for 1 h in PBS containing 5% nonfat dry milk and 0.1% Tween-20, and then incubated overnight with primary antibody at 4℃. The dilutions of the primary antibodies were as follows: 1:500 for anti-NS5A, 1:1000 for anti-Bach1, 1:2000 for anti-HCV core and anti-GAPDH antibodies. The membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (dilution 1:10 000). Finally, the bound antibodies were visualized with the ECL-Plus chemiluminescence system according to the manufacturer's protocol (Amersham, Piscataway, NJ). A Kodak 1DV3.6 computer-based imaging system (Eastman-Kodak, Rochester, NY) was used to measure the relative optical density of each specific band obtained after western blotting. Data are expressed as percentages of the controls (corresponding to the value obtained with the vehicle-treated cells), which were assigned values of one.

Cellular reactive oxygen species (ROS) production assay

Levels of cellular oxidative stress were measured using DCF assay. Briefly, cells were seeded into 24-well plates. The following day, the media were removed, and the cells were washed with PBS (PBS supplemented with 1 mmol/L $CaCl_2$ and 0.5 mmol/L $MgCl_2$), and then incubated with 100 μmol/L 2',7'-dichlorodihydrofluorescein (H_2DCF-DA) or 2',7'-dichlorofluorescein diacetate (DCF-DA) in DMEM without phenol red for 30 min at 37℃ in the dark. The cells were washed twice with PBS, and then treated with selected concentrations of FeNTA for 1 h. Intracellular ROS levels were measured as an increase in fluorescence of the oxidized product of DCF-DA on a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT) at the excitation and emission wavelengths of 488 and 525 nm, respectively. The oxidation-insensitive analog of H_2DCF-DA served as a control to correct for possible changes in cellular uptake, ester cleavage, and efflux. It showed no changes in fluorescence in these studies.

Statistical analysis

Experiments were repeated at least three times with similar results. Except for Western blots, all experiments included at least triplicate samples for each treatment group. Representative results from single experiments are presented. Statistical analyses were performed with JMP 6.0.3 software (SAS Institute, Cary, NC). Initial interpretation of data showed that they were normally distributed. Therefore, appropriate parametric statistical procedures were used: Student's *t*-test for comparisons of two means and analysis-of-variance (*F* statistics) for comparisons of more than two, with pair-wise comparisons by the Kruskal-Wallis test. Values of $P < 0.05$ were considered significant.

RESULTS

Iron up-regulates HMOX1 mRNA levels in Huh-7 and cell lines expressing HCV proteins

As shown in Figure 1, *HMOX1* gene expression was significantly increased in CNS3 cells, which express HCV core to NS3, even without exposure to iron or hydrogen peroxide, compared to 9-13 cell lines, which express NS3 to NS5B, or parental Huh-7 cells. Iron, in the form of FeNTA and hydrogen peroxide (another known oxidative stressor), further up-regulated the *HMOX1* gene expression in CNS3 cells. Increase of *HMOX1* gene expression by iron in Huh-7 (6.7 fold) and 9-13 cells (5.2 fold) was greater than in CNS3 cells (1.9 fold).

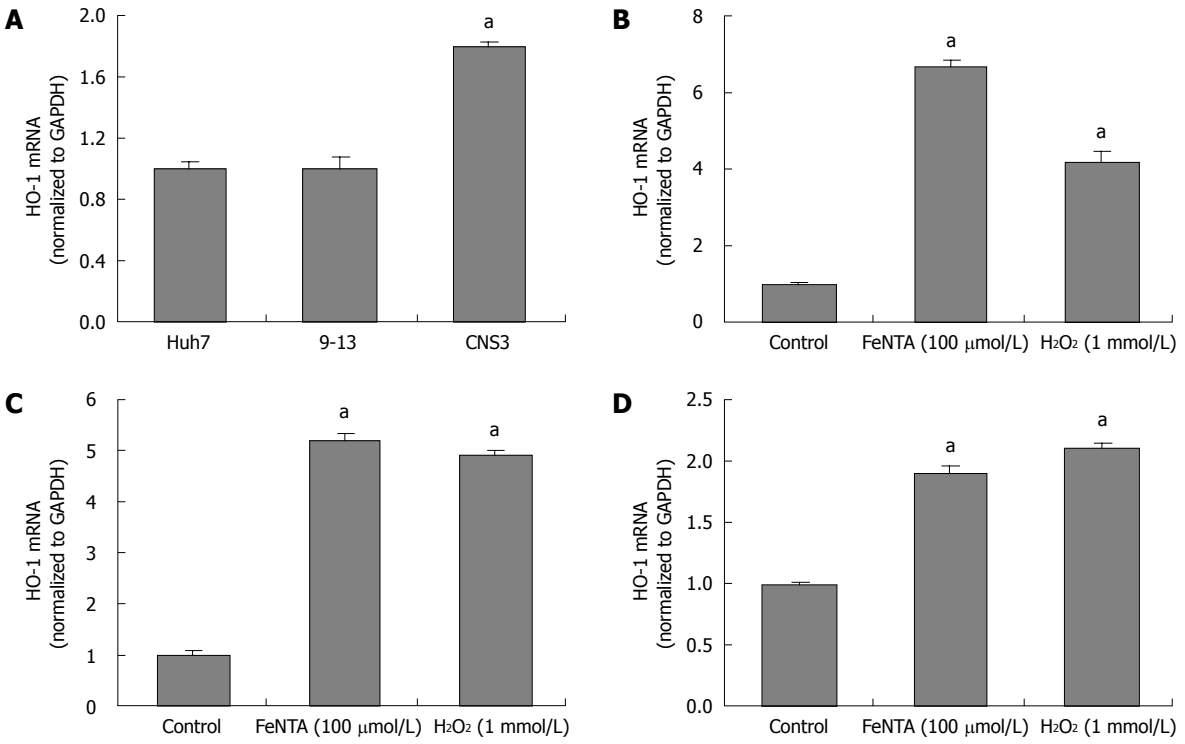

Figure 1 Iron up-regulates HMOX1 mRNA levels in Huh-7, 9-13 and CNS3 cells. A: HMOX1 mRNA levels in Huh-7, 9-13 and CNS3 cells; B: HMOX1 mRNA levels in Huh-7 cells treated with 100 μmol/L FeNTA or 1 mmol/L H_2O_2 for 6 h; C: HMOX1 mRNA levels in 9-13 cells treated with 100 μmol/L FeNTA or 1 mmol/L H_2O_2 for 6 h; D: HMOX1 mRNA levels in CNS3 cells treated with 100 μmol/L FeNTA or 1 mmol/L H_2O_2 for 6 h. Values for cells without any treatment were set equal to 1. HMOX1 mRNA data are presented as means ± SE from triplicate samples, all normalized to GAPDH in the same samples. $^aP < 0.05$ *vs* control. Huh-7, 9-13 or CNS3 cells were cultured as described in Materials and Methods, and treated with 100 μmol/L FeNTA or 1 mmol/L H_2O_2 for 6 h. Cells were harvested and total RNA was extracted. Levels of mRNA for HMOX1 and GAPDH were measured by quantitative RT-PCR.

Effects of Iron on Nrf2 and Bach1 protein levels in Huh-7 and cell lines expressing HCV proteins

Previous studies from our and other laboratories have demonstrated that Bach1 and Nrf2 act as transcriptional factors that regulate *HMOX1* gene expression in mammalian cells[34-36], and that Huh-7 cells expressing HCV proteins show significant up-regulation of the *HMOX1* gene, and reciprocal down-regulation of the *Bach1* gene[41]. To determine whether iron affected the *Nrf2* or *Bach1* gene expression, parental Huh-7 and cell lines (9-13 and CNS3) expressing HCV proteins were treated with FeNTA, and Nrf2 and Bach1 protein levels were measured by Western blots, as described in Materials and Methods. Cells exposed to 50 and 100 μmol/L FeNTA showed significant accumulation of Nrf2 protein (Figure 2A-C), whereas 50 or 100 μmol/L NaNTA did not affect Nrf2 protein levels (data not shown). In contrast, there were no detectable changes of Bach1 protein levels in either Huh-7 cells or cell lines expressing HCV proteins, suggesting that Bach1 is not involved in up-regulation of the *HMOX1* gene expression by iron (Figure 3A-C).

Nrf2-siRNA abrogates up-regulation of the HMOX1 gene expression by iron in 9-13 cells

To further establish the role of Nrf2 in up-regulation of the *HMOX1* gene expression by iron, we silenced *Nrf2* gene expression by Nrf2-siRNA as we did previously in Huh-7 cells[35]. In comparison with control, 20 nmol/L Nrf2-siRNA significantly reduced Nrf2 protein expression, and 100 nmol/L Nrf2-siRNA repressed Nrf2 protein expression by 92% (Figure 4A). We also successfully silenced the *Nrf2* gene expression in CNS3 cells (data not shown). HMOX1 mRNA levels were significantly induced by iron in cells without Nrf2-siRNA transfection, and this effect was blocked in cells transfected with 100 nmol/L Nrf2-siRNA, indicating that Nrf-2 siRNA plays a key role in up-regulation of the *HMOX1* gene expression by iron (Figure 4B).

Increased ROS, induced by iron, in the form of ferric nitrilotriacetate, in the cell lines expressing HCV proteins

Oxidative stress is one of the key stressors inducing the *HMOX1* gene expression[30,31], occurring due to iron-catalyzed formation of reactive oxygen species (ROS)[42]. We observed that the cells exposed to 50 μmol/L FeNTA exhibited significant increases in the fluorescence intensity of H_2DCF-DA (by 1.4 fold in Huh-7, 1.7 fold in 9-13 and 1.6 fold in CNS3 cells), which are similar to the increases produced by hydrogen peroxide (1 mmol/L). 100 μmol/L FeNTA further increased fluorescence intensity by 2.1 fold in 9-13 and 1.9 folds in CNS3 cells (Figure 5A-C), whereas 50 or 100 μmol/L NaNTA did not affect fluorescence intensity (data not shown). The results of the same experiment done with the oxidation-insensitive analogue of the probe (DCF-DA) in CNS3 (Figure 5D), Huh-7 and 9-13 cells (data not shown) indicated no significant

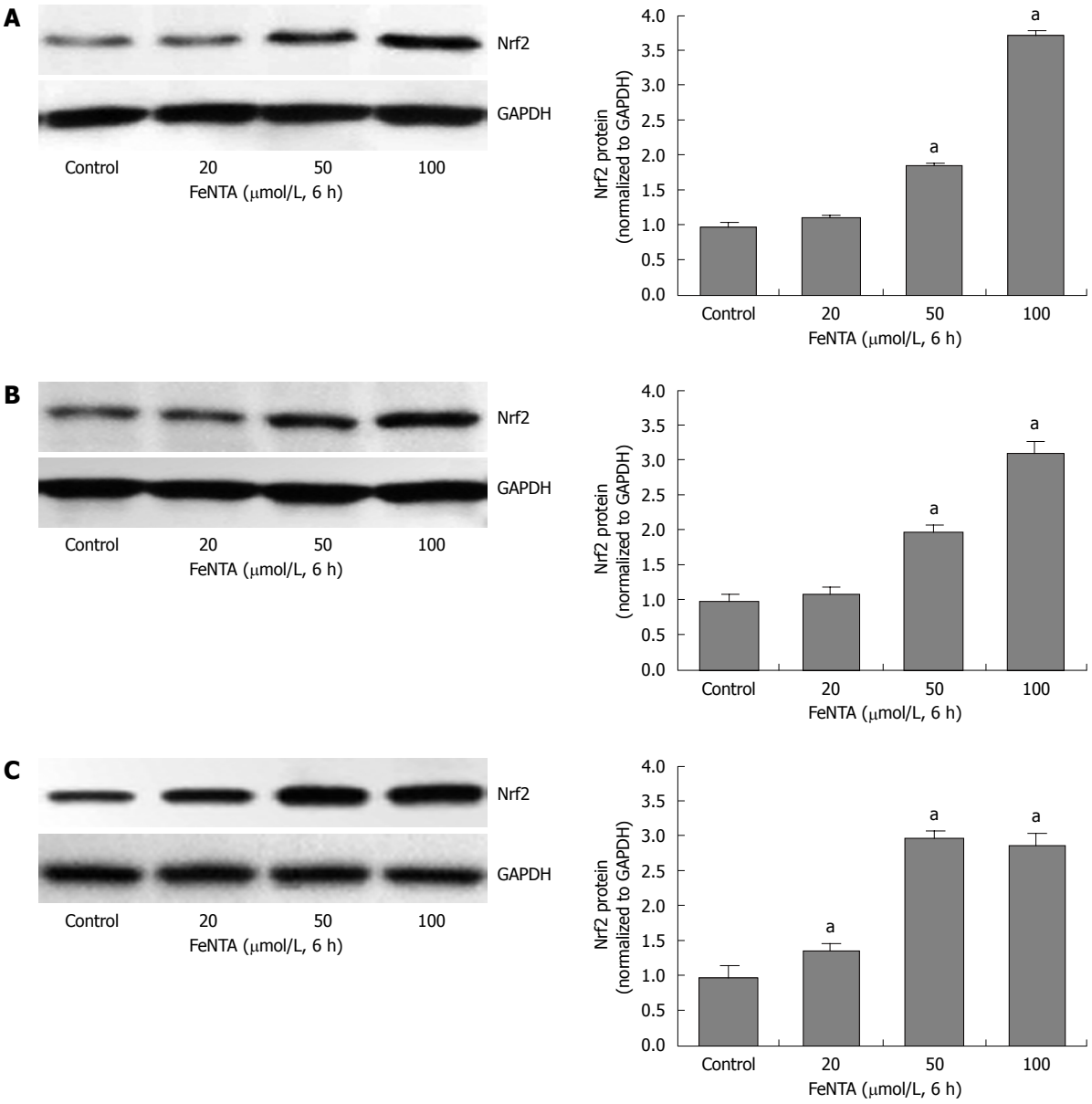

Figure 2 Effects of FeNTA on Nrf2 protein levels in Huh-7, 9-13, and CNS3 cells. A: Nrf2 protein levels in Huh-7 cells; B: Nrf2 protein levels in 9-13 cells; C: Nrf2 protein levels in CNS3 cells. [a]*P* < 0.05 *vs* control. Huh-7, 9-13 or CNS3 cells were treated with different concentrations of FeNTA (0, 20, 50, 100 μmol/L) or 100 μmol/L NaNTA for 6 h, after which cells were harvested and total protein was isolated, as described in Materials and Methods. Proteins were separated on 4%-15% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-human Nrf2 and GAPDH specific antibodies. The relative amounts of Nrf2 proteins were normalized to GAPDH.

difference between control cells and cells treated with FeNTA or H_2O_2. Therefore, the increased fluorescence intensity seen with the oxidation sensitive probe H_2DCF-DA (Figure 5A-C) can be directly ascribed to changes in the oxidation of the probe in the cells. We also changed the order of adding the H_2DCF-DA and FeNTA or H_2O_2 and observed the same pattern of results (data not shown).

The iron chelator DFO blocks increased ROS induced by iron in the cell lines expressing HCV proteins

DFO and deferasirox (Exjade) are widely used iron chelators to remove excess iron from the body. They act by binding iron at 1:1 (deferoxamine:iron) and 2:1 (deferasirox:iron) ratios and enhancing its elimination. By removing excess iron, these agents reduce the damage done by iron to various organs and tissues such as the liver. In this study, DFO was used to examine whether the effects of FeNTA were blocked by DFO chelation. In comparison with 100 μmol/L FeNTA alone, 50 μmol/L DFO (deferoxamine:iron 1:2) significantly decreased DCF fluorescence intensity in 9-13 and CNS3 cells (Figure 6A-C). Indeed, ROS induced by iron were completely blocked by DFO in all three cell lines treated with 50 μmol/L FeNTA and increasing concentrations of DFO (50, 100 and 200 μmol/L) (Figure 6A-C). To confirm we were truly measuring changes in H_2DCF-DA oxidation and not changes in its uptake, ester cleavage, or efflux, we repeated experiments shown in Figure 6A-C with the oxidation-insensitive analogue of the probe (DCF-DA). No significant differences between control

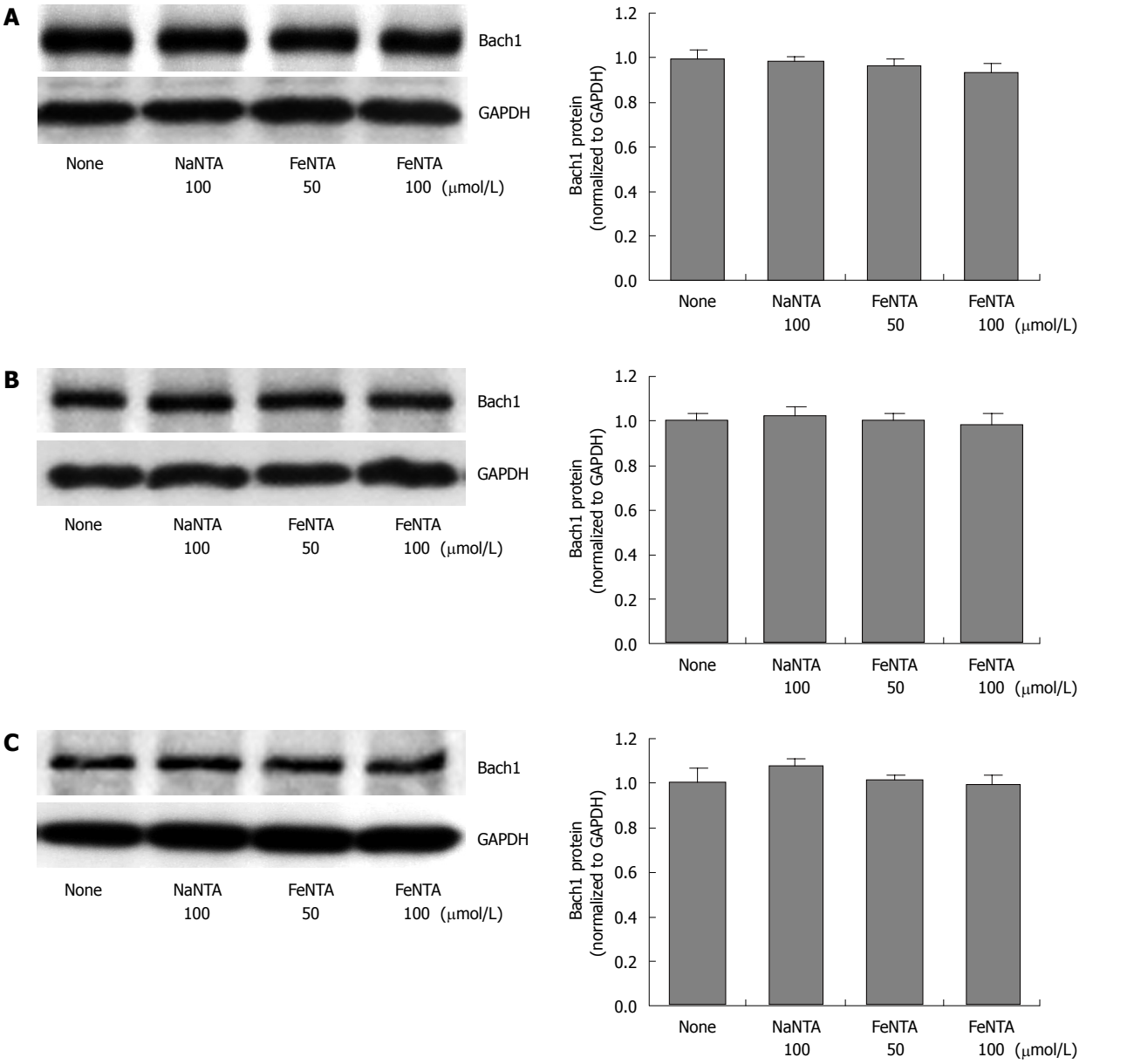

Figure 3 Effects of FeNTA on Bach1 protein levels in Huh-7, 9-13, and CNS3 cells. A: Bach1 protein levels in Huh-7 cells; B: Bach1 protein levels in 9-13 cells; C: Bach1 protein levels in CNS3 cells. Huh-7, 9-13 or CNS3 cells were treated with different concentrations of FeNTA (0, 50, 100 μmol/L) or 100 μmol/L NaNTA for 16 h, after which cells were harvested and total protein was isolated, as described in Material and Methods. Proteins were separated on 4%-15% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-human Bach1 and GAPDH specific antibodies. The relative amounts of Bach1 were normalized to GAPDH.

and treated cells were found in CNS3 (Figure 6D), Huh-7 or 9-13 cells (data not shown).

Iron decreases HCV protein expression in cell lines expressing HCV proteins

To evaluate the effect of iron in the form of FeNTA on HCV RNA and protein expression, Con1 full length HCV replicon cells were exposed to FeNTA and with or without DFO. Treatment with FeNTA resulted in a 80%-90% reduction in HCV core mRNA and protein levels (Figure 7A and B), and decreased expression of HCV NS5A mRNA by about 90% and protein by about 50% (Figure 7C and D), whereas no significant effects were produced by NaNTA, establishing that the effects are due to iron and not to the nitrilotriacetate anion (data not shown). These down-regulatory effects were abrogated by DFO (200 μm).

DISCUSSION

The major findings of this work are as follows: (1) Iron, in the form of FeNTA, up-regulates *HMOX1* gene expression in human Huh-7, and cell lines (9-13 and CNS3) stably expressing HCV proteins (Figure 1); (2) Iron significantly increases Nrf2 protein levels in human hepatoma cells, and silencing the *Nrf2* gene with Nrf2-specific siRNA abrogates the up-regulation of HMOX1 by iron (Figures 2 and 4); (3) Iron does not significantly change Bach1 protein levels in human hepatoma cells (Figure 3); (4) Iron increases ROS (Figures 5 and 6) but

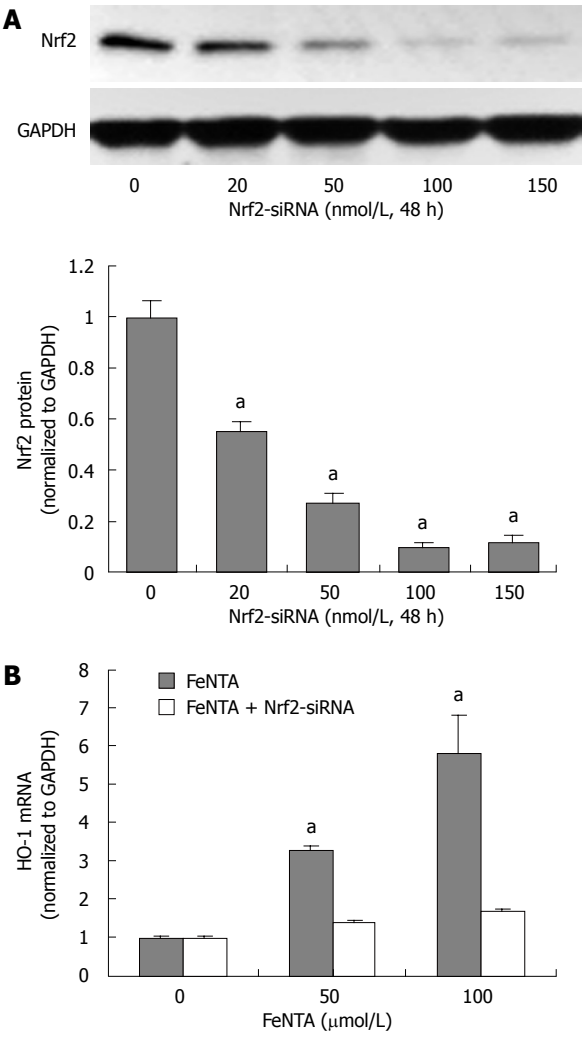

Figure 4 Silencing the Nrf2 gene abrogates up-regulation of the HMOX1 gene by iron. A: Dose-response effect of Nrf2-specific siRNA on Nrf2 protein levels; B: Effect of Nrf2-specific siRNA on FeNTA up-regulated levels of HMOX1 mRNA. $^{a}P < 0.05$ *vs* control. 9-13 cells were transfected with selected concentrations of Nrf2-siRNA (0, 20, 50, 100, 150 nmol/L). After 48 h of transfection, cells were treated with different concentrations of FeNTA (0, 50, 100 μmol/L) for 6 h, after which cells were harvested and total RNA was isolated. The HMOX1 mRNA levels were measured by quantitative RT-PCR as described in Materials and Methods.

decreases HCV gene expression (Figure 7) in human hepatoma cells; and (5) These effects are blocked by the selective iron chelator DFO (Figures 5-7). However, none of the effects is produced by Na_3NTA, establishing that they are due to iron and not to the NTA anion. These results show clearly that iron is capable of acting directly on hepatoma cells and on HCV gene expression in hepatoma cells, without the need for mediation of effects by other tissues, organs, or cell types. Thus, it appears that iron exerts manifold effects on HCV-infected hepatocytes. On the one hand, it increases ROS and oxidative stress, acting in concert with HCV proteins, especially the core protein. On the other hand, it induces HMOX1 by increasing expression and activity of Nrf2 (Figures 1, 2 and 4), and it decreases levels of CNS3 or NS5A mRNA and protein expression (Figure 7). These latter effects are likely mediated by the recently described iron-dependent inactivation of the HCV RNA polymerase NS5B[43].

HMOX1 is a heat shock protein (also known as HSP 32), which can be induced to high levels, not only by heat shock, but also by a large number of physiological or pathological stressors[30-33]. Nrf2 is a basic leucine zipper transcriptional activator[44,45]. It protects cells against oxidative stress through antioxidant response element (ARE)-directed induction of several phase 2 detoxifying and antioxidant enzymes, including HMOX1[35,46]. $Nrf2^{-/-}$ mice displayed a dramatically increased mortality associated with liver failure when fed doses of ethanol that were tolerated by wild type mice, establishing a central role of Nrf2 in the natural defense against ethanol-induced liver injury[47]. Cobalt protoporphyrin (CoPP)-mediated induction of HMOX1 involves increased Nrf2 protein stability in human hepatoma Huh-7 cells[35]. In this study, silencing Nrf2 by Nrf2-siRNA markedly abrogated FeNTA-mediated up-regulation of HMOX1 mRNA levels. Therefore Nrf-2 plays a central role in up-regulation of *HMOX1* gene expression by FeNTA (Figure 4B).

Bach1, a member of the basic leucine zipper family of proteins, has been recently shown to be a transcriptional repressor of HMOX1, and to play a critical role in heme-, CoPP-, SnMP- and ZnMP-dependent up-regulation of the *HMOX1* gene[35,36,48-53]. Upon exposure to heme, heme binds to Bach1 and forms antagonizing heterodimers with proteins in the Maf-related oncogene family. These heterodimers bind to MAREs, also known as AREs, and suppress expression of genes that respond to Maf-containing heterodimers and other positive transcriptional factors. Surprisingly, ZnMP does not bind to Bach1, but it still produces profound post-transcriptional down-regulation of Bach1 protein levels by increasing proteasomal degradation and transcriptional up-regulation of HMOX1[53]. In contrast, iron does not affect levels of Bach1 mRNA (data not shown) or protein (Figure 3), suggesting that Bach1 is not involved in up-regulation of the *HMOX1* gene by iron.

Expression of HMOX1 was recently reported to be decreased in human livers from patients with chronic hepatitis C[54,55] including some with only mild fibrosis. The reasons for this are not known currently. It is known that levels of expression of the *HMOX1* gene depend in part upon genetic factors (lengths of GT repeats in the promoter[56-59] and a functional polymorphism (A/T) at position -413 of the promoter[60,61]. Higher expression and/or induction of HMOX1 are probably beneficial to mitigate liver cell injury in HCV infection, as well as in other liver diseases. This may be a therapeutic goal, achieved by treatment with heme or CoPP or with silymarin[62] or other herbal products or compounds that combine anti-oxidant, iron-chelating and HMOX1-inducing effects.

Recently, we showed that HCV expression in CNS3 cells increases the levels of HMOX1 mRNA and protein[41]. This induction is likely in response to oxidative stress. More recently, we showed that micro RNA-122, which is expressed at a high level in hepatocytes, causes down-regulation of Bach1, which, as already described,

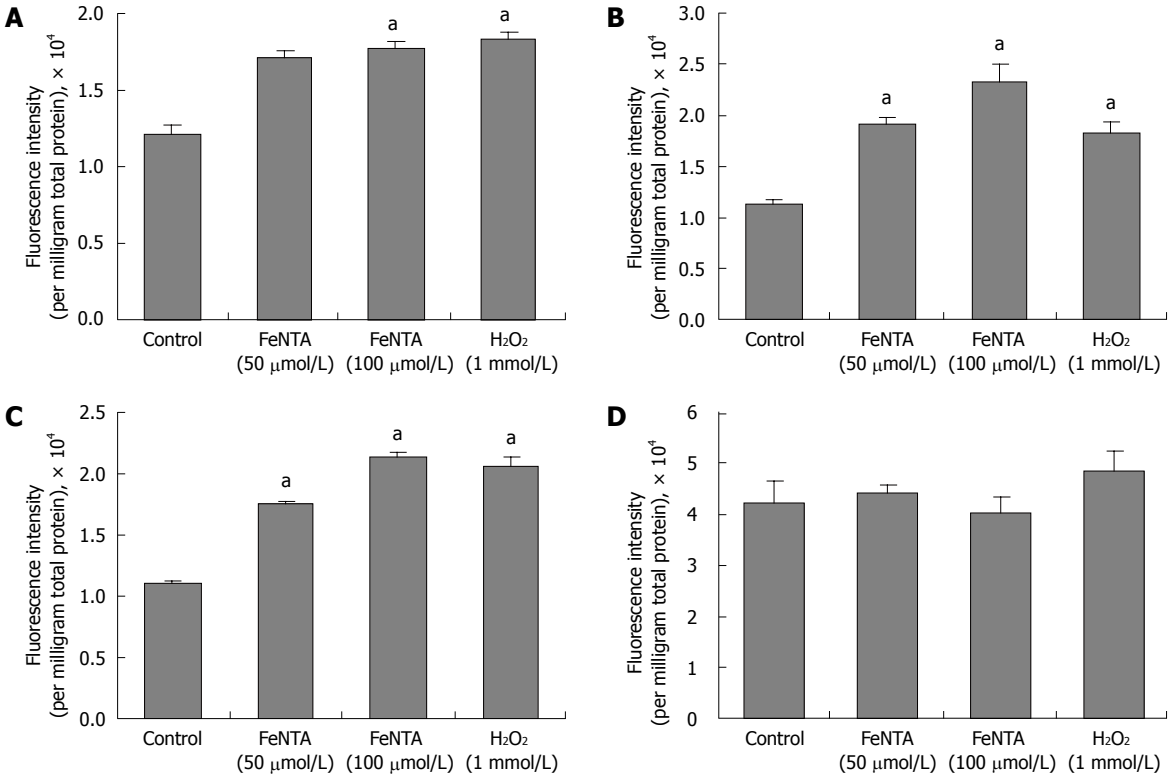

Figure 5 Effects of FeNTA on intracellular ROS in Huh-7, 9-13, and CNS3 cells. A: Fluorescence intensity with the H_2DCF-DA probe in Huh-7 cells; B: Fluorescence intensity with the H_2DCF-DA probe in 9-13 cells; C: Fluorescence intensity with the H_2DCF-DA probe in CNS3 cells; D: Fluorescence intensity with the (control) DCF-DA in CNS3 cells. Cells were preincubated with 100 μmol/L H_2DCF-DA or DCF-DA for 30 min, and then exposed to selected concentrations of FeNTA (0, 50, 100 μmol/L) for 1 h. Intracellular ROS production was measured as described in Materials and Methods. Data represent fluorescence intensity measured and expressed as relative fluorescence units per milligram total protein (mean ± SE, n = 3 experiments). $^aP < 0.05$ *vs* control.

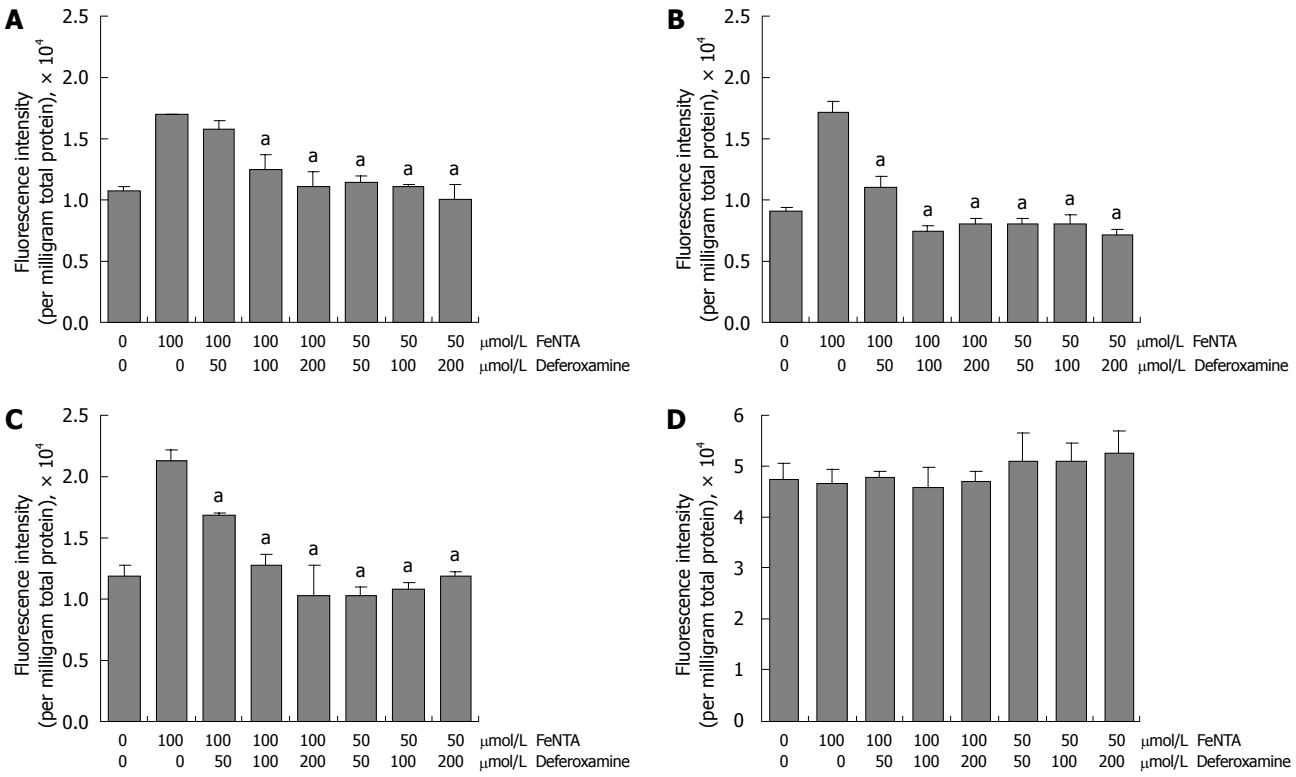

Figure 6 Effects of deferoxamine on intracellular ROS induced by FeNTA in Huh-7, 9-13, and CNS3 cells. A: Fluorescence intensity with the H_2DCF-DA probe in Huh-7 cells; B: Fluorescence intensity with the H_2DCF-DA probe in 9-13 cells; C: Fluorescence intensity with the H_2DCF-DA probe in CNS3 cells; D: Fluorescence intensity with the (control) DCF-DA in CNS3 cells. Cells were loaded with 100 μmol/L H_2DCF-DA for 30 min, treated with different concentrations of deferoxamine (50, 100, 200 μmol/L) for 30 min, and then exposed to different concentrations of FeNTA (50, 100 μmol/L) for 1 h. Intracellular ROS production was measured as described in Materials and Methods.

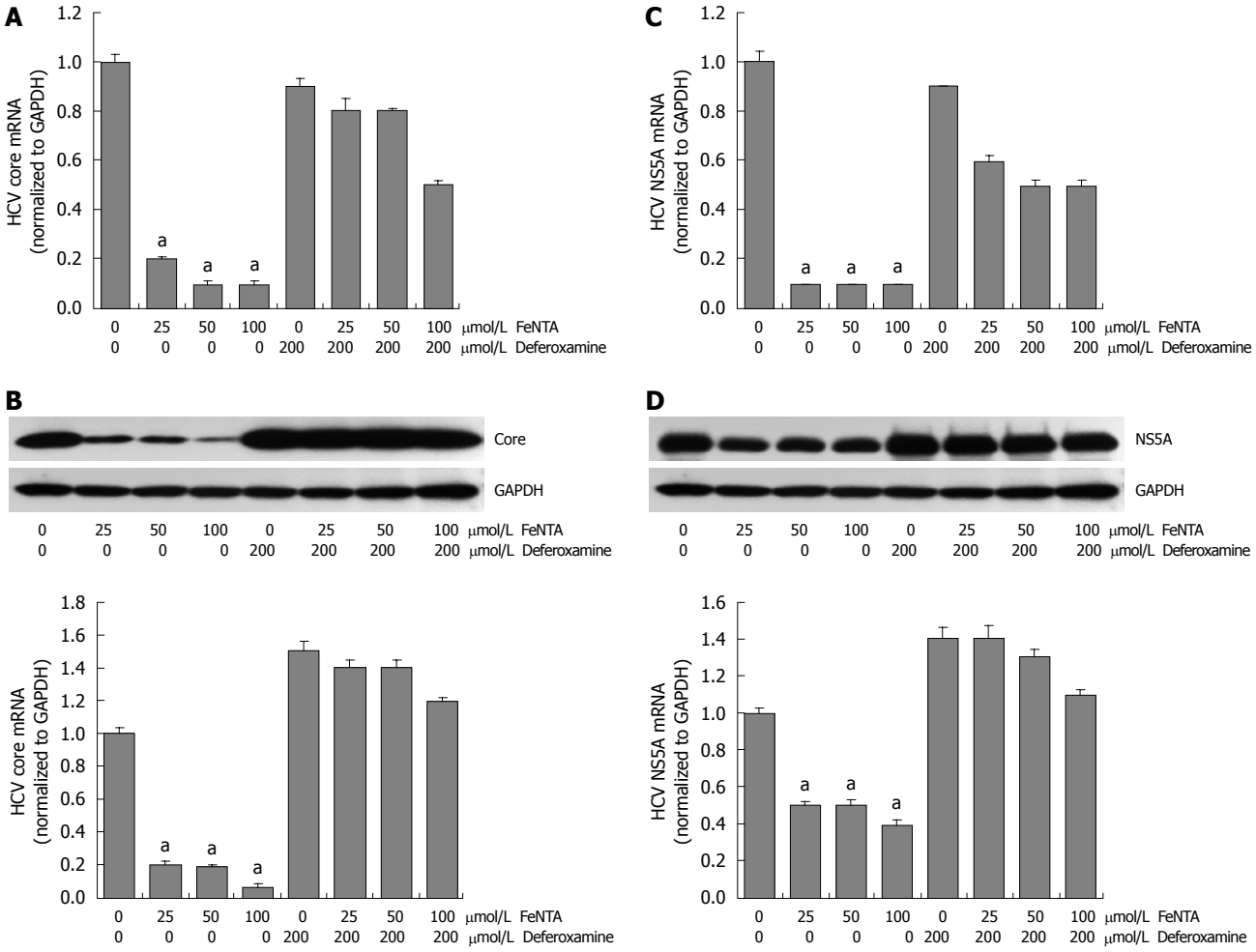

Figure 7 Effects of FeNTA on HCV core and NS5A mRNA and protein levels. A: Core mRNA levels in Con1 cells treated with FeNTA and with or without deferoxamine; B: Core protein levels in Con1 cells treated with FeNTA and with or without deferoxamine; C: NS5A mRNA levels in Con1 cells treated with FeNTA and with or without deferoxamine; D: NS5A protein levels in Con1 cells treated with FeNTA and with or without deferoxamine. Data are presented as mean ± SE from triplicate samples, all normalized to GAPDH in the same samples. $^{a}P < 0.05$ *vs* control. The Con1 full length HCV replicon cells were treated with indicated concentrations of FeNTA and with or without deferoxamine. After 24 h, cells were harvested and total RNA and proteins were extracted. Levels of mRNA were measured by quantitative RT-PCR, and protein levels were determined by Western blots as described in Materials and Methods. Values for cells without any treatment were set equal to 1.

tonically down-regulates the *HMOX1* gene[40]. In addition, we and others have shown that expression of micro RNA-122 is required for HCV replication in human hepatoma cells[40,63,64]. Whether iron affects levels of micro RNA-122 has not yet been assessed, to our knowledge.

Others recently reported that iron binds to NS5B, the RNA dependent RNA polymerase of HCV, and inhibits its enzymatic activity[43]. The HCV replicon system used in that study showed changes in the gene expression of certain genes involved in iron metabolism, including down-regulation of ceruloplasmin and transferrin receptor 1 but up-regulation of ferroportin thus producing an iron-deficient phenotype[65]. The authors speculated that the HCV genes and proteins somehow produced these changes in order to diminish the effects of iron to inhibit NS5B RNA polymerase activity and to decrease HCV protein expression.

Regardless of these results in cell culture models, the preponderance of clinical evidence[10-15,24-29] supports the view that iron acts as a co-morbid or synergistic factor in chronic hepatitis C infection. Because both iron and HCV infection increase oxidative stress within hepatocytes, one attractive mechanistic explanation for the additive or synergistic affects of these two perturbations is that they act, at least in part, by increasing oxidative stress in the form of highly reactive oxygen species. These considerations provide additional rationale for the notion that reduction of iron and anti-oxidant therapy[62] may be of benefit in the management of difficult to cure chronic hepatitis C[10-15,24-29,66-68]. Iron reduction has usually been achieved with therapeutic phlebotomies. However, deferasirox (Exjade) recently has been approved in the USA and other countries as oral chelation therapy for iron overload states. Thus, studies of deferasirox for therapy of chronic hepatitis C are timely and important[69], especially because the therapy of chronic hepatitis C currently is fraught with side effects, difficulties of adherence and rates of response that are not better than about 50%[70-72].

In conclusion, iron can cause or exacerbate liver damage, including viral hepatitis. In the work reported in this paper we assessed effects of iron and iron chelators on liver cells, some of which also expressed genes and proteins of the HCV. Iron increased oxidative stress

and led to up-regulation of the *HMOX1* gene, a key cytoprotective gene. A mechanism for this action was to increase expression of the positive transcription factor Nrf-2. In contrast, iron did not affect expression of Bach1. Iron decreased expression of HCV genes and proteins. All the effects of iron were abrogated by DFO. The induction of HMOX1 helps to protect liver cells from the damaging effects of the HCV.

ACKNOWLEDGMENTS

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COMMENTS

Background

Iron overload is known to be toxic to many organs. The most common form of iron overload is hereditary hemochromatosis. In this disease, iron overload results in damage to many organs including the heart, pancreas and liver. In fact, the main site of iron deposition is in the liver. Recently it has been learned that iron plays a role in non-hemochromatotic liver disease. By insight into the mechanisms of how iron leads to this damage, novel ways to improve outcomes and success in treating these liver diseases may be achieved. One such liver disease is chronic hepatitis C.

Research frontiers

Currently chronic hepatitis C affects more than 170 million people worldwide. Standard therapy consists of a combination of pegylated alpha interferon and ribavirin. This is a difficult treatment regimen consisting of almost 1 year of therapy in many cases. Unfortunately, there is only a 50% success rate for treatment overall. There is much ongoing research seeking to improve this success rate. Until recently there were no tissue culture models for investigating hepatitis C, but cell lines have been developed which support hepatitis C viral (HCV) replication. These models allow for a unique and new way to investigate HCV replication and pathogenicity.

Innovations and breakthroughs

This article examines the role of iron in inducing heme-oxygenase 1 (HMOX1) in a tissue culture model of hepatitis C. HMOX1 is a heat shock protein that is induced by physiologic and pathologic stressors. Oxidative stress is one such stressor. The authors have shown that HMOX-1 is up regulated in cell lines that express HCV proteins. The addition of iron in the form of ferric nitrilotriacetate (FeNTA) to these cell lines further upregulates *HMOX-1* gene expression. This up regulation is independent of Bach1, a protein which functions to suppress HMOX-1. The addition of iron increased oxidative stress in these cell lines as measured by a fluorescence assay and they feel it is this oxidative stress that results in further up regulation of *HMOX1* gene expression. Conversely the expression of HCV proteins was down-regulated when HMOX1 was induced. The induction of HMOX1 likely helps to protect liver cells from the damaging effects of the HCV. The iron chelators deferoxamine (Desferal) and deferasirox (Exjade) blocked the effects of FeNTA in generating reactive oxidative stress as measured by fluorescence.

Applications

Clinical evidence supports the view that iron acts as a co-morbid factor in chronic hepatitis C infection. This may be a result of the increased oxidative stress caused by both iron and HCV infection. Therefore the use of anti-oxidant therapy and iron chelators could be of benefit in the treatment of chronic HCV infection. Recently, deferasirox (Exjade) has been approved in the USA and other countries to treat iron overload states. Studies using deferasirox as an adjunct to the treatment of hepatitis C may be an aid to advance the therapy for chronic hepatitis C.

Peer review

The manuscript is a very well written and well-designed study. In this study authors have shown the critical role of iron on HCV expression and potential use of anti-chelating agents to treat the HCV patients. The study is novel.

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ORIGINAL ARTICLES

EGFR and HER2 expression in advanced biliary tract cancer

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Abstract

AIM: To analyze the pathogenetic role and potential clinical usefulness of the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) in patients with advanced biliary tract cancer (BTC).

METHODS: EGFR and HER2 expression was studied in biopsy samples from 124 patients (51% women; median age 64.8 years), with advanced BTC diagnosed between 1997 and 2004. Five micrometers sections of paraffin embedded tissue were examined by standard, FDA approved immunohistochemistry. Tumors with scores of 2+ or 3+ for *HER2* expression on immunochemistry were additionally tested for HER2 gene amplification by fluorescence *in situ* hybridisation (FISH).

RESULTS: 34/124 patients (27.4%) had gallbladder cancer, 47 (37.9%) had intrahepatic BTC and 43 (34.7%) had extrahepatic or perihilar BTC. EGFR expression was examined in a subset of 56 samples. EGFR expression was absent in 22/56 tumors (39.3%). Of the remaining samples expression was scored as 1+ in 12 (21.5%), 2+ in 13 (23.2%) and 3+ in 9 (16%), respectively. HER2 expression was as follows: score 0 73/124 (58.8%), score 1+ 27/124 (21.8%), score 2+ 21/124 (17%) and score 3+ 4/124 (3.2%). *HER2* gene amplification was present in 6/124, resulting in an overall amplification rate of 5%.

CONCLUSION: Our data suggest that routine testing and therapeutic targeting of HER2 does not seem to be useful in patients with BTC, while targeting EGFR may be promising.

Key words: Cholangiocarcinoma; Gallbladder cancer; Chemotherapy; Targeted therapy

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INTRODUCTION

Biliary tract cancer (BTC) is a heterogeneous tumor entity consisting of an intrahepatic mass forming type cholangiocarcinoma, perihilar Klatskin tumors, extrahepatic BTC, also termed intraductal growth type, and gallbladder cancer. More than 50% of tumors are diagnosed at an advanced stage with these patients having a dismal prognosis with a mean overall survival of 7-8 mo[1,2]. Chemotherapy is widely used but of only little benefit. Therefore, new treatment options are urgently needed. Growth factor inhibitors or antibodies and small molecules such as erlotinib, gefitinib, cetuximab, panitumu-

mab, trastuzumab and lapatinib targeting the epidermal growth factor receptor (EGFR) or the human epidermal growth factor receptor 2 (HER2) have been successfully used for the treatment of colorectal, breast, lung and head and neck cancers among others[3-7]. While there are some case reports and a phase Ⅱ trial reporting promising results targeting EGFR in BTC there are no data regarding the use of trastuzumab or lapatinib also targeting HER2[8-10]. For pancreatic carcinoma, a malignancy somewhat related to BTC, trastuzumab has been successfully used in one study and at least one further multicenter study is currently under way[11,12].

EGFR and HER2 are receptor tyrosine kinases encoded by proto-oncogenes. Growth factors such as epidermal growth factor (EGF) or transforming growth factor (TGF) bind to these receptors at their extracellular ligand-binding domain and initiate intracellular signalling cascades, leading to tumor cell proliferation, migration, invasion, resistance to apoptosis and angiogenesis[3,4]. In an experimental tumor model a high proportion of ErbB-2 (HER2) transgenic mice develop BTC, suggesting a role of ErbB-2 signalling in biliary carcinogenesis[13].

Overall, overexpression of EGFR and HER2 in tumor cells has been associated with a poor prognosis, but also offers the therapeutic option of pharmacologically targeting these receptors. To date, EGFR and HER2 overexpression has been reported in up to about 80% of BTC, mostly in small patient cohorts[14-19]. Nevertheless, there are no data regarding the correlation of immunohistochemical scores, determined by standardized methods, with clinical findings, including the overall survival of patients with advanced BTC treated by chemotherapy. The aim of this study, therefore, was to asses the clinical significance of the expression of EGFR and HER2 proteins and their potential as therapeutic targets in advanced BTC.

MATERIALS AND METHODS

Patients

Expression of EGFR and HER2 was analyzed in biopsy samples from 124 patients with advanced or relapsed, unresectable BTC (51% women, median age 64.8 years). The patients had been consecutively diagnosed at the Tumorzentrum Ludwig Heilmeyer-Comprehensive Cancer Center Freiburg between 1997 and 2004, and were followed for a median of 71 mo. All BTC cases were histologically proven adenocarcinoma. Written informed consent was obtained from all patients. Primary gastrointestinal cancers other than BTC were excluded by upper endoscopy, colonoscopy and a multislice computed tomography (CT) scan according to the consensus guidelines published in 2002[20]. Sixty-one patients (49.2%) had been treated by chemotherapy and could be restaged for response. The chemotherapy regimens were based on 5-fluorouracil or gemcitabine, often in combination with cisplatin or oxaliplatin. The treatment response was measured by CT, magnetic resonance imaging or ultrasound according to the standard World Health Organization (WHO) criteria (WHO, 1979). The patients subgroup analyzed for EGFR was randomly selected. The different tumor types were similar between the subgroup and the whole study population. The baseline characteristics of the study population are given in Table 1.

Table 1 Characteristics of patients with advanced biliary tract cancer

Patients (n = 124)	n (%)
Age (yr)	
Range	32.8-84.8
Median	64.8
mean ± SD	63.4 ± 11.0
Male:female	61:63 (49:51)
Gallbladder cancer	33 (26.6)
Mass forming type	47 (37.9)
Intraductal growth type	44 (35.5)
Histological type	
Well differentiated	8 (6.5)
Moderately differentiated	80 (64.5)
Poorly differentiated	36 (29)
Stage[1]	
Ⅰ	9 (7.3)
Ⅱ	20 (16.1)
Ⅲ	31 (25)
Ⅳ	64 (51.6)
Chemotherapy	62 (50)
Partial response	9 (14.5)
Stable disease	27 (43.5)
Progressive disease	26 (42)

[1]According to AJCC/UICC classification 2002.

Immunohistochemistry

Biopsy samples were fixed in 10% formalin, embedded in paraffin, cut in 5 μm sections and stained with hematoxylin/eosin for histological typing and grading. Immunohistochemistry (IHC) was performed on adjacent freshly cut deparaffinized sections using the peroxidase-labelled streptavidin-biotin technique, EGFR pharmDx™ for EGFR and Dako REAL™ detection system for HER2 staining (both Dako Glostrup, Denmark). Immunostaining was performed according to the package insert of the two FDA-approved detection systems, using only the supplied reagents and procedures.

IHC results were scored independently by two pathologists (Waiz O and Schmitt-Graeff A) blind to all clinical data. As recommended by the manufacturer additional tissue controls were performed along with the cell line controls.

The EGFR pharmDx™ kit is based on the dextran technology. First sections were treated with proteinase K at 37℃ for 5 min (epitope retrieval) prior to incubation with the EGFR antibody for 1 h at room temperature. Following incubation with the primary antibody, the visualization reagent consists of both labeled polymer and HRP (Horseradish peroxidase). Subsequently added chromogen DAB+ results in a visible reaction product (brown) at the antigen site. Staining was performed using the Dako Autostainer® (Dako Glostrup, Denmark) au-

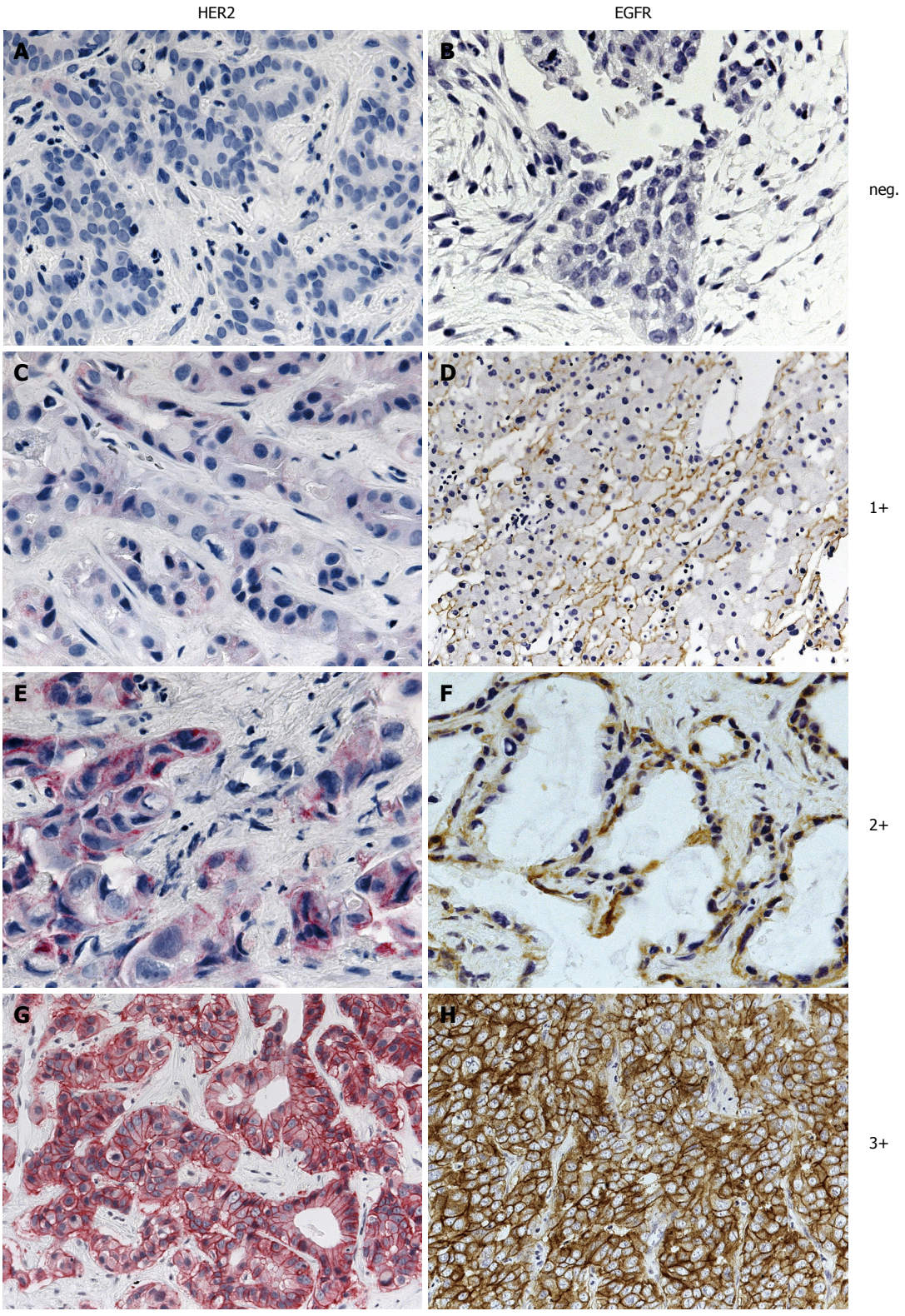

Figure 1 Immunohistochemistry of biliary tract cancer specimens for expression of HER2 (A, C, E and G) and EGFR (B, D, F and H). A: HER2 neg.; B: EGFR neg.; C: HER2 1+; D: EGFR 1+; E: HER2 2+; F: EGFR 2+; G: HER2 3+; H: EGFR 3+.

tomated system. As recommended for the interpretation of EGFR pharmDx™, both the intensity and percentage of tumor cells exhibiting membranous and/or cytoplasmic staining were assessed. If specific membrane staining was observed in less than 1% of tumor cells, the specimen was reported to be EGFR negative. The four categories negative, weak (1+), moderate (2+) and intense (3+) were used as recommended by the manufacturer and approved by the FDA (Figure 1).

The detection of HER2 was performed with heat induced epitope retrieval (HIER) in 10 mmol/L citrate buffer (Target Retrieval Solution, pH 6.1, Dako) in a

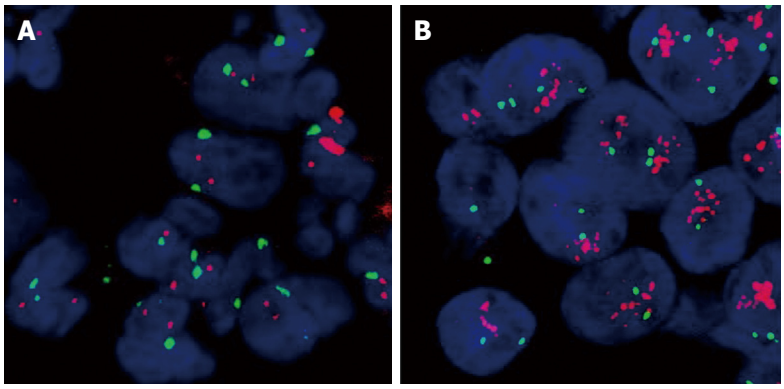

Figure 2 Dual color FISH analysis of HER2 in cholangiocarcinoma specimens. A: Nonamplified tumor with single *HER2* gene copy status. The red probe is specific for *HER2*. The green probe is specific for chromosome 17 centromere; B: Tumor with high amplification of the *HER2* gene. There are multiple red *HER2* specific signals in this tumor in relation to the green chromosome 17 signals.

water bath at 98℃ for 45 min and anti-HER2 primary antibody (Polyclonal Rabbit Anti Human c-erbB-2 oncoprotein, Dako Glostrup, Denmark). Following incubation with the primary antibody, the visualization is based on the sequential application of biotinylated link antibody and streptavidin labeled with alkaline phosphatase antibody. Subsequently added chromogen Fast RED results in a visible reaction product (red) at the antigen site. HER staining was also performed using the Dako Autostainer® (Dako Glostrup, Denmark). Tumors were counted negative if less than 10% of carcinoma cells were stained, irrespective of membrane signal intensity. If 10% or more of carcinoma cells showed membrane staining, the staining intensity was classified as weak (1+), moderate (2+) or intense (3+) as recommended by the manufacturer and approved by the FDA (Figure 1). Cytoplasmic staining was discounted. Images provided by DAKO were used to define these staining categories.

Fluorescence in situ hybridization (FISH)

The PathVysion™ detection kit (Abbott, Illinios, USA) was used for FISH analysis in cases of IHC expression scores 2+ or 3+ for HER2. FISH staining was carried out according to the manufacturer's manual. The presence of gene amplification was determined using the supplied fluorescence-labelled DNA probe for chromosomal locus 17q11.2-q12 for the detection of *HER2*. FISH-stained sections were scanned at × 1000 magnification and in each of three separate carcinoma areas. Twenty nuclei were analyzed. *HER2* and chromosome 17 copy number were counted for all cells and the ratio of *HER2* to chromosome 17 was calculated. A normal *HER2*:17 ratio was defined as ≤ 2; a ratio > 2 was interpreted as gene amplification. A normal *HER2* copy number was attested at < four signals per cell (Figure 2).

Statistical analysis

Data are presented as mean ± SD or medians with range for continuous variables and as absolute and relative frequencies for categorical variables. Overall survival estimates and median survival times were calculated by the Kaplan-Meier method followed by Cox models estimating relative risks. All statistical tests were two-sided using a 5% significance level. SASS software was used for the calculations.

RESULTS

Expression of EGFR and HER2 protein

From the 124 patients examined 34 (27.4%) had gallbladder cancer, 47 (37.9%) had intrahepatic mass forming type BTC (IHCC) and 43 (34.7%) had extrahepatic or perihilar, intraductal growth type BTC (EHCC). Because EGFR expression was expected to be present two fold more frequently than HER2 expression in BTC only a subset of 56 samples was examined. EGFR expression was negative in 39.3% (22/56) of the patients. Weak (1+) positive EGFR staining was found in 21.5% (12/56), moderate (2+) in 23.2% (13/56) and intense (3+) in 16% (9/56) of the tumors. Overall 39.2% of the samples showed EGFR overexpression (scores 2+ and 3+), being significantly ($P = 0.028$) more frequent in EHCC (57.9%) than in IHCC (25%). HER2 expression was as follows: 72/124 (58%) were negative, 26 (21%) 1+, 22 (18%) 2+ and 4 (3%) 3+. Representative examples of EGFR and HER2 immunohistochemical staining and *in situ* hybridization are shown in Figures 1 and 2.

A close correlation between treatment response and gene amplification has been shown for HER2 in previous studies[4]. However, unlike the case of trastuzumab and HER2 in breast cancer, *EGFR* gene amplification detected by FISH has not been approved as being as useful for deciding on an EGFR targeted therapy yet. Therefore, we did not study *EGFR* gene amplification in our patient cohort. Regarding HER2, based on published data and the manufacturer's recommendation, tumors with no or 1+ HER2 immunostaining were not further investigated for gene amplification. Of the 124 patients samples tested 25 were examined for *HER2* gene amplification. HER2 FISH was performed in 2+ and 3+ samples and was successfully performed in all but one tumor examined. All specimens exhibiting 3+ immunostaining (4/4) showed gene *HER2* amplification while amplification was present in 2/21 (10%) of 2+ samples. Taken together, *HER2* gene amplification could be detected in 6/124 (5%) tumors.

Correlation of EGFR and HER2 expression with clinicopathological factors

Among the 124 patients 80 (64.5%) had moderately differentiated tumors, 36 (29%) had poorly differentiated and

Table 2 EGFR and HER2 expression and clinicopathological factors (Cox's model)

Parameter	EGFR 2+ and 3+ tumors	*P*	HER2 2+ and 3+ tumors	*P*
Gallbladder cancer	5/13		8/34	
Mass forming type	6/24	0.088	7/47	0.517
Intraductal growth type	11/19		10/43	
Histological type				
Well differentiated	2/3		1/8	
Moderately differentiated	16/39	0.511	20/80	0.202
Poorly differentiated	4/14		4/36	
Stage[1]				
Ⅰ	3/4		4/9	
Ⅱ	2/6	0.317	4/20	0.059
Ⅲ	8/17		9/31	
Ⅳ	9/29		8/64	
Chemotherapy				
Partial response	2/3		4/9	
Stable disease	3/13	0.296	4/27	0.156
Progressive disease	5/13		5/26	

[1]According to AJCC/UICC classification 2002.

8 (6.5%) had well differentiated tumors. The majority of patients (64/124, 51.6%) had stage Ⅳ disease, 31 (25%) had stage Ⅲ, 20 (16.1%) stage Ⅱ and 9 stage Ⅰ (7.3%). The patients had not undergone surgery because of unresectability, comorbidity or patients' wish. Half of the patients (62/124) had been treated with chemotherapy, resulting in tumor control in 59% (14.7% PR, 44.3% SD). Median overall survival was 13 mo with a median OS of 14 mo for patients treated with chemotherapy compared to 9 mo for patients not treated with chemotherapy. There was no statistical association between protein expression and grade, stage, overall survival and treatment response for EGFR and HER2, respectively. The frequencies of EGFR and HER2 overexpression and clinicopathological variables are summarized in Table 2. In univariate analysis EGFR and HER2 expression could not be shown to be of prognostic relevance for overall survival ($P = 0.06$ and $P = 0.49$).

DISCUSSION

Expression of the two ErbB family growth factor receptors EGFR and HER2 has been intensively studied in different tumor entities and led to the use of targeted therapy with specific inhibitors or antibodies of these receptors in colorectal, breast, lung as well as head and neck cancer[4]. To date in other cancers monoclonal antibodies and small molecule tyrosine kinase inhibitors such as cetuximab, trastuzumab, erlotinib, gefitinib and lapatinib are under investigation. Expression of EGFR and HER2 as potential therapeutic targets has been reported in various tumors[4,7,21,22]. For BTC, data for EGFR and HER2 overexpression have been presented in mostly small patient cohorts[14,17,19,23]. Recently Yoshikawa *et al*[24] described an unselected large cohort of 236 cases of resected BTC. In this study, we investigated EGFR and HER2 expression in a large cohort of patients with advanced, unresectable BTC.

In BTC the percentage of EGFR overexpressing tumors in previously reported series ranged from 8.1% to 81%. Yoshikawa *et al*[24] showed EGFR overexpression in 26.4% of EHCC and 17.7% of IHCC. Similarly, in our study EGFR overexpression was more frequent in EHCC (57.9%) than in IHCC (25%, $P = 0.028$). In these reports by Ito *et al*[23] and Yoshikawa *et al*[24] EGFR overexpression was associated with biological aggressiveness of BTC. In addition, there is increasing evidence that EHCC and IHCC respond differently to chemotherapy suggesting different biological properties of these two tumor subtypes of "cholangiocarcinoma"[25,26].

HER2 overexpression and amplification has been found in a range between 5% and 76% in BTC[14-19,24,27]. This wide range may in part reflect the lack of standardized methodologies used within the different studies to assess HER2 status. In our cohort with advanced, unresectable BTC, HER2 overexpression was present in 20% and treatment relevant *HER* amplification in 5%. Some authors suggest that HER2 overexpression is due to gene deregulation rather than gene amplification because in some reports there is no strict correlation between protein expression and gene amplification[19]. Overall, the highest concordance between HER2 expression and gene amplification was demonstrated for breast cancer[7,28,29]. Our data suggest that in advanced BTC with high HER2 expression there is also a good correlation between overexpression and amplification, since all samples with a 3+ HER2 score showed gene amplification as compared to only 2/21 (10%) with a 2+ HER2 score. Similar correlations have been reported for other malignancies, including breast cancer with a tendency towards lower therapeutic relevant expression rates with improved, standardized detection methods. It has to be assumed that earlier reports overestimated HER2 expression in BTC, explaining the discrepancy between HER2 expression and gene amplification[28,30,31]. Despite the fact that HER2 transgenic mice frequently develop BTC, HER2 does not seem to play a major role in human biliary tract malignancies. This animal tumor model may therefore be of limited value as a model for human BTC.

In recent reports in early stage, resected BTC overall HER2 amplification was about 5%-10%[24,32]. In our cohort of patients with advanced BTC, HER2 amplification was merely 5%. Since targeted therapy with the anti-HER2 antibody trastuzumab in breast cancer is only effective when the HER2/neu receptor is overexpressed *via* gene amplification HER2 seems not to be a promising target in BTC. Therapeutic trials targeting HER2 should therefore not further be initiated.

Since EGFR expression does not predict its therapeutic usefulness, future clinical trials have to evaluate the advantage of Anti-EGFR therapy in comparison with standard treatment in patients with BTC.

In summary, our findings demonstrate that EGFR overexpression is frequent in BTC, especially in EHCC. In contrast, HER2 overexpression and gene amplification is a rare event. While therapeutic targeting

of HER2 seems to be not promising, future clinical trials in patients with BTC should focus on EGFR.

COMMENTS

Background

The epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) are involved in the carcinogenesis of many malignancies. Therapeutic molecules targeting EGFR and HER2 have been successfully used for the treatment of colorectal, breast, lung and head and neck cancers among others. It is unknown if EGFR and HER2 are overexpressed in advanced biliary tract cancer (BTC) and therefore may serve as therapeutic targets in these cancers.

Research frontiers

As the so called targeted therapies are most effective when the corresponding receptor or signalling pathway is activated, previous studies have focused on EGFR and HER2 in various tumors. There are conflicting data about overexpression of EGFR and HER2 in BTC and about the therapeutic importance of the two growth factor receptors in these tumors. Because of the low incidence, clinical trials on BTC are difficult and mostly performed in small patient cohorts. A possible selection bias might additionally explain these conflicting data.

Innovations and breakthroughs

While EGFR is significantly overexpressed in advanced BTC, HER2 overexpression and amplification is rare and therefore seems not to play a role in the carcinogenesis of BTCs.

Applications

Because of these expression data on EGFR and HER2 in BTC, and the correlation of expression and therapeutic effectiveness, especially with HER2 in other tumors, future clinical trials in BTC should focus on EGFR as a therapeutic target.

Terminology

EGFR and HER2 are receptor tyrosine kinases encoded by proto-oncogenes. Growth factors such as epidermal growth factor or transforming growth factor bind to these receptors and initiate tumor cell proliferation, migration, invasion, resistance to apoptosis and angiogenesis. BTC is a heterogeneous tumor entity with rising incidence, consisting of intrahepatic mass forming type cholangiocarcinoma, perihilar Klatskin tumors, extrahepatic bile duct tumors, also termed intraductal growth type cholangiocarcinoma, and gallbladder cancer.

Peer review

Harder *et al* describe low expression and gene amplification of HER2 in biopsy samples of advanced BTC, as assessed by immunohistochemistry and fluorescence *in situ* hybridisation, respectively. The authors suggest that deviating numbers of some earlier studies may be due to non-standardized techniques. It is convincing that in the present paper results for protein expression and gene amplification correlated well.

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ORIGINAL ARTICLES

Protein interaction network related to *Helicobacter pylori* infection response

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Abstract

AIM: To understand the complex reaction of gastric inflammation induced by *Helicobacter pylori* (*H pylori*) in a systematic manner using a protein interaction network.

METHODS: The expression of genes significantly changed on microarray during *H pylori* infection was scanned from the web literary database and translated into proteins. A network of protein interactions was constructed by searching the primary interactions of selected proteins. The constructed network was mathematically analyzed and its biological function was examined. In addition, the nodes on the network were checked to determine if they had any further functional importance or relation to other proteins by extending them.

RESULTS: The scale-free network showing the relationship between inflammation and carcinogenesis was constructed. Mathematical analysis showed hub and bottleneck proteins, and these proteins were mostly related to immune response. The network contained pathways and proteins related to *H pylori* infection, such as the JAK-STAT pathway triggered by interleukins. Activation of nuclear factor (NF)-κB, TLR4, and other proteins known to function as core proteins of immune response were also found. These immune-related proteins interacted on the network with pathways and proteins related to the cell cycle, cell maintenance and proliferation, and transcription regulators such as BRCA1, FOS, REL, and zinc finger proteins. The extension of nodes showed interactions of the immune proteins with cancer-related proteins. One extended network, the core network, a summarized form of the extended network, and cell pathway model were constructed.

CONCLUSION: Immune-related proteins activated by *H pylori* infection interact with proto-oncogene proteins. The hub and bottleneck proteins are potential drug targets for gastric inflammation and cancer.

Key words: Gastric cancer; *Helicobacter pylori*; Inflammation; Pathway; Protein interaction network

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a gram negative bacterium which infects about 50% of the world population[1-3]. It is known to cause various gastroduodenal diseases such as chronic active gastritis in experimental animals and in humans. In human volunteers, *H pylori* caused gastritis and hypochlorhydria[4]. Mongolian gerbils infected by *H pylori* also developed symptoms such as intestinal metaplasia and adenocarcinoma[5-9]. Many scholars have demonstrated a relationship between *H pylori* and gastric carcinoma[3], and the World Health Organization (WHO) and the International Agency for Research on Cancer consensus group have classified *H pylori* as a definite biological carcinogen[10].

H pylori colonization causes a strong systemic immune response[11]. It induces the production of interleukins (ILs) (Korean Society for Medical Microbiology, 2004), tumor necrosis factor (TNF)[12,13], and proinflammatory

cytokines[14]. It also causes activation of nuclear factor κB (NF-κB)[15], activator protein-1 (AP-1), c-Jun, NH_2-terminal kinase, mitogen-activated protein kinase/extracellular signal-regulated kinase, and other cell proliferation and survival factors[16]. Bacterial toxins, high levels of superoxides, radicals, and singlet oxygen are known to induce carcinogenesis in gastric cells. Bacterial virulence factors such as CagA and VacA[1,17,18] induce cell hyperproliferation and the expression of oncogenes. However, the exact mechanism between *H pylori* and gastric carcinoma is unclear[19].

Various tools have been employed to identify the relationship between *H pylori* and gastric cancer, including c-DNA microarrays[4,20]. However, most of these methods did not consider the systematic interaction of biological components. As an alternative, a network construction and analysis of protein-protein interactions[21] were applied to examine the inflammatory response to *H pylori* infection in a systematic manner.

MATERIALS AND METHODS

The research method used in this study mainly consisted of three steps. Step one: extraction of the genes which changed significantly during *H pylori* infection from the database and by querying web databases to gather protein-protein interactions. Step two: construction of a network and summarizing the constructed network. Step three: analysis and extension search of the network. A flow chart showing the data flow is described in Figure 1.

Searching genes related to H pylori infection (Step 1)

Genes related to *H pylori* infection were collected by searching PubMed. The expression of genes significantly changed ($P < 0.05$) by *H pylori* infection in the microarray[4,11,13,20] data was examined, and genes related to the immune response were identified and collected. A total of 39 filtered genes (Table 1) were obtained.

Scanning protein interactions and construction of protein interaction networks (Step 2)

The protein interaction networks were constructed based on statistical prediction through the analysis of microarray data. Selected genes were queried to the Uniprot database to convert into proteins. The proteins were scanned by a human Protein-protein Interaction Prediction (PIPs) database (http://www.compbio.dundee.ac.uk/www-pips/). Protein links were then extracted from the Human Protein Reference Database reference (HPRD, http://www.hprd.org/index_html). Without HPRD references, any further search of the protein links was stopped. An extended network was constructed by integrating all results extracted from the PIPs server (Figure 2). Pajek (http://vlado.fmf.uni-lj.si/pub/networks/pajek/) was used for the construction of extended networks. Then, a core network showing simplified main pathways, major proteins, and subcellular location information was extracted from the extended network using Cytoscape (http://www.cytoscape.org/).

Table 1 List of proteins extracted from the literary database showing significant change after *H pylori* infection

Protein/gene name	Uniprot ID	HPRD reference
ITGB2	P05107	
LY96	Q9Y6Y9	X
TLR10	Q9BXR5	
TLR2	O60903	
TLR3	O15455	X
VCAM1	P19320	
HCK	P08631	
MAPK8	P45983	
RAC2	P15153	
SOCS2	O14508	X
STAT6	P42226	
C2	P06681	X
C3	P01024	
C4A	P0C0L4	X
CCL18	P55774	X
CCL19	Q99731	X
CCL3	P10147	X
CCL4	P13236	X
CRP	P02741	
CXCL13	O43927	
CXCL2	P19875	X
CXCL9	Q07325	X
HLA-DMA	P28067	X
HLA-DPB1	Q30154	X
HLA-DQB1	P03992	X
HLA-DRB5	Q30154	X
HSPH1	Q92598	
C11TA	P33076	
PLAT	P00750	
IFITM1	P13164	X
IRF4	Q15306	
MADCAM1	Q13477	
ALOX5	P09917	X
TLR5	O60602	X
CD53	P19397	X
TLR6	Q9Y2C9	X
SLAMF1	Q13291	
PTPRC	P08575	
FAIM3	O60667	X
CD180	Q99467	
TLR4	O00206	
TLR1	Q15399	
CXCL3	P19876	X
CD47	Q08722	
IFNGR1	P15260	
IL10RA	Q13651	X
IL18RAP	O95256	X
ITGAX	P20702	X
IL8	P10145	

Nodes with no HPRD reference were marked with x. HPRD: Human protein reference database.

Analysis of protein interaction network (Step 3)

The protein interactions of an extended network were examined whether or not the network contained known pathways related to *H pylori* infection, inflammation, and carcinogenesis. The core network was not analyzed because it was just the simplified form of the extended network.

Four factors: Shortest paths, degree (connectivity), betweenness centrality (BC), and closeness centrality (CC), were adopted to analyze general mathematical properties of the extended network and to search

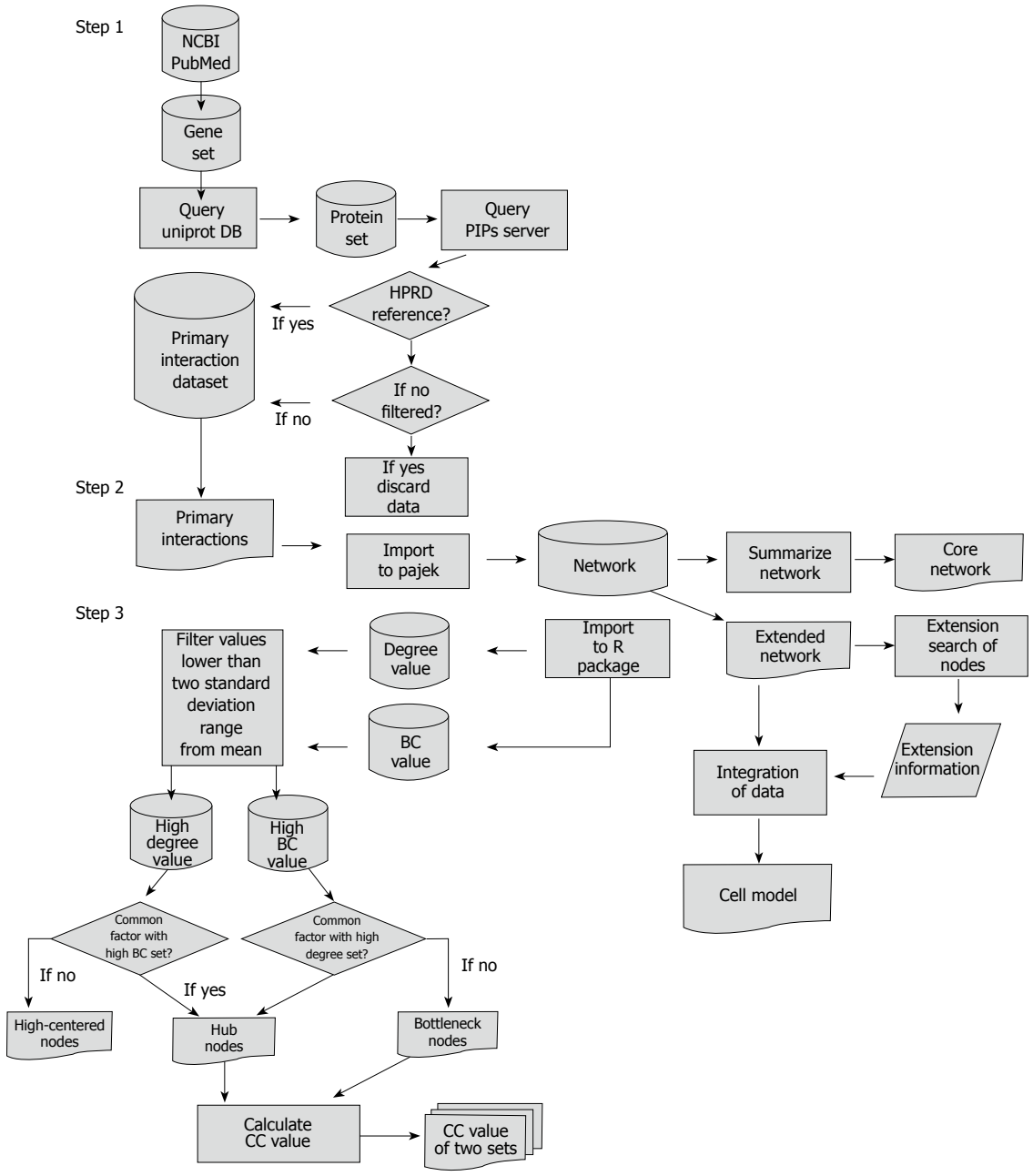

Figure 1 Flow chart showing overall methods and data flow used in this study.

topologically important proteins[21].

Degree, the most basic characteristic of a node, is defined as the number of links the node has with other nodes. Degree distribution is obtained by counting the number of nodes with a fixed degree value, which is variable from minimum to maximum degree, and dividing it by the total number of nodes of a network[22]. Highly concentrated nodes play a major role as a hub in a network. Degree was also used to check if an extended network was scale-free, which is frequently found in cellular networks[22,23]. The scale-free network follows a power-law degree distribution[22]. Power law is defined as: $P(x) = Cx^{-a}$

$C = e^c$ and $P(x)$ is a probability that a selected node has exactly x links (degree value)[23]. α is the degree exponent which determines some properties of the network. Most of the networks found in nature are known to have degree exponent values between two and three[22]. In this study, cumulative distribution function, a superior method of plotting data[23], was used. The plot of log transformed probability distribution function $P(x)$ in which x has a degree value greater than or equal to x, was drawn. $P(x)$ is defined mathematically as[23]:

$$P(x) = \int_x^\infty p(x')dx'$$

As the distribution follows power law,

$$P(x) = C\int_x^\infty x'^{-a}dx' = \frac{C}{a-1}x^{-(a-1)}$$

A cumulative plot also follows power law, but the degree exponent of the plot is one less than the original distribution[23]. The degree exponent was calculated by measuring the slope of the regression line and adding one to the exponent value. Other factors such as R

square, standard error, and *P*-value were also computed.

BC for node k is defined as:

$$b(k) = \sum_{i,j} b_{i\to j}(k) = \sum_{i,j} \frac{g^k_{i\to j}}{g_{i\to j}}$$

$g_{i\to j}$ is the number of shortest paths from node i to j, while $g^k_{i\to j}$ is the number of geodesics among $g_{i\to j}$ that passes through node κ[21,24]. The BC value of all nodes in the network was examined to check for bottlenecks in the network.

CC is defined as the inverse of the average length of the shortest paths to/from all the other vertices in the graph[25]. It tells us the topological center of the network[25]. CC was calculated by adopting the core algorithm of the R igraph package (http://www.r-project.org/). CC values of the protein set with either large BC value or degree were measured and compared to total CC values to check topological centrality of hubs and bottlenecks in the network.

The shortest path (geodesics) is calculated by measuring the length of all the geodesics from or to the vertices in the network. The average shortest path was measured to see how many average steps were required to link two randomly selected nodes in the network.

After computing BC and the degree of all the nodes, nodes under two standard deviation ranges from the mean were filtered out and CC values of nodes larger than two standard deviation ranges from the mean were measured. As a result, nodes with a large BC value, a large degree, both a large BC and degree, and CC value were obtained. The R package was used to calculate and analyze these values.

The network was constructed by scanning primary interactions of significantly and differentially expressed genes compared to control. Thus, it may not include hidden interactions of protein nodes between the two major nodes. For example, only the primary interaction between node A and B is available by ordinary network analysis, although the two proteins are linked *via* node C in reality. However, by extending the network, a pathway passing through node C between A and B can be found.

RESULTS

Protein interaction networks

By integrating scanned primary interactions of previously selected nodes from the PIPs server, the extended network was constructed. A core network was then derived from the extended network.

The extended network was composed of 604 nodes, connected *via* 808 edges (Figure 2). One giant network with 599 nodes and 805 edges, and two separate interactions were observed. Examining the shortest paths of the network showed that two randomly selected nodes on the network were connected *via* 4.89 links. This suggests that the nodes were very closely linked. In addition, a small world effect can be found[26]. The distribution of the shortest paths was plotted using histograms (Figure 3A). The average value (4.89) was similar to other values of human protein networks[21,26].

Table 2 List of proteins with a large degree value and their CC values

Protein	Degree	CC value
RELA/NF-κB3	105	0.047675522
MAPK8	68	0.046693511
NFKBIA	63	0.047164646
HCK	49	0.046599691
PTPRC	43	0.046388184
ITGB2	40	0.045643782
MAP2K1	36	0.045681818
PLAT	25	0.045451119
STAT6	24	0.046513422
HLA-DMA	24	0.04396646
TRAF4	24	0.044062843
TLR4	24	0.046527778
HLA-DRB5	23	0.04573032
TLR2	22	0.046083301
IL10RA	18	0.046206897
ALOX5	18	0.044056404

CC: Closeness centrality.

The cumulative distribution plot showed clear evidence that the extended network follows scale-free distribution (Figure 3B). By measuring the slope of the regression line of the plot drawn on the basis of log transformed cumulative data, the α value of 1.1968 in the power law distribution was determined. As the degree exponent of the cumulative plot is one less than original distribution[23], the true degree exponent value should be 2.1968 (standard error = 0.04, coefficient of determination R square = 0.97, and *P*-value = nearly zero by the least square fit)[27]. It is known that networks with a degree exponent larger than three do not have features that scale-free networks have[22]. The degree exponent value of the extended network (2.1968) was lower than 3, which was similar to other networks following a scale-free distribution, rather than a random distribution.

Important nodes in the network

One of the properties of networks following scale-free distribution is the existence of a small number of highly connected nodes, called hubs which are more important than other less connected nodes[22,28]. The hub nodes are more critical to the survival of cells (Tables 2 and 3). The scale-free networks are prone to breakdown into fragments when nodes are attacked[29]. Other important nodes also have a large BC value. The node with a large BC functions as a bottleneck in the network, even when the node's degree is low. Nodes with a degree or BC value larger than the mean plus two standard deviations were selected. Sixteen nodes were determined to have a large degree (Table 2) and 19 nodes had a large BC (Table 4). Twelve nodes had both a large degree and a large BC (Table 3). Six nodes: NF-κB3 (Nuclear factor κB p65 subunit), MAPK8 (Mitogen-activated protein kinase 8), NFKBIA (NF-κB inhibitor α), HCK (Hemopoietic cell kinase), PTPRC (Leukocyte common antigen CD45), and ITGB2 (Integrin β-2) were the top six nodes on both degree and BC values.

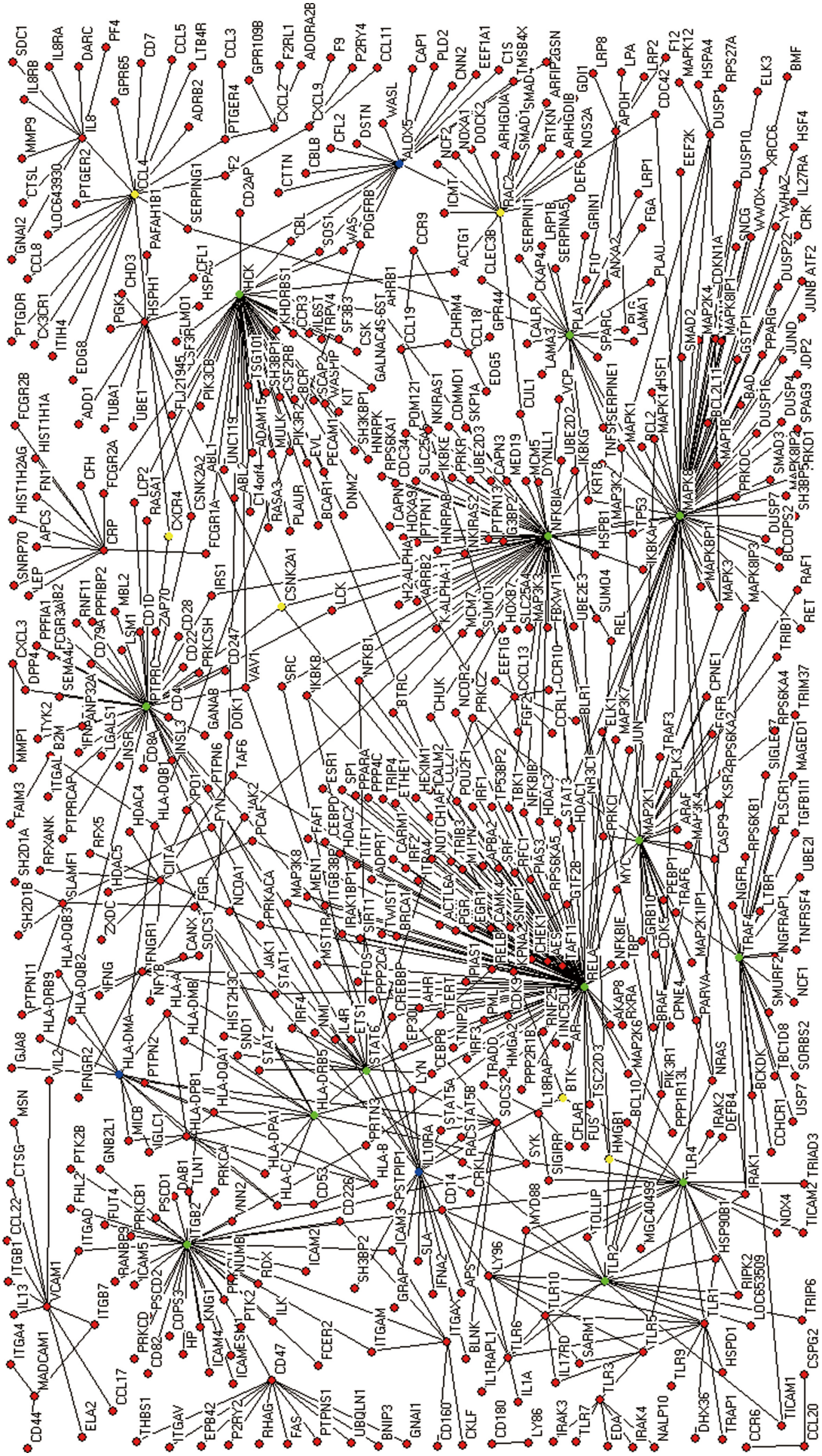

Figure 2 The extended protein interaction network of a cell infected by *H pylori*. Green nodes (a large BC and degree), blue nodes (only a large degree), yellow nodes (only a large BC).

Table 3 List of proteins with both a large BC and degree, and their functions

Protein name	Function
RELA/NF-κB3	NF-κB is a pleiotropic transcription factor which is present in almost all cell types and is involved in many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis
MAPK8	Responds to activation by environmental stress and pro-inflammatory cytokines by phosphorylating a number of transcription factors, primarily components of AP-1 such as JUN, JDP2 and ATF2 and thus regulates AP-1 transcriptional activity
NFKBIA	Inhibits the activity of dimeric NF-κB/REL complexes by trapping REL dimers in the cytoplasm, masking their nuclear localization signals
HCK	May serve as part of a signaling pathway coupling the Fc receptor to activation of the respiratory burst. May also contribute to neutrophil migration and regulate the degranulation process of neutrophils
PTPRC	Required for T-cell activation through the antigen receptor
ITGB2	Receptor for ICAM1, ICAM2, ICAM3 and ICAM4
TLR4	Cooperates with LY96 and CD14 to mediate the innate immune response to bacterial lipopolysaccharide (LPS). Acts *via* MYD88, TIRAP and TRAF6, leading to NF-κB activation, cytokine secretion and the inflammatory response
PLAT	Converts the abundant, but inactive, zymogen plasminogen to plasmin by hydrolyzing a single Arg-Val bond in plasminogen. By controlling plasmin-mediated proteolysis, it plays an important role in tissue remodeling and degradation, in cell migration and many other physiopathological events
TRAF4	Adapter protein and signal transducer that links members of the tumor necrosis factor receptor family to different signaling pathways by association with the receptor cytoplasmic domain and kinases
MAP2K1	Catalyzes the concomitant phosphorylation of a threonine and a tyrosine residue in a Thr-Glu-Tyr sequence located in MAP kinases. Activates ERK1 and ERK2 MAP kinases
TLR2	Cooperates with LY96 to mediate the innate immune response to bacterial lipoproteins and other microbial cell wall components
STAT6	Carries out a dual function: signal transduction and activation of transcription. Involved in interleukin-4 signaling

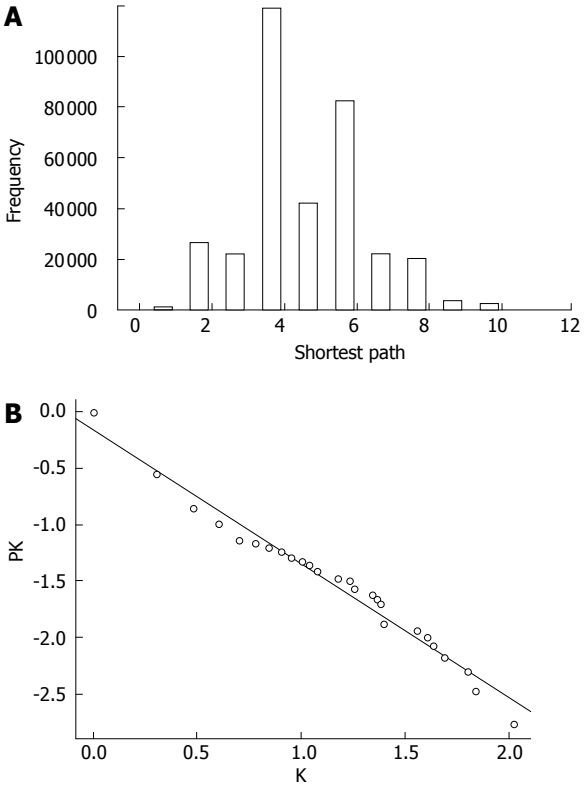

Figure 3 Properties of the extended network. A: Histogram showing distribution of the shortest path. Two randomly selected nodes were connected *via* 4.9 links; B: Cumulative degree distribution plot of the extended network showing that degree distribution follows the power law. Line indicates the degree exponent of 1.2, which is one lower than the true degree exponent of 2.2.

STAT6 (Signal transducer and activator of transcription 6), TLRs (Toll-like receptor), TRAF4 (TNF receptor-associated factor 4), and PLAT (TPA, Tissue-type plasminogen activator) also had both a large BC and degree (Table 3). NF-κB3, NFKBIA, PTPRC, TLRs, and HLA-DRB5 are already well-known for having

Table 4 List of proteins with a large BC value and their CC values

Protein	BC	CC
RELA/NF-κB3	62240.75685	0.047675522
MAPK8	37123.82691	0.046693511
PTPRC	34614.23746	0.046388184
HCK	33484.99898	0.046599691
NFKBIA	28771.33132	0.047164646
ITGB2	27901.91607	0.045643782
TLR4	19587.75501	0.046527778
PLAT	19339.65216	0.045451119
CCL4	18441.86414	0.043654528
HMGB1	15263.85652	0.046466826
MAP2K1	14047.72141	0.045681818
STAT6	13376.61128	0.046513422
CXCR4	11471.71166	0.044769471
CSNK2A1	11279.76894	0.046574496
HLA-DRB5	11088.56681	0.045730320
BTK	11016.20767	0.046621308
TLR2	10824.15886	0.046083301
RAC2	10692.38019	0.044872749
TRAF4	10472.80579	0.044062843

BC: Betweenness centrality.

biological functions related to immune response[2,3,21]. STAT6 is related to the JAK-STAT pathway which sends signals from ILs directly to the nucleus[30]. MAPK8 of the MAPK signaling pathway can be found in the signaling of other inflammatory responses in asthma, and is related to cell proliferation[31]. TRAF4 is involved in tumor necrosis and TPA (PLAT) in plasminogen activation, respectively. Most of these nodes are related to immune response and signal transduction, suggesting that these nodes perform major functions against *H pylori* infection.

Not only nodes with both a large degree and BC, but also nodes with a large BC and a small degree were considered important in previous research[21], since these nodes function as bottlenecks in the network,

Table 5 List of proteins with only a large BC and their functions

Protein name	Function
HMGB1	Binds to preferentially single-stranded DNA and unwinds double-stranded DNA
BTK	Plays a crucial role in B-cell ontogeny
CSNK2A1	Casein kinases are operationally defined by their preferential utilization of acidic proteins such as caseins as substrates
CXCR4	Transduces a signal by increasing the intracellular calcium ion level
CCL4	Monokine with inflammatory and chemokinetic properties
RAC2	Plasma membrane-associated small GTPase which cycles between an active GTP-bound and inactive GDP-bound state. In active state binds to a variety of effector proteins to regulate cellular responses, such as secretory processes, phagocytosis of apoptotic cells and epithelial cell polarization. Seems to be involved in the regulation of NADPH oxidase

even without the role of hubs. Six nodes: HMGB1 (High mobility group protein B), BTK (Bruton tyrosine kinase), CSNK2A1 (CSK2A1, Casein kinase Ⅱ subunit alpha), RAC2 (Ras-related C3 botulinum toxin substrate 2), CCL4 (C-C motif chemokine 4), and CXCR4 (C-X-C chemokine receptor type 4) had a large BC but a low degree. Large BC nodes such as CXCR4, CCL4, BTK, CSNK2A1, and RAC2 with the exception of HMGB1 are related to immune response and signal transduction (Table 5). HMGB1 unwinds double-stranded DNA and binds preferentially to single-stranded DNA, which may be related to the gene regulation of immune response. As expected these large BC nodes were linked to important nodes, such as hubs. HMGB1 was linked to NF-κB3, TLR4, TLR2, and PLAT, which have a large BC degree (Figure 2). BTK interacted with NFKBIA, TLR4, HCK, and IL10RA. NFKBIA, TLR4, and HCK had a large BC and degree, while IL10RA had a large degree only. CSNK2A1 was linked to NF-κB3, NFKBIA, PTPRC, and HSPH1. RAC2 interacted with NFKBIA, HCK, and ALOX5. Lastly, CCL4 and CXCR4 were linked to PTPRC and PLAT. Thus, it was demonstrated that the nodes with large BC play important roles in the connection and communication of nodes including hubs.

The CC values of nodes with a large degree or BC were checked to see if these proteins were near to the topological center of the network. The larger the CC value is, the closer the node is to the center of the network[21]. NF-κB3 was closest to the topological center, and NFKBIA was the second closest in the network (Tables 2 and 4).

Biological functions of pathways and nodes in the network

Pathways related to immune response and other biological phenomena were observed in the network (Figures 4 and 5). The network contained previously known pathways which were involved in *H pylori* infection and inflammation.

The network (Figures 2 and 4) showed interactions of IL 1, 4, 8, 10, 13, 17, and 18 receptors with JAKs and STATs that send signals from cell-surface receptors to the nucleus[30]. IL 8 increases significantly during *H pylori* infection, thus it was used as a standard to determine the pathogenicity of different *H pylori* strains[19]. IL 1, 10, and 18 changed significantly, which was demonstrated by microarray analysis or Western blotting data[11,13,32]. IL 4 and 13 are proinflammatory cytokines. While IL 4 induces eosinophilic inflammation and differentiation of Th2 cells, IL 13 produces immunoglobulin E (IgE)[33].

Interactions of Toll-like receptors (TLRs), also known to be immune-related, were observed. The TLR4 signaling pathway is associated with an immune response by interacting with MYD88 and IRAK1[34,35] in the network (Figure 4). They were linked to proteins in the nucleus through MAPKs.

Another pathway in the network was found among the MAPKs. Interactions among MAPK 1, 3, and 8 in the network were observed. In immune-related diseases such as asthma, the activation of MAPK due to infection has also been reported[21,36,37].

Besides full pathways, the presence of single or a few interactions having biological functions were informative. NF-κB and AP-1 are two key regulatory factors of inflammation[38-40]. NF-κB1-NF-κB3 linkage and JAK-NFKBIA-STAT linkage were found (Figure 5). The regulation of NF-κB by AP-1(JUN) and NFKBIA was also observed (Figure 4).

Although activation of TNFα[13] was not found in the network, TNFSF11 (Tumor necrosis factor ligand superfamily member 11) and TRAFs (TNF receptor associated factor), related to TNF, were found. Tumor necrosis factors induce cell proliferation by activating anti-apoptosis[16]. Cell proliferation and carcinogenesis are one of the well-known characteristics of cells infected by *H pylori*[19]. In addition, BRCA1 (Breast cancer type 1 susceptibility protein), FOS (*c-fos*, Proto-oncogene protein)[41], REL (C-Rel proto-oncogene protein), and VAV1 (Proto-oncogene vav), which are oncogenes, were found. The presence of TNF and the oncogenes in the network suggests that *H pylori* infection may be related to carcinogenesis.

SRC (Proto-oncogene tyrosine-protein kinase) in the network is involved in cell maintenance and communication[21]. CDK5 (Cyclin-dependent kinase 5), RASA1 (Ras GTPase-activating protein 1) and RASA3 are related to cell growth effect[30].

Not only protein nodes related to inflammation and carcinogenesis, but also proteins related to stress resistance were found. Infection of *H pylori* increases levels of superoxide and singlet oxygen. The stress-resistance protein, HSPH1 (Heat shock protein 105 kDa), HSPA8 (Heat shock cognate 71 kDa protein), and HSPB1 (Heat shock protein β-1) were found.

Generally, stimulation and regulation of the immune

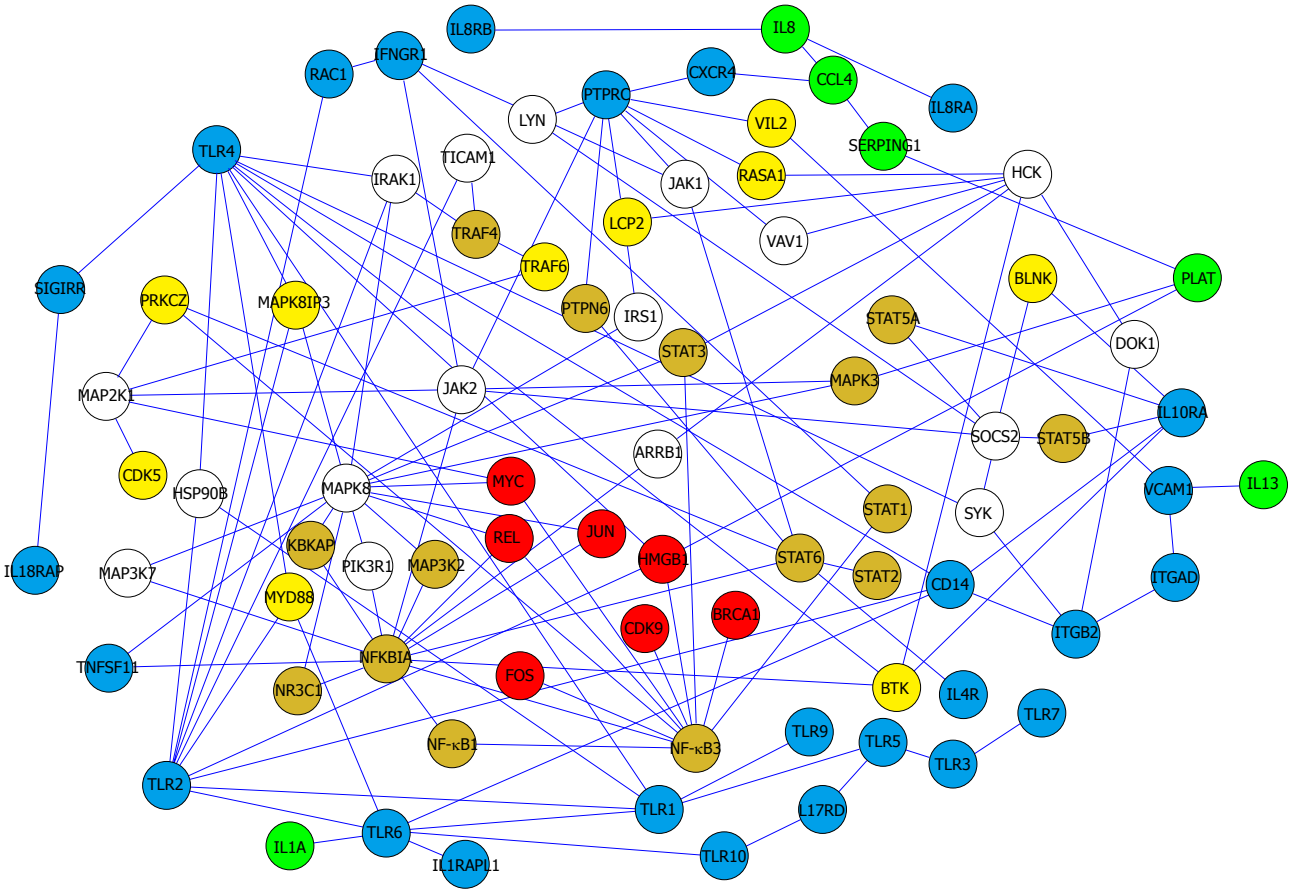

Figure 4 Core network showing simplified interactions and major pathways. Color of the nodes indicates subcellular location. Red (nucleus), orange (nucleus and cytoplasm), yellow (cytoplasm), blue (membrane), green (extracellular), and white (unknown).

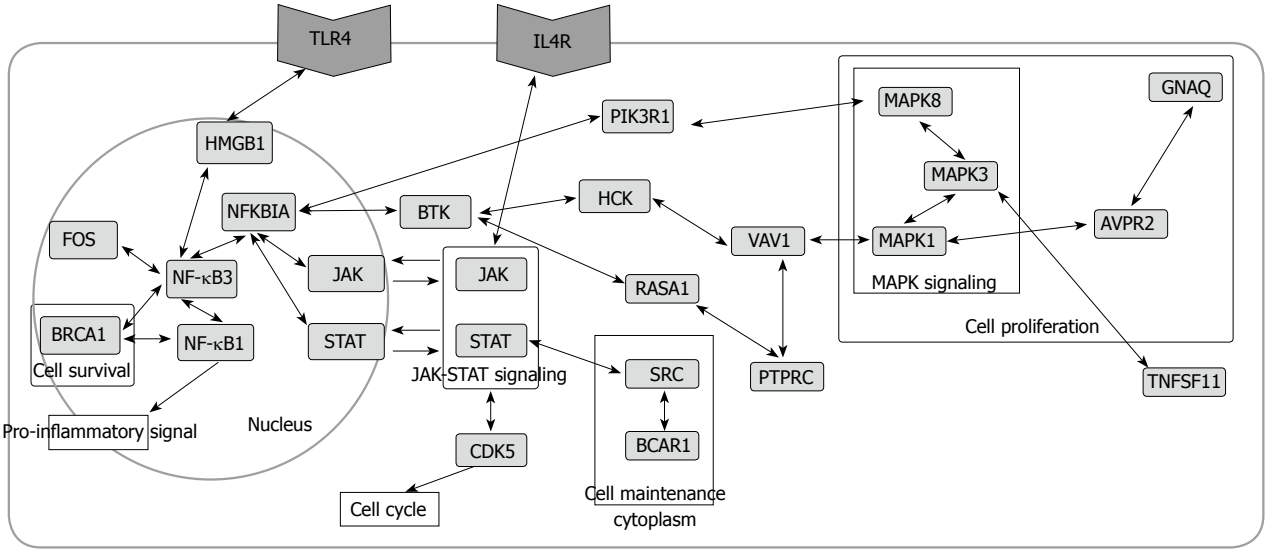

Figure 5 Cell model showing major interactions after *H pylori* infection.

system through their receptors were found in the network. Activation of cell signaling, cell proliferation, cell survival, proto-oncogenes, and stress resistance were observed. These functions are reminiscent of the observed response of cells infected by *H pylori*. The virtual network analysis in this study reflects the real protein-interaction-network in the cell.

Extension search of the network

A biologically important protein can be missed, as the network is constructed by searching only the primary interactions of selected genes. To overcome this problem, further interactions of nodes regardless of their degree or BC were examined. The extension of SRC led to BCAR1 (Figure 5). Thus, the role of cell maintenance[42]

was connected with that of carcinogenesis in BCAR1. ADRB2 (β-2 adrenergic receptor) extension was linked to PRKCE, PRKACA, MAPK1, and MAPK3 (Figure 2). This pathway has not previously been reported in *H pylori* infection, but has been found in immune-related diseases such as asthma[21]. BRCA1 was further linked to CDK2, 4, 7, and CDC2 (Cell division control protein 2 homolog) (Figure 5). CDKs are activated proceeding to the cell cycle. The extension of BRCA1 was linked to JUND (transcription factor jun D), which binds to an AP-1 site and stimulates its promoter activity. BRCA1 extension led to ZNF467 (Zinc finger protein 467), a transcription regulator which has a possible relationship with cancer (Figure 2). The extension of MAPK1 led to GNAQ (Guanine nucleotide-binding protein G(q) subunit α) *via* GNAS (Guanine nucleotide-binding protein G(s) subunit α isoforms short) and AVPR2 (Vasopressin V2 receptor)[21] (Figure 5). The proteins in this pathway contribute to cell proliferation, a well-known characteristic of cells infected by *H pylori*[19]. STAT1-CREBBP (CREB-binding protein) linkage was related to G1 arrest of a cell[21] (Figure 5).

DISCUSSION

The correlation between inflammation caused by *H pylori* infection and gastric cancer has been studied and supported by many researchers. It is important to understand the relationship between inflammation and the carcinogenesis mechanism. Microarray data were used to determine the global gene expression of infected cells. Microarray data showed up/down regulation of gene expression related to immune response, cell cycle, cell growth, and signal transduction, which may support the hypothesis that *H pylori* infection causes cancer development[4,11,13,20]. However, the data did not present a clear mechanism of carcinogenesis in a systematic manner. In this study, network analysis methods were applied to integrate previous data and construct the network model which shows the relationship between inflammation and cancer development.

The extended network showing primary interactions of significantly expressed genes (proteins in the network) was constructed. The network contained many protein nodes related to immune response and signal transduction induced by extracellular signals such as cytokines. The important nodes selected based on large BC and degree values were mostly involved in immune response and signal transduction. For example, the p65 subunit of NF-κB (NF-κB3), one of the most important regulatory factors of inflammation, was the node with the largest degree and BC value. Large BC nodes, the bottlenecks in the network, were linked to important nodes with a large degree, a large BC, or both. Like large BC and degree nodes, a large BC node was mostly related to immune response and signal transduction, with the exception of HMGB1. The constructed network also contained many pathways related to immune response and signal transduction. TLR4, JAK-STAT, and MAPK8 pathways are major pathways found in the network. Not only the pathways, but important nodes such as NF-κB and AP-1 (JUN) were also found in the network.

The network also showed many nodes related to carcinogenesis. Tumor related proteins such as BRCA1, FOS, REL, VAV1, TNFSF11, and TRAFs were found. The extension search of nodes was also linked to pathways related to cell proliferation, cell survival, and the cell cycle. The extracellular signal from ILs and TLRs goes to NF-κB, NFKBIA, and AP-1 in the nucleus *via* the JAK-STAT and MAPK signaling pathways. The signal then goes to proteins in the cytoplasm *via* the JAK-STAT pathway and BTK, promoting cell proliferation and proceeding to the cell cycle. These activated processes are one of the characteristics of cells infected by *H pylori*. In addition, *H pylori* infection is known to increase levels of radicals and oxides. Radicals and oxides are widely thought to be possible mutagens. Oxidative stress may be an additional mechanism of carcinogenesis.

Another important factor of hub and bottleneck protein nodes is that they are potential drug targets. By inhibiting the functions of hubs and bottlenecks by small molecules, the function of the network can be shut down, meaning that the inflammatory and carcinogenesis processes can be stopped, theoretically. Traditionally, antibiotics have been used to treat gastric inflammation caused by *H pylori* infection[14]. However, this treatment has the potential problem of antibiotic resistance in the bacteria. As a potential alternative, this study presented the hub and bottleneck nodes as a drug target of gastric inflammation, cancer, and other diseases caused by *H pylori* infection.

The analysis of protein network interactions showed immune response and carcinogenesis-related cell responses in a bigger picture. The extension search of nodes also demonstrated key signal transductions linking inflammatory response and carcinogenesis. This study showed how a systematic approach such as the network construction produces meaningful information. It also offered a relatively easy and simple framework to understand the complexity of cellular interactions having functional importance. Therefore, the application of this tool may be an alternative to find important genes and drug targets in other diseases and in complex biological systems.

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COMMENTS

Background

The correlation between inflammation caused by *Helicobacter pylori* (*H pylori*) infection and gastric cancer has been studied and supported by many researchers.

Research frontiers

To explain the relationship between *H pylori* infection and cancer development,

microarray analysis was used. Microarray data showed the regulatory patterns of gene expression related to immune response, cell cycle, cell growth, and signal transduction. However, the data obtained did not show the mechanism of carcinogenesis in a systematic manner.

Innovations and breakthroughs

In this study, protein network analysis, one of the bioinformatic tools, was applied to integrate previous microarray data, and a network model was constructed showing the relationship between inflammation and cancer development. The network contained many proteins related to immune response and signal transduction induced by extracellular cytokines. Some tumor-related proteins (BRCA1, FOS, REL, VAV1, TNFSF11, TRAF) were found.

Applications

This article offered a relatively easy and simple framework to understand the complexity of cellular interactions having functional importance. This tool may be used as an alternative to find important genes and drug targets in gastric inflammation and cancer and in complex biological systems.

Peer review

This study about protein interaction network in *H pylori* infection is potentially interesting and informative.

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ORIGINAL ARTICLES

Attenuation of portal hypertension by natural taurine in rats with liver cirrhosis

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Abstract

AIM: To investigate the inhibitory effect of natural taurine (NTau) on portal hypertension (PHT) in rats with experimentally-induced liver cirrhosis (LC).

METHODS: Experimentally-induced LC Wistar rats (20 rats/group) were treated with either oral saline or oral NTau for 6 consecutive weeks. Evaluation parameters included portal venous pressure (PVP), portal venous resistance (PVR), portal venous flow (PVF), splanchnic vascular resistance (SVR) and mean arterial pressure (MAP). Vasoactive substance levels including nitric oxide (NO), nitric oxide synthase (NOS) and cyclic guanosine monophosphate (cGMP) were also measured. Histological investigation of type Ⅰ and Ⅲ collagen (COL Ⅰ and Ⅲ) and transforming growth factor-β_1 (TGF-β1) was also performed.

RESULTS: Treatment with NTau (1) significantly decreased PVP, PVR and PVF, and increased MAP and SVP; (2) markedly increased the vascular compliance and reduced the zero-stress of the portal vein; (3) markedly decreased the amount of NO and cGMP and activity of NOS; and (4) improved the pathological status of the liver tissue and reduced the expression of COL Ⅰ, COL Ⅲ and TGF-β1.

CONCLUSION: NTau inhibited the LC-induced PHT by improving hyperdynamic circulation, morphology of liver and biomechanical properties of the portal vein in experimentally-induced LC rats.

Key words: Taurine; Liver cirrhosis; Portal hypertension; Rat

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INTRODUCTION

Liver cirrhosis (LC)-induced portal hypertension (PHT), also referred to as hepatocirrhotic portal hypertension, is highly susceptible to life-threatening complications such as esophageal and fundus ventriculi variceal bleeding, as well as ascites and hepatic encephalopathy (HE), resulting in high mortality among this group of patients. Currently, several treatment modalities are commonly employed for the management of LC-induced PHT, including systemic drug treatment, surgical intervention, endoscopic ligation, and liver transplantation. Although endoscopic ligation is useful in preventing initial hemorrhage in the upper digestive tract, it is less effective when dealing with recurring bleeding[1]. Transjugular intrahepatic portosystemic stent shunt (TIPSS) can, to a certain extent, reduce various complications of PHT. It may, however, augment the incidence of HE. Therefore, TIPSS is not generally the first therapeutic option for patients with PHT owing to its inability to improve the survival rate[2]. On the other hand, liver transplantation is regarded as the last option to combat advanced liver cirrhosis. However, the inherent risk

associated with this radical surgical procedure and the inevitable high costs of organ transplantation, together with the long-standing disturbance of the systemic hemodynamics after transplantation, practically prevents it from being a routinely used method. Presently, long-term pharmacological treatment is still the mainstay for LC-induced PHT. To date, to develop an effective therapeutic approach for the management of LC-induced PHT remains a formidable challenge to many researchers in the field.

Our previous study showed that natural taurine (NTau) markedly inhibited the contraction and collapse of hepatic stellate cells (HSCs)[3]. It has been well established that contraction of HSCs significantly contributes to the initiation and progression of hepatocirrhotic portal hypertension[4,5]. As a continuation of our previous efforts to evaluate the therapeutic potential of NTau for LC-induced PHT, the present study aims at investigating the inhibitory effects of NTau on PHT, specifically focusing on the perspectives of hepatic "forward" and "backward" flow theories as well as on portal venous biomechanics and hemodynamics.

MATERIALS AND METHODS

Animals and reagents

Male specific-pathogen-free (SPF) Wistar rats weighing 215 ± 18 g were provided by the Centre of Experimental Animals, Guangxi Medical University, Nanning, Guangxi Province, China. Immunohistochemical staining kits for type I collagen (COL I), type Ⅲ collagen (COL Ⅲ) and transforming growth factor-β1 (TGF-β1) were purchased from the Wuhan Boster Biological Technology Ltd (Wuhan, Hubei Province, China). Assay kits for nitric oxide (NO), nitric oxide synthase (NOS) and cyclic guanosine monophosphate (cGMP) were obtained from the Nanjing Jiangcheng Bioengineering Institute (Nanjing, Jiangsu Province, China). The instruments used in the current project included an electromagnetic flowmeter (MFV-3200; Nihon Kohden, Japan), an 8-channel physiology recorder (RM-6000; Nihon Kohden, Japan), an ultraviolet spectrophotometer (80-2106-20; Pharmacia, UK), a UV-spectrophotometer (9100; Beijing Rayleigh Analytical Instrument Corporation, Beijing, China), and a light microscope (BX51, Olympus, Japan).

Extraction of NTau

The natural taurine (2-aminoethyl-sulfonic acid) used in the present study was extracted from black clams (*Meretrix meretrix* L.). Briefly, after the clam meat was cleaned and weighed (500 g), it was then minced in an electrical blender (4000 r/min) ten times, with each process lasting for about 10 s. Distilled water (1 L) was then added into the mince and further homogenized for 30 min. The mixture was then boiled in a water bath at 100℃ for 30 min, followed by filtering the mixture through four layers of gauze. The residue on the top of the gauze was discarded and the filtrate was then centrifuged to obtain the supernatant, which was then de-acidified with HCl ($HCl:H_2O$ = 3). After centrifugation, the proteins were adjusted to pH 10 with NaOH (20%) aqueous solution to yield the de-alkalined proteins. After the pH value was adjusted to 5, the supernatant was further condensed. The other unwanted amino acids and pigments were removed by column chromatography using strong acid cation exchange resin as the solid phase and eluting with distilled water. The resultant NTau was quantitatively measured by high performance liquid chromatography (HPLC) and the purity of the NTau was determined to be 98.8%.

Establishment of animal model and treatment protocol

An animal model of LC was established following a previously described protocol[6]. In brief, rats were fed with animal chow consisting of 80% corn flour, 19.5% animal fat and 0.5% cholesterol. The animals were only allowed to drink 15% aqueous alcohol. After an initial subcutaneous injection of a 40% CCl_4-olive oil solution at a dose of 5 mL/kg, the subcutaneous injections were repeated once every 3 d at a dose of 3 mL/kg for a total duration of 42 d. All rats were kept at room temperature under 12-h dark/light cycles and received humane care in accordance with the Guidelines of the Guangxi University of Chinese Medicine for the Care of Laboratory Animals.

Forty Wistar rats were randomized into 2 groups, with 20 rats in each group: a model group without NTau treatment (LC - NTau), and a model group treated with NTau (LC + NTau). During establishment of the model, rats in the LC + NTau group were concomitantly administered with 600 mg/kg NTau by gavage once daily, while LC - NTau group received only saline. Another 20 Wistar rats which received only normal animal chow and no CCl_4-olive oil solution injection were also used as the normal healthy control (NML) in the experimental design.

Influence of NTau on the "forward flow" theory of LC

Measurement of hemodynamic parameters: Measurement of the hemodynamic parameters was conducted according to the methods described previously[7] and was performed by an experienced technician affiliated to the first author's research group. Briefly, the animals were fasted for 8 h prior to measurement. Pentobarbital sodium (30 mg/kg) was injected through an ear vein to induce anesthesia. Then, a median epigastric incision was made in each animal which was placed in a supine position. The main portal vein (about 2 cm in length) was dissociated and exposed by blunt dissection. An incision was made followed by placement of an electromagnetic probe with an appropriate caliber connected with the electromagnetic flowmeter into the portal vein to measure the portal venous flow (PVF). Similar catheterizations were made into the portal vein and carotid artery for the measurement of the portal venous pressure (PVP) and mean arterial pressure (MAP) with the aid of a physiology recorder. All data were recorded after the hemodynamic parameters were stabilized. Portal venous resistance

(PVR) and splanchnic vascular resistance (SVR) were also calculated separately using the following equations PVR = PVP/PVF and SVR = MAP/PVF, respectively.

Determination of NO, NOS and cGMP in portal venous blood: Heparinized portal venous blood (5 mL) was obtained and centrifuged at 3000 r/min for 10 min. The serum was extracted for determination of the concentrations of NO, NOS and cGMP using the nitrate reductase method, chemical colorimetry and radioimmunoassay respectively according to the manufacturers' instructions.

Influence of NTau on the "backward flow" theory of LC

Histological investigation of the liver tissue: Twenty-four hours after the last dosing of the experiment, the rats were anesthetized with ethyl ether, and the livers were quickly removed from the etherized animals. Tissue mass of a size measuring about 1 cm × 1 cm × 1 cm was collected from a site about 0.5 cm distant from the hepatic margin of the left lobe and then placed in a 4% paraformaldehyde solution for fixation for 24 h. The tissue mass was dehydrated in increasing concentrations of ethanol. After hydration, wax-impregnation, embedding and sectioning, HE and Masson staining were sequentially performed. Morphological changes and the degree of fiber hyperplasia of the liver tissue in rats with LC were observed under a light microscope. The grading of LC was performed according to the Knodell HAI, Scheuer, METAVIR, modified Ishak HAI and Chevallier grading systems[8,9].

Quantitative detection of COL Ⅰ, COL Ⅲ and TGF-β1 in liver tissue: Liver tissue was fixed with 10% formalin and embedded in paraffin. Then, serial sections (4 μm) were cut. Immunohistochemical staining was performed by the Streptavidin-biotin complex (SABC) method. Briefly, the paraffin sections were baked in an oven at 60℃ for 1 h and then placed into a pure xylene solution for deparaffinage twice, with each lasting for 15 min. The sections were then placed into a 3% hydrogen peroxide solution at room temperature for 30 min to inactivate endogenous peroxidase, followed by boiling in a 0.01 mol/L citrate buffer under high temperature and pressure conditions for 2 min. After this, the tissues were covered with a normal goat serum blocking buffer and placed in an incubator at 37℃ for 30 min. Subsequently, anti-COL Ⅰ, COL Ⅲ and TGF-β1 primary antibodies were separately added at a dilution of 1:100 to the tissues. The sections were incubated at 37℃ for 30 min. After being kept at 4℃ overnight, the sections were washed thoroughly in PBS (5 min × 3 times), and then biotin secondary antibody was added before incubation at 37℃ for 30 min. The sections were washed again in PBS (5 min × 3 times) and in distilled water (3 min × 3 times), followed by incubation with avidin-peroxidase at 37℃ for 20 min. 3,3'-diaminobenzidine (DAB) was added after smearing. The color developing was monitored under a light microscope. The staining was stopped by washing the sections in distilled water. Following staining with hematoxylin for 1 min and washing in distilled water, the sections were sequentially dehydrated in 95% and 100% ethanol for 5 min each. After air drying, the sections were sealed by neutral gum and observed under a light microscope. Mias-2000 image analysis software (Institute of Image and Graphics, Sichuan, China) was used for quantitative measurement. The ratio of the positive area of COL Ⅰ, COL Ⅲ and TGF-β1 to the overall visual field area was calculated.

Determination of biomechanics of the portal vein: After sacrifice of the rats, the main portal vein was immediately removed and connected to a three-way baroceptor. The pressure was amplified by a dynamic electric resistor which was linked to a computer. The biomechanics of the portal vein were evaluated by measuring the corresponding pressure when the relative volume of blood vessels was changed. The vascular compliance, denoted as C, was calculated using the following formula: $C = 2\pi R \cdot \Delta R/\Delta P$, where R represents the radius of the blood vessel, ΔR and ΔP are the change of the radius and portal vein pressure respectively.

To measure the zero-stress state of the blood vessels, cross-sections were made across the portal vein and arterial rings were obtained. A cut along the ventral margin of the arterial ring was made and the expanded angle of the arteriae aorta was observed under an anatomical microscope. The photographic recording was carried out, followed by printing out on paper. The included angle, i.e. the opening angle, which was formed from the midpoint of the inner lining of the arterial ring to the two broken ends of the inner lining, was measured and used to represent changes in the zero-stress state of the blood vessels. In each rat, 5 open angles of the arterial ring were measured and their mean value was calculated.

Statistical analysis

Data were expressed as mean ± SE. Statistical comparisons among NTau-treated animals, non-treated model controls, and healthy controls were carried out using one-way analysis of variance (ANOVA), followed by *post-hoc* Dunnett's test using the appropriate group as the control. Comparison of degrees of LC between groups was conducted by rank sum test. The analysis was performed on the SPSS for Windows (version 14.0). Differences were considered significant at $P < 0.05$ or $P < 0.01$.

RESULTS

Effect of NTau on hemodynamic alterations

Table 1 summarizes the hemodynamic data of the normal healthy animals, those having undergone LC induction and LC animals treated with NTau for six consecutive weeks. Compared with the normal healthy control group, the LC rats had very significantly higher PVP ($P < 0.01$), and the PVF and PVR were also markedly elevated in the experimentally-induced LC animals, while the MAP and SVR were considerably lower in these model animals

Table 1 Comparison of the portal hemodynamics in different groups of animals

	MAP (mmHg)	PVP (mmHg)	PVF (mL/min per kg)	SVR (mmHg/mL per min/kg)	PVR (mmHg/mL per min/kg)
NML	105.12 ± 8.56	5.26 ± 0.72	43.15 ± 1.23	2.1 ± 0.23	0.07 ± 0.03
LC - NTau	90.23 ± 4.51[a]	7.85 ± 0.91[a]	59.65 ± 4.32[a]	1.61 ± 0.26[b]	0.10 ± 0.04[a]
LC + NTau	93.42 ± 3.25[c]	5.38 ± 0.65[d]	48.21 ± 2.25[c]	1.81 ± 0.35[c]	0.07 ± 0.05[c]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the normal healthy control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* the non-treatment model group.

Table 2 Effects of NTau on the concentrations of NO, cGMP and activity of NOS in portal venous blood

	n	NO (μmol/L)	cGMP (nmol/L)	NOS (U/L)
NML	20	21.41 ± 2.32	0.21 ± 0.02	2.88 ± 1.35
LC - NTau	20	48.56 ± 3.61[a]	0.34 ± 0.04[a]	6.45 ± 0.42[a]
LC + NTau	20	33.14 ± 5.33[b]	0.26 ± 0.01[c]	3.81 ± 1.21[c]

[a]$P < 0.05$ *vs* the control group; [b]$P < 0.01$, [c]$P < 0.05$ *vs* the non-treated model group.

Table 3 Degrees of liver cirrhosis of rats in different experimental groups

	n	Grading of LC[1]						
		S_0	S_I	S_{II}	S_{III}	S_{IV}	S_V	S_{VI}
NML	20	0	0	0	0	0	0	0
LC - NTau[b]	20	0	0	0	0	5	12	3
LC + NTau[d]	20	0	0	2	6	7	5	0

[1] Grading criteria: S0, normal structure of liver tissue, which is characterized by an absence of deposition of abnormal collagen fibers; S_I, occurrence of fibrous septa (FS) due to hyperplasia of collagen fibers in portal areas (P) or areas surrounding the central vein (C); extension of FS within 50% of the distance between two portal areas (P-P) or between portal and central vein areas (P-C); S_{II}, extension of fibrous septa beyond 50% of the P-P or P-C; connection of fibrous septa is either incomplete or complete; absence of completely enclosed or separated hepatic lobules; S_{III}, presence of completely enclosed hepatic lobules by FS; S_{IV}, separation of hepatic lobules by extensive hyperplasia of collagen fibers; destruction of the normal lobular structure; pseudo-lobules formed are mainly large-square-shaped (> 50%); S_V, concomitant presence of large-square- and small-round-shaped pseudo-lobules (both less than 50%); and S_{VI}, intrahepatic pseudo-lobules are mainly small-round-shaped (> 50%). [b]$P < 0.01$ *vs* the normal healthy control; [d]$P < 0.01$ *vs* the non-treated LC group.

($P < 0.05$). The treatment with NTau significantly attenuated PVP ($P < 0.01$), PVF and PVR ($P < 0.05$) when compared with the non-treated model group. Accordingly, the NTau treatment enhanced the MAP and SVR ($P < 0.05$). These experimental data suggest that NTau was able to improve the hemodynamic conditions in animals with LC.

Effects of NTau on the concentrations of NO and cGMP and activity of NOS in the portal venous blood

The effects of NTau on the concentrations of NO and cGMP and NOS activity are summarized in Table 2. When compared with the healthy control group, the amount of NO and cGMP and the activity of NOS were significantly increased in animals with LC. Treatment with NTau caused a significant reduction in NO ($P < 0.01$) and cGMP ($P < 0.05$) content in the blood and in the activity of NOS ($P < 0.05$) when compared with that of the non-treated LC animals. The data derived from this experiment indicate that treatment with NTau significantly mitigates the release and activation of vasoactive substances such as NO, cGMP and NOS.

Histological investigation of the liver tissue

Histological observation of normal rat liver under HE staining was characterized by intact and distinct structure of liver tissue, normal structure of hepatic lobules, and radial distribution of a cord-like arrangement of hepatocytes around the central vein (Figure 1A). In experimentally-induced LC rats, abnormal histological features were observed such as the destruction of the normal structure of hepatic lobules; extensive fibroplasia of interstitial tissue of the liver, which divided the hepatic lobules into different sizes of hepatocellular mass (i.e. formation of pseudo-lobules); extensive fatty degeneration of hepatocytes with some necrosis; and infiltration of many inflammatory cells into the portal areas and hepatic lobules. In the NTau-treated group, the damage of the structure of hepatic lobules was still observed, but with milder fibroplasia, fewer infiltrations of inflammatory cells and only modest foamy degeneration of hepatocytes when compared with the non-treated LC rats. Under Masson staining, only a small amount of collagen was observed in the normal healthy group. In contrast, a large amount of collagen fibrils were found in the LC model rats; however, only thin collagen fibrils were observed in the NTau-treated animals.

The degree of LC in all three animal groups is presented in Table 3. It is evident that the experimental procedure involved in establishing the model, essentially the high fat diet and injection of CCl_4, succeeded in inducing LC in all rats, and the degree of LC ranged from S_{IV} to S_{VI}. The treatment with NTau succeeded in ameliorating the extent of LC.

COL Ⅰ, COL Ⅲ and TGF-β1 changes in the liver tissue

Figure 2 is a bar chart presentation of the ratio of the positive area of COL Ⅰ, COL Ⅲ and TGF-β_1 in the different animal groups. It is evident that the induction of LC in the model rats significantly increased the amount of COL Ⅰ, COL Ⅲ and TGF-β_1, with COL Ⅰ increasing from 9.41 ± 1.36 to 30.63 ± 8.25, COL Ⅲ from 10.55 ± 2.46 to 33.65 ± 7.52, and TGF-β_1 from 15.37 ± 4.62 to 31.28 ± 7.85, respectively. The

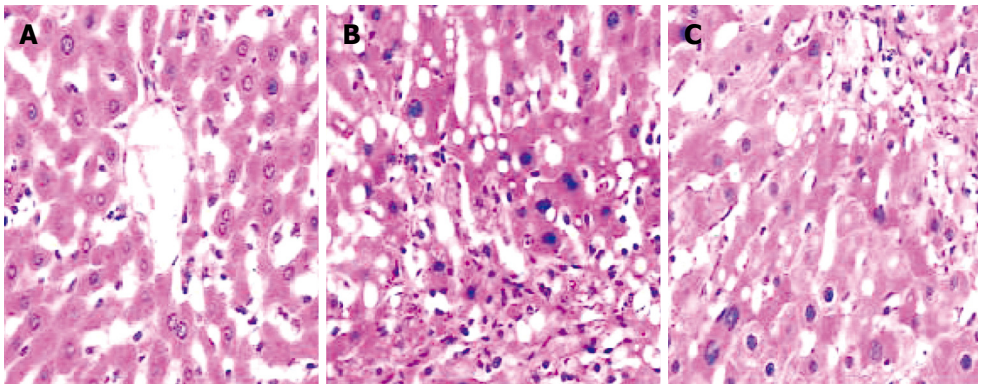

Figure 1 Histological fingdings of liver tissue of the rats in different experimental groups. A: Normal healthy rats; B: LC rats; C: NTau-treated groups. (magnification, 10 × 20).

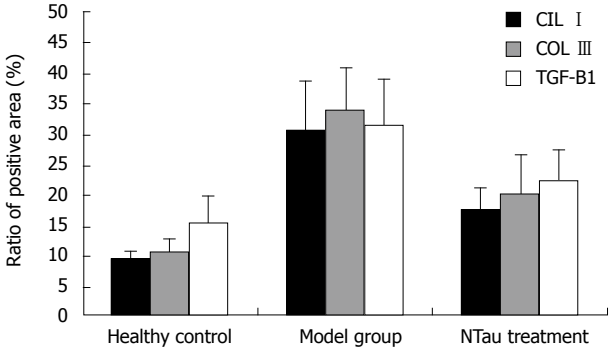

Figure 2 Bar chart presentation of the amount of COL Ⅰ, COL Ⅲ and TGF-β1 in the liver tissue of rats in different experimental groups.

intervention with NTau was capable of reducing the amount of all these parameters in the LC rats, with COL I decreasing from 30.63 ± 8.25 to 17.58 ± 3.6, COL Ⅲ from 33.65 ± 7.52 to 20.34 ± 6.41 and TGF-β1 from 31.28 ± 7.85 to 22.17 ± 5.43, respectively. Figures 3-5 present the immunohistochemical analysis data of the expression of COL Ⅰ, COL Ⅲ and TGF-β1 in the liver tissue of the different experimental groups.

COL I was only slightly expressed in the basement membrane of the central vein of the hepatic lobules in the normal healthy rats. Its expression was significantly elevated in the LC rats with thicker collagen fibrils clearly detectable in the connective tissue surrounding the hepatocytes. Compared with the non-treated model group, the rats in the NTau treatment group had conspicuously reduced expression of COL I and thinner collagen fibrils in the connective tissue surrounding the hepatocytes. There was mild expression of COL Ⅲ in the interstitial connective tissue surrounding hepatocytes in the normal healthy rats. However, significantly increased and thickened COL Ⅲ fibrils were observed in the interstitial connective tissue surrounding hepatocytes in the model group. Treatment with NTau was capable of markedly attenuating the expression of COL Ⅲ, with thinner collagen fibrils being observed. Similarly, TGF-β1 was only expressed mildly in the hepatocellular cytoplasm in normal rats, and there was a strongly positive expression of TGF-β1 in hepatocellular cytoplasm in the LC rats. Similarly to expression of COL I and Ⅲ, treatment with NTau showed a significant reduction in the expression of TGF-β1 in hepatocellular cytoplasm.

Biomechanical and biodynamic changes in the portal vein

Experiments on portal vein biomechanics showed that, in general, the portal compliance decreased as the pressure in the portal vein increased (Figure 6). Among the different groups of rats, the portal compliance in the model group was significantly lower than that of the normal control group ($P < 0.05$), while the NTau treatment was able to markedly improve the portal compliance when compared with the non-treated model group ($P < 0.05$).

In addition, there was a substantial difference ($P < 0.01$) in the opening angle of the portal ring between the model group (89.23 ± 10.47 degree) and the healthy control group (76.25 ± 9.45 degree). The NTau treatment significantly reduced the opening angle of the portal ring (82.61 ± 6.31) when compared to the animals in the model group ($P < 0.05$). The direct proportion of zero-stress to opening angle indicated that the LC rats had a very significant increase in zero-stress level, and the NTau treatment was able to mitigate this abnormal elevation.

DISCUSSION

Taurine, a sulfur-containing β-amino acid [H_2N-$(CH_2)_2$-SO_2OH], is ubiquitously distributed in tissues of mammalian and marine organisms. Taurine was once thought to be a non-functional terminal metabolite of sulfur-containing amino acids in the body. However, recent studies have confirmed that taurine has a wide variety of biological functions including maintaining homeostasis and regulating physiological functions of different systems. Taurine also possesses a wide spectrum of pharmacological activities such as antipyretic, anticonvulsant, antiplatelet-aggregation, hypotensive, immunity-enhancing, liver-protecting and angiotasis-regulating effects[10-17]. It is known that the liver is the main

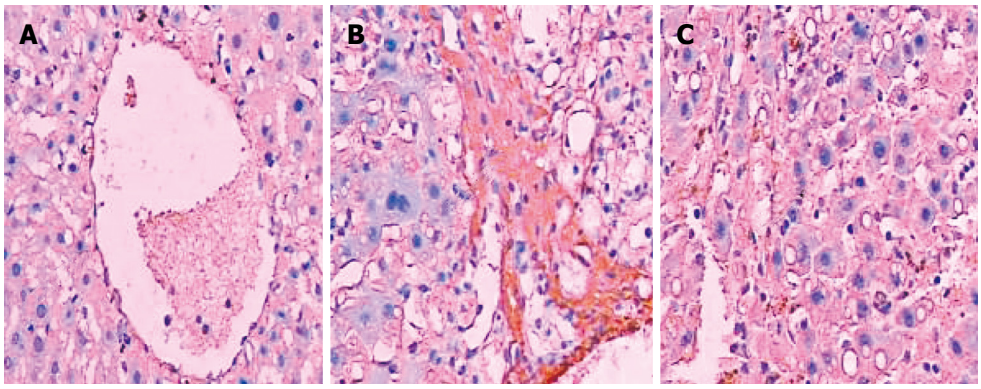

Figure 3 Immunohistochemical analysis of the expression of COL I in liver tissue of rats in different experimental groups. A: Normal healthy rats; B: LC Rats. Note the expression of a large amount of COL Ⅰ; C: NTau-treated rats. Note the reduction of the expression of COL Ⅰ. (magnification, 10 × 20).

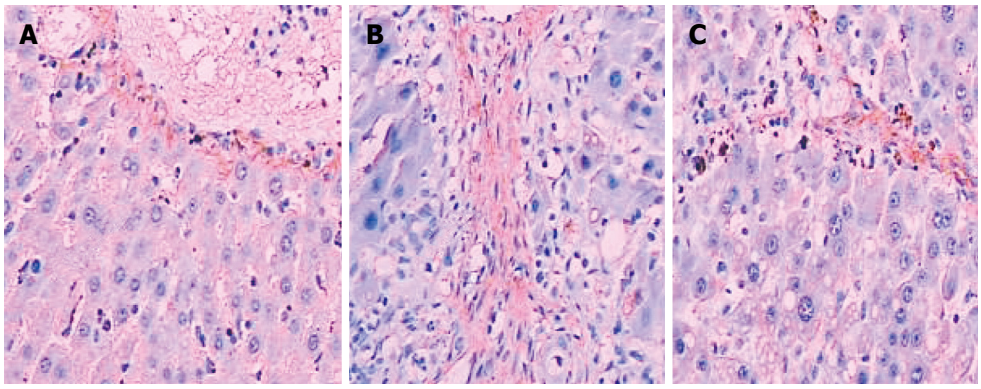

Figure 4 Immunohistochemical analysis of the expression of COL Ⅲ in liver tissue of rats in different experimental groups. A: Normal healthy rats; B: LC rats. Note the abundance in the expression of COL Ⅲ; C: NTau-treated rats. The expression of COL Ⅲ was decreased. (magnification, 10 × 20).

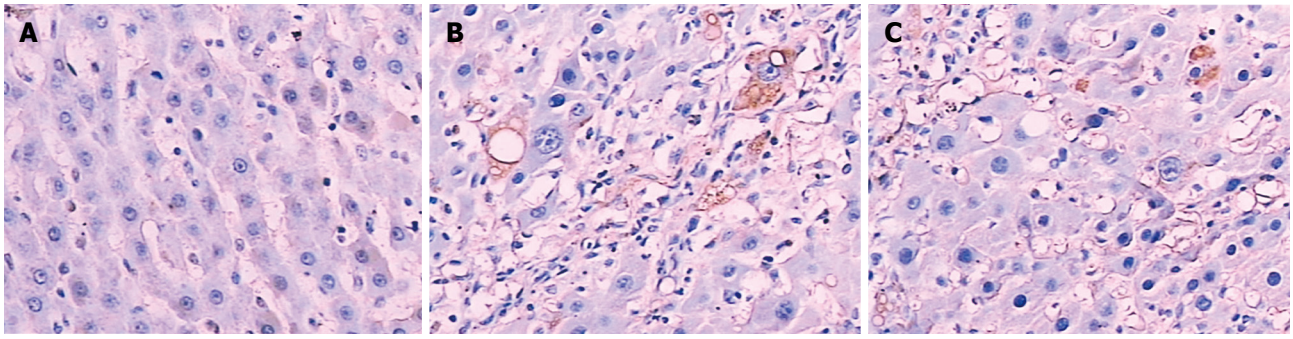

Figure 5 Immunohistochemical analysis of the expression of TGF-β1 in liver tissue of rats in different experimental groups. A: Normal healthy rats; B: LC rats. Note the increased expression of TGF-β_1; C: NTau-treated rats. Note that the expression of TGF-β_1 was reduced. (magnification, 10 × 20).

organ for taurine biosynthesis and also an important target organ for taurine's many biological activities. Interestingly, the amount of taurine in the liver tissue of rats with chronic liver disease falls below the normal range[18].

As a free amino acid, taurine can either be synthesized through chemical reactions or extracted from natural sources. Studies in China and other countries have demonstrated that synthesized taurine may inhibit hepatic fibrosis (HF) by inhibiting collagenation and proliferation of HSCs[19,20]. However, the role of taurine in inhibiting PHT has hitherto not been systematically investigated. Our previous study found that NTau promoted apoptosis of HSCs in a more marked manner than that of synthesized product[21], and that NTau could lower PHT of LC through inhibiting contraction of HSCs[22].

Two seemingly contradictory theories, i.e. the "backward flow" theory and the "forward flow" theory, have been put forward to explain the development of PHT in LC. In the "backward flow" theory, an increase in intrahepatic resistance is thought to be the main reason for the occurrence of PHT. The "forward flow" theory, on the other hand, considers systemic hyperdynamic circulation (SHC) as the primary cause for PHT. Recent studies have shown that both mechanisms are involved in the pathogenesis of PHT[23]. In fact, patients with LC

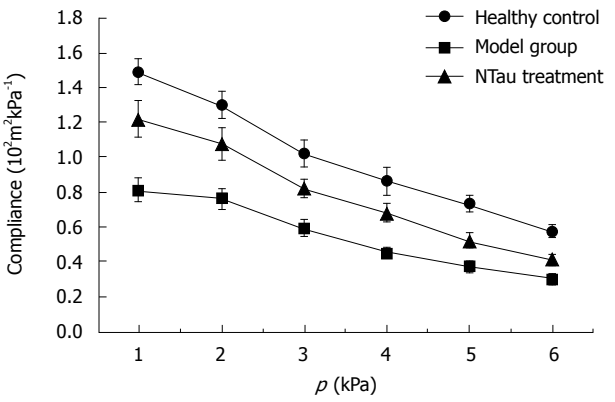

Figure 6 Comparison of the portal ring compliance among rats in different experimental groups.

may present both disturbances of blood circulation (i.e. increased resistance of "backward flow" theory) in liver tissue and abnormality in vasoactive substances (i.e. hyperdynamic circulation of "forward flow" theory).

By combining the "backward flow" and the "forward flow" theory together, this would furnish a better understanding of the pathogenesis of PHT[24,25]. In the "forward flow" theory, the pathogenesis of PHT may be primarily attributed to initiation factors, including dilation of peripheral vessels, decrease in peripheral vascular resistance and mean arterial pressure, increase in blood volume, splanchnic blood flow and cardiac output, and development of systemic hyperdynamic circulatory syndrome (HCS)[23,26]. HCS plays an important role in the maintenance of PHT and is also a primary cause for the development of sodium-water retention, ascites, hepato-renal and hepato-pulmonary syndromes. Our experimental study showed that NTau was capable of decreasing portal blood flow and improving systemic HCS by reducing PVF and increasing SVR and MAP. All these experimental observations indicate that NTau is able to reduce portal pressure through acting on the "forward flow" mechanism.

An increase in endogenous vasodilators is regarded as the most significant factor for peripheral arterial vasodilatation. Consequently, an increase of vasodilators may elicit a wide spectrum of pathophysiological alterations including vasodilatation of peripheral and splanchnic tertiary arterioles, decreased resistance, deficiency of effective arterial blood volume, activation of neurohumoral systems for pressure and water-sodium retention, compensatory expansion of plasma volume, increase in returned blood volume and cardiac output, and splanchnic active hyperemia. All these alterations can lead to hyperdynamic systemic and splanchnic circulation. Among the common vasodilators, NO, glicentin, prostacyclin and calcitonin gene-related peptides are the most important ones. NO is produced by L-arginine in the presence of NOS and may exert biological effects by increasing cGMP *via* activation of guanylate cyclase. It is also an important signaling molecule involved in various physiological processes such as vasodilatation. NO has also been found to be involved in the development of a hyperdynamic circulatory state in LC[27,28]. In our experiments, decreased activity of NOS and reduced amounts of NO and cGMP were observed after the NTau treatment, suggesting that NTau attenuates PHT of LC most probably *via* regulating the NO system.

In the "backward flow" theory, the most critical initiating determinants of portal pressure include the progression of HF, passive structural disorder in the liver, formation of pseudo-lobules and interrupted supply of blood circulation to hepatocytes, which further contribute to hepatocellular necrosis, proliferation of collagen, and formation of regenerative nodules. Consequently, compression and traction of peripheral vessels and bile duct by regenerative nodules and proliferated collagen fibrils may lead to increased resistance to blood flow of portal and hepatic veins, and subsequently result in PHT[25,29,30]. In our study, the treatment with NTau led to the improvement of the structure of liver tissue and significantly lowered the amounts of COLI and COL Ⅲ, indicating that NTau can decrease portal pressure by acting on the "backward flow" mechanism, namely by improving the pathological structure of liver tissue and inhibiting HF.

TGF-β_1 is a multifunctional peptide with a wide range of potential influences on the growth and differentiation of cells, aggregation of extracellular matrix (ECM), and immune response. It is also one of the mediators that is most closely associated with the production of fibrils. In the occurrence and development of HF, TGF-β_1 has an ability to activate HSCs and promote the gene expression of collagen as well as the synthesis and deposition of ECM[31,32]. In our experiments, the level of TGF-β_1 in the NTau treatment group was significantly lower than that of the LC model group, suggesting that NTau inhibits the development of HF, possibly through inhibiting the expression of TGF-β_1.

Increased resistance to PVF may elicit biomechanical changes in the portal vein by causing vascular reconstruction characterized by thickening of vessel walls, narrowing of vessel lumen and proliferation of smooth muscle cells, resulting in further maintenance and exacerbation of PHT. Such alterations in biomechanics subsequently make treatment of LC-induced PHT ever more difficult[33,34]. Therefore, to achieve a favorable therapeutic response in the treatment of PHT of LC, terminating this vicious circle becomes particularly important, and lowering the portal pressure should not be the sole target for intervention. Vessel wall is a viscoelastic tissue with unique biomechanical properties including creep, stress-relaxation and hysteresis. Compliance and zero-stress state are usually used to describe the biomechanical properties of vessels. Indeed, reduced portal compliance and enlarged opening angle were found in rats with LC. The NTau treatment, on the other hand, was able to improve the portal compliance and decrease the opening angle, indicating that NTau could inhibit the development of LC-induced PHT by improving the biomechanical properties of the portal vein.

The pathomechanism of LC-induced PHT is complex,

for it involves SHC, structural alterations in the liver, increased resistance to PVF and biomechanical changes in the portal vein. Our present experimental data derived from the LC rat model unequivocally demonstrate that NTau can inhibit PHT of LC by improving hyperdynamic circulation, structure of liver tissue, and the biomechanical properties of the portal vein by delaying the progression of HF. Given that taurine is an important nutrient in the body with a deficiency in chronic liver diseases, the supplementation of an adequate amount of taurine may improve the functional status of the body. In view of its pharmacological and nutritional values, we believe that treatment with taurine may provide an additional dimension for the management of portal hypertension associated with liver cirrhosis.

COMMENTS

Background

Portal hypertension which acts as the main manifestation of patients in the compensatory stage of cirrhosis of the liver is the material cause of death. Treatment of portal hypertension caused by liver cirrhosis can not only enhance the prognosis of the disease but also improve the patients' quality of life. Hence, the concept of implementing treatment of portal hypertension is gradually attracting more and more attention, changing the passive state of the past which practised symptomatic treatment when complications developed.

Research frontiers

Intensive literature review shows that taurine can suppress the course of liver cirrhosis. This project investigates the function of taurine in inhibiting the course of liver cirrhosis and portal hypertension caused by liver cirrhosis from different aspects.

Innovations and breakthroughs

This study proposes the concept of the integration of the three theories for the formation of liver cirrhosis: the backward flow theory, the forward flow theory and the biomechanics of portal vein theory, then systematically explores the inhibiting effect and possible mechanism of action of taurine on portal hypertension caused by liver cirrhosis.

Applications

The treatment of portal hypertension mainly embodies medication treatment, surgical treatment, interventional treatment, liver transplant *etc*. At present, long-term medication treatment takes the center stage. Though there are plenty of medicines available for portal hypertension caused by liver cirrhosis, there is still a lack of effective medication. As a result, there is sound justification for investigating the therapeutic functions of taurine in portal hypertension caused by liver cirrhosis. Pure natural taurine widely exists in marine fauna. The ocean, which occupies 70.8% of the total earth's surface, is a natural medicinal resource with huge potential. It makes great sense to explore new areas of marine life's application in the search for new medicinal resources.

Terminology

Forward flow theory is one of the theories of the formation of portal hypertension caused by liver cirrhosis. The pathogenesis of PHT may be primarily attributed to initiation factors, including dilation of peripheral vessels, decrease in peripheral vascular resistance and mean arterial pressure, increase in blood volume, splanchnic blood flow and cardiac output, and development of systemic hyperdynamic circulatory syndrome. For backward flow theory, the main cause of portal hypertension caused by liver cirrhosis is the formation of diffuse fibrous septa and regenerative nodules in liver which is followed by hepatic sinus narrowing. Then intra-liver vessels constrict and the resistance of blood flow in the portal system increases which results in passive congestion of the portal system. Finally, portal hypertension develops. As vasodilatation increases, the reactivity of vessels to endogenous vascular-constriction substances falls, then functional arteriovenous fistula and portal-systemic shunting develop and result in hyperdynamic circulation over the whole body while blood flow of the portal vein increases.

Peer review

This is a small but reasonably designed experimental study to examine the impact of natural taurine in a rat model of cirrhosis. The paper particularly concentrates on the effects of this agent on the level of portal hypertension and clearly shows that this is attenuated following repeat dosage by gavage.

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ORIGINAL ARTICLES

Induction of apoptosis and cell cycle arrest in human HCC MHCC97H cells with *Chrysanthemum indicum* extract

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Abstract

AIM: To investigate the effects of *Chrysanthemum indicum* extract (CIE) on inhibition of proliferation and on apoptosis, and the underlying mechanisms, in a human hepatocellular carcinoma (HCC) MHCC97H cell line.

METHODS: Viable rat hepatocytes and human endothelial ECV304 cells were examined by trypan blue exclusion and MTT assay, respectively, as normal controls. The proliferation of MHCC97H cells was determined by MTT assay. The cellular morphology of MHCC97H cells was observed by phase contrast microscopy. Flow cytometry was performed to analyze cell apoptosis with annexin V/propidium iodide (PI), mitochondrial membrane potential with rhodamine 123 and cell cycle with PI in MHCC97H cells. Apoptotic proteins such as cytochrome C, caspase-9, caspase-3 and cell cycle proteins, including P21 and CDK4, were measured by Western blotting.

RESULTS: CIE inhibited proliferation of MHCC97H cells in a time- and dose-dependent manner without cytotoxicity in rat hepatocytes and human endothelial cells. CIE induced apoptosis of MHCC97H cells in a concentration-dependent manner, as determined by flow cytometry. The apoptosis was accompanied by a decrease in mitochondrial membrane potential, release of cytochrome C and activation of caspase-9 and caspase-3. CIE arrested the cell cycle in the S phase by increasing P21 and decreasing CDK4 protein expression.

CONCLUSION: CIE exerted a significant apoptotic effect through a mitochondrial pathway and arrested the cell cycle by regulation of cell cycle-related proteins in MHCC97H cells without an effect on normal cells. The cancer-specific selectivity shown in this study suggests that the plant extract could be a promising novel treatment for human cancer.

Key words: Apoptosis; Cell cycle; Chinese traditional medicine; *Chrysanthemum indicum*; Hepatocellular carcinoma; Herbal medicine

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Li ZF, Wang ZD, Ji YY, Zhang S, Huang C, Li J, Xia XM. Induction of apoptosis and cell cycle arrest in human HCC MHCC97H cells with *Chrysanthemum indicum* extract. *World J Gastroenterol* 2009; 15(36): 4538-4546 Available from: URL: http://www.wjgnet.com/1007-9327/15/4538.asp DOI: http://dx.doi.org/10.3748/wjg.15.4538

INTRODUCTION

Hepatocellular carcinoma (HCC) is known as a common and aggressive malignant tumor worldwide. In China, HCC accounts for 90% of primary liver cancer, which is the second most common cause of death[1]. Chemotherapy plays an important role in the treatment of cancer, but it is limited to a significant extent by its toxicities, significant

resistance to available chemotherapeutic agents and side effects, including myelosuppression, neutropenia and thrombocytopenia[2,3]. One possible way to increase the efficacy of anticancer drugs and to decrease toxicities or side effects is to develop traditional medicines, especially from medicinal plants[4-7].

Natural products have become increasingly important for new pharmaceutical discoveries, and among all the uses for natural products in biomedical science, traditional Chinese herbology has been a pioneering specialty[8]. This is particularly evident in the treatment of cancers, in which more than 60% of drugs are of natural origin[9]. Hence a new medicinal plant with anticancer activities could be a valuable substance in cancer treatment. The flowers of *Chrysanthemum indicum* (*Chrysanthemi Indici Flos*), a *Compositae* plant, is a traditional Chinese medicine and medicinal plant distributed widely in China. Oriental *Chrysanthemum indicum* traditional medicine has been used to treat vertigo, hypertensive symptoms and several infectious diseases such as pneumonia, colitis, stomatitis and carbuncles[10]. A series of studies have demonstrated that *Chrysanthemum indicum* possesses antimicrobial[11], antiinflammatory[11-13], immunomodulatory[12], and neuroprotective effects[14]. Recently, much attention has been devoted to the anticancer activity of *Chrysanthemum indicum* on human PC3, HL 60 and HeLa cancer cells in a dose- and time-dependent manner[15-17]. However, its anticancer mechanism of action is still not clear and needs further investigation.

Apoptosis induced by herbs has become a principal mechanism by which anticancer therapy exerts its effect[7,18]. Upstream initiator caspases including caspase-9 activate downstream effector caspases such as caspase-3, playing a pivotal role in the induction of apoptosis. Caspase-9, triggered by chemotherapeutic drugs, is the apical caspase in the mitochondria-initiated apoptosis pathway, which requires the release of cytochrome C from the mitochondria as well as interaction with Apaf-1[19]. This pathway, associated with changes in the permeability of the outer mitochondrial membrane and the collapse of the membrane potential ($\Delta\psi_m$), results in release of cytotoxic proteins and caspase activation[19].

Cell cycle regulation, a fundamental mechanism determining cell proliferation, is tightly mediated through a complex network of positive factors, such as cyclin-dependent kinases (CDKs) and cyclin, and negative factors, including CDK-inhibitor (CDKI) regulatory molecules. The activated CDK4-cyclin complexes are inactivated by binding to P21, a CDKI[20]. Plant extracts which arrest the cell cycle in cancer cells *via* regulation of CDK and CDKI proteins also can be used for therapeutic intervention[21-23].

The aim of the present study was to examine the anticancer activities of *Chrysanthemum indicum* and related mechanisms in MHCC97H cell lines, typical human HCC cell lines, which are commonly used in the study of antitumor cells[24]. In order to compare the actions of *Chrysanthemum indicum* on normal hepatocytes and endothelial cells, the effects of the extract were examined in rat hepatocytes and a human umbilical vein endothelial cell ECV304 cell line. Furthermore, we investigated the effect of the extract in human MHCC97H cells and the mechanisms underlying its effect on inhibition of proliferation.

MATERIALS AND METHODS

Plant material and extraction

Fresh, ripe fruits of high quality flowers of *Chrysanthemum indicum* were procured from Xi'an traditional medicine group (Shaanxi, China) in March 2006 and the characteristics were consistent with that described in the Pharmacopeia of the People's Republic of China. Moreover, *Chrysanthemum indicum* was also authenticated by Professor Wang Jun-Xian, a taxonomist in the Department of Pharmacy in Xi'an Jiongtong University. The plant materials were air dried at room temperature and then powdered. The dried and powdered fruit of *Chrysanthemum indicum* (500 g) were extracted with 95% ethanol (EtOH) twice under refluxed temperature. After evaporation of organic solvent under reduced pressure, the resultant *Chrysanthemum indicum* EtOH extract (CIE) was concentrated under reduced pressure to give 66.5 g (13.3%) EtOH extract. The dry extract was stored in a refrigerator at -20℃ until use in the experiments. CIE was dissolved in phosphate buffered solution (PBS) and diluted in cultured medium before use, and the control group was made up of medium, PBS and the cells.

Reagents, antibodies, cells, and culture medium

A human MHCC97H HCC cell line was purchased from the Liver Cancer Institute of Fudan University (Shang Hai, China). A human ECV304 cell line was obtained from the Cell Bank of Academia Sinica (Shang Hai, China). MHCC97H cells and ECV304 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) in a humidified incubator containing 5% CO_2 in air at 37℃ before use and subcultured with 0.25% trypsin-0.02% EDTA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123 (Rh123), annexin V and propidium iodide (PI) were purchased from Sigma Corporation (Sigma, St.Louis, MO, USA). DMEM, FBS and trypsin were obtained from GibcoBRL, Grand Island, NY, USA. Anti-caspase-3, anti-caspase-9, anti-cytochrome C, and anti-β-actin were purchased from Santa Cruz Company. Anti-CDK4 and P21 were obtained from eBioscience Corporation.

Hepatocyte preparation, culture and viability assay

Hepatocytes were isolated by the collagenase perfusion method[9] from 10-wk-old male Sprague-Dawley rats anesthetized with intraperitoneal administration of ketamine. The viability of the isolated hepatocytes was over 90% as determined by 0.2% trypan blue exclusion. The cells were plated in 35 mm plastic dishes at a density of 3×10^5 cells/mL in 2 mL of Williams' Medium E supplemented with 10% FBS, and were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37℃ overnight. After overnight incubation, the culture medium was changed to fresh medium, and

cultures were incubated with varying concentrations of CIE 400, 800 and 1200 μg/mL for 24 h. According to a previous report[25], the cells were then trypan blue stained and a hemocytometer was used to determine the total cell count and viable cell number. Viability of cells were determined as follows: viability (%) = viable cell number/total cell count × 100%.

Cell viability assay

ECV304 cells were seeded in a 96-well plate (5×10^4 cells/well). After 24 h seeding, cells were treated with CIE (400, 800 and 1200 μg/mL) for 24 h, in 3 parallel wells each, with untreated cells serving as a control, then the MTT assay as described by Xiao *et al*[26] was performed. At 24 h, 20 μL MTT solution (5 mg/mL) was added to each well and incubated for a further 4 h. The medium was removed and 200 μL DMSO was added to each well. Absorbance (*A*) at 570 nm was measured using a microculture reader. The percentage of viable cells was calculated as follows: (*A* of experimental group/*A* of control group) × 100%.

MHCC97H cells were seeded into 96-well plates at a density of 5×10^4/well and were then incubated with different concentrations of CIE (400, 800 and 1200 μg/mL) for 24, 48 and 72 h, then the MTT assay was performed. At 24, 48 and 72 h, 20 μL MTT solution (5 mg/mL) was added to each well, and the cells were further incubated at 37℃ for 4 h. The MTT assay and calculation of the percentage of viable cells were the same as described above.

Apoptosis assays

In accordance with the study of Chen *et al*[27], the apoptotic rates were analyzed by flow cytometry using an annexin V-FITC/PI kit. Staining was performed according to the manufacturer's instructions, and flow cytometry was conducted on a FACS Caliber (Becton Dickinson, Mountain View, NJ, USA). Cells that were annexin V (-) and PI (-) were considered viable cells. Cells that were annexin V (+) and PI (-) were considered early apoptotic cells. Cells that were annexin V (+) and PI (+) were considered late apoptotic cells.

Cell cycle assays

Cell cycle analyses were carried out by the method of Vinodhkumar *et al*[28]. Briefly, cells were incubated in culture media alone or culture media containing 400-1200 μg/mL of CIE, at 37℃ for 48 h. Cells were harvested in cold PBS, fixed in 70% EtOH, and stored at 4℃. Fixed cells were washed with PBS once and suspended in 1 mL of PI staining reagent 50 mg/mL containing 100 μg/mL Rnase, and were then incubated in the dark for 30 min. The distribution of the cell cycle was measured by a Becton Dickinson FACS analysis system and quantitation of cell cycle distribution was carried out using Multicycle Software.

Detection of mitochondrial membrane potential ($MMP\Delta\psi_m$)

Loss of $MMP\Delta\psi_m$ was assessed by flow cytometry, using a fluorescent indicator Rh123, as described by Tang *et al*[29] and Li *et al*[30]. Briefly, cells were treated with different concentrations of CIE. Then, Rh123 working solution was added to the culture at a final concentration of 2 μg/mL and then incubated in the dark at 37℃ for 30 min. Cells were then washed with PBS, and fluorescence of Rh123 was detected immediately using a FACS Caliber, at an excitation wavelength of 488 nm and emission wavelength of 525 nm.

Western blotting

Cancer cells (2.5×10^7/well) were treated with different concentrations of CIE for 24 or 48 h. To extract cytoplasmic protein as by the method of Li *et al*[31], cells were collected by centrifugation at 200 r/min for 10 min at 4℃. The cells were washed twice with ice-cold PBS, followed by centrifugation at 200 r/min for 5 min. The cell pellet was then suspended in ice-cold cell extraction buffer for 30 min on ice. In addition, as described by Hsu *et al*[32], cells were then lysed in a sample buffer, followed by sonication and denaturation. Protein concentrations were measured using DC Protein Assay (Bio-Rad, Hercules, CA) and equal amounts of protein (50 μg) were subjected to SDS-PAGE on 12% gel. The proteins were then electrophoretically transferred to nitrocellulose membranes and processed for immunoblotting. Membranes were first blocked with 5% non-fat dry milk overnight at 37℃ and immunolabeled using primary antibodies. Goat anti-rabbit horseradish peroxidase-conjugated antibodies (Cell Signaling Technology, Beverly, MA) were used as secondary antibodies and detected with enhanced chemiluminescence (Amersham, USA). Equal loading of each lane was evaluated by immunoblotting using the same membranes with β-actin antibodies after detachment of previous primary antibodies. The band density for the target protein in each sample was measured with image analysis software (Gene Genus, Gene Company) and normalized to β-actin expression.

Statistical analysis

All data were expressed as mean ± SE. Statistical analysis was performed with analysis of variance (ANOVA) using the statistical software SPSS 11.0. *P*-values < 0.05 were regarded as statistically significantly.

RESULTS

Effect of CIE on numbers of viable rat hepatocytes and ECV304 cells

In order to compare the effects of CIE on rat hepatocytes and human ECV304 cells, the numbers of viable cells were measured. As shown in Figure 1A, CIE did not decrease the number of viable rat hepatocytes, used as a normal cell model. To confirm the activity of CIE in human cells, we measured the number of viable cells treated with varying concentrations of CIE in human endothelial cells (ECV304). CIE did not reduce the number of viable ECV304 cells at any dose (Figure 1B). Therefore, the effect of CIE in inhibiting

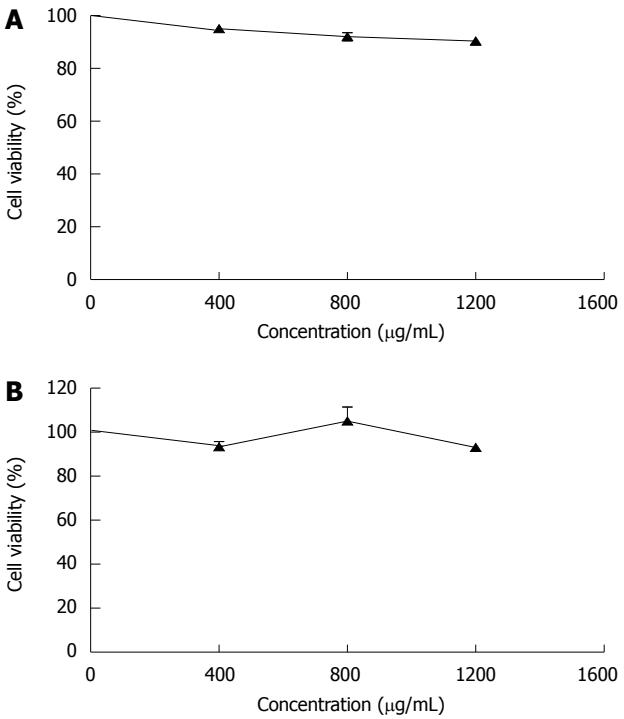

Figure 1 Effects of *Chrysanthemum indicum* extract (CIE) on cell viability of normal cells. A: Rat hepatocytes; B: Human umbilical vein endothelial cell line ECV304. Various concentrations of CIE were added, and the cells were incubated for 24 h. Cell viability was measured by 0.2% trypan blue exclusion (A) and MTT assay (B) respectively. Results presented are representative of 3 independent experiments.

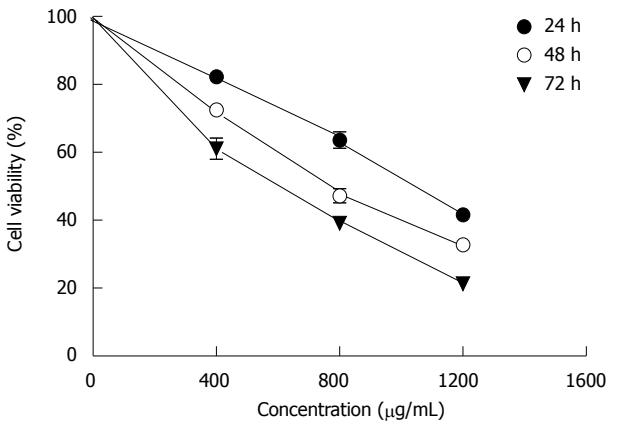

Figure 2 Dose-response curves for CIE in MHCC97H cells, following 24 h, 48 h and 72 h incubation, as assessed by MTT assay. CIE produced a concentration- and time-dependent decrease in cellular proliferation. Results presented are representative of 3 independent experiments.

proliferation of MHCC97H cells and its mechanism were examined in subsequent experiments.

Cytotoxic activities of CIE against human HCC cells

When MHCC97H cells were incubated with 400-1200 μg/mL CIE for 24, 48, 72 h, as shown in Figure 2, there was a significant dose-dependent reduction in cell viability. The IC_{50} value at 24 h was 1009 ± 130 μg. When 1200 μg/mL CIE was incubated with cancer cells for 72 h, viable cells amounted to only 25% of control. These findings indicated that CIE significantly decreased

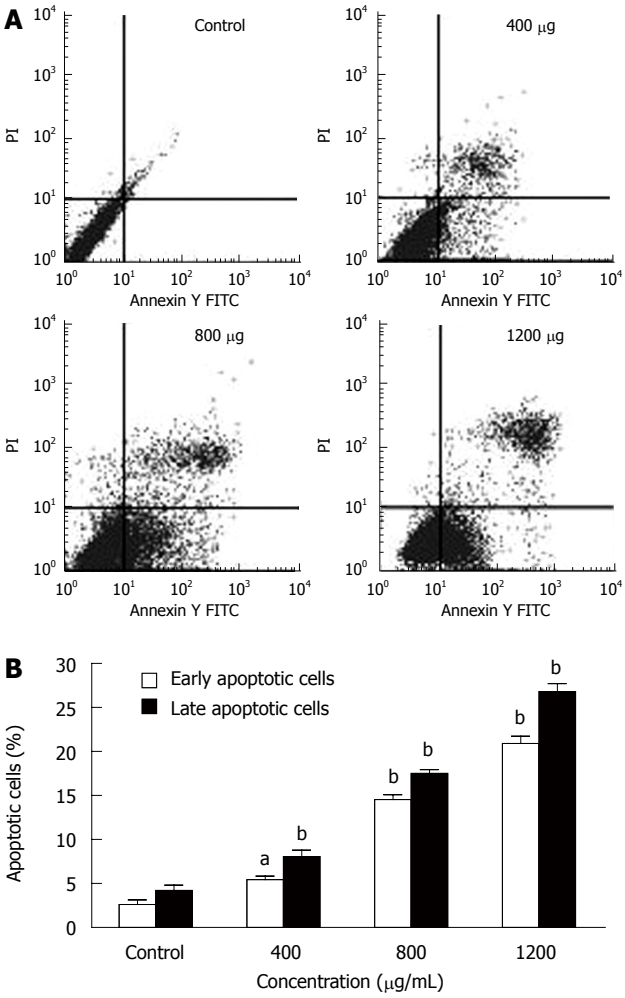

Figure 3 Effect of CIE on the induction of MHCC97H cell apoptosis. A: The apoptosis of MHCC97H cells induced by CIE were determined by flow cytometry at 24 h; B: CIE induced a concentration-dependent increase in early and late cellular apoptosis. Results presented are representative of 3 independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

proliferation of MHCC97H cells in a dose- and time-dependent manner. Hence, the proliferation inhibitory effect of CIE on MHCC97H and its mechanisms were tested in the following experiments.

CIE induces MHCC97H cell apoptosis

MHCC97H cells were incubated with different CIE concentrations (400, 800 and 1200 μg/mL) for 24 h and were analyzed by flow cytometry. Pretreatment of MHCC97H cells with various concentrations of CIE induced significant apoptosis (Figure 3A). The numbers of early and late apoptotic cells were significantly increased compared with the control group (Figure 3B). The proportion of early and late apoptotic cells in the 1200 μg/mL treatment group was more than 10 times higher than in the drug-free cells.

Effects of CIE on cell morphology

After incubation with CIE at different concentrations (400, 800, 1200 μg/mL), the cells were examined by phase contrast microscopy for evidence of morphological apoptosis induced by CIE (Figure 4). The control cells

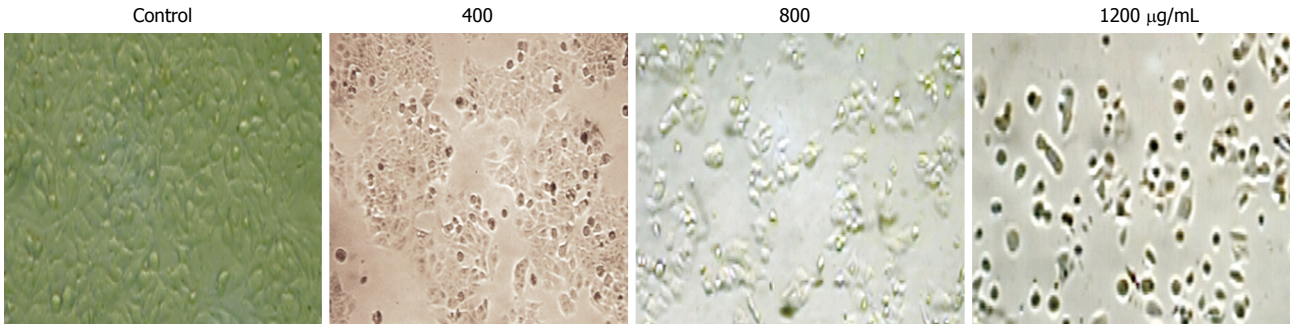

Figure 4 Morphological changes in MHCC97H cells with CIE. Cells were observed by phase contrast microscopy in controls and after treatment with 400, 800, 1200 μg/mL CIE (× 200).

showed a typical polygonal and intact appearance (control), whereas the CIE-treated cells displayed morphological changes with preapoptotic characteristics, such as cellular shrinkage (400, 800 μg/mL), rounding (800 μg/mL), and poor adherence (1200 μg/mL), as well as round floating shapes (1200 μg/mL).

CIE causes loss of MMPΔψm

To explore whether CIE-induced apoptosis involved the MMPΔψm, we used a fluorescent indicator, Rh123 to detect the MMPΔψm when MHCC97H cells were treated with 400-1200 μg/mL of CIE for 24 h. As shown in Figure 5A and B, after exposure to different CIE doses, cells exhibited much lower Rh123 staining (236.7 ± 9.3, 170.7 ± 13.9, 105 ± 10.5) than controls (275 ± 14.5: $P < 0.05$ or $P < 0.01$), indicating that CIE can significantly decrease MMPΔψm associated with cancer cell apoptosis.

CIE-induced apoptosis is caspase-dependent

To determine whether apoptosis induced by CIE was a mitochondrial-dependent caspase pathway, we further tested whether cytochrome C could be released from the mitochondria into the cytoplasm. As shown in Figure 6A, although there was no detectable cytochrome C in the cytosolic fraction of continuously growing MHCC97H cells, the level of cytochrome C released from the mitochondria increased dose-dependently in the presence of CIE concentrations ranging from 400 to 1200 μg/mL. In accordance with mitochondrial cytochrome C release into the cytoplasm, caspase-9 protein expression was increasingly detected. Accordingly, caspase-3 protein expression was also increased dose-dependently on exposure to CIE (Figure 6A and B). Taken together, these findings suggest that CIE exerted a significant apoptotic effect on MHCC97H cells in a concentration-dependent manner through the mitochondrial pathway, and was accompanied by a decrease in MMPΔψm, release of cytochrome C, and activation of caspase-9 and caspase-3.

Cell cycle analysis

The cell cycle of cancer cells was also determined by flow cytometry. MHCC97H cells treated with CIE 400, 800, 1200 μg/mL for 48 h showed an accumulation of cells in the S phase of the cell cycle. In contrast, the population of cells in G0-G1and G2/M phases was

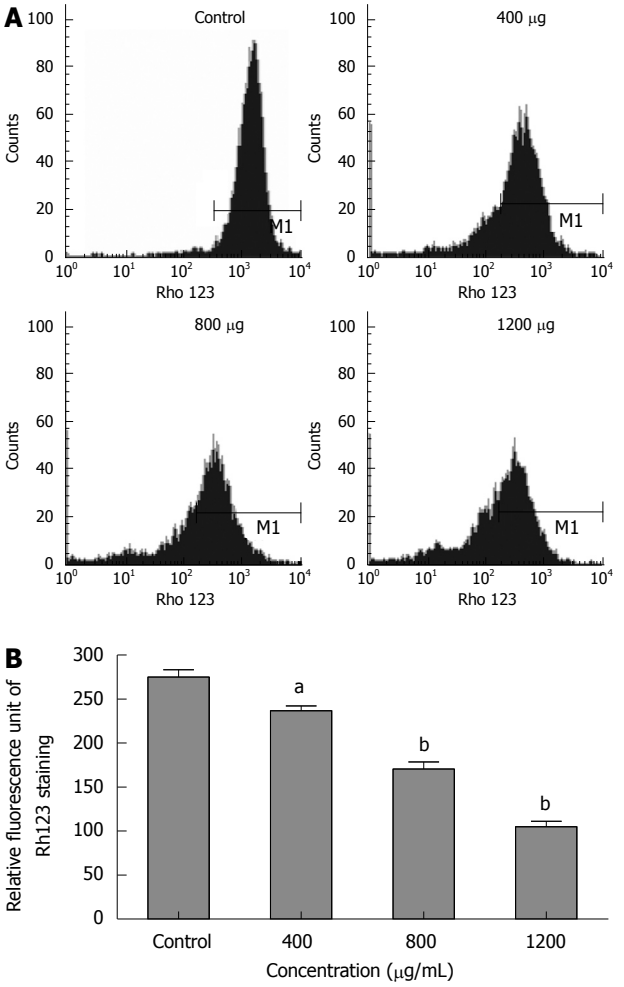

Figure 5 Effect of CIE on MHCC97H cellular mitochondrial membrane potential (MMPΔψm). A: The MMPΔψm of MHCC97H cells were determined by flow cytometry at 24 h after CIE; B: Results presented are representative of 3 independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

significantly decreased, especially at 400 μg/mL CIE (Figure 7A-C). In addition, as shown in Figure 7A, cancer cells incubated with higher doses of CIE for 48 h also showed a sub-G1 peak indicating apoptosis. These observations suggest that a small number of cancer cells escape from the S phase and undergo apoptosis, particularly at the 1200 μg/mL CIE concentration. Therefore, with the dose-dependent increase in cancer cell apoptosis, the proportion of cells arrested in the

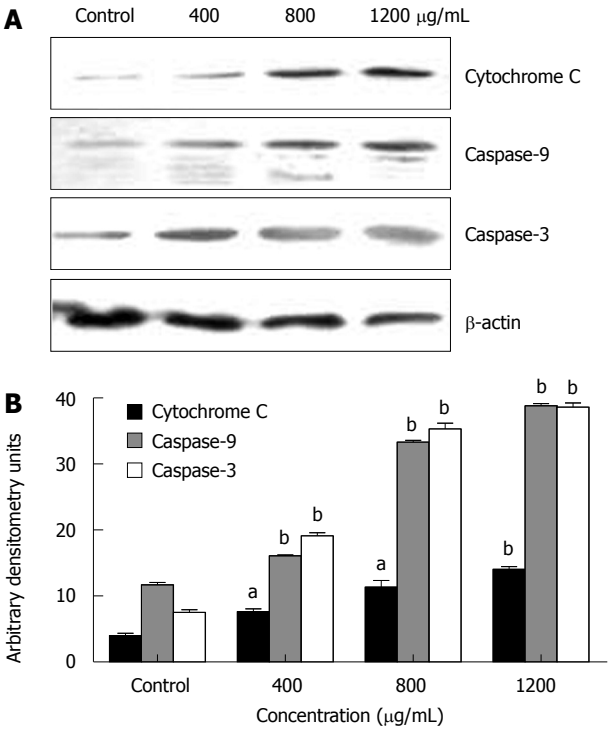

Figure 6 Effect of CIE on apoptosis-related protein expression. A: The expressions of cytochrome C, caspase-9 and caspase-3 were assessed by Western blotting; B: Results presented are representative of 3 independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

S phase by CIE decreased as the CIE concentration increased from 400 to 1200 μg/mL.

The mechanism of action of CIE on the cell cycle

To determine the mechanism by which CIE arrested the cell cycle in the S phase, Western blotting was used to determine the expression levels of cell cycle-regulating proteins including P21 and CDK4. P21 protein expression was markedly higher than that of the control group at all CIE doses tested. In contrast, CDK4 levels were significantly lower than that of the control group, as shown in Figure 8A and B. The results suggested that CIE could arrest the cell cycle *via* upregulation of P21 and downregulation of CDK4.

DISCUSSION

So far, the underlying mechanisms of the pharmacological effect of *Chrysanthemum indicum* in cancer therapy have been unclear, and this study examined the effect of CIE and its underlying mechanisms on inhibition of tumor cell proliferation. In the present study, we have demonstrated that CIE potently inhibits the proliferation of MHCC97H cells by inducing apoptosis (Figures 3 and 4) and arresting the cell cycle (Figure 7) but has no cytotoxicity in rat hepatocytes and human endothelial cells (ECV304) that were used as representatives of normal cells (Figure 1).

Morphological changes in apoptotic characteristics, such as cellular shrinkage, rounding, poor adherence, and round floating shapes in CIE-treated cells were also observed by phase-contrast microscopy (Figure 4). The

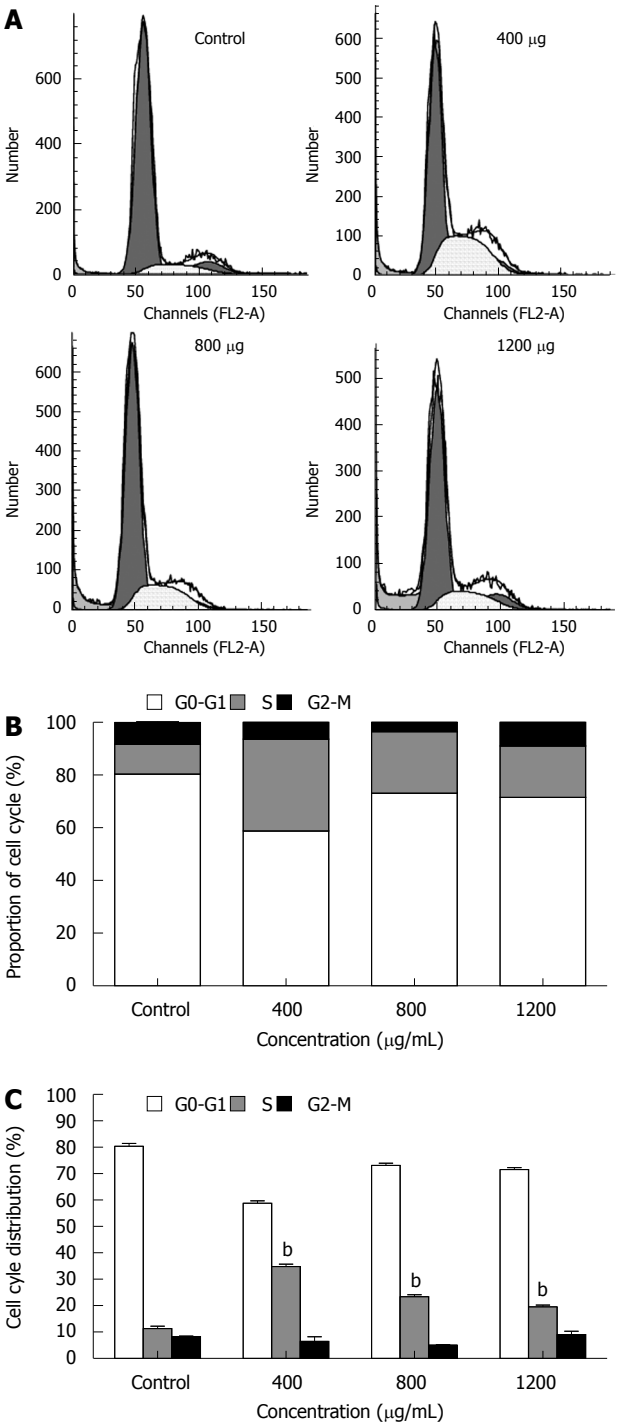

Figure 7 Effect of CIE on MHCC97H cell cycle. A: The cell cycle of MHCC97H cells in the presence of CIE was determined by flow cytometry at 48 h; B and C: Results presented are representative of 3 independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

induction of cancer cell apoptosis without side effects is recognized as an important target in cancer therapy. Apoptosis triggered by activation of the mitochondrial-dependent caspase pathway represents the main programmed cell death mechanism[19]. The mitochondrial-dependent apoptosis pathway is activated by various intracellular stresses that induce permeabilization of the mitochondrial membrane, leading to cytochrome C release[33]. Flow cytometry with Rh123 staining showed

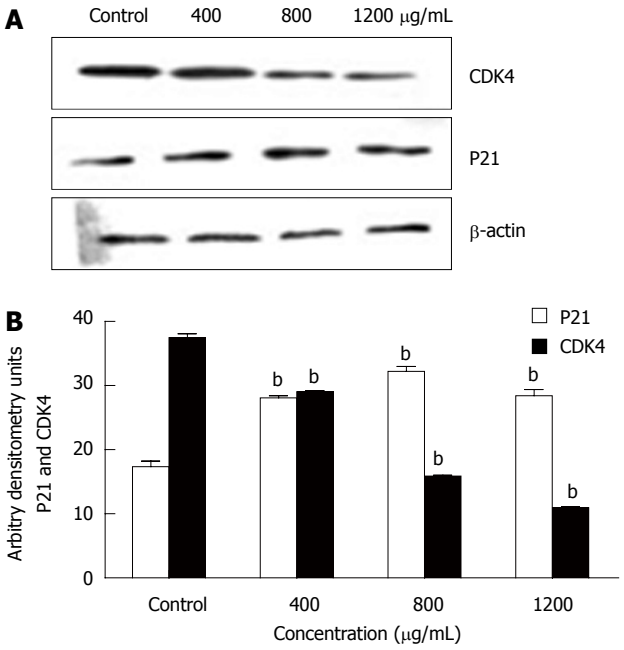

Figure 8 Effect of CIE on cell cycle-related protein expression. A: The expression of CDK4 and P21 was assessed by Western blotting; B: Results presented are representative of 3 independent experiments. $^{b}P < 0.01$ *vs* control group.

disruption of MMPΔψm in the CIE-treated cells (Figure 5), indicating that the mitochondrial apoptotic pathway played a pivotal role in CIE-induced apoptosis of MHCC97H cells. Furthermore, cytosolic cytochrome C activates pro-caspase-9 by binding to Apaf-1 and in the presence of dATP, and contributes to activation of caspase-9 and caspase-3, thus triggering apoptosis[34,35]. In this study, caspase-9 and caspase-3 levels reached a maximum at about 24 h in the cells after exposure to CIE 1200 μg/mL (Figure 6). These results indicated that CIE-induced apoptosis of MHCC97H cells was mediated by loss of MMPΔψm, increased cytosolic translocation of cytochrome C, and activation of caspase-9 and caspase-3.

The inhibition of tumor cell growth without toxicity in normal cells has attracted attention as an important target in cancer therapy. Dysregulation of the cell cycle mechanism has also been shown to play an important role in various cancer cell growths, including HCC. In this study, CIE inhibited MHCC97H cell proliferation partly as a result of accumulation of cells in the S phase of the cell cycle. The present study, to the best of our knowledge, is also the first to demonstrate that CIE induced arrest of the cell cycle in the S phase in HCC cells (Figure 7). The S phase is associated with DNA synthesis and plays a crucial role in cell cycle progression. Recently, a series of S phase chemotherapeutic agents such as *Smilax glabra Roxb*[36], baicalein from *Scutellariae radix* roots[37] and others[38] have been found to inhibit cancer cells, including HCC. Furthermore, in accordance with these results, CIE upregulated P21 and downregulated CDK4 (Figure 8), indicating that cell cycle-related proteins were involved in the CIE-induced cell cycle arrest in MHCC97H cells. One of the CDKI proteins, P21 can perform a key function in controlling cell cycle progression by negatively regulating CDK4 activity[38]. Inappropriate expression of cell cycle-related proteins, such as CDK4 and P21, could be one of the major factors contributing to HCC development[39]. Moreover, CDK4 and P21 play important roles in regulation of the S phase of the cell cycle[37,38,40,41]. These findings, taken together with the present study, suggest that upregulation of P21 and downregulation of CDK4 are likely to be involved in the S phase arrest induced by CIE in HCC cells.

Additionally, in clinical studies, *Chrysanthemum indicum* can be used in combination with other chemotherapeutic agents or traditional Chinese medicines in treatment of other cancers. Xiang *et al*[42] found that patients with metastatic breast cancer postoperatively receiving *Chrysanthemum indicum* as one of the main components, in combination with other traditional Chinese medicines, had a 5-year overall survival rate of 70% and a complete response rate of 60%, and in combination with chemotherapeutic agents, had a 5-year overall survival rate of 77% and a complete remission rate of 80%, without adverse effects. Bi *et al*[43] demonstrated that *Chrysanthemum indicum,* in combination with traditional Chinese medicines, achieved a response rate of 67% in advanced stage esophageal carcinoma patients, without myelosuppression or toxicities of the liver and kidney.

In conclusion, different effects of CIE treatment were observed in cancer and normal cells. CIE exerted a significant apoptotic effect on MHCC97H cells through the mitochondrial-dependent caspase-3 pathway. It arrested the cell cycle of cancer cells in the S phase by upregulation of P21 and downregulation of CDK4. In addition, the cancer-specific selectivity shown in this study suggests that the herb could be a promising novel plant with potential in the treatment of human cancer without side effects.

COMMENTS

Background

Ethnopharmacology used in folk medicine continues to be an important source of discovery and development of novel therapeutic agents in cancer. The flowers of *Chrysanthemum indicum, a Compositae* plant, is common in ethnopharmacology, and has long had wide spread use in the treatment of hypertension, colitis, pneumonia and carbuncles by traditional Chinese practitioners. Recently, much attention has been devoted to the anticancer activity of *Chrysanthemum indicum,* especially in hepatocellular carcinoma (HCC). However, the underlying mechanisms of the pharmacological effect of the plant extract in cancer therapy have been largely undetermined.

Research frontiers

Induction of apoptosis and arrest of the cell cycle by plant extracts has become a principal mechanism by which anticancer therapy is effective. Apoptosis triggered by the activation of the mitochondrial-dependent caspase pathway represents the main programmed cell death mechanism. Permeabilization of the outside mitochondrial membrane plays a vital role in cell apoptosis, during which loss of the mitochondrial membrane potential and release of cytochrome C into the cytosol, followed by caspase-9-dependent activation of caspase-3 occurs, resulting in apoptosis. Dysregulation of the cell cycle mechanism has also been shown to perform an important function in growth of various cancer cells, including HCC. The S phase is associated with DNA synthesis and plays a crucial role in cell cycle progression. One of the CDKIs, P21, can influence key functions in the control of the cell cycle by negatively regulating CDK4 activity, and plays an important role in regulation of the S phase of the cell cycle.

Innovations and breakthroughs

So far, there has been no evidence found to show that the mitochondrial

pathway is involved in induction of apoptosis and cell cycle arrest by *Chrysanthemum indicum* extract (CIE) in human HCC cells. Therefore, the present study examined the anticancer activities of CIE and related mechanisms in MHCC97H cell lines. The data showed that CIE could induce apoptosis and arrest the cell cycle of MHCC97H cells. CIE exerted a significant apoptotic effect on MHCC97H cells through the mitochondrial-dependent caspase-3 pathway, and arrested the cell cycle in the S phase in cancer cells by upregulation of P21 and downregulation of CDK4.

Applications

This study suggests that *Chrysanthemum indicum* could be a promising plant with potential in the novel treatment of human cancer, particularly HCC.

Peer review

The manuscript written by Li ZF *et al* describes that *Chrysanthemum indicum* extract can induce apoptosis and cell cycle arrest in a hepatoma cell line. Many patients with HCC still die each year, and novel therapeutic strategies are needed. The data are encouraging and promising.

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ORIGINAL ARTICLES

Effects of lysophosphatidic acid on human colon cancer cells and its mechanisms of action

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Abstract

AIM: To study the effects of lysophosphatidic acid (LPA) on proliferation, adhesion, migration, and apoptosis in the human colon cancer cell line, SW480, and its mechanisms of action.

METHODS: Methyl tetrazolium assay was used to assess cell proliferation. Flow cytometry was employed to detect cell apoptosis. Cell migration was measured by using a Boyden transwell migration chamber. Cell adhesion assay was performed in 96-well plates according to protocol.

RESULTS: LPA significantly stimulated SW480 cell proliferation in a dose-dependent and time-dependent manner compared with the control group ($P < 0.05$) while the mitogen-activated protein kinase (MAPK) inhibitor, PD98059, significantly blocked the LPA stimulation effect on proliferation. LPA also significantly stimulated adhesion and migration of SW480 cells in a dose-dependent manner ($P < 0.05$). Rho kinase inhibitor, Y-27632, significantly inhibited the up-regulatory effect of LPA on adhesion and migration ($P < 0.05$). LPA significantly protected cells from apoptosis induced by the chemotherapeutic drugs, cisplatin and 5-FU ($P < 0.05$), but the phosphoinositide 3-kinase (PI3K) inhibitor, LY294002, significantly blocked the protective effect of LPA on apoptosis.

CONCLUSION: LPA stimulated proliferation, adhesion, migration of SW480 cells, and protected from apoptosis. The Ras/Raf-MAPK, G12/13-Rho-RhoA and PI3K-AKT/PKB signal pathways may be involved.

Key words: Lysophosphatidic acid; Colon cancer; Proliferation; Apoptosis; Adhesion; Migration; Signal pathway

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INTRODUCTION

Colorectal cancer (CRC) is a common form of cancer and a major cause of cancer death. The incidence of CRC has been rapidly increasing in recent years. Although the incidence of CRC was substantially lower in Asia than in the USA in the mid-twentieth century, the incidence in Japan and China has been rapidly increasing[1,2]. Thus, CRC is now a leading cancer killer worldwide.

Lysophosphatidic acid (LPA) was first found in the ascitic fluid from ovarian cancer patients. It is a bioactive glycerophospholipid generated and released by platelets, macrophages, epithelial cells, and some tumor cells. Studies have shown the presence of high levels of LPA in the ascitic fluid of patients with ovarian cancer[3] and LPA is known to be an "ovarian cancer activating factor", which exerts growth factor-like effects through four specific G protein-coupled receptors (LPA1-4).

LPA is a potent mediator with a broad range of cellular responses, including regulation of cell proliferation, protection from apoptosis, modulation of chemotaxis and transcellular migration[4,5], which mediates survival of ovarian cancer cells, macrophages, fibroblasts, and neonatal cardiac myocytes. Some of these cellular responses indicate that LPA is a mediator of tumor progression.

In a recent study we found that plasma levels of several LPAs, including 18:1-LPA and 18:2-LPA, were significantly increased in CRC patients compared with controls[6]. This is the first report of high levels of LPA in plasma of CRC patients. It implies that LPA may play roles in CRC development. In order to clarify these roles of LPA in CRC development, the LPA effect on the CRC cell line, SW480, was studied *in vitro*.

LPA was found firstly to be increased in the body fluids of ovarian cancer patients, so the roles of LPA in ovarian cancer have been widely studied. A few preliminary studies of LPA in CRC have been reported, but not in the cell line SW480. Our previous study revealed a high expression of LPA receptors on SW480 cells, especially LPA receptor 2[7,8]. This study aimed to investigate the effect of LPA on proliferation, migration, adhesion, and apoptosis in the CRC cell line, SW480.

MATERIALS AND METHODS

1-Oleoyl LPA (18:1 LPA) was purchased from Avanti Polar Lipids (Birmingham, Al, USA). Inhibitor of phosphoinositide 3-kinase (PI3K), LY290042, and inhibitor of mitogen-activated protein kinase (MAPK), PD98059, are from Cell Signaling (Beverly, MA, USA). Rho kinase inhibitor, Y-27632, was from Biomol (Beverly, MA, USA). Boyden transwell migration chambers and 24-well plates were from Corning Costar Corporation (Cambridge, MA).

SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mL/L fetal bovine serum, streptomycin (100 mg/L), and penicillin (100 kU/L) at 37℃ in 50 mL/L CO_2 incubator. Cells were serum starved for 12 h before LPA treatment.

Cell proliferation assay

Methyl tetrazolium (MTT) colorimetry assay was employed to measure cell proliferation. SW480 cells (2×10^3/well) were seeded in 96-well plates. After cells were starved for 12 h, DMEM containing LPA supplemented with 1 g/L bovine serum albumin was put into the wells. After 24, 48, 72 and 96 h of culture, 20 μL of MTT solution (5 g/L) was added to each well. Four hours later, the medium was removed and 150 μL of dimethyl sulfoxide was added to each well. Absorbance value was measured at 490 nm on a Microplate Reader (EXL800). Each assay was performed in quintuplicate.

Annexin V staining

After treating with cisplatin or 5-FU, LPA and/or inhibitors, the cells were resuspended in binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$). Then the cells were stained with 5 μL of annexin-FITC and 5 mg/L propidium iodide (PI), and then analyzed by flow cytometry (FACSCalibur cytometer, BD Biosciences), and CellQuest (BD Biosciences) was used to quantify the apoptotic cells. Experiments were performed in triplicate.

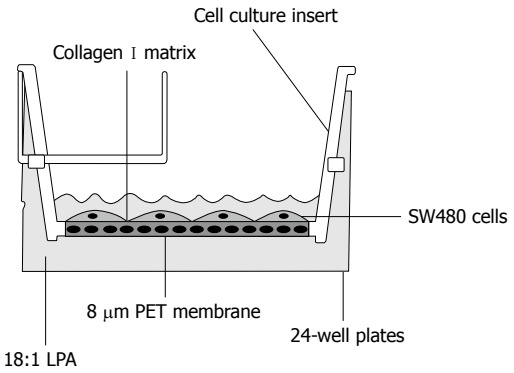

Figure 1 Costar transwell cell culture chamber inserts.

Cell adhesion assay

Flat-bottom 96-well plates were coated with 2 μg of collagen I (0.04 g/L) (Sigma Chemical Co.) in phosphate-buffered saline overnight at 4℃. Plates were blocked with 2 g/L BSA for 2 h at room temperature followed by washing three times with DMEM. SW480 cells (4×10^4/well) were added to each well. Four hours later, unbound cells were removed, washing twice with DMEM. Bound cells were fixed by methanol and stained with crystal blue. Stained cells were counted with a phase contrast microscope. Experiments were performed in triplicate.

Cell migration assay

Migration assays were performed in Costar transwell cell culture chamber inserts (coated with collagen I ; Corning Costar Corporation, Cambridge, MA) with an 8 μm pore size as described as Figure 1. Briefly, SW480 cells (5×10^4 cells in 100 μL of starvation medium) were used for cell migration, which was conducted for 4 h at 37℃. Migrated cells were fixed, stained and counted in five randomly (× 200) selected fields with a phase contrast microscope, and the average numbers of cells per field were counted.

Administration of LPA and inhibitors

SW480 cells were starved in serum-free DMEM for 12 h and treated with LPA at different doses, and then reconstituted in DMEM containing 10 g/L BSA. All inhibitors including LY294002 (50 μmol/L), PD98059 (10 μmol/L), and Y-27632 (10 μmol/L) were applied to cells 30 min before the action of LPA.

Statistical analysis

Statistical significance was assessed by one-way ANOVA using SPSS software. Data are presented as the mean ± SE.

RESULTS

LPA stimulation of proliferation of SW480 cells

SW480 cells were starved in serum-free DMEM for 12 h and treated with LPA at different doses. After different time periods, MTT assay was performed to evaluate the

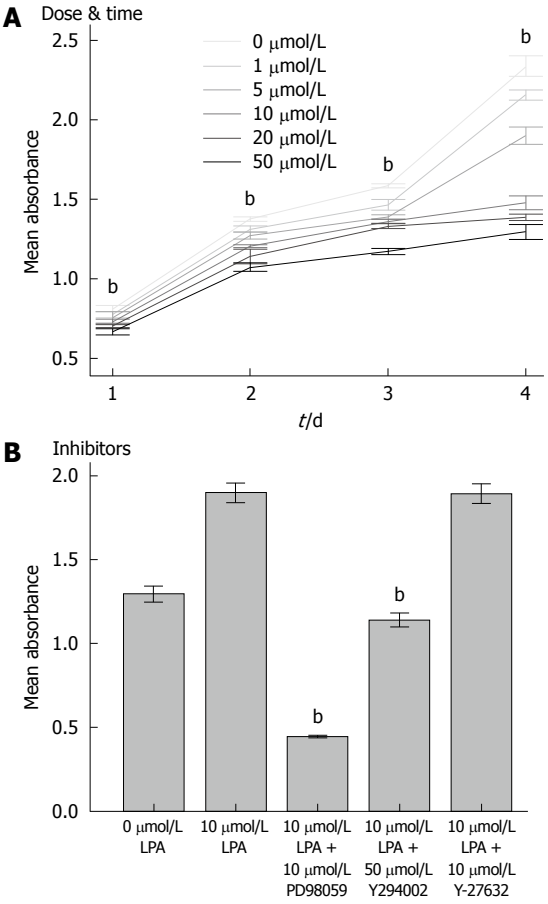

Figure 2 LPA effect on SW480 cell proliferation. Results presented as mean ± SE, $n = 5$. A: Dose and time effect of LPA on the proliferation of SW480 cells. SW480 cells were starved in serum-free DMEM for 12 h and treated with LPA at different doses. At different time points, MTT assay was performed to evaluate cell numbers. $^bP < 0.001$ *vs* 0 μmol/L LPA; B: Inhibitors blocked LPA-induced cell proliferation. SW480 cells were starved in serum-free DMEM for 12 h and treated with LPA (10 μmol/L). Inhibitors including LY294002 (50 μmol/L), PD98059 (10 μmol/L), and Y-27632 (10 μmol/L) were applied to cells 30 min before the action of LPA. ninety-six hours later, MTT assay was performed to evaluate cell growth. $^bP < 0.001$ *vs* 10 μmol/L LPA.

activity of cell growth. It was found that LPA significantly stimulated the proliferation of SW480 cells in a dose- and time-dependent manner ($P < 0.001$, Figure 2A). LPA, especially when the concentration was ⩾ 10 μmol/L, remarkably stimulated cell growth compared with the control group.

In order to investigate the signal pathways which mediated the stimulation effect of LPA on SW480 cells, inhibitors against key molecules of several signal transduction pathways were applied to the LPA-treated group. Three inhibitors were employed including PI3K inhibitor (LY290042), MAPK inhibitor (PD98059), and Rho kinase inhibitor (Y-27632). It was found that after applying the inhibitors, the stimulation effect of LPA on cell growth was significantly blocked by PD98059 and LY290042 ($P < 0.001$, Figure 2B); especially PD98059. This indicated that the Ras/Raf-MAPK signal pathway and the PI3K-AKT/PKB signal pathway may be involved in the LPA stimulation effect on proliferation of SW480 cells.

LPA induction of migration of SW480 cells

SW480 cells (1×10^5 cells in 100 μL of starvation medium) were seeded on the transwell inserts with an 8 μm pore size. Different doses of LPA in DMEM were added to the lower chamber of the transwell. Cells were then incubated at 37℃ for 4 h. Cells migrated to the lower surface of inserts were fixed, stained, and quantified. It was found that LPA significantly enhanced SW480 cell migration toward the lower chamber of the transwell in a dose-dependent manner compared with the control ($P < 0.001$, Figure 3A and B). This indicates that LPA has a significant chemotactic effect on SW480 cells.

In order to investigate the signal pathways which mediated the chemotactic effect of LPA on SW480 cells, some inhibitors against key molecules of signal transduction pathways were employed. It was demonstrated that Rho kinase inhibitor (Y-27632 at 10 μmol/L) dramatically blocked the chemotactic effect of LPA on SW480 cells ($P < 0.001$, Figure 3C). This indicated that Rho kinase and G12/13-Rho-RhoA signal pathways may mediate the LPA effect on SW480 cell migration.

LPA induction of adhesion of SW480 cells

SW480 cells were seeded in 96-well plates. After the cells had undergone 12 h of starvation, LPA at different doses was added to the cells. SW480 cells were allowed to adhere to the plates for 4 h at 37℃ in the incubator. Unbound cells were washed away twice. Adhered cells were fixed, stained, and quantified. Images of adhered cells under different doses of LPA were taken (Figure 4A). It was demonstrated that LPA significantly increased SW480 cell adhesion to extracellular matrix (ECM) in a dose-dependent manner compared with controls ($P < 0.001$, Figure 4B).

Some inhibitors were used to determine the mechanisms which mediated the LPA effect on adhesion. It was found that Rho kinase inhibitor (Y-27632) and LY294002 dramatically inhibited LPA upregulation of adhesion; especially Y-27632 ($P < 0.001$, Figure 4C and D). This indicated that the G12/13-Rho-RhoA signal pathway and the PI3K-AKT/PKB signal pathway may participate in the LPA effect on the adhesion of SW480 cells.

LPA protected SW480 cells from apoptosis

SW480 cells (1×10^5/well) seeded in 24-well culture plates were starved for 24 h and then treated with cisplatin (10 mg/L) or 5-FU (8 mg/L) for 24 h in the absence or presence of LPA (20 μmol/L). Inhibitors including LY294002 (50 μmol/L) and PD98059 (10 μmol/L) were added to the LPA-treated group.

Apoptotic cells were detected by flow cytometry after Annexin V and PI staining. Apoptotic cells were defined as Annexin-positive, PI-negative (Figure 5). After cells were exposed to cisplatin and 5-FU, the apoptotic population was 20.2% ± 2.3% and 14.2% ± 2.6%, respectively. However, after the action of LPA, the apoptotic population dropped to 14.6% ± 2.1% in the cisplatin-treated group and 10% ± 2.8% in the 5-FU-treated group. LPA protected 27.7% of cells from cisplatin-induced apoptosis and protected 29.6% of cells

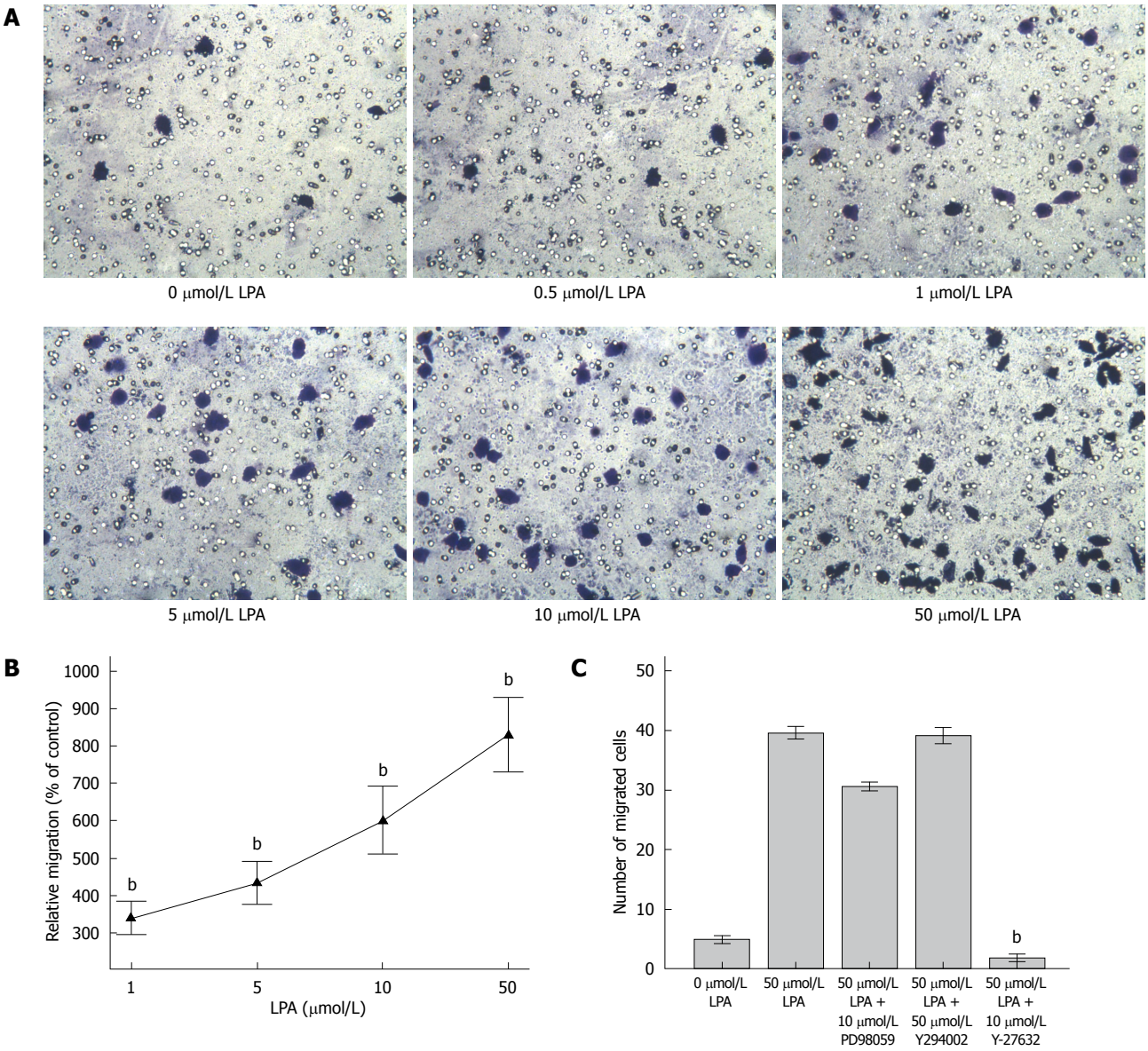

Figure 3 LPA stimulated migration of SW480 cells. A: LPA stimulated migration of SW480 cells (× 200). SW480 cells (1 × 10^5/100 μL) were seeded into the inserts of transwell chambers after starvation for 8 h. Cells were incubated at 37℃ for 4 h. Cells on the outside surface of inserts were fixed and stained. Typical images are presented; B: Migrated cells were quantified and relative migration rates (mean ± SE) are presented. $^bP < 0.001$ *vs* 0 μmol/L LPA; C: Effect of inhibitors on LPA-induced cell migration. 50 μmol/L LPA in 600 μL medium was added to the lower chamber of transwell. Inhibitors including 10 μmol/L PD98059, 50 μmol/L LY294002 and 10 μmol/L Y-27632 were added to cells in the upper chamber. Cells on the outside surface of inserts were fixed, stained, and quantified. Data were analyzed using one-way ANOVA with post-hoc *t*-tests. $^bP < 0.001$ *vs* 50 μmol/L LPA.

from 5-FU-induced apoptosis. This suggests that LPA effectively protected SW480 cells from apoptotic death induced by the chemotherapeutic agents.

In order to elucidate the mechanisms of LPA protection from apoptosis, LY294002 and PD98059 were added to the LPA-treated group. Apoptotic population increased to 50.2% ± 3.2% and 32.5% ± 3.6% respectively after exposure to LY294002 (PI3K inhibitor) and PD98059 (MAPK inhibitor). This indicated that the PI3K, MAPK, PI3K-AKT/PKB signal pathways and the Ras/Raf-MAPK signal pathway may be involved in the LPA apoptotic-protection effect.

DISCUSSION

LPA, the simplest glycerophospholipid, was initially found in the ascites of ovarian cancer patients at significant levels (2-80 μmol/L), and plays an important role in the development of ovarian cancer. LPA exerts growth factor-like effects through four specific G protein-coupled receptors (LPA_{1-4}). The effects include mitogenesis, secretion of proteolytic enzymes, and migration activity, which are accompanied by stress fiber formation and focal adhesion assembly in ovarian cancer cells.

We found recently that the level of LPA increases not only in the body fluid of ovarian cancer patients, but also in the plasma of patients with CRC. There are many studies focused on the LPA role in ovarian cancer cells. It has been found that LPA plays important roles in the progression of ovarian cancer and acts as an ovarian cancer promoter. Since LPA also increases in the plasma of CRC patients, what are the roles of LPA in CRC?

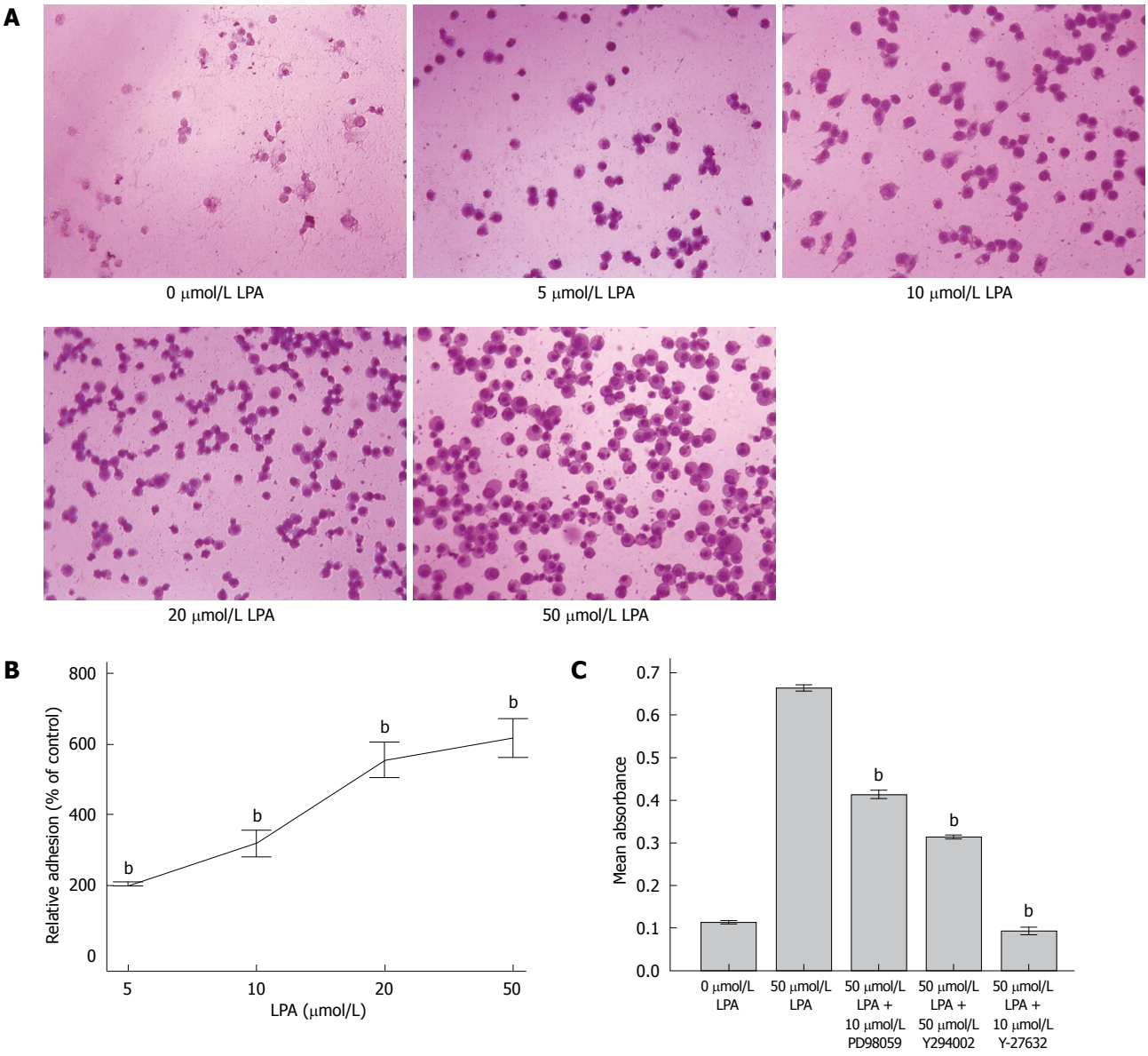

Figure 4 LPA stimulated adhesion of SW480 cells. A: Typical image of stained adhered cells (× 200); B: Adhered cells were quantified and relative adhesion rates (mean ± SE) are presented. $^{b}P < 0.001$ *vs* 0 μmol/L LPA; C: Effect of inhibitors on LPA induced adhesion. 50 μmol/L LPA or LPA plus inhibitors (including 10 μmol/L PD98059, 50 μmol/L LY294002 and 10 μmol/L Y-27632) were added to cells in 96 well plate. Adhered cells were quantified and presented. Results presented as mean ± SE, $n = 5$. $^{b}P < 0.001$ *vs* 50 μmol/L LPA.

There are few studies regarding LPA effects on CRC. Furthermore, our previous study has shown that LPA receptors ($LPA_{2, 4}$) are highly expressed in SW480 cells. In the present study, we preliminarily investigated the roles of LPA in the proliferation, migration, adhesion and apoptosis of SW480 cells and its mechanisms of action. We found that LPA significantly stimulated the proliferation of SW480 cells in a dose-dependent and time-dependent manner. This is consistent with the reports in ovarian cancer, in which LPA promotes growth of ovarian cancer similar to growth factor[9].

We found that the MEK1 inhibitor, PD98059, significantly inhibited the LPA effect on the proliferation of SW480 cells. MEK1, a MAPK, is a key molecule of the Ras/Raf1/MEK/ERK signal pathway[10]. We also found that the PI3K inhibitor, LY290042, partially inhibited the effect of LPA on the cell proliferation. This indicated that LPA stimulates the proliferation of SW480 cells through the Ras/Raf1/MEK/ERK pathway, and that the PI3K-AKT/PKB signal pathway may also be partially involved in the LPA effect on proliferation.

MAPK transfers major cell proliferation signals from the cell surface to the nucleus. There are three major subfamilies of MAPK, including the extracellular-signal-regulated kinase (Ras/Raf1/MEK/ERK or ERK MAPK), the c-Jun N-terminal or stress-activated protein kinase (JNK or SAPK), and MAPK14[11]. The Ras/Raf1/MEK/ERK pathway is one of the most important pathways for cell proliferation. Several lines of evidence suggest that, in CRC, the Ras/Raf1/MEK/ERK pathway, but not the JNK pathway or the p38 MAPK pathway, is the major regulator of cell proliferation. There is growing evidence that activation of the Ras/Raf1/MEK/ERK pathway is involved in the

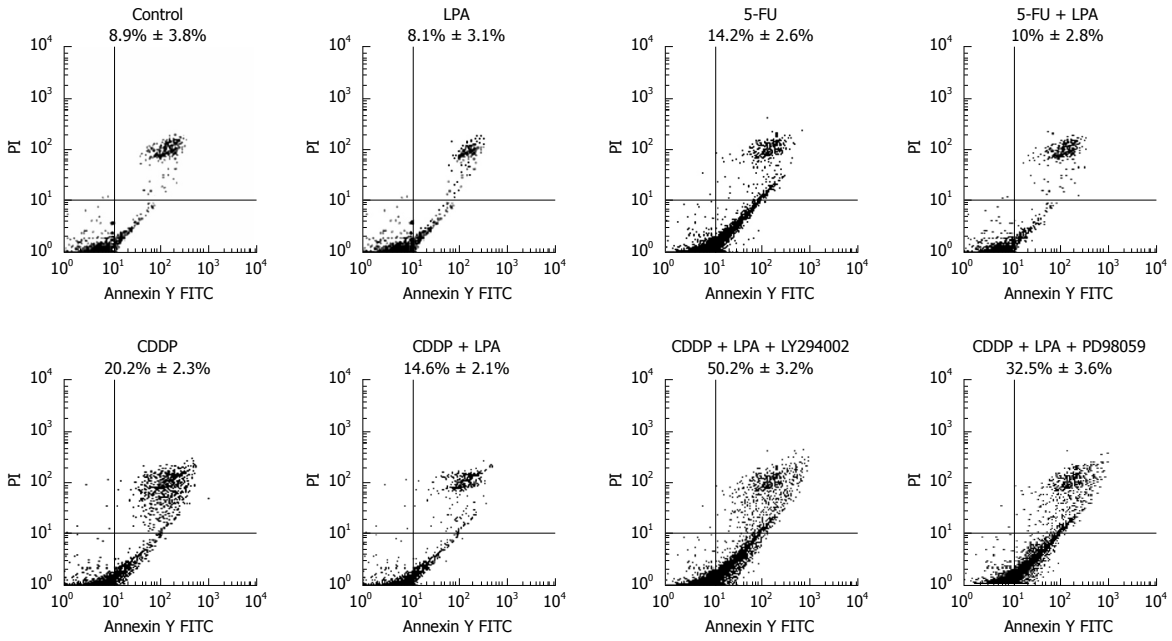

Figure 5 LPA protected cells from cisplatin- & 5-FU-induced apoptosis. SW480 cells were serum-starved for 24 h and treated for 24 h with 10 μg/mL cisplatin (CDDP); 10 μg/L cisplatin + 20 μmol/L LPA; 8 μg/mL 5-FU; 8 μg/mL 5-FU + 20 μmol/L LPA; 10 μg/mL cisplatin + 20 μmol/L LPA + 50 μmol/L LY294002; 10 μg/mL cisplatin + 20 μmol/L LPA + 10 μmol/L PD98059, respectively. Cells were harvested, stained with Annexin V/PI, and analyzed by FACS analysis. The Annexin V-positive and PI-negative population was defined as apoptotic cells. Representative results from three separate experiments are presented.

pathogenesis, progression, and oncogenic behavior of human CRC[12]. This supports our observations that LPA stimulated growth of CRC cell line SW480 through the Ras/Raf1/MEK/ERK pathway.

There are several studies reporting LPA's effect on the proliferation of CRC cells. Zhang *et al*[13] has found that LPA facilitates proliferation of colon cancer cells *via* induction of Krüppel-like Factor 5 (KLF5). KLF5 is a transcriptional factor highly expressed in the crypt compartment of the intestinal epithelium. LPA stimulated the KLF5 expression in colon cancer cells, SW480 and HCT116. Moreover, LPA-mediated KLF5 induction was partially blocked by inhibition of MAPK kinase and protein kinase C (PKC). This also indirectly indicated that the MAPK signaling pathway is involved in the proliferation of SW480 cells.

Yang *et al*[14] reported that LPA-induced colon cancer cell proliferation requires the β-catenin signaling pathway. LPA activated the main signaling events in the β-catenin pathway, but inhibition of PKC blocked the effects, suggesting PKC involvement in LPA-induced activation of the β-catenin pathway. This also indirectly indicated that the PKC signaling pathway is involved in the proliferation of SW480 cells.

Balavenkatraman *et al*[15] reported that DEP-1 protein tyrosine phosphatase inhibits proliferation and migration of colon carcinoma cells and is upregulated by protective nutrients. Upregulation of DEP-1 expression, and in turn inhibition of cell growth and migration, may present a previously unrecognized mechanism of chemoprevention by nutrients. This result contradicts other reports.

In our study, it was found that LPA significantly enhanced the migration and adhesion of SW480 cells in a dose-dependent manner. The stimulation effect of LPA on cell adhesion, invasion and migration has been reported in other cancer types, including ovarian cancer[16-19], pancreatic cancer[18], and breast cancer[20,21]. Enhanced migration activity and increased adherence to ECM are two major factors which contribute to tumor metastasis. Cell-ECM adhesions can alter the cell's capacity to attach and migrate through surrounding tissues. Changes of the expression and activities of the components of such adhesions could make an important contribution to preventing cancer invasion. Our study showed that LPA stimulated both migration and adhesion to ECM of colon cancer SW480 cells. This means that LPA significantly promotes the metastatic potential of SW480 cells. In our study, Rho kinase inhibitor, Y-27632, significantly inhibited cell migration and adhesion induced by LPA. LY294002 partially inhibited the LPA effect on adhesion. This indicated that the G12/13-Rho-GEFs-RhoA signal pathway may mediate the effect of LPA on both migration and adhesion, and that the PI3K-AKT/PKB pathway may partially mediate the LPA effect on adhesion.

Rho GTPase family proteins, including Rho, Rac1, and Cdc42, control a wide variety of cellular processes, such as cell adhesion, motility, proliferation, differentiation, and apoptosis[22]. One of the best effectors of Rho is Rho-associated kinase (ROCK). ROCK is a target effect molecule downstream of RhoA. Rho activates ROCK by phosphorylation of Ser854 and Thr697, and induces a series of actions downstream to stimulate adhesion and migration. Y-27632 is a novel and specific inhibitor of ROCK, which is cell permeable and inhibits ROCK- Ⅰ and ROCK-Ⅱ by competing with ATP.

It has been reported in other tumors that LPA

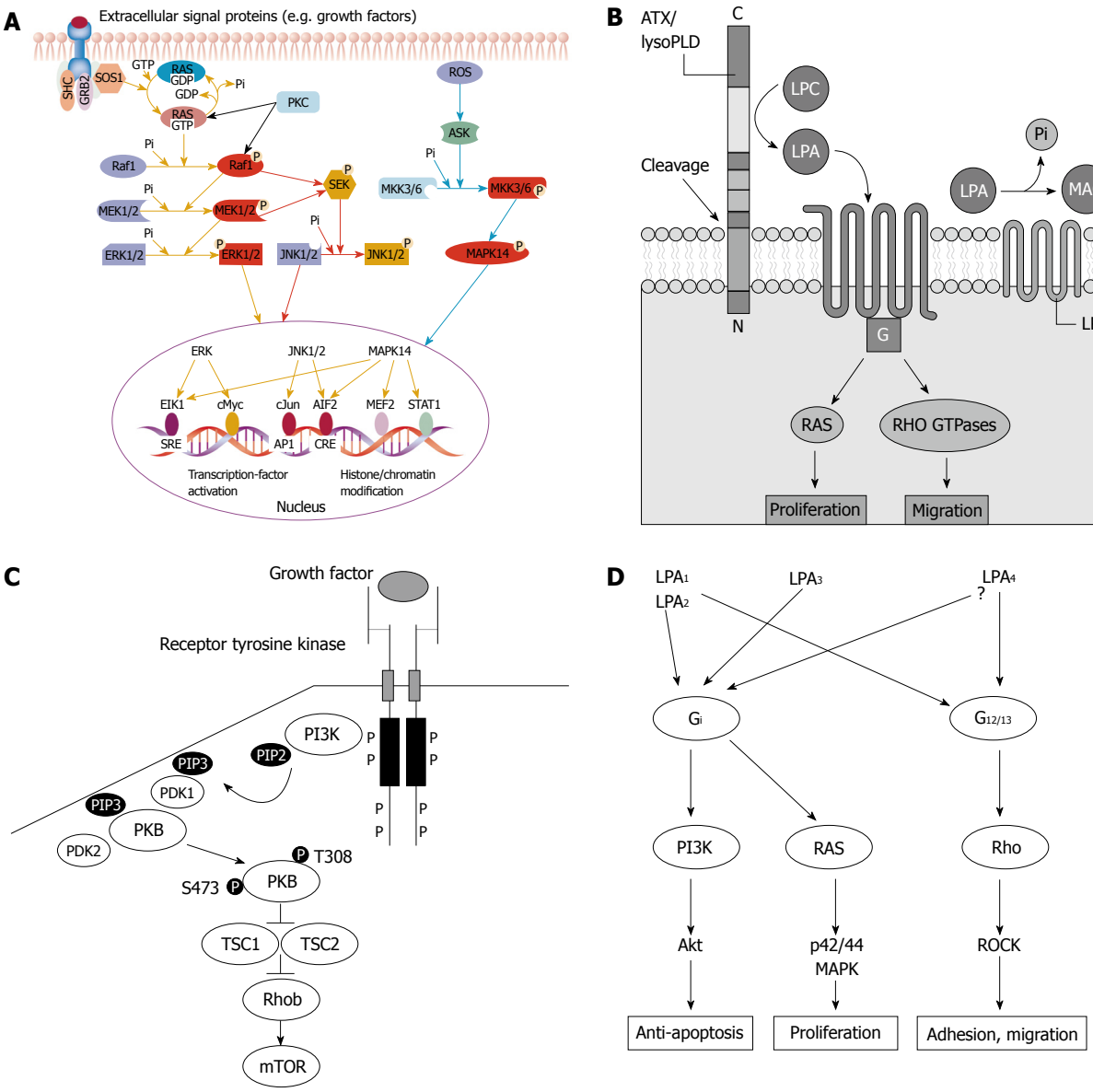

Figure 6 LPA effect and signal transduction pathway. A: Ras/Raf1/MEK/ERK signal pathway[27]; B: G12/13-Rho-GEFs-RhoA signal pathway; C: PI3K-AKT/PKB signal pathway; D: Possible signal pathways mediated by LPA effect on SW480 cells.

drives the formation of focal adhesions, the tyrosine phosphorylation of focal adhesion proteins, and ROCK. Our results suggested that in colon cancer SW480 cells, the G12/13-Rho-GEFs-RhoA pathway may mediate migration and adhesion induced by LPA.

Several studies have also found that LPA promotes the metastasic potential of CRC.

Tatsuta *et al*[23] found that LPA significantly increased the peritoneal and pleural metastases of intestinal adenocarcinomas induced by azoxymethane through RhoA activation. This is consistent with our study, in which the Rho signaling pathway was found to be involved in the LPA effect on metastasis. Other mechanisms have also been found.

Shiada *et al*[24] found that cross-talk between LPA1 and epidermal growth factor receptors mediates up-regulation of sphingosine kinase 1 to promote gastric cancer cell motility and invasion. Down-regulation of SphK1 attenuated LPA-stimulated migration and invasion of MNK1 cells. Shiada *et al*[25] also reported that LPA acts as a potent stimulator of colon cancer progression, although the binding to LPA1 and LPA2 induced slightly different responses. Komuro *et al*[26] found that LPA1 expression was increased in the early stage of adenoma.

We also found that LPA significantly protected SW480 cells from apoptosis induced by chemotherapeutic drugs, while LY294002 and PD98059 effectively blocked the LPA effect on apoptosis, indicating that the PI3K-AKT/PKB and the Ras/Raf-MAPK signal pathways may mediate the LPA effect on apoptosis; especially the PI3K-AKT/PKB pathway. The apoptosis protection roles of LPA have been reported in ovarian cancer cells.

PI3K can be divided into three classes. Class I PI3K is the most studied class of PI3K, consisting of an 110 kDa catalytic subunit and a regulatory subunit of 85 kDa. The activity of PI3K protein family is associated with cytoskeletal organization, cell division, inhibition of apoptosis and glucose uptake. The phospholipid

products of PI3K activate downstream targets, including PDK, Akt and PKC. LY294002 blocks PI3 kinase-dependent Akt phosphorylation and kinase activity. In SW480 cells we observed that the PI3K-AKT/PKB pathway may partially mediate the effect of LPA on proliferation.

In this study, it has been found that LPA significantly stimulated the proliferation, adhesion, and migration of human colon cells, SW480, and protected their apoptosis. The Ras/Raf-MAPK signal pathway may be involved in the LPA effect on proliferation. The G12/13-Rho-RhoA signal pathway may be associated with the LPA effect on adhesion and migration. The PI3K-AKT/PKB signal pathway may participate in the anti-apoptotic effect of LPA. This indicates that LPA probably acts as a promoter of the development of CRC. To decrease the LPA level in CRC patients and to block the LPA action (Figure 6) could be the aim of new strategies of treatment and prevention of CRC. The pathways involved in the LPA effects which we have discovered in this study could be new treatment targets of CRC.

COMMENTS

Background

Colorectal cancer (CRC) is a major cause of cancer death worldwide. The incidence has been rapidly increasing in recent years. Lysophosphatidic acid (LPA) was initially found in the ascites of ovarian cancer patients. Recently the authors group found that LPA levels increase not only in the body fluid of ovarian cancer patients, but also in the plasma of patients with CRC. There are many studies of LPA roles in ovarian cancer cells which have found that LPA stimulates the progression of ovarian cancer. Since LPA increases in the plasma of CRC patients as well, what are the roles of LPA in CRC? This study preliminarily investigated the roles of LPA in the proliferation, migration, adhesion and apoptosis of SW480 cells and its mechanisms of action.

Research frontiers

LPA was firstly found in the ascitic fluid from ovarian cancer patients. It is a bioactive glycerophospholipid generated and released by platelets, macrophages, epithelial cells, and some tumor cells. Studies have shown the presence of high levels of LPA in the ascitic fluid of patients with ovarian cancer and LPA is known to be an "ovarian cancer activating factor", which exerts growth factor-like effects through four specific G protein-coupled receptors (LPA_{1-4}). LPA is a potent mediator with a broad range of cellular responses, including regulation of cell proliferation, protection from apoptosis, modulation of chemotaxis and transcellular migration, which also mediates survival of ovarian cancer cells, macrophages, fibroblasts, and neonatal cardiac myocytes. Some of these cellular responses indicate that LPA is a mediator of tumor progression.

Innovations and breakthroughs

Since LPA was found in the ascites of ovarian cancer patients, there have been many studies on LPA, but most of the studies have focused on ovarian cancer. There are some studies of LPA in colon cancer, but this present study has some different findings from other studies. Firstly, because the expression level of LPA receptor varies in different colon cancer cell lines, and different LPA receptors mediate different responses to LPA, so LPA effects on SW480 cells are different from other colon cancer cell lines. Secondly, some mechanisms found in this study are not completely the same as findings in other studies. They found some pathways which mediate the LPA effect on proliferation, migration, and adhesion which are different from other studies.

Applications

In order to completely block the growth, metastasis and progression of CRC, the mechanisms for its development need to be clarified. The provided some information about the LPA effects on colon cancer cells and some mechanisms of action. These results will help to design targeted strategies to block LPA's stimulation effect on colon cancer.

Peer review

LPA is associated with inflammation and has been thought to be one of the mediators of inflammation-induced promotion of cancer. LPA is one of the possible keys for inflammation-induced carcinogenesis in GI tract. Actually there are at least 511 papers on LPA and cell proliferation, among which there are nine papers on LPA and colon cancer. In this particular manuscript, the study was conducted carefully on many aspects including proliferation, apoptosis, cell adhesion, migration *etc*, of colon cancer cells. Title, abstract, methods and results were carefully written.

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BRIEF ARTICLES

Early graft dysfunction following adult-to-adult living-related liver transplantation: Predictive factors and outcomes

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Abstract

AIM: To describe a condition that we define as early graft dysfunction (EGD) which can be identified preoperatively.

METHODS: Small-for-size graft dysfunction following living-related liver transplantation (LRLT) is characterized by EGD when the graft-to-recipient body weight ratio (GRBWR) is below 0.8%. However, patients transplanted with GRBWR above 0.8% can develop dysfunction of the graft. In 73 recipients of LRLT (GRBWR > 0.8%), we identified 10 patients who developed EGD. The main measures of outcomes analyzed were overall mortality, number of re-transplants and length of stay in days (LOS). Furthermore we analyzed other clinical pre-transplant variables, intra-operative parameters and post transplant data.

RESULTS: A trend in favor of the non-EGD group (3-mo actuarial survival 98% *vs* 88%, $P = 0.09$; 3-mo graft mortality 4.7% *vs* 20%, $P = 0.07$) was observed as well as shorter LOS (13 d *vs* 41.5 d; $P = 0.001$) and smaller requirement of peri-operative Units of Plasma (4 *vs* 14; $P = 0.036$). Univariate analysis of pre-transplant variables identified platelet count, serum bilirubin, INR and Meld-Na score as predictors of EGD. In the multivariate analysis transplant Meld-Na score ($P = 0.025$, OR: 1.175) and pre-transplant platelet count ($P = 0.043$, OR: 0.956) were independently associated with EGD.

CONCLUSION: EGD can be identified preoperatively and is associated with increased morbidity after LRLT. A prompt recognition of EGD can trigger a timely treatment.

Key words: Small-for-size graft dysfunction; Living-related liver transplantation; Graft-to-recipient body weight ratio; Partial liver transplantation; Allograft dysfunction

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INTRODUCTION

Small-for-size graft dysfunction (SFSGD) is one of the greatest limiting factors for the expansion of segmental liver transplantation from living donors[1], and is characterized by: (1) onset within 2 wk after living-

related liver transplantation (LRLT); (2) a graft-to-recipient body weight ratio (GRBWR) below 0.8%; (3) total bilirubin higher than 5 mg/dL, and/or output of ascites through abdominal drainages of more than 1 L/d; and (4) exclusion of technical (e.g. arterial or portal occlusion, outflow congestion, bile leak), infective (e.g. sepsis) and immunological (e.g. acute cellular rejection) complications.

By definition, SFSGD can be diagnosed only in the presence of a GRBWR of less than 0.8%, or a ratio of graft volume (GV) relative to the standard liver volume (SLV) of the recipient (GV/SLV) of less then 30%[1-4]. However, despite a GRBWR above 0.8%, some recipients of LRLT may have a worse clinical course.

The aim of this study was to analyze a group of LRLT recipients in order to identify those who developed a clinical picture of SFSGD in the absence of a GBWR of < 0.8% and with a GV/SLV ratio highest than 30%. Those patients were defined as affected by early graft dysfunction (EGD).

MATERIALS AND METHODS

We evaluated the rate of EGD in 73 consecutive recipients of adult-to-adult LRLT performed at our institute between July 2004 and September 2008, and whose GRBWR was > 0.8% and with a GV/SLV ratio higher than 30%. Follow-up in months was 27.34 ± 13.77.

There were 43 males and 30 females, with a median age of 57 years (range 18-68 years). The etiology of the liver disease was related to hepatitis C virus infection in 47 cases, to hepatitis B virus infection in nine patients, to both B and C virus infection in three patients, and to non-viral causes in 14 patients. Twenty-two patients had hepatocellular carcinoma (HCC). Donor liver resection resulted in 73 right hepatectomies (liver segments 5-8). Graft implantation was performed with the piggy back technique and, in all cases, with the use of veno-venous bypass. Details of surgical procedures are reported elsewhere[5,6]. Volumetric computed tomography (CT) scan was used to calculate liver and spleen volumes.

The main measures of outcomes analyzed were overall mortality, number of re-transplants and length of stay in days (LOS).

In order to identify predictors of EGD, epidemiologic pre-transplant variables such as age of the recipient and donor, sex of the recipient and donor, recently reported as markers of graft function[7], were evaluated (Table 1).

Furthermore, we analyzed other clinical pre-transplant variables such as: serum bilirubin, serum albumin, serum sodium, INR, platelets count, WBC count, Child-Pugh score, MELD score, Meld-NA score, recently described[8-10], percentage of donor liver steatosis, liver volume and spleen volume evaluated using CT, spleen/liver volume ratio (S/LVR), GBWR and GV/SLV (Table 2).

Then we observed the following intra-operative parameters: mean arterial pressure, systemic vascular resistance, cardiac output, cardiac index, units of transfused packed red blood cells, units of transfused platelets, and units of transfused fresh frozen plasma (Table 3).

Finally as post transplant data we looked at the LOS.

Table 1 Univariate analysis of epidemiologic data in the group with EGD *vs* the group without EGD, median (range)

	With EGD (10 pts)	Without EGD (63 pts)	*P* value
Age recipient	52.72 (38-61)	57.6 (18-68)	NS
Age donors	29.5 (26-54)	30 (18-53)	NS
Sex recipient (M/F)	5/5	38/25	NS
Sex donors (M/F)	5/5	39/24	NS

EGD: Early graft dysfunction.

Statistical analysis

Survival analysis was performed using the Kaplan-Meier analysis with SPSS (SPSS Inc., Chicago, Ill, United States), and a descriptive analysis was used for the outcome. Normality was tested with the Wilk-Shapiro test. Differences between the two groups were tested using the unpaired Student's *t*-test, Mann-Whitney test, χ^2 test; $P < 0.05$ were considered significant. Multivariate analysis was performed to identify independent determinants for occurrence of EGD (logistic regression stepwise backward procedure).

RESULTS

Ten out of 73 patients (13.7%) fit our criteria for EGD. No statistically significant differences were found between EGD and non-EGD recipients in terms of 3-mo patient and graft mortality [one patient out of ten (10%) *vs* one patient out of 63 (1.6%), $P = 0.13$; two patients out of ten (20%) *vs* three patients out of 63 (4.7%), $P = 0.07$], number of re-transplants during the first 3 mo after LRLT [one patient out of ten (10%) *vs* two patients out of 63 (3.2%), $P = 0.33$] and 3-mo and 1-year actuarial patient survival (88% *vs* 98%: $P = 0.09$ by the log-rank test; 80% *vs* 94%, $P = 0.12$ by the log-rank test).

The 4-year actuarial patient survival was 77.78% *vs* 88.01%, ($P = 0.201$ by the log-rank test) (Figure 1). Although the statistical analysis doesn't indicate any statistical significance, probably due to the small size of the sample examined, the survival analysis points out a lower survival rate (77.78%) on the EGD patient *vs* non-EGD patient (88.01%); this is clinically relevant.

In the EGD patients, we observed two deaths: one because of sepsis and the second one due to multiorgan failure. In the non-EGD group, we observed six deaths: three because of neoplastic recurrence of HCC and three due to multiorgan failure. HCC recurrence could be explained by the advanced stage of the tumor at the pathologic examination, although the patients were classified within Milan criteria.

We did observe a significant difference between the two groups in terms of LOS, with the EGD group having

Table 2 Univariate analysis of pre-transplant clinical data in the two groups: EGD *vs* non-EGD

	EGD (10 pts)	Non-EGD (63 pts)	*P* value
Pre-transplant serum bilirubin (mg/dL)	8.71 (1.27-29.21)	2.01 (0.28-24.82)	0.013
[1]Pre-transplant serum albumin (g/dL)	2.6 (2.2-3.3)	2.8 (1.31-4)	NS
Pre-transplant serum sodium (mEq/L)	133 (122-145)	138 (126-144)	NS
Pre-transplant INR	1.38 (1.27-2.55)	1.22 (0.81-2.55)	0.001
Pre-transplant platelets (mmc)	48 000 (22 000-60 000)	71 000 (24-400)	0.007
Pre-transplant WBC (mmc)	4575 (1700-7200)	4200 (1500-15 500)	NS
Child-Pugh score, points	10.00 (8-12)	8.0 (5-12)	NS
MELD score	20.50 (12-40)	15.0 (6-28)	NS
Meld-Na score	24.25 ± 7.9	18.13 ± 5.8	0.004
Steatosis (No/Macro 1%-2%/Macro 10%-20%/Macro 25%-30%)	(6/2/2/0)	(35/15/9/4)	NS
Liver volume (mL)	780 (590-1186)	1016 (557-1482)	NS
Spleen volume (mL)	983 (648-1382)	709 (161-2711)	NS
S/LVR	0.96 (0.55-2.34)	0.78 (0.13-2.95)	NS
GRDWR	1.26 (0.79-1.59)	1.48 (0.81-2.96)	NS
GS/SLV	59.52 (37.34-70.19)	68.5 (38.7-132.6)	NS

[1]Neither group of patients received albumin supplementation before transplant. S/LVR: Spleen/liver volume ratio; GRDWR: Graft-to-recipient body weight ratio; GS/SLV: Graft-to-recipient standard liver volume. Data are expressed as mean (range), or mean ± SD.

Table 3 Univariate analysis of intraoperative parameters in the two groups: EGD *vs* non-EGD

	EGD (10 pts)	Non-EGD (63 pts)	*P* value
MAP, mmHg	73.8 ± 13	76.7 ± 12	NS
SVR dyn·s·cm^{-5}	676 (350-1429)	704 (308-1249)	NS
C/O, L/min	6.9 (3.7-12)	9.4 (6-11.8)	NS
C/I, L/min per meter^2	4.5 (3.6-7.5)	4.2 (2.2-6.4)	NS
PRBC	12 (0-47)	3 (0-34)	NS
Units plasma transfused	14 (0-47)	4 (0-34)	0.036

MAP: Mean arterial pressure; SVR: Systemic vascular resistance; C/O: Cardiac output; C/I: Cardiac index; PRBC: Units of transfused packed red blood cells. Data are expressed as mean (range), or mean ± SD.

Table 4 Multivariate analysis of pre-transplant epidemiologic and clinical data in the two groups: EGD *vs* non-EGD (logistic regression, backward stepwise procedure)

	P value	OR	95% CI per OR
Meld-Na score	0.025	1.175	1.021; 1.352
Pre-transplant platelets, mmc	0.043	0.956	0.915; 0.999

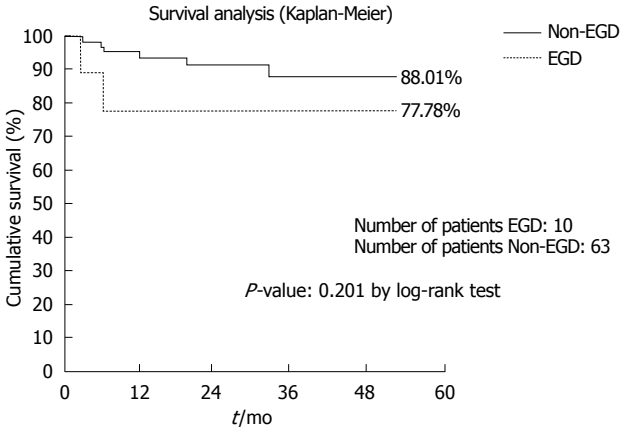

Figure 1 Survival analysis.

a longer median LOS (13 d *vs* 41 d, $P = 0.001$) and greater median number of units of plasma transfused during surgery (4 *vs* 14, $P = 0.036$).

At univariate analysis of the variables collected, INR, platelet count, serum bilirubin and Meld-Na score, were identified as predictors of EGD (Table 3).

In the multivariate analysis (logistic regression, backward stepwise procedure), we analyzed INR, platelet count, serum bilirubin and Meld-Na score. Meld-Na score ($P = 0.025$, OR: 1.175) and pre-transplant platelet count ($P = 0.043$, OR: 0.956) were the variables independently associated with occurrence of EGD (Table 4).

In conclusion, the main clinical outcomes of the two groups were not statistically significant in terms of both early and late patient survival, probably because of the small size of the sample. In fact, as the survival rate was 77.78% *vs* 88.01% for EGD and non-EGD patients, we can hypothesize that survival rate acquires a statistically significant difference by enrolling a larger number of patients.

DISCUSSION

A GRBWR below 0.8% is considered mandatory for the diagnosis of SFSGD. Despite these findings in the literature, there are few patients who fully develop SFSGD by classic definition.

On the other hand, there are many patients who do not do well immediately after LRLT. We observed a clinical picture similar to that of SFSGD in patients who received partial livers that could not be described as small (GRBWR > 0.8).

In this study, the relevant clinical impact of EGD is suggested by the reduced 3-mo and 1-year patient survival and the increased graft-loss rate in the group of patients with this condition, even though there was no statistically significant difference, which is probably

due to an insufficient sample size (and a small number of events). The increased LOS in the EGD group reflects the increased time of recovery. Those patients who developed EGD were in fact those with worse INR, platelet count and total bilirubin. In addition, as previously reported by Yoshizumi *et al*[11], we noted that patients with a higher MELD score, higher Child Pugh score and hyponatremia, tended to have a worse outcome.

In fact, in the EGD group (Table 3), these parameters were higher than in the non-EGD patient.

Our data, although not significant in accordance to others[10], are clinically relevant especially at the time of selection of donors and recipient.

Our study was also aimed at finding objective criteria for identifying those patients who had a worse clinical course in the 2 wk after LRLT, and with a GRBWR above 0.8%. Our data support the hypothesis that SFSGD and EGD have a multi-factorial genesis in which the combination of the donor's factors (GV and quality of the graft) and the recipient's factors (portal hypertension and stage of liver disease) lead to allograft dysfunction after partial liver transplantation[3,9,10,12].

The clinical variables identified at the univariate analysis as predictors of EGD confirmed the relevant roles of liver disease and portal hypertension in graft dysfunction.

Serum bilirubin, INR, and Meld-Na score are markers of liver function and platelet count is a marker of portal hypertension. However, at the multivariate analysis, the only variables independently associated with occurrence of EGD were Meld-Na score and pre-transplant platelet count.

The transplant community is now focused on the possibility of detecting predictive factors based on simple biochemical and imaging assessments which could allow physicians to treat those patients at risk of EGD immediately after surgery.

It has been demonstrated that in patients with cirrhosis and severe portal hypertension, the occlusion of the splenic artery causes a significant reduction in portal pressure, which is directly related to the spleen volume and indirectly related to the liver volume[13]. This concept is at the center of our strategy for performing early splenic artery embolization for the treatment of SFSGD following LRLT[14].

EGD can be identified preoperatively and is associated with increased morbidity after LRLT. Obviously, a prompt recognition of EGD can trigger a timely and appropriate treatment.

COMMENTS

Background

Small-for-size graft dysfunction (SFSGD) following living-related liver transplantation (LRLT) is characterized by early graft dysfunction (EGD) when the graft-to-recipient body weight ratio (GRBWR) is below 0.8%. However, patients transplanted with GRBWR above 0.8% can develop dysfunction of the graft.

Research frontiers

The study was aimed at finding objective criteria for identifying those patients who had a worse clinical course in the 2 wk after LRLT and had a GRBWR above 0.8%. They describe a condition that they define as EGD which can be identified preoperatively and seems to be associated with increased morbidity after LRLT.

Innovations and breakthroughs

A GRBWR below 0.8% is considered mandatory for the diagnosis of SFSGD. Despite the findings in the literature, there are few patients who fully develop SFSGD by classic definition. The authors observed a clinical picture similar to that of SFSGD in patients who received partial livers that could not be described as small (GRBWR > 0.8).

Applications

A prompt recognition of EGD can trigger a timely and appropriate treatment.

Terminology

The authors describe a condition that they define as EGD which can be identified preoperatively and seems to be associated with increased morbidity after LRLT.

Peer review

This is an important study which impacts on the field. Gruttadauria and colleagues report herein the parameters which allow preoperative identification of a condition defined as EGD i.e. the transplant Meld-Na score and pre-transplant platelet count. The study is original, well designed and performed.

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COX-2 polymorphisms *-765G→C* and *-1195A→G* and colorectal cancer risk

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Abstract

AIM: To determine the possible modulating effect of the *COX-2* polymorphisms, *-765G→C* and *-1195A→G*, on the risk of colorectal cancer (CRC) in a Dutch population.

METHODS: This case-control study includes 326 patients with CRC and 369 age- and gender-matched controls. Genotypes of the *COX-2* polymorphisms *-765G→C* and *-1195A→G* were determined by polymerase chain reaction-based restriction fragment length polymorphism. *COX-2* genotypes and haplotypes were analyzed and odds ratios with 95% confidence intervals were estimated by logistic regression.

RESULTS: The *-765GG* genotype was associated with an increased risk of developing CRC (OR, 1.45; 95% CI, 1.03-2.04). No significant difference was observed in the genotype distribution of the *-1195A→G* polymorphism between patients and controls. The *GG/AC* haplotype was present significantly less often in patients than in controls (OR 0.44; 95% CI, 0.22-0.85). When the *AC*, *AG* and *GG* haplotypes were investigated separately, the *AC* haplotype showed a tendency to be less frequent in patients than in controls ($OR_{(AG/AC)}$ 0.78; 95% CI, 0.57-1.06).

CONCLUSION: The *-765GG* genotype is associated with an increased risk of developing CRC and the *GG/AC* haplotype seems to protect against CRC. These findings suggest a modulating role for the *COX-2* polymorphisms *-765G→C* and *-1195A→G* in the development of CRC in a Dutch population.

Key words: Colorectal carcinoma; Cyclooxygenase-2; Genetic polymorphism

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INTRODUCTION

Colorectal cancer (CRC) is a common disease in both men and women. CRC includes cancerous growths in the cecum, colon, sigmoid and rectum. In Western countries, 5% of the population ultimately develop CRC, thus this disease is an important public health issue[1]. CRC is ranked the third most common form of cancer worldwide in terms of incidence[2]. In the Netherlands, CRC is the second most common form of cancer affecting women and the third most common form of cancer affecting men. In 2003 in the Netherlands 9898 new cases of CRC were diagnosed[3].

CRC is usually observed in one of three specific patterns: sporadic, inherited or familial. The sporadic form accounts for approximately 70% in the population and is most common in individuals older than 50 years of age, probably as a result of interactions between low penetrance genes and environmental factors. Fewer than 10% of the population has an inherited predisposition to colon cancer. Inherited colon cancer is usually the result of a single germ line mutation. The third pattern, familial colon cancer, includes those families in which CRC develops too frequently to be considered as sporadic colon

cancer and which are not in a pattern consistent with an inherited syndrome. Up to 25% of all cases of CRC are estimated to fall into this category[1].

Cyclooxygenase (COX), also known as prostaglandin endoperoxidase H synthase, is a modifier gene and key enzyme in the conversion of arachidonic acid into prostaglandins.

The COX family consists of two isozymes: COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and is involved in the homeostasis of various physiological functions. *COX-1* is well known as the housekeeping gene. COX-2 is an inducible form and its expression can be induced by mitogenic and proinflammatory stimuli. Increased expression of COX-2 is observed in many types of cancers. COX-2 is also associated with many stages of cancer development, e.g. invasion, metastasis, hyperproliferation, transformation and tumor growth[4,5].

Recent studies suggest that single nucleotide polymorphisms (SNPs) in the *COX-2* promoter may alter the enzyme function of COX-2 by differential regulation of COX-2 expression. A differential COX-2 expression may influence the risk of the development of gastrointestinal adenocarcinomas, including CRC[6-9].

In a study of African-Americans, an inverse association was found between the Val511Ala polymorphism and the risk of CRC[8]. In two studies the promoter polymorphisms *-765G→C* and *-1195A→G* were associated with an increased risk of CRC[9,10], whereas Ulrich *et al*[11] reported a reduced risk of CRC associated with the *-765G→C* polymorphism. The inconsistent results may indicate that the *COX-2* polymorphisms *-765G→C* and *-1195A→G* may play a role in carcinogenic processes in combination with specific life-style conditions or dependent on the racial composition of a particular population.

The purpose of our study was to determine the possible modulating effect of the *COX-2* polymorphisms *-765G→C* and *-1195A→G* on the risk of sporadic CRC in a Dutch population. The results of this research will lead to a better understanding on the role of SNPs in the *COX-2* promoter in colon cancer carcinogenesis. Such knowledge in future may eventually lead to better preventive measures for CRC.

MATERIALS AND METHODS

Patients and controls

This case-control study included 326 patients with CRC (59.8% men, 40.2% women) and 369 cancer-free controls (59.1% men, 40.9% women). In the patient group, 31.0% had a proximal tumor and 68.1% had a distal tumor, whereas in 0.9% of cases localization of the tumor was unknown (see legend of Table 1). All subjects were of Caucasian origin with a mean age of 63.7 years and were recruited at Radboud University Nijmegen Medical Center, the Netherlands. The patient and control groups were matched for gender and age. The characteristics of patients and controls are summarized in Table 1.

Table 1 Characteristics of patients with colorectal cancer (CRC) and controls (mean ± SD)

	Patients with CRC (n = 326)	Controls (n = 369)
Age (yr)	62.7 ± 11.7	64.5 ± 10.7
Male gender	195 (59.8%)	218 (59.1%)
Female gender	131 (40.2%)	151 (40.9%)
Localization of tumor[1]		
Proximal[2]	101 (31.0%)	
Distal[3]	222 (68.1%)	

[1]Note that the localization of the tumor was unknown in 3 patients; [2]Proximal tumor: cecum, ascending and transverse colon; [3]Distal tumor: descending colon, sigmoid, rectosigmoid junction and rectum.

Genotyping

DNA from patients and controls was isolated from whole blood using the Pure Gene DNA isolation kit (Gentra Systems, Minneapolis, MN) and stored at 4℃. Genotypes of the *COX-2 -765G→C* and *-1195A→G* polymorphisms were determined by polymerase chain reaction (PCR)-based restriction fragment length polymorphism, according to the method of Zhang *et al*[5].

First, PCR was used to amplify the *COX-2* promoter region containing the polymorphism *-765G→C* and *-1195A→G*. The primers used to amplify the *COX-2* promoter region were 765F5'-TATTATGAGGAGAATTTACCTTTCGC-3'/765R5'GCTAAGTTGCTTTCAACAGAAGAAT-3', and 1195F5'CCCTGAGCACTACCCATGAT-3'/1195R5'-GCCCTTCATAGGAGATACTGG-3'. PCR was performed using a 25 μL reaction mixture containing 100 ng of DNA, 10 mmol/L of Tris/HCl (pH 9.0), 50 mmol/L of KCl, 0.1% of Triton X-100, 2 mmol/L of $MgCl_2$, 200 nmol/L of each primer, 250 μmol/L of deoxyribonucleotide triphosphates and 2.5 U Taq DNA polymerase. The PCR profile for the *-1195A→G* polymorphism consisted of an initial melting step of 3 min at 95℃, followed by 40 cycles of 30 s at 95℃, 30 s at 58℃, 30 s at 72℃ and a final elongation step of 7 min at 72℃. Cycle conditions for the *-765G→C* polymorphism were 4 min at 95℃, followed by 40 cycles of 30 s at 95℃, 30 s at 54℃, 30 s at 72℃ and finally the same elongation step as for the *-1195A→G* PCR assay. The samples were then analyzed by agarose gel electrophoresis for control of the PCR products.

The PCR products (10 μL) were incubated with 10 U of restriction enzymes *Pvu*Ⅱ and *Hha*1 at 37℃ for determination of the *-1195A→G* and *-765G→C* genotypes, respectively. Finally, the samples were analyzed by agarose gel electrophoresis. The *-765G→C* and *-1195A→G* genotypes that could be detected were: *765CC* (100 bp fragment), *765GC* (100 + 74 + 26 bp fragments), *765GG* (74 + 26 bp fragments), *1195AA* (273 bp fragment), *1195GA* (273 + 220 + 53 bp fragments) and *1195GG* (220 + 53 bp fragments), respectively.

Statistical analysis

The data analysis was performed using SPSS software

Table 2 Genotype distribution and OR of the *COX-2 -1195A→G* and *-765G→C* polymorphisms in patients with CRC and controls

Genotype	Patients with CRC (*n* = 326)	Controls (*n* = 369)	OR (95% CI)
-1195AA	213 (65.3%)	232 (62.9%)	Reference
-1195GA	101 (31.0%)	124 (33.6%)	0.90 (0.66-1.23)
-1195GG	12 (3.7%)	13 (3.5%)	1.01 (0.45-2.25)
-765GG	241 (73.9%)	249 (67.5%)	Reference
-765GC	75 (23.0%)	112 (30.4%)	0.69 (0.49-0.97)
-765CC	10 (3.1%)	8 (2.2%)	1.29 (0.50-3.33)

OR: Odds ratio; CI: Confidence interval.

(Version 14.0, SPSS, Chicago, IL, USA). Logistic regression was used to assess the association between the genotypes and the risk of CRC. The statistical significance of the *-1195A→G* and *-765G→C* genotype distributions between the patient and control groups was determined by Chi-square analysis. A *P*-value of < 0.05 was used as the criterion of statistical significance and all analyses were adjusted for age and sex. A test for deviation from the Hardy-Weinberg equilibrium, by comparing the expected to observed genotype frequencies, was used. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated. Based on the two polymorphisms tested, a haplotype analysis was performed. In the two populations studied, seven different haplotypes could be distinguished: *AC/AC, AG/AC, AG/AG, GC/AC, GG/AC, GG/AG* and *GG/GG*. The localization of the tumor, distal or proximal, was also included in the database analyses.

RESULTS

Using cancer-free controls as a reference we tested for an association of the two *COX-2* polymorphisms with CRC. The genotype distributions in patients and controls of the two *COX-2* polymorphisms investigated are summarized in Table 2. The observed genotype distributions for the *-765G→C* and *-1195A→G* polymorphisms in patients with CRC and controls were in accordance with the Hardy-Weinberg equilibrium, with *P*-values of 0.19 and 0.99 for patients with CRC and 0.24 and 0.46 for controls, respectively. When both polymorphisms were investigated separately, there was no significant difference in the *-765G→C* or *-1195A→G* allele frequency between the patient and control group. However, the *-765GG* genotype was more frequent in patients than in controls (OR, 1.45; 95% CI, 1.03-2.04). There was no significant difference in the genotype distribution of the *-1195A→G* polymorphism among patients and controls.

Next, the potential association of genotype distribution of the two *COX-2* polymorphisms with tumor localization was investigated. We distinguished proximal and distal tumor localization. Proximal included the cecum, colon ascendens and colon transversum and distal included the rectum, sigmoid, colon descendens and flexura lienalis. No association between the *-765G→C* and *-1195A→G* polymorphisms and tumor localization was detected.

Table 3 *COX-2* haplotypes in patients with CRC and controls

Haplotype	Patients with CRC (*n* = 326)	Controls (*n* = 369)	OR (95% CI)
AC/AC	9 (2.8%)	8 (2.2%)	1.16 (0.40-3.42)
AG/AC	59 (18.1%)	74 (20.1%)	0.83 (0.54-1.27)
AG/AG	145 (44.5%)	150 (40.7%)	Reference
GC/AC	1 (0.3%)	-	-
GG/AC	16 (4.9%)	38 (10.3%)	0.44 (0.22-0.85)
GG/AG	84 (25.8%)	86 (23.3%)	1.01 (0.68-1.50)
GG/GG	12 (3.7%)	13 (3.5%)	0.92 (0.38-2.24)

Also no association of the genotype distribution of the *-765G→C* and *-1195A→G* polymorphisms in the patient group was found with gender and age.

Based on the two polymorphisms tested, a haplotype analysis was performed in the two populations studied and seven haplotypes could be distinguished (Table 3). A significant difference between the *COX-2* haplotypes was observed. The *GG/AC* haplotype was less frequent in patients (OR, 0.44; 95% CI, 0.22-0.85). When the *AC*, *AG* and *GG* haplotypes were investigated separately; the *AC* haplotype tended to occur less frequently in patients than in controls ($OR_{(AG/AC)}$ 0.78; 95% CI, 0.57-1.06).

DISCUSSION

The COX-2 protein was detected in 70% of all colorectal cancer tissues. In adjacent normal colorectal tissue in the same slide the COX-2 protein was not observed. These results suggest that increased expression of COX-2 is associated with CRC[12]. SNPs in the *COX-2* promoter may alter the enzyme activity of COX-2 by differential regulation of COX-2 expression, which may influence the risk of the development of CRC[7-9]. It has been recently demonstrated that the polymorphisms *-765G→C* and *-1195A→G* may have a functional effect on COX-2 expression and enzyme activity[7-9]. Both the *-765G→C* and *-1195A→G* polymorphisms were shown to display a lower *COX-2* promoter activity, which may result in a lower expression of the COX-2 enzyme[5,13].

We investigated the potential association of the *COX-2* polymorphisms *-765G→C* and *-1195A→G* and the risk of developing CRC, and found that the *-765GG* genotype was present more often in patients than in controls. As demonstrated by Zhang *et al*[5] the reporter gene expression driven by the *-765G*-containing *COX-2* promoter was higher as compared to the *-765C*-containing counterpart. This indeed could mean a higher COX-2 expression in *-765GG* individuals.

A study in American Caucasians reported a reduced risk of colorectal adenomas in individuals bearing the *-765GG* genotype, but this lower risk was found only among users of non-steroidal antiinflammatory drugs (NSAIDs). In addition, a lower risk of adenoma among *-765CC* genotypes was found only in non-users of NSAIDs[11]. Zhang *et al*[5] and Tan *et al*[9] reported that the *-765GC* genotype was associated with an increased

risk of esophageal squamous cell carcinoma (ESCC) and CRC, in Chinese populations. The findings of Tan *et al*[9] and Zhang *et al*[5] seem in contrast with our results, since we found a reduced risk of CRC with the *-765GC* genotype. However, racial differences in the study populations may explain these apparent contradictory results, since the distribution of the *COX-2* polymorphisms studied here differs considerably between the Chinese and Dutch study populations. The genotype frequencies found in our Dutch patients with CRC for the *-765G→C* and *-1195A→G* polymorphisms were: 73.9% *GG*, 23.0% *GC*, 3.1% *CC* and 65.3% *AA*, 31.0% *GA*, 3.7% *GG*, respectively. Zhang *et al*[5] in a Chinese population reported genotype frequencies of 90.6% *GG*, 9.4% *GC*, 0% *CC* and 30.5% *AA*, 52.9% *GA* and 16.6% *GG*. Tan *et al*[9] in Chinese patients with CRC recently reported approximately the same genotype frequencies as Zhang *et al*[5]: 91.6% *GG*, 8.4% *GC*, 0% *CC* and 34.5% *AA*, 49.4% *GA* and 16.1% *GG*. These findings suggest that ethnic differences in genotype frequencies of *COX-2* polymorphisms may have a significantly different modulating effect on disease phenotypes in different ethnic populations.

According to Zhang *et al*[5] and Tan *et al*[9] in a Chinese population, the *-1195GA* and *-1195AA* genotypes were associated with an increased risk of ESCC and CRC, respectively. This again is not in line with our findings, since we could not demonstrate a significant difference in the allele distribution of the *-1195A→G* polymorphism between our Dutch patients with CRC and controls.

We also investigated the potential association of the genotype distributions of the *-1195A→G* and *-765G→C* polymorphisms with tumor localization. No association between the two polymorphisms and tumor localization was found, which is in accordance with the results of Tan *et al*[9] who found a very similar distribution of both *COX-2* genotypes in patients with colon (n = 403) or rectal (n = 597) cancer.

The *COX-2 GG/AC* haplotype (*-1195G-765G/-1195A-765C)* was found to be present less frequently in patients. When the *AC*, *AG* and *GG* haplotypes were investigated separately, the *AC* haplotype tended to be less frequently present in patients with CRC than in controls ($OR_{(AG/AC)}$ 0.78; 95% CI, 0.57-1.06). This is in line with the findings of Zhang *et al*[5] who demonstrated that the luciferase expression of the *AG* constructs was higher than the expression of the *AC* constructs, suggesting that the *AC* haplotype was associated with a lower COX-2 expression and a decreased risk of CRC.

However, Zhang *et al*[5], Tan *et al*[9] and Moons *et al*[14] found an association of the *AC* haplotype with an increased risk of ESCC, CRC and esophageal adenocarcinoma (EAC). These findings are in contrast with our results, as described above. In addition, the predicted expression levels of the COX-2 protein are higher in *AG* versus *AC* haplotype individuals, according to Zhang *et al*[5], which is not in agreement with the hypothesis that high expression of COX-2 is a risk factor for colorectal or esophageal carcinoma. It should be noted however that haplotype frequencies of *AC* are very low in the patient and control populations studied by Zhang *et al*[5] and Tan *et al*[9], being 4.5% *vs* 1.6% and 3.8% *vs* 1.8%, respectively, compared to 21.2% *vs* 32.6% in our study. In the study of Moons *et al*[14] the *AC* haplotype occurred in 25.0% of the total study population, who were patients with esophageal adenocarcinoma, Barrett's esophagus and reflux esophagitis, a proportion which is very close to our data. In the study of Moons *et al*[14] unfortunately no cancer-free controls were included, but patients with Barrett's esophagus or reflux esophagitis were used as controls, both of which would confer a risk of esophageal adenocarcinoma.

In summary, we found a significant difference in the *-765G→C* polymorphism distribution between the patients with CRC and the control group; the *-765GG* genotype was associated with an increased risk for CRC. The *GG/AC* haplotype was found less frequently in patients with CRC and may be associated with a reduced risk of CRC. These findings suggest a modulating role for the *COX-2* polymorphisms *-1195A→G* and *-765G→C* in the development of CRC in a Dutch population.

COMMENTS

Background

Cyclooxygenase-2 (COX-2) is a key enzyme in the development and progression of neoplasms. COX-2 was found to be over-expressed in gastrointestinal tumors, including those of the colon/rectum. The corresponding *COX-2* gene is polymorphic and two single nucleotide polymorphisms (SNPs; *-1195A→G* and *-765G→C*) were demonstrated to influence the expression of COX-2. Therefore, these polymorphisms may modulate the risk for gastrointestinal cancers, such as cancers of the colon/rectum.

Research frontiers

In this study, the *-765GG* genotype was associated with an increased risk of developing CRC and the *GG/AC* haplotype seemed to protect against CRC.

Innovations and breakthroughs

The *COX-2* polymorphisms *-765G→C* and *-1195A→G* may modulate the development of CRC in a Dutch population.

Applications

Screening for the *COX-2 -765GG* genotype in a population at risk of colorectal cancer may be valuable in future in order to select the high risk patients. Information and prevention programs can than be focused on these patients.

Terminology

COX-2 is an enzyme that catalyzes the conversion of arachidonic acid in prostaglandin H2, the precursor of other prostaglandins, prostacyclin and thromboxanes. These regulatory compounds play a role in many biological processes such as cell proliferation, angiogenesis, immune function and inflammation, which are all crucial in the development and progression of neoplasms.

Peer review

The manuscript, reported by Hoff *et al*, analyzes COX-2 polymorphisms *-765G→C* and *-1195A→G* and colorectal cancer (CRC) risk in a Dutch population. This manuscript contributes in the effort to characterize some molecular signatures as risk factors for colon cancer in different ethnic populations.

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BRIEF ARTICLES

Lack of correlation between *p53* codon 72 polymorphism and anal cancer risk

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Abstract

AIM: To investigate the potential role of *p53* codon 72 polymorphism as a risk factor for development of anal cancer.

METHODS: Thirty-two patients with invasive anal carcinoma and 103 healthy blood donors were included in the study. *p53* codon 72 polymorphism was analyzed in blood samples through polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing.

RESULTS: The relative frequency of each allele was 0.60 for Arg and 0.40 for Pro in patients with anal cancer, and 0.61 for Arg and 0.39 for Pro in normal controls. No significant differences in distribution of the codon 72 genotypes between patients and controls were found.

CONCLUSION: These results do not support a role for the *p53* codon 72 polymorphism in anal carcinogenesis.

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Key words: Anus neoplasms; Arginine; Genetic polymorphism; Polymerase chain reaction; Proline; Tumor suppressor protein *p53*

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INTRODUCTION

Squamous cell carcinoma (SCC) of the anus is a relatively uncommon malignancy, affecting approximately 4600 patients per year in the United States[1]. Globally, annual incidence rates of invasive anal cancer range from 0.1 to 2.8 cases per 100 000 among men and 0.0-2.2 cases per 100 000 among women[2]. In particular, anal cancer rates among men who have sex with men are notably higher[3]. Invasive anal cancer, like invasive cervical cancer, has been causally linked to high-risk human papillomavirus (HPV) infection[4,5]. According to a recent review, HPV is detected in 71% of invasive anal cancers, with approximately 72% of the HPV-positive cases being associated with HPV 16 and/or 18 infection[6]. This estimate of HPV 16 and 18 prevalence is similar to that found in invasive cervical cancer[7].

Although many risk factors for the development of anal cancer have been identified, such as the practice of receptive anal intercourse and immunodeficiency, the molecular mechanisms related to anal carcinogenesis remain unclear. Mutations in the *p53* gene are the most common genetic alterations in human cancer and they can be found in up to 80% of anal carcinomas[8]. In addition to gene mutations, some polymorphisms in the *p53* gene have been suggested to play a role in different malignancies[9-11]. Recent studies have focused on a common single-base-pair polymorphism at codon

72, which results in a Pro (CCC) or Arg (CGC) residue at this position. The two polymorphic variants have been shown to have not only structural differences, as reflected by distinct electrophoresis patterns of migration, but also different biological properties[12-14]. The Arg variant has been demonstrated to be more susceptible to degradation by the HPV E6 protein than the Pro variant, with individuals who are homozygous for Arg having a higher risk of being affected by HPV-associated malignant tumors[15].

In this article, we present the results of what is believed to be the first study to investigate the potential association of *p53* codon 72 polymorphism with invasive carcinoma of the anal canal.

MATERIALS AND METHODS

Cases and controls

Thirty-two patients with histologically confirmed primary SCC of the anal canal (mean age 60.3 years, range 30-81 years) were enrolled prospectively in the study. As a non-malignant control group, we studied 103 consecutive healthy blood donors with no previous history of cancer (mean age 47.7 years, range 40-72 years). Demographic characteristics of cases and controls are shown in Table 1. After pretreatment assessment, including a complete medical history and physical examination, colonoscopic examination, computed tomography of the abdomen and pelvis, and chest radiography, the patient's AJCC (American Joint Committee on Cancer) tumor stage was determined[16]. The distribution was as follows: 6.2% stage Ⅰ (n = 2), 53.1% stage Ⅱ (n = 17), 34.3% stage Ⅲ (n = 11) and 6.2% stage Ⅳ (n = 2).

The study was approved by the Ethics and Scientific Committee of the Santa Casa Hospital Complex and Hospital de Clínicas de Porto Alegre. Informed consent was obtained from all patients and controls before being enrolled in the study.

DNA extraction and genotyping

p53 codon 72 polymorphism was studied in blood samples collected by venous puncture. Genomic DNA was extracted from peripheral lymphocytes using Ultra Clean DNA Bloodstain Kit (MoBiolabs, Solana Beach, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of codon 72, modified from the technique described by Ara *et al*[17], was used to identify *p53* genotypes. The forward primer used was 5'-TTGCCGTCCCAAAGCAATGGATGA-3', and the reverse primer was 5'-TCTGGGAAGGGACA GAAGATGAC-3'. Each PCR reaction mixture (50 mL) contained 10 pmol each primer, 1.5 mmol/L $MgCl_2$, 200 mmol/L each dNTP, 1 U Platinum®*Taq* DNA polymerase (Invitrogen, Sao Paulo, Brazil), and 100-300 ng genomic DNA. Reaction mixtures were preincubated for 5 min at 94℃. PCR conditions were 94℃ for 1 min and 55℃ for 1 min, followed by 72℃ for 1 min for 35 cycles. The final extension was at 72℃ for 10 min.

Table 1 Demographic characteristics of cancer patients and controls *n* (%)

Characteristics	Cases	Controls	*P*
Gender			
Male	7 (22)	22 (21)	NS
Female	25 (78)	81 (79)	
Race			
White	28 (87)	96 (93)	NS
Non-white	4 (13)	7 (7)	
Age (yr); mean (range)	60.3 (30-81)	47.7 (40-72)	< 0.001
Total	32 (100)	103 (100)	

NS: Not significant.

After confirmation of an amplified fragment of the expected size (199 bp) on an agarose gel, 10 μL PCR product was digested with 6 U restriction enzyme *Bst*UI (New England Biolabs, Ipswich, MA, USA) at 60℃ for at least 4 h. DNA fragments were electrophoresed through a 2.5% agarose gel and stained with ethidium bromide. RFLP results were confirmed by sequencing the PCR fragments from nine randomly selected samples (three of each genotype) using an automated sequencing system (ABI Prism 310 Genetic Analyzer; Applied BioSystems, Foster City, CA, USA). Sequencing reactions were performed using the BigDye® Terminator V3.1 cycle sequencing reaction kit (Applied BioSystems) according to the manufacturer's instructions. Forward and reverse primers were utilized as sequencing primers.

Statistical analysis

Univariate statistics were used first to compare cases and controls for demographic variables and genotype prevalence. The χ^2 test was used to analyze categorical variables and ANOVA was used to compare the continuous variable age. The association between the *p53* polymorphism and anal cancer was determined using the logistic regression method to assess ORs and 95% CI. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of *p53* codon 72 polymorphism by PCR-RFLP was performed in all cases and controls. The Arg allele was cleaved by *Bst*UI, which yielded two small fragments (113 and 86 bp). The Pro allele was not cleaved by *Bst*UI, which had a single 199-bp band. Heterozygotes contained three bands, which corresponded to 199, 113 and 86 bp. The PCR results were confirmed by DNA sequencing.

The distribution of the codon 72 genotypes in patients and controls did not deviate from the Hardy-Weinberg equilibrium. The genotype frequencies in cases and controls are presented in Table 2, with no association with anal cancer risk being observed. The relative frequency of each allele was 0.60 for Arg and 0.40 for Pro in patients with anal cancer, and 0.61 for Arg and 0.39 for Pro in normal controls.

We also analyzed the codon 72 polymorphism of the healthy controls according to their age. The genotype

Table 2 Distribution of *p53* codon 72 polymorphism in cancer patients and controls *n* (%)

	Total	Arg/Arg	Arg/Pro	Pro/Pro	OR[1]	CI	*P*
Anal cancer	32	10 (31.2)	19 (59.4)	3 (9.4)	1.6	0.6-4.9	0.325
Controls	103	31 (30.1)	62 (60.2)	10 (9.7)			

[1]Adjusted for age. Arg/Arg *vs* Arg/Pro and Pro/Pro.

distribution in the 78 controls under 50 years old was as follows: 25 Arg/Arg (32.1%), 6 Pro/Pro (7.7%), and 47 Arg/Pro (60.3%). The distribution in the 25 controls over 50 years old was: 6 Arg/Arg (24.0%), 4 Pro/Pro (16.0%), and 15 Arg/Pro (60.0%). No significant difference in the genotype distribution was found between these two age groups ($P = 0.407$).

DISCUSSION

High-risk HPV infection has been implicated in the pathogenesis of different malignancies[17-21]. Several biochemical and genetic studies have shown that HPV E6 and E7 proteins exert a cooperative effect on cellular transformation and immortality by interfering with the function of cellular tumor suppressor proteins[22-24].

A common polymorphism has been known in codon 72 of the *p53* gene, with two alleles encoding either Arg (*p53*Arg) or Pro (*p53*Pro)[13,14]. Storey *et al*[15] have investigated the effect of this polymorphism on the susceptibility to E6-mediated degradation and found that individuals homozygous for Arg are seven times more susceptible to HPV-associated cervical carcinogenesis than heterozygotes are. Since then, the effect of codon-72 polymorphism of *p53* on cervical cancer has been studied, with contradictory results being reported. Overall, as demonstrated in a recent meta-analysis, compared with the heterozygous genotype (Pro/Arg), the homozygous genotype (Arg/Arg) of codon-72 of *p53* is associated with an approximately 20% increased risk of cervical cancer[25].

Invasive anal cancer, like invasive cervical cancer, has well-documented precursors, known as anal intraepithelial neoplasia 2-3 (histology) or high-grade squamous intraepithelial lesions (cytology)[6]. Anal cancer also has been causally linked to high-risk HPV infection, therefore, we decided to evaluate, perhaps for the first time, the potential role of codon 72 polymorphism as a risk factor for development of this type cancer.

In order to minimize sources of bias and avoid misinterpretation of the results, standard safeguards were adopted. Patients and controls were matched ethnically and derived from a population living in the same geographic region (Southern Brazil), and were enrolled consecutively in a single institution. All PCR results were confirmed by DNA sequencing.

We investigated the allele and genotype frequencies at *p53* codon 72 in 32 patients with anal cancer and 103 healthy individuals from southern Brazil. No significant differences in the relative allele frequency and in the distribution of genotypes were found between patients and controls. These results are in line with several studies that failed to demonstrate a correlation of the *p53* codon 72 polymorphism with development of non-cervical HPV-associated epithelial malignant tumors, such as head and neck and oral SCCs[26,27].

The association between codon 72 polymorphism and risk of cancer has been reported in different populations[28]. Studies have been conducted to evaluate this polymorphism as a risk factor for different types of cancer, such as gastric[29], lung[9] and breast carcinomas[11]. So far, the published data have been inconclusive. The conflicting results found in the literature might be attributed to variations in protocols among different laboratories, or to poor selection of control groups[29]. They also might have been caused by the inherent characteristics of the population being analyzed, as there are considerable variations in the distribution of the codon 72 genotypes in various populations. This polymorphism seems to be maintained by natural selection influenced by environmental factors, such as the degree of exposure to the UV-B component of sunlight[30]. The resulting North-South Arg/Pro gradient has been reported in different geographical regions. Population-based studies have indicated that the Arg allele is most prevalent in individuals with light complexion and least prevalent in those with darker complexion, with a clear and consistent decline in the prevalence of the Pro allele, with increasing northern latitude[30-32].

The population from Southern Brazil, in contrast with other regions of the country, is composed mainly of Caucasian individuals who are descended from European immigrants[33]. Although most of these immigrants came from Portugal, Germany and Italy[34], the genotype distribution found in our healthy controls was notably different from the genotype distribution observed in those countries[35-37]. This can be explained partially by the process of miscegenation among different ethnic groups (Caucasians, Amerindians and Afro-Brazilians) that took place during Brazilian colonization[38]. Each specific population seems to have its own characteristic genotype distribution that can differ markedly from the polymorphic frequencies found in other populations, even when neighboring countries are compared.

We believe, however, that the lack of correlation between the codon 72 genotype distribution and anal cancer risk observed in our study cannot be interpreted solely as a result of population ethnicity. In a previous study of cancer patients and normal individuals from Southern Brazil, we were able to detect a significant

association of *p53* codon 72 polymorphism with breast cancer risk[39]. We analyzed blood samples collected from 118 women with primary breast carcinoma and from 202 female blood donors (healthy controls) through PCR-RFLP and DNA sequencing. The Arg/Arg genotype was significantly associated with an increased risk for breast cancer (OR 2.9; 95% CI: 1.43-3.6; $P < 0.002$). The relative frequency of each allele was 0.75 for Arg and 0.25 for Pro in patients with cancer, and 0.62 for Arg and 0.38 for Pro in normal controls ($P < 0.001$). In the present study, the relative frequency of each allele observed within the control group (0.61 for Arg and 0.39 for Pro) was therefore very similar to our previous observation in normal controls derived from the same population.

In summary, we did not detect significant differences in the allele distribution at codon 72 of *p53* between patients with invasive anal cancer and healthy controls. Our results do not support the hypothesis that *p53* codon 72 polymorphism is associated with anal cancer susceptibility. The role of the genetic susceptibility to high-risk HPV infection and anal cancer, however, merits further investigation.

COMMENTS

Background

A common Arg/Pro polymorphism at codon 72 of the *p53* gene has been studied as a risk factor for human papilloma virus (HPV)-associated malignancies. Although anal cancer has been associated repeatedly with high-risk HPV infection, this polymorphism has not been investigated in this type of cancer up until now.

Research frontiers

Although several risk factors for the development of anal cancer have been determined, the molecular mechanisms involved in anal carcinogenesis remain unclear. In this context, the identification of new factors involved in progression of the anal carcinoma represents a critical step towards development of new anticancer strategies in this malignancy.

Innovations and breakthroughs

This is believed to be the first study to investigate *p53* codon 72 polymorphism in patients with anal cancer. The authors did not detect differences in the allele distribution at codon 72 of *p53* between patients with invasive anal cancer and healthy controls. In contrast to previous observations with cervical cancer, this polymorphism does not seem to be associated with anal cancer susceptibility. The results, however, are in line with several studies that failed to demonstrate a correlation of the *p53* codon 72 polymorphism with development of non-cervical HPV-associated epithelial malignant tumors, such as head and neck and oral squamous cell carcinomas.

Applications

In the process of identifying genetic causes of cancer, it is important to determine precisely which elements of a biological pathway are responsible for affecting tumor suppression or development. Then, treatments and preventive measures can be directed to those individuals who would benefit most. Previous studies have identified *p53* codon 72 polymorphism as a potential contributing factor in HPV-associated carcinogenesis. This study found that *p53* codon 72 polymorphism is not a likely risk factor for anal cancer, and future research should focus on other parts of the *p53* gene pathway to understand its role in development of this type of cancer.

Terminology

Mutations in the *p53* gene are the most common genetic alterations in human cancer. In addition to gene mutations, some polymorphisms in the *p53* gene have been suggested to play a role in different malignancies. A polymorphism is known in codon 72 of the *p53* gene, which results in a Pro or Arg residue at this position. These two polymorphic variants have been shown to have different biological properties, including differences in cancer susceptibility.

Peer review

Contu *et al* Have described the lack of a functional role of a specific *p53* polymorphism in the pathogenesis of anal cancer. The major point of this paper and the spectrum of methods used are rather confined, but the point is clear and the paper is well written. Although this paper presents negative data, I feel that the attempt to perform genetic analysis on a relatively uncommon malignancy (anal cancer) may be viewed favorably clinically.

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Hemoperfusion with polymyxin B-immobilized fiber column improves liver function after ischemia-reperfusion injury

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Abstract

AIM: To investigate the usefulness of direct hemoperfusion with a polymyxin B-immobilized fiber column (DHP-PMX therapy) for warm hepatic ischemia-reperfusion (I/R) injury after total hepatic vascular exclusion (THVE) using a porcine model.

METHODS: Eleven Mexican hairless pigs weighing 22-38 kg were subjected to THVE for 120 min and then observed for 360 min. The animals were divided into two groups randomly: the DHP-PMX group (n = 5) underwent DHP-PMX at a flow rate of 80 mL/min for 120 min (beginning 10 min before reperfusion), while the control group did not (n = 6). The rate pressure product (RPP): heart rate × end-systolic arterial blood pressure, hepatic tissue blood flow (HTBF), portal vein blood flow (PVBF), and serum aspartate aminotransferase (AST) levels were compared between the two groups.

RESULTS: RPP and HTBF were significantly (P < 0.05) higher in the DHP-PMX group than in the control group 240 and 360 min after reperfusion. PVBF in the DHP-PMX group was maintained at about 70% of the flow before ischemia and differed significantly (P < 0.05) compared to the control group 360 min after reperfusion. The serum AST increased gradually after reperfusion in both groups, but the AST was significantly (P < 0.05) lower in the DHP-PMX group 360 min after reperfusion.

CONCLUSION: DHP-PMX therapy reduced the hepatic warm I/R injury caused by THVE in a porcine model.

Key words: Ischemia-reperfusion injury; Total hepatic vascular exclusion; Polymyxin B-immobilized fiber column

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INTRODUCTION

In ischemia-reperfusion (I/R), the generation of reactive oxygen species on reoxygenation inflicts tissue damage and initiates a cascade of deleterious cellular responses leading to inflammation, cell death, and ultimately organ failure[1]. Hepatic I/R injury occurs in various clinical settings, such as transplantation, trauma, liver or bowel resection, and hemorrhagic shock[2]. Severe hepatic I/R injury can lead to liver or multiple organ failure and is associated with increased morbidity and mortality[3]. Total hepatic vascular exclusion (THVE), which involves the total occlusion of the liver vasculature at the hepatoduodenal ligament (i.e. Pringle's maneuver) and the occlusion of the inferior vena cava below and above the liver, is used during the resection of large and posterior portions of the liver clinically[4,5]. As this technique induces hepatic I/R injury, inhibition of this injury caused by THVE is necessary to obtain a better postoperative course.

The Toraymyxin polymyxin B-immobilized fiber column (PMX cartridge; Toray Industries, Tokyo, Japan) was developed in Japan in 1994 as an extracorporeal

hemoperfusion device that uses polymyxin-B fixed to α-chloroacetamide-methyl polystyrene-derived fibers packed in the cartridge. Direct hemoperfusion with PMX (DHP-PMX) therapy can remove circulating endotoxin and reduce various cytokines, even in patients with high plasma cytokine levels[6]. This method has been used to treat endotoxemia[7] and was reported to lower inflammatory cytokine and plasminogen activator inhibitor-1 (PAI-1) levels immediately[8]. DHP-PMX therapy has also been found effective for severe sepsis secondary to intra-abdominal infection[9] and acute lung injury or acute respiratory distress syndrome caused by sepsis[10]. Recently, we reported the efficacy of DHP-PMX therapy in normothermic cardiopulmonary bypass[11], a pulmonary warm I/R injury model[12], and a small intestine warm I/R injury model[13].

In this study, we evaluated the usefulness of DHP-PMX therapy in warm hepatic I/R injury with a porcine THVE model.

MATERIALS AND METHODS

Animals

All animals were cared for in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the guidelines set forth in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication 85-23, revised 1985). The study was performed under the supervision of the Animal Care and Experimental Committee of Gunma University, Showa campus, Japan.

Operative procedure

Eleven Mexican hairless pigs (both sexes, weighing 22-38 kg) were used in this study. They were not allowed access to food for 24 h before the experiment. After administering ketamine hydrochloride (250 mg) and atropine (0.5 mg) intramuscularly, the pigs were intubated endotracheally and ventilated mechanically at a tidal volume of 25 mL/kg and a rate of 12 breaths/min. During the experiment, general anesthesia was maintained with a mixture of 1%-2% isoflurane and 100% oxygen. Lactated Ringer's solution (20 mL/kg per hour) was infused *via* a catheter inserted into the right subclavian vein. A laparotomy was performed *via* a midline incision. The liver was skeletonized completely by dividing all of the suspensory ligaments and dissecting the retrohepatic vena cava from the posterior abdominal wall. The portal vein, hepatic artery, and common bile duct were isolated and their collaterals were occluded separately. THVE was achieved by clamping the infrahepatic and suprahepatic vena cava after clamping the portal vein and hepatic artery. An active venovenous (v-v) bypass system was started as a portosystemic shunt just before THVE to prevent congestion of the portal vein and lower body. This system consisted of a centrifugal pump system (Lifestream; St. Jude Medical, Chelmsford, MA) and venous cannulas. The blood-contact surfaces of these components were heparin-coated. The v-v bypass system was established with drains (12 Fr) inserted into the splenic and right external iliac veins for blood removal, with another drain inserted into the right external jugular vein (12 Fr) for blood return. Blood from the portal vein and infrahepatic vena cava was bypassed into the right external jugular vein *via* a Y-shaped shunt. The bypass blood flow was maintained at more than 20 mL/kg per minute with systemic heparinization (200 U/kg). Liver ischemia was induced by total exclusion of hepatic inflow for 120 min. After releasing the clamps to end the ischemia, the bypass system was removed. The splenic, right external iliac, and right external jugular veins were ligated after removing the cannulas. The parameters described below were measured and the animals were observed for 360 min after reperfusion.

Experimental groups

The experimental study involved two groups: the DHP-PMX (n = 5) and control (n = 6) groups. The animals were assigned randomly to either group. In the DHP-PMX group, a double-lumen catheter was positioned in the right atrium through the left subclavian vein and DHP-PMX was performed through the catheter at a flow rate of 80 mL/min for 120 min (beginning 10 min before reperfusion). Direct hemoperfusion was not performed in the control group.

Monitoring and sampling

The external iliac artery was cannulated for monitoring the arterial blood pressure and collecting blood samples. Arterial blood pressure and heart rate (HR) were monitored directly through a catheter connected to a transducer (Spectramed TA 1017; San-ei, Tokyo, Japan). The rate pressure product (RPP: HR × end-systolic arterial blood pressure) was also calculated. Blood samples were collected from the same catheter before and after the procedure [before ischemia and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion]. All samples were centrifuged at 900 × g for 15 min at 4℃, and the serum or plasma was frozen at -80℃ for later measurement.

Hepatic tissue blood flow (HTBF)

HTBF was measured with a laser Doppler flowmeter (Laser Blood Flow Monitor MBF 3; Moor Instruments, Devon, UK) before ischemia and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion. The laser probe was always placed on the right median lobe of the liver. HTBF is expressed as a percentage of the flow before ischemia.

Portal vein blood flow (PVBF)

PVBF was measured before ischemia and 30, 60, 120, 240 and 360 min after reperfusion using an electromagnetic blood flowmeter (Model MFV-3100; Nihon Kohden, Tokyo, Japan). PVBF is expressed as a percentage of the flow before ischemia.

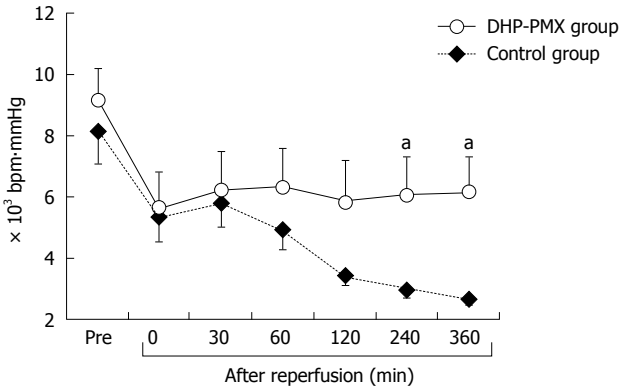

Figure 1 The rate pressure product (RPP: heart rate × systolic arterial blood pressure) before ischemia (pre) and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion. Data are expressed as the mean ± SE. $^aP < 0.05$ *vs* the control group.

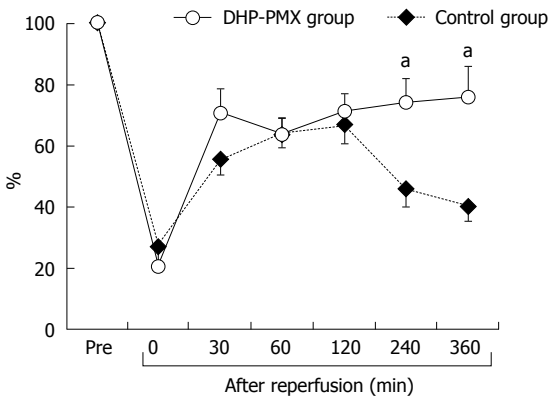

Figure 2 The hepatic tissue blood flow (HTBF) before ischemia (pre) and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion. The HTBF was evaluated as a percentage of the flow before ischemia. Data are expressed as the mean ± SE. $^aP < 0.05$ *vs* the control group.

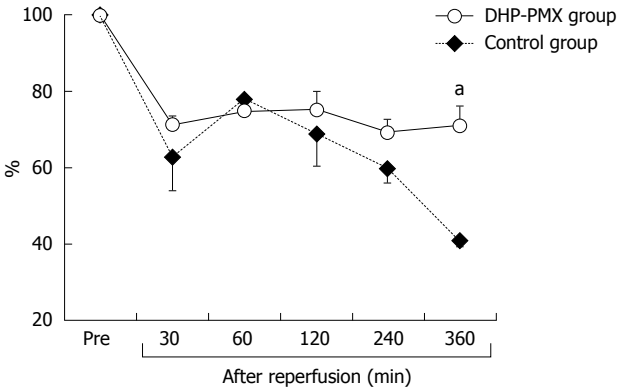

Figure 3 The portal vein blood flow (PVBF) before ischemia (pre) and 30, 60, 120, 240 and 360 min after reperfusion. The PVBF was evaluated as a percentage of the flow before ischemia. Data are expressed as the mean ± SE. $^aP < 0.05$ *vs* the control group.

Serum aspartate aminotransferase (AST) assays

Serum AST levels were measured at 37℃ using an ultraviolet rate assay on an autoanalyzer (Hitachi 736-60; Hitachi, Tokyo, Japan) with blood samples collected and preserved using the method described above.

Statistical analysis

The results are expressed as the mean ± SE. StatView ver. 5.0 (Abacus, Berkeley, CA) was used for the statistical analyses. Statistical comparisons were made using repeated measure analysis of variance followed by Fisher's protected least significant difference. $P < 0.05$ were considered to be statistically significant.

RESULTS

All animals survived until the endpoint of the study (360 min after reperfusion).

The changes in RPP

The changes in RPP were similar in both groups until reperfusion. The RPP in the control group decreased gradually until 360 min after reperfusion, while that in the DHP-PMX group was maintained at about 6000 bpm·mmHg and differed significantly ($P < 0.05$) from the RPP in the control group 240 and 360 min after reperfusion (Figure 1).

The changes in HTBF

The HTBF decreased to about 20% of the baseline immediately after reperfusion in both groups (Figure 2). After reperfusion, the HTBF in the DHP-PMX group was maintained above 60% of the baseline, while no improvement was seen in the control group; the HTBF in the control group was significantly ($P < 0.05$) lower than that in the DHP-PMX group 240 and 360 min after reperfusion (Figure 2).

The changes in PVBF

The PVBF decreased after reperfusion in both groups (Figure 3). The PVBF was similar in both groups 30 and 60 min after reperfusion. Subsequently, the PVBF in the DHP-PMX group was maintained at about 70% of the flow before ischemia, while that in the control group decreased gradually beginning 120 min after reperfusion; a significant ($P < 0.05$) difference was observed between the two groups 360 min after reperfusion (Figure 3).

The changes in serum AST

As shown in Figure 4, the serum AST before ischemia did not differ significantly between the two groups. The serum AST increased gradually after reperfusion in both groups, although the increment in the DHP-PMX group was smaller than in the control group and differed significantly 360 min after reperfusion (Figure 4).

DISCUSSION

Polymyxin B binds to endotoxin, which is an outer membrane component of Gram-negative bacteria and is thought to be an important pathogenic trigger for the production of inflammatory mediators. Several preclinical studies have demonstrated that hemoperfusion or plasmapheresis over immobilized polymyxin B removes

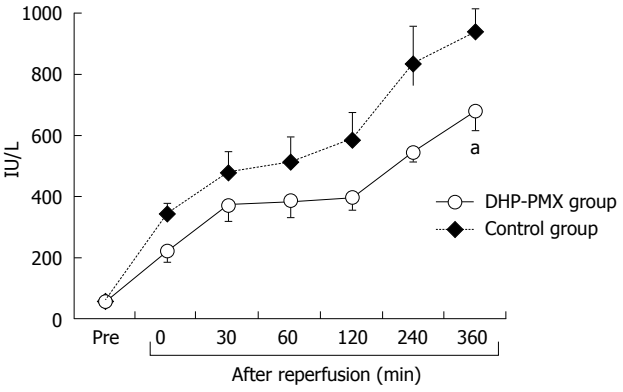

Figure 4 The serum AST before ischemia (pre) and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion. Data are expressed as the mean ± SE. $^aP < 0.05$ *vs* the control group.

endotoxin from blood[14-16]. Recently, some studies have shown improved hemodynamic status[17] and survival[18] in patients with sepsis who were treated with PMX. Moreover, DHP-PMX therapy was found to be effective for patients with septic shock infected with either Gram-negative or Gram-positive bacteria, which do not release endotoxins[19]. Therefore, DHP-PMX therapy is used in patients with severe sepsis or septic shock and its clinical effect has been described. In addition, studies have reported on the mechanism of DHP-PMX action. Kushi *et al*[20] showed that the adsorption of pathogenic bacteria prevented the release of inflammatory cytokines and reduced the stimulation of vascular endothelial cells to lower the PAI-1 level, rather than direct inhibition of PAI-1 production by DHP-PMX therapy. Tani *et al*[8] postulated that the reduction in plasma endotoxins by endotoxin adsorption contributed to the cessation of cytokine gene expression and the excretion of cytokines. In addition, DHP-PMX therapy improved the PaO_2/FiO_2 ratio in patients with acute lung injury or acute respiratory distress syndrome caused by sepsis, and this appeared to be related to decreases in the blood neutrophil elastase and IL-8 levels[10]. We hypothesized that DHP-PMX therapy might be effective in various inflammatory situations and have evaluated the utility of DHP-PMX therapy in normothermic cardiopulmonary bypass in a pig model[11], pulmonary warm I/R injury in a canine model[12], and small intestine warm I/R injury in a canine model[13] with satisfactory results. In this study, we evaluated whether DHP-PMX therapy could reduce hepatic I/R injury in a THVE model.

We found that RPP, PVBF, and HTBF in the DHP-PMX group were preserved significantly ($P < 0.05$) better than in the control group after reperfusion. In addition, the increase in serum AST was significantly ($P < 0.05$) lower in the DHP-PMX group. These results demonstrate that DHP-PMX therapy reduces the hepatic warm I/R injury caused by the THVE technique.

The natural ligands of cannabinoid receptors are lipid-like substances called endocannabinoids, and include arachidonoyl ethanolamine or anandamide and 2-arachidonoylglycerol. Cannabinoid-2 receptor agonists have been reported to have a protective effect against I/R injury in the liver and other organs by reducing endothelial cell activation, the expression of adhesion molecules such as intercellular adhesion molecule and vascular cell adhesion molecule, the levels of tumor necrosis factor-α and chemokines, neutrophil infiltration, lipid peroxidation, and apoptosis[21,22]. In addition, Wang *et al*[23] demonstrated that the absorption of anandamide during DHP-PMX therapy eliminated the diverse negative effects of anandamide, such as hypotension, immunosuppression, and cytotoxicity. Considering these results, DHP-PMX treatment appears not only to remove endotoxins, but also to reduce the inflammatory reaction by inhibiting various inflammatory cascades and to have an effective role in I/R injury. Further studies are required, including the suitable timing and duration and the detailed mechanisms of DHP-PMX therapy in I/R injury.

In conclusion, DHP-PMX therapy reduced the hepatic warm I/R injury caused by the THVE method using a porcine model.

COMMENTS

Background

Total hepatic vascular exclusion (THVE) is used during the resection of large and posterior portions of the liver clinically. This technique induces hepatic ischemia-reperfusion (I/R) injury, and severe hepatic I/R injury is associated with increased morbidity and mortality. The inhibition of the hepatic I/R injury caused by THVE is necessary to obtain a better postoperative course.

Research frontiers

The Toraymyxin polymyxin B-immobilized fiber column (PMX cartridge) was developed as an extracorporeal hemoperfusion device. Direct hemoperfusion with PMX (DHP-PMX) therapy can remove circulating endotoxin. The authors had already demonstrated the efficacy of DHP-PMX therapy in a pulmonary warm I/R injury model and a small intestine warm I/R injury model. In this study, the authors investigated whether DHP-PMX therapy reduced the hepatic warm I/R injury caused by THVE in a porcine model.

Innovations and breakthroughs

The authors found that systemic hemodynamics, blood flow for liver and liver function were preserved significantly better in the group treated with DHP-PMX therapy than in the group with no treatment after reperfusion.

Applications

Their results demonstrate that DHP-PMX therapy reduces the hepatic warm I/R injury caused by the THVE technique.

Terminology

The PMX cartridge (Toray Industries, Tokyo, Japan) was developed in Japan in 1994 as an extracorporeal hemoperfusion device that uses polymyxin-B fixed to α-chloroacetamide-methyl polystyrene-derived fibers packed in the cartridge. DHP-PMX therapy can remove circulating endotoxin and reduce various cytokines, even in patients with high plasma cytokine levels. This method has been used to treat endotoxemia and was reported to lower inflammatory cytokine and plasminogen activator inhibitor-1 levels immediately. DHP-PMX therapy has also been found effective for severe sepsis secondary to intra-abdominal infection and acute lung injury or acute respiratory distress syndrome caused by sepsis.

Peer review

This is an interesting study that investigates the utility of direct hemoperfusion with a polymyxin B-immobilized fiber column (DHP-PMX therapy) on warm hepatic I/R injury with THVE using a porcine model. The title accurately reflects the major topic and contents of the study. The abstract gives a clear delineation of the research background, objectives, materials and methods, results and conclusions. Materials and Methods are very well described. The results are clearly presented and the conclusions are scientifically reliable and valuable.

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BRIEF ARTICLES

Epithelioid angiomyolipoma of the liver: Cross-sectional imaging findings of 10 immunohistochemically-verified cases

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Abstract

AIM: To retrospectively evaluate the computed tomography (CT)/magnetic resonance imaging (MRI) imaging features of epithelioid angiomyolipoma of the liver (Epi-HAML), with pathology as a reference.

METHODS: The CT/MRI findings (number, diameter, lobar location, and appearance of lesions) in a series of 10 patients with 12 pathologically proven epithelioid angiomyolipomas of the liver were retrospectively analyzed. The imaging features, including attenuation/signal intensity characteristics, presence of fat, hypervascular, outer rim, and vessels within lesion, were evaluated and compared with that of non-Epi-HAML in 11 patients (13 lesions). The Fisher exact test was used to compare difference in probability of imaging features between the two types.

RESULTS: For 21 patients, CT images of 15 patients and MR images of six patients were available. No patient underwent two examinations. For the 15 patients with a CT scan, all HAML lesions in the two groups (10 Epi-HAML and seven non-Epi-HAML) manifested as hypoattenuation. For the six patients with MRI, all lesions (two Epi-HAML and six non-Epi-HAML) were hypointense on T1WI (fat suppression) and hyperintense on T2WI. There were 10 non-Epi-HAML, but only two Epi-HAML lesions showed the presence of fat, which significantly different between the two types (P = 0.005). On the dynamic contrast enhancement (DCE) imaging, eight Epi-HAML, and 13 non-Epi lesions manifested as hypervascular. Punctate or curved vessels were displayed in 10 Epi-HAML as well as in nine non-Epi lesions and outer rim enhancement could be found with eight Epi-HAML as well as six non-Epi lesions.

CONCLUSION: Little or no presence of adipose tissue was found to be an imaging feature of Epi-HAML, compared with the non-Epi type. In addition, hypervascularity with opacification of central punctiform or filiform vessels on DCE would be a characteristic enhancement pattern for Epi-HAML.

Key words: Epithelioid angiomyolipoma; Liver; Immunohistochemical staining; Magnetic resonance imaging; Computed X-ray tomography

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INTRODUCTION

Hepatic Angiomyolipoma (HAML) is a rare benign tumor and the etiology is unclear. Some cases have been associated with the tuberous sclerosis complex (TSC). HAML belongs to a family of tumors that have collectively been called "PEComa"[1-3]. Histologically, HAML is composed of a heterogeneous mixture of blood vessels, smooth muscle, and adipose cells, of varying proportions and distributions, not only among different tumors, but from area to area within the same tumor. Thus, according to the line of differentiation and the predominance of tissue components, HAML is usually subcategorized into mixed,

lipomatous (≥ 70% fat), myomatous (≤ 10% fat), and angiomatous types. The smooth muscle cell component is the most specific for the diagnosis. Depending on the dominant cell type, HAML can be subcategorized into epithelioid, spindle, and intermediate forms[1]. Epithelioid AML (Epi-AML) was first described in the liver in 2000[4], diagnosis can be difficult, for example the differentiation of Epi-AML from hepatocellular carcinoma and the metastatic sarcomatoid variant of renal cell carcinoma. Only one case report of Epi-AML has been mentioned or reviewed in the literature[4-7]. In this paper, 10 immunohistochemically verified cases were retrospectively analyzed and the CT/MR imaging findings were summarized and compared with those of non-epi-HAML.

MATERIALS AND METHODS

Patients

The imaging examinations of 21 cases (10 Epi-HAML and 11 non-Epi-HAML) with pathologically proven HAML were included in this study. Proof of diagnosis was based on findings at liver resection and pathological manifestations, including immunohistochemical staining. Cases were collected from one university hospital over a seven-year period and were identified by reviewing pathology databases. In our patients, AML were incidentally detected on cross-sectional imaging performed for various reasons, such as abdominal pain (n = 15), suspected gallbladder stone (n = 3), or urinary (n =3) diseases. Institutional review board approval and patient consent were not required for this retrospective study because patient privacy was maintained and patient care was not impacted.

Imaging protocols and methods

Helical multiphasic CT was performed in 15 patients using a Siemens Sensation 16 (Siemens Medical Solutions) or a PHILIPS Marconi MX8000 4 slice CT unit with 5 to 7 mm contiguous sections. After non-enhanced acquisitions of the liver, patients underwent helical multiphase CT that included both hepatic arterial phase and portal venous phase imaging (30-35 s and 80-85 s, respectively), after *i.v.* infusion of 90-100 mL nonionic contrast material (iopromide, Ultravist 300, Bayer Schering Pharma). Contrast material was injected at a rate of 3 mL/s with a power injector (Envision CT, MEDRAD).

MRI was performed in six patients using 1.5-T MR units (Magnetom avanto, Siemens Medical Solutions) with the combination of a phased-array body coil and spine array coil for signal reception. Baseline MR images, including a respiratory-navigated T2-weighted turbo spin-echo sequence [TR/TE, 2000/104 ms; slice thickness, 7 mm; flip angle, 150°; matrix, 207 (phase) × 384 (read); FOV, 33-38 cm] and a breath-hold T1-weighted fast low angle shot (FLASH) sequence [TR/TE, 112/4.76 ms; slice thickness, 7 mm flip angle, 70°; matrix, 114 (phase) × 256 (read); FOV, 33-38 cm]. Dynamic imaging, breath-hold T1-weighted FLASH sequence was performed using the following parameters: TR/TE, 230/2.47 ms; flip angle, 70°; matrix, 135 (phase) × 256 (read); effective slice thickness, 7 mm; and FOV, 33-38 cm. Dynamic imaging was performed before and after administration of gadopentate dimeglumine (Magnevist; Bayer Schering Pharma), consisting of late arterial (delay time 20-25 s), portal (70-90 s), and equilibrium (180 s) phases. The contrast-enhanced imaging was acquired after a bolus injection of 30-35 mL of contrast with a fixed delay. The contrast material was injected into the antecubital vein at a rate of 2.5 mL/s *via* a power injector (Spectris, Medrad, Indianola, PA, USA). Three dynamic phases were repeated for 18-21 s during a single breath-hold.

Imaging analysis

Imaging studies were evaluated on film by two abdominal radiologists (with experience ranging from 5 to 10 years) in consensus. Readers were not blinded to the pathology results.

The following imaging criteria were analyzed: number of lesions; lesion diameter; lesion location according to the hepatic segment numbering system of Couinaud; attenuation at non-enhanced CT, classified as hypoattenuating, isoattenuating, or hyperattenuating to the adjacent liver parenchyma; signal intensity characteristics of the lesions at non-enhanced T1-weighted (including T1WI and T1WI with fat suppression) and T2-weighted MRI; presence of fat tissue, hypoattenuating foci (-20 to -120 Hu) on non-enhanced CT images or hyperintense on T1WI but hypointense on T1WI with fat suppression; enhancement pattern at contrast-enhanced CT or MRI with regard to three-phase dynamic enhancement; presence of the central vessels in lesion at contrast-enhanced imaging; and presence of outer rim enhancement.

Pathology

All tissues were reviewed independently by one pathologist. Histopathological diagnosis was made according to the World Health Organization's classification of tumors of the liver and intrahepatic bile ducts[8]. The most important diagnostic criterion was the presence of HMB-45-positive myoid cells. All tumor tissues had been fixed in neutral buffered formalin and were routinely embedded in paraffin. Hematoxylin-eosin stained sections were evaluated and immunohistochemical studies were performed on representative blocks by the EnVision Plus system (DAKO, Glostrup, Denmark) with a panel of antibodies (HMB-45, SMA, S-100, CD34, A103, MSA, Vimentin, CD68, CD117, HepPar-1, AFP, AE1/AE3, and CK8).

Statistical analysis

Statistical analysis was performed by using software (Intercooled Stata, version 9.0 for Windows, 2005; Stata Corp, College Station, TX, USA). We used the Fisher exact test to compare probability of these imaging features for Epi-HAML and non-Epi lesion. A P value less than 0.05 was considered statistically significant.

RESULTS

General data

The data are summarized in Tables 1 and 2. Age at diagnosis of hepatic angiomyolipoma varied between

Table 1 Synopsis of demographics and imaging findings in 10 patients with Epi-HAML

No.	Age (yr)/sex	Size (cm), segment	Unenhanced CT/MR	Fat	Vascularity	Outer rim	Vessels in lesion
1	51/F	6.5, 4.0 Ⅵ/Ⅶ, Ⅳ	Hypo	No	Hypovascular	Yes	Yes
2	42/F	4.2, Ⅰ	Hypo	No	Hypovascular	No	No
3	35/F	7.5, Ⅵ	Hypo	No	Hypervascular	No	Yes
4	36/F	1.5, Ⅳ	Hypo	No	Hypervascular	Yes	Yes
5	17/F	10.0, Ⅴ/Ⅶ/Ⅷ	Hypo	No	Hypervascular	Yes	Yes
6	55/F	5.0, Ⅱ/Ⅲ	Hypo	No	Hypervascular	Yes	Yes
7[1]	33/F	6.0, 1.0, Ⅱ/Ⅲ/Ⅳ, Ⅶ	Hypo	Yes	Hypervascular	No	Yes
8	36/F	3.0, Ⅳ	Hypo	No	Hypervascular	Yes	Yes
9	46/F	4.0, Ⅱ/Ⅲ	T1 hypo, T2 hyper	No	Hypervascular	Yes	Yes
10	47/F	2.5, Ⅵ	T1 hypo, T2 hyper	No	Hypervascular	Yes	Yes

[1]The demonstration of hypervascularity and vessels with lesion were only for one lesion (large) of No. 7 patient. Hyper: Hyperattenuation; Hypo: Hypoattenuation; T1 hypo: Hypointense on T1WI (fat suppression); T2 hyper: Hyperintense on T2WI.

Table 2 Synopsis of demographics and imaging findings in 11 patients with non-Epi-HAML

No.	Age (yr)/sex	Size (cm), segment	Unenhanced CT/MR	Fat	Vascularity	Outer rim	Vessels in lesion
1	45/F	6.0, Ⅵ/Ⅶ	Hypo	Yes	Hypervascular	No	No
2	34/F	4.5, Ⅱ/Ⅲ	Hypo	No	Hypervascular	No	Yes
3	37/F	5.5, Ⅷ/Ⅳ	T1 hypo, T2 hyper	Yes	Hypervascular	Yes	Yes
4	40/F	12.0, Ⅶ/Ⅷ/Ⅳ 2.0, 1.0, Ⅱ/Ⅲ	T1 hypo, T2 hyper	Yes	Hypervascular	Yes	Yes
5	50/F	6.5, Ⅳ	T1 hypo, T2 hyper	Yes	Hypervascular	Yes	Yes
6	43/M	2.0, Ⅵ	Hypo	Yes	Hypervascular	No	No
7	47/M	2.0, Ⅳ	Hypo	Yes	Hypervascular	No	No
8	46/M	3.0, Ⅷ	Hypo	Yes	Hypervascular	No	Yes
9	44/F	4.0, Ⅱ	Hypo	No	Hypervascular	No	No
10	50/F	3.0, Ⅵ	Hypo	Yes	Hypervascular	No	Yes
11	21/F	12.0, Ⅵ/Ⅵ	T1 hypo, T2 hyper	No	Hypervascular	Yes	Yes

Table 3 Comparison of imaging features between Epi-HAML and non-Epi-HAML[1]

	Epi-HAML, n = 12 (10)	Non-Epi-HAML, n = 13 (11)	P value
Fat	2 (1)	10 (8)	0.005
Hypervascular	8 (8)	13 (11)	0.082
Vessels in lesions	10 (9)	9 (7)	0.645
Outer rim	8 (7)	6 (4)	0.428

[1]Data without parentheses are the number of lesions and data in parentheses are the number of patients. The Fisher exact test was used to analyze difference in per-lesion probability of imaging features between two types.

17 and 55 years (mean 40.7 years). In the Epi-HAML group, there was history of TSC for one patient, and a combination of left kidney AML for another one. Among 10 patients, eight had solitary lesions and two had two lesions. Lesions had a mean diameter of 4.6 (range, 1.5-10.0). In the non-Epi-HAML group, there was right kidney AML in one patient, 10 had solitary lesions, and one had three lesions. Lesions had a mean diameter of 4.9 (range, 1.0-12.0).

Comparison of CT/MRI findings between Epi-HAML and non-Epi-HAML

The CT/or MR images of 21 patients (CT images for 15 patients and MR for six) were available for retrospective analysis. The results are show in Tables 1-3.

At non-enhanced CT, 10 Epi-HAML and seven non-Epi HAML were hypoattenuating to the surrounding liver. Two Epi-HAML and six non-epi lesions were hypointense on T1WI (fat suppression) and hyperintense on T2WI. The presence of fat was detected in 10 non-Epi HAML but only two Epi type lesions (Figure 1) and there were significant differences between the two lesion types ($P < 0.01$, Table 3).

On arterial phase, eight Epi-HAML and 13 non-Epi-HAML showed obvious enhancement (Figures 1-3). Punctate or curved vessels could be seen within 10 Epi-HAML as well as nine non-Epi type lesions (Figures 1-4) on arterial or/and portal phase. Outer rim enhancement could be found in eight Epi-HAML as well as in six non-Epi-HAML on enhanced imaging (Figures 3 and 4).

Pathology

Immunohistochemical studies showed that tumor cells were positive for HMB-45 and A103, but negative for cytokeratin (HepPar-1, AFP, AE1/AE3, CK8.) in all cases. Smooth muscle actin (SMA) and desmin staining were weak to moderate in epithelioid cells. MSA staining was similar to desmin but was more weak when positive. The endothelial cells lining the blood vessels were positive for CD34. CD117 and S-100 were negative in all cases.

Follow-up

Imaging and clinical follow-up was available in 10 patients with Epi-HAML and ranged from one to six years.

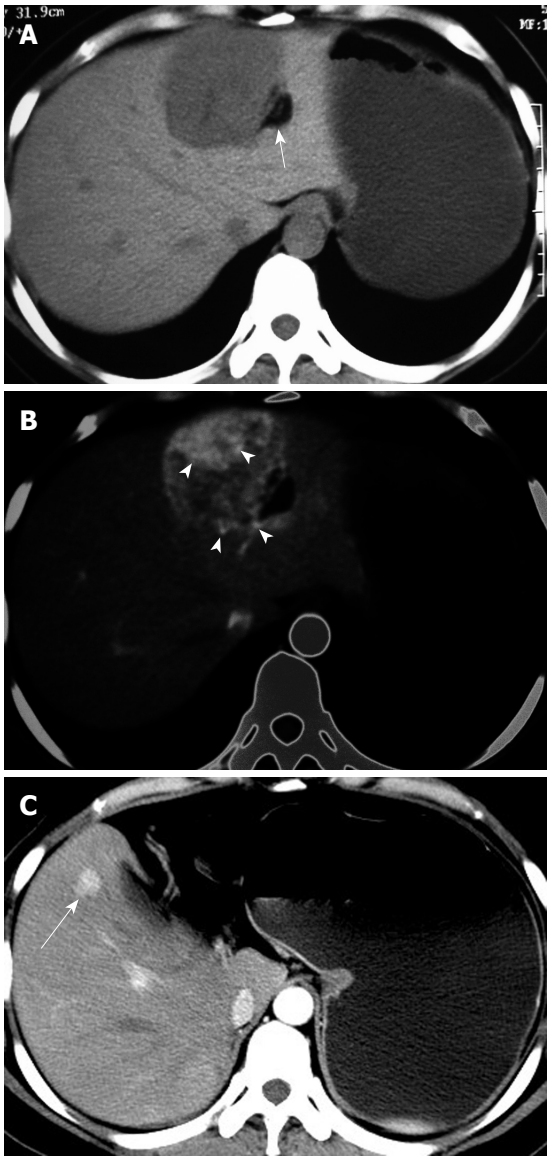

Figure 1 33-year-old woman with epithelioid angiomyolipoma in left lobe of liver (patient 7). A: Non-enhanced CT scan shows hypoattenuating lesion with fat components (arrow, CT value mean-35 Hu) in segments Ⅱ/Ⅲ/Ⅳ; B: Contrast-enhanced CT scan shows inhomogeneous enhancement lesion with punctiform or filiform enhanced vessels (arrowheads, the window width and level was adjusted) on arterial phase; C: Contrast-enhanced CT scan at 1-year follow-up shows enhanced recurrent nodule (arrow) on arterial phase image after the left lobe surgery.

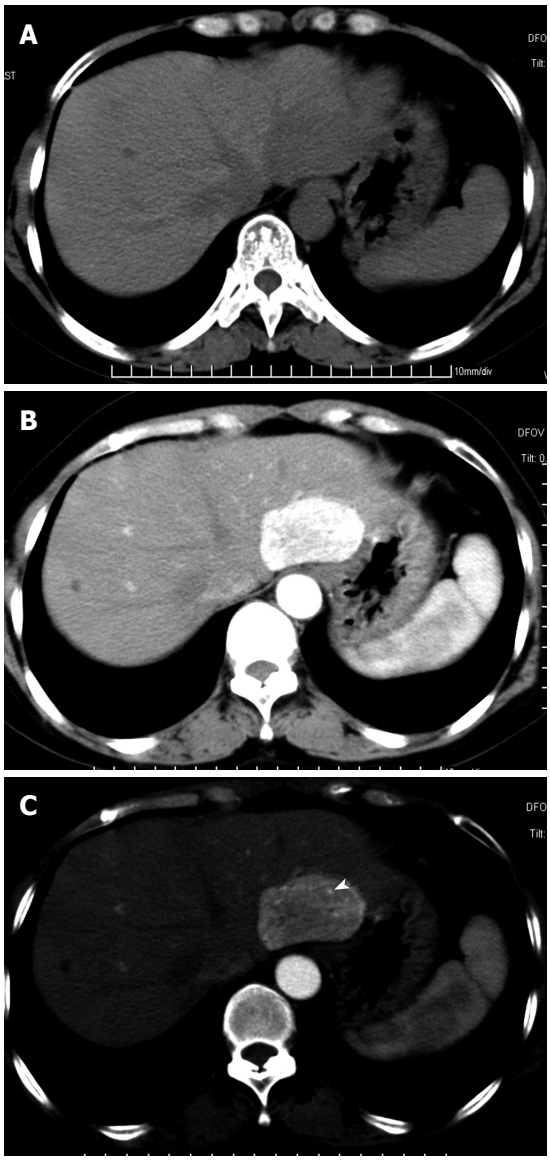

Figure 2 55-year-old woman with epithelioid angiomyolipoma in left lobe of liver (patient 6). A: Non-enhanced CT scan shows hypoattenuating lesion in segment Ⅱ; B: contrast-enhanced CT scan shows obviously enhanced lesion on arterial phase; C: the punctate enhanced vessels (arrowhead) within the lesion is show on an arterial phase image by adjusting the window width and level.

Recurrent hepatic lesions were found in one patient (Figure 1), pubic bone destruction and metastatic nodule in body soft tissue were proved with biopsy in another.

DISCUSSION

Previous literatures showed variable imaging appearances for HAML[9-12]. The imaging characteristics of HAML are correlated with its histological components, and demonstration of blood vessels and mature adipose tissue are the most important radiographic features. Color Doppler sonography shows a punctiform or filiform vascular distribution pattern. Contrast-enhanced CT shows marked enhancement of the soft tissue components in the arterial phase. MRI is the most specific imaging entity for the detection of lipomatous components; however, because hepatic AML can have such variation in the amount of adipose tissue present, detection varies based on the percentage of the lesion that is composed of adipose tissue. MRI findings include hypointensity or hyperintensity on T1WI, slight hyperintensity on T2WI, dense enhancement in the arterial phase, and hypointensity in the delayed phase.

In our study, most of the Epi-AML tumors (10/12) were completely devoid of adipose tissue, which is the characteristic finding compared with that of non-Epi type. The result was consistent with a previous report[1]. After *i.v.* contrast administration, there was obvious enhancement on the arterial phase for most of these lesions, suggesting that Epi-AML was a hypervascular tumor. Most lesions (seven) manifested as hypoattenuation or hypointensity on portal/equilibrium phase. By adjusting the window

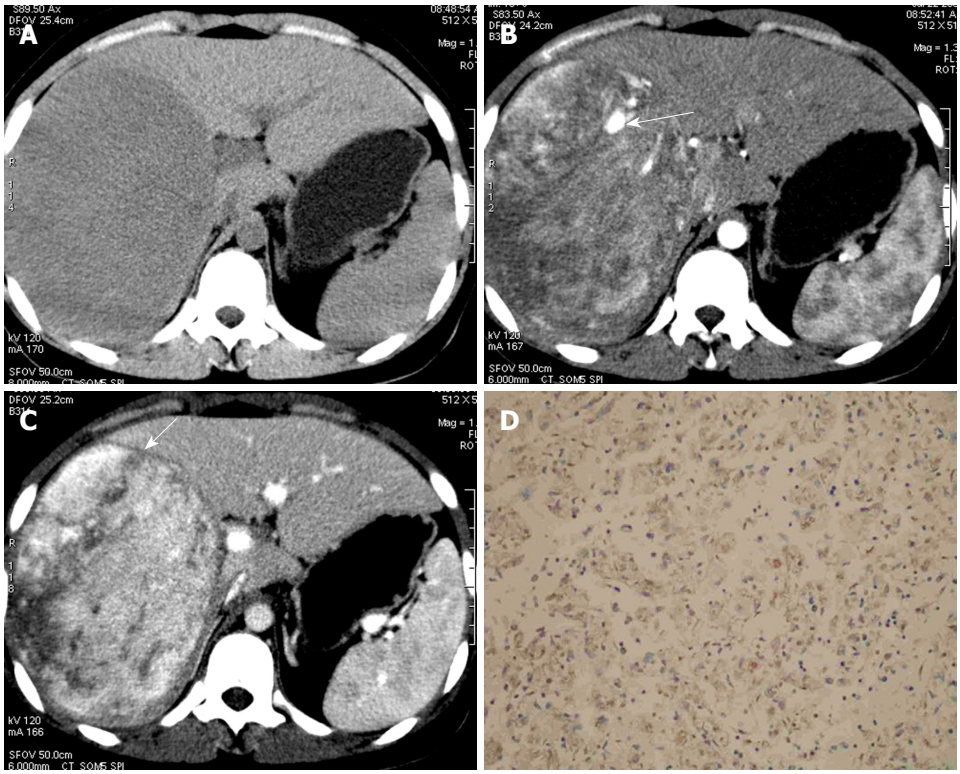

Figure 3 17-year-old girl with epithelioid angiomyolipoma in right lobe of liver (the patient had a history of tuberous sclerosis complex, patient 5). A: Non-enhanced CT scan shows homogeneous hypoattenuating lesion in segment Ⅴ/Ⅶ/Ⅷ; B: Contrast-enhanced CT scan shows inhomogeneous enhanced lesion with opacification of central vessels (arrow) on the arterial phase; C: The slight enhanced outer rim (arrow) around the inhomogeneous enhancement lesions is shown on portal phase contrast-enhanced CT; D: Immunohistochemical staining for HMB-45 shows diffusely positive staining in tumor cells within the cytoplasm (EnVision, × 100).

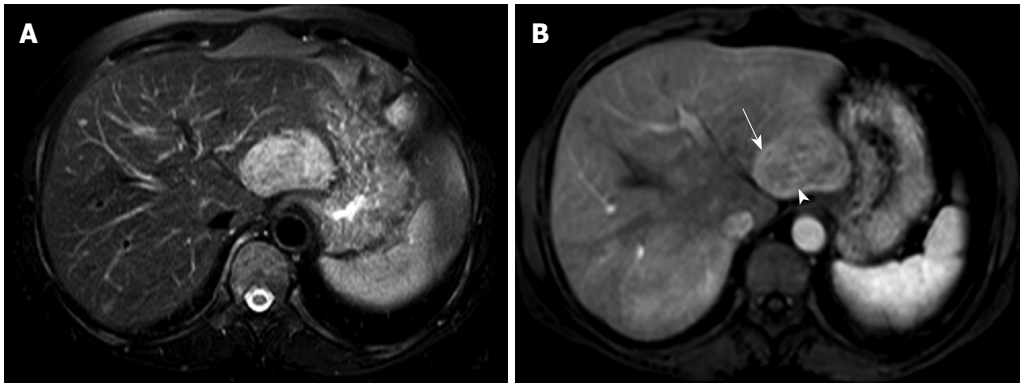

Figure 4 46-year-old woman with epithelioid angiomyolipoma in left lobe of liver (patient 9). A: Non-enhanced MRI T2WI shows hyperintense lesion in segment Ⅱ/Ⅲ; B: Contrast-enhanced MRI shows obvious enhanced lesions with outer rim enhancement (arrow) and opacification of punctate or curved vessels (arrowhead) on arterial phase.

width and level, punctate or curved vessels could be seen within 10 lesions on enhanced scanning. In addition, intact or discrete outer rim enhancement within 8 lesions was observed. According to the pathology specimens and literature report, it is not true capsular but a pseudocapsule, which is composed of the compressed liver parenchyma and sparse fibrosis tissues with small vessels, resulting in delayed enhancement on late phase[13]. A similar manifestation can be found in other hypervascular lesion, such as hepatocellular carcinoma(HCC) and focal nodular hyperplasia (FNH). The distinction between these lesions can be difficult. However, the enhancement patterns were somewhat different among Epi-AML, HCC, and FNH. Most of the HCC enhanced markedly in the arterial phase and decreased rapidly on the portal venous/equilibrium phase. Additionally, capsule could be found in most of the HCC, so the margins of HCC were more clear than those of AML in the portal venous phase, a suggestive finding for correct diagnosis[14]. The enhancement pattern of FNH was similar to AML, in both of them prolonged enhancement could be shown in the portal venous phase. However, most of FNH enhanced homogeneously on the arterial phase except the central scar, which was characteristic of FNH and could be enhanced on the portal venous phase/delayed phase[15].

Five cases of hepatic malignant angiomyolipoma have been reported[5,16-19]. Strict histology criteria for defining hepatic angiomyolipoma as malignant have not been put

forward, but it should be suspected, especially in tumors with many mitoses. Those with necrosis and significant cellular pleomorphism might show aggressive behavior. There is clinical evidence of aggressive behavior such as recurrence and metastases beyond the liver, for two cases in these 10 Epi-AML cases. However, there are no characteristic findings for malignant HAML on cross-sectional imaging. Therefore, although most HAMLs are biologically benign, this tumor should be considered to have malignant potential, especially for Epi-AML. So resection and careful follow-up are recommended.

It is important to recognize the limitations of our study. Firstly, the study is retrospective; secondly, the numbers reported are limited and no one patient had both CT and MRI. A further limitation is that not every patient underwent regular imaging follow-up and documentation was incomplete.

In summary, little or no adipose tissue was found to be an imaging feature of Epi-HAML, *vs* non-Epi-HAML. In addition, hypervascularity with opacification of central punctiform or filiform vessels on DCE would be a characteristic enhancement pattern for Epi-HAML.

COMMENTS

Background

Hepatic Angiomyolipoma (HAML) is a rare benign tumor and belongs to a family of tumors that have collectively been called "PEComa". The immense variability of the morphological appearance is due to the different heterogeneous mixtures of vessels, epithelioid cells, and lipocytes. As a specific form, the epithelioid HAML (Epi-HAML) has characteristics in pathology and diagnosis that can be difficult, e.g. differentiation of Epi-AML from hepatocellular carcinoma and the metastatic sarcomatoid variant of renal cell carcinoma.

Research frontiers

Previous studies showed variable imaging appearances for HAML. The imaging characteristics of HAML are correlated with its histological components. Both CT and MRI, for the most part, do not allow the definitive differentiation of HAML from hepatocellular carcinoma, adenoma, liposarcoma, lipoma, hamartoma, and sometimes even from focal nodular hyperplasia, especially if the fat content is low. The Epi-HAML has morphological characteristics of tumor cells. In addition, the tumors are devoid of fat or only scattered fat cells distribution in pathology. However, the imaging features of Epi-HAML have not been unequivocally described and evaluated. In this study, the authors summarized CT/MRI imaging features of Epi-HAML and compared with those of non-Epi-HAML.

Innovations and breakthroughs

Previous reports have showed demonstration of mature adipose tissue is the most important radiographic features and highlighted the importance in the diagnosis of HAML, in particular for mixed, lipomatous types of HAML. This is the first study to report that blood vessels are also an imaging feature in Epi-HAML. Furthermore, the study would suggest that little or no adipose tissue might be imaging characteristics of Epi-HAML, *versus* non-Epi-HAML.

Applications

By understanding the imaging characteristics, this study could help radiologists familiarize themselves with the appearance of Epi-HAML, and improve the confidence in the diagnosis of incidental liver tumor, especially in cases where the fat content is low and the interpretation of histological findings is difficult.

Terminology

Immunohistochemistry is a method of analyzing and identifying cell types based on the binding of antibodies to specific components of the cell. The most important diagnostic criterion for HAML is the presence of HMB-45-positive myoid cells.

Peer review

The manuscript reported the imaging characteristics of Epi-HAML, only a few reports had been published about the lesion before. It will be helpful for radiologists to obtain knowledge of the lesion.

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BRIEF ARTICLES

Effect of preoperative transcatheter arterial chemoembolization on angiogenesis of hepatocellular carcinoma cells

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Abstract

AIM: To evaluate the effects of four types of preoperative transcatheter arterial chemoembolization (TACE) on angiogenesis of hepatocellular carcinoma (HCC) cells.

METHODS: A total of 136 patients with HCC underwent liver resection. One to five courses of TACE prior to liver resection were performed in 79 patients (TACE group), in which one to four courses of chemotherapy alone were performed in 11 patients (group A); one to five courses of chemotherapy combined with iodized oil were performed in 33 patients (group B); one to three courses of chemotherapy combined with iodized oil and gelatin sponge were performed in 23 patients (group C); one to three courses of chemotherapy combined with iodized oil, ethanol and gelatin sponge were performed in 12 patients (group D). The other 57 patients only received liver resection (non-TACE group). The microvessels were marked by CD31. The expression of CD31 and vascular endothelial growth factor (VEGF) protein were detected by immunohistochemical methods.

RESULTS: The mean microvessel density (MVD) in HCC cells was significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). The expression of VEGF protein in HCC cells were significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). MVD and the expression of VEGF protein were positively correlated. Mean MVD and the expression of VEGF protein were closely related to the number of courses of TACE and the interval of TACE.

CONCLUSION: Four different types of preoperative TACE regimens enhanced angiogenesis in HCC cells by up-regulating the expression of VEGF protein. It is necessary to repress angiogenesis of liver cancer after TACE.

Key words: Angiogenesis; Hepatocellular carcinoma; Immunohistochemistry; Transcatheter arterial chemoembolization; Vascular endothelial growth factor

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asian countries. It is responsible for more than 250 000 deaths worldwide each year, 40% of which occur in China ranking HCC second after gastric carcinoma[1-3]. Surgical resection is recognized as the most effective treatment method for patients with HCC[4]. Although recent advances in treatment have helped prolong the survival of patients with HCC, they consequently increase the risk of intrahepatic recurrence and extrahepatic metastasis. Only a minority of patients currently diagnosed with HCC may benefit from this radical option[5].

Transcatheter arterial chemoembolization (TACE) has become one of the most popular and effective palliative methods for patients with HCC[6-11]. Various mixtures of anticancer drugs, lipiodol and gelatin sponge have been used as TACE agents. There have been few reports comparing the efficacy of different TACE regimens in patients with HCC[12-14].

There is ample evidence that tumor angiogenesis is the pathological basis and a necessary condition for solid tumor growth and metastasis[15]. Vascular endothelial growth factor (VEGF) is a strong angiogenesis factor in HCC[16], and plays an important role in the development and prognosis of liver cancer. In the present study, we examined the effects of the four main types of TACE used clinically (pure intra-arterial chemotherapy, chemotherapy plus lipiodol, chemotherapy plus lipiodol plus gelatin sponge, and chemotherapy plus lipiodol plus alcohol plus gelatin sponge) on angiogenesis of HCC cells *in vivo*.

MATERIALS AND METHODS

Patients

From February 1992 to February 2001, a total of 136 patients with HCC were referred to our hospital for surgery, of which, 122 were men and 14 were women with a mean age of 45 years (range: 20 to 70 years). A diagnosis of HCC was obtained for all patients by preoperative ultrasound (US) and/or computed tomography (CT) and/or magnetic resonance imaging (MRI) and/or plasma AFP levels, and was then confirmed by biopsy.

Surgical procedure

The patients were randomly divided into two groups. In the TACE group, 79 patients underwent 1-5 courses of chemoembolization prior to liver resection, in which one to four courses of chemotherapy alone were performed in 11 patients (group A), one to five courses of chemotherapy combined with iodized oil were performed in 33 patients (group B), one to three courses of chemotherapy combined with iodized oil and gelatin sponge were performed in 23 patients (group C), and one to three courses of chemotherapy combined with iodized oil, ethanol and gelatin sponge were performed in 12 patients (group D). Fifty patients underwent one course of TACE, 19 patients underwent two courses of TACE and 10 patients underwent three or more courses of TACE during an interval of 52.8 ± 12.2 d (mean ± SD). Twenty-five patients had an interval of one month or less, 29 patients had an interval of two months or less, 16 patients had an interval of three months or less and 9 patients had an interval of more than three months. In the non-TACE group, 57 patients received initial liver resection without preoperative TACE. The extent of liver resection carried out was based on the location of the tumor, the severity of concomitant liver cirrhosis and preoperative liver reserve function.

Immunohistochemical methods

The formalin-fixed, paraffin-embedded specimens were examined immunohistochemically using anti-CD31 antibody and anti-VEGF antibody (LSAB Kit, Dako). Breast cancers were used as positive controls. Negative controls were generated by substituting the primary antibody with phosphate-buffered saline (PBS). CD31 and VEGF immunostained cells showed a brownish-yellow color in the cytoplasm (Figure 1A and B). Microvessel density (MVD) counting followed the method of Weidner *et al*[17], i.e. first, the regions with the highest density of CD31-positive cells were chosen and counted under a low-power microscope (× 40). Then microvessel numbers were counted under a high-power microscope (× 400). Each isolated brown vascular endothelial cell or cluster of endothelial cells was counted as a vascularization. VEGF counting followed the method of Park *et al*[18], i.e. negative (-) was < 5% of positively stained cells, weakly positive (+) was 5% to 15% of positively stained cells, moderately positive (++) was 15% to 50% of positively stained cells and strongly positive (+++) was > 50% of positively stained cells. All slides were reviewed and scored in a blind test by two observers without knowledge of the corresponding clinical data. A few cases with discrepant scoring were jointly re-evaluated until agreement was reached.

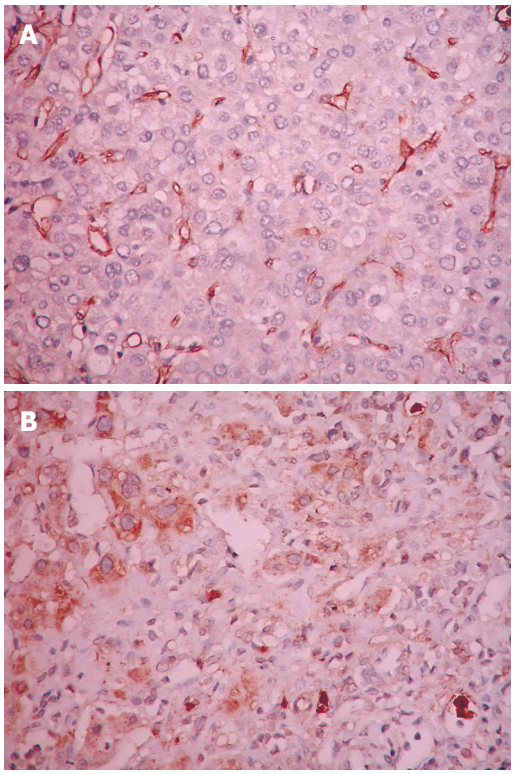

Figure 1 Expression of CD31 (A) and VEGF (B) protein detected by immunohistochemical methods after chemotherapy combined with ethanol, iodized oil and gelatin sponge (Dako Envision, peroxidase method × 400).

Statistical analysis

MVD was expressed as mean ± SD and analyzed using the two-sample *t*-test for the two groups, and by analysis of variance for multiple comparisons. VEGF was analyzed using table χ^2 test. The correlation between MVD and VEGF was analyzed using Pearson correlation analysis. *P*-value < 0.05 was considered statistically significant.

RESULTS

Correlation between methods of TACE and expression of CD31 and VEGF protein

The mean MVD was (47.71 ± 23.33), (56.05 ± 22.45),

Table 1 Expression of VEGF protein in groups A, B, C, D, and the non-TACE group

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
A	11	2 (18.2)	4 (36.4)	3 (27.3)	2 (18.2)
B	33	5 (15.2)	6 (18.2)	9 (27.3)	13 (39.4)
C	23	2 (8.7)	6 (26.1)	4 (17.4)	11 (47.8)
D	12	1 (8.3)	2 (16.7)	4 (33.3)	5 (41.7)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

Table 2 Correlation between expression of CD31 and VEGF protein

Group	Cases	MVD
VEGF (-)	27	35.47 ± 17.35
VEGF (+)	34	44.12 ± 15.84
VEGF (++)	33	52.56 ± 17.29
VEGF (+++)	42	60.72 ± 23.46

(54.36 ± 24.46), (51.90 ± 19.41) and (44.36 ± 17.67) in groups A, B, C, D and the non-TACE group, respectively. The mean MVD was significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was significantly higher in groups B, C and D than in group A or the non-TACE group ($\chi^2 = 12.63$, $P < 0.05$) (Table 1). When the expression of VEGF protein was increased, MVD increased significantly, and both were positively correlated (Pearson correlation, $r = 0.445$, $P < 0.05$) (Table 2).

Correlation between courses of TACE and expression of CD31 and VEGF protein

The mean MVD was (44.36 ± 17.67), (54.01 ± 23.83), (53.38 ± 22.64) and (51.94 ± 22.64) in the non-TACE group, the one-course TACE group, the two-course TACE group and the three-, four- and five-course TACE group, respectively. The mean MVD was significantly higher in the one-course TACE group than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was higher in the TACE groups than in the non-TACE group ($\chi^2 = 16.786$, $P > 0.05$) and decreased as the courses of TACE increased (Pearson correlation, $r = 0.331$) (Table 3).

Correlation between interval of TACE and expression of CD31 and VEGF protein

The mean MVD was (44.36 ± 17.67), (49.20 ± 19.84), (55.30 ± 23.31), (61.48 ± 26.63) and (44.25 ± 17.52) in the non-TACE group, the 1 mo-interval TACE group, the 1-2 mo interval TACE group, the 2-3 mo interval TACE group, and the > 3 mo interval TACE group, respectively. A comparison between the groups showed that the mean MVD was higher in the 1 mo-interval TACE group, the 1-2 mo interval TACE group and the 2-3 mo interval TACE group than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was higher in the TACE interval groups than in the non-TACE group ($\chi^2 = 12.488$, $P = 0.407$) and was highest in the 1-2 mo interval TACE group (Table 4).

Table 3 Correlation between courses of TACE and expression of VEGF protein

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
One-course	50	5 (10.0)	14 (28.0)	12 (24.0)	19 (38.0)
Two-course	19	3 (15.8)	4 (21.1)	3 (15.8)	9 (47.4)
Three-, four- or five-course	10	2 (20.0)	0 (0.0)	5 (50.0)	3 (30.0)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

Table 4 Correlation between interval of TACE and expression of VEGF protein

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
1 mo interval	27	4 (14.8)	7 (25.9)	7 (25.9)	9 (33.3)
1-2 mo interval	28	2 (7.1)	7 (25.0)	6 (21.4)	13 (46.4)
2-3 mo interval	16	3 (18.8)	2 (12.5)	5 (31.3)	6 (37.5)
> 3 mo interval	8	1 (12.5)	2 (25.0)	2 (25.0)	3 (37.5)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

DISCUSSION

TACE is one of the most common and effective palliative treatments. The prognosis of patients treated with TACE depends not only on the use of an effective TACE regimen but also on tumor factors[12]. According to the literature, very limited data are currently available regarding the molecular mechanism of TACE treatment in patients with HCC[13-15]. We believe that the current study is the first to detail the correlations between the expression of CD31 and VEGF protein and different TACE regimens, courses of TACE and interval of TACE.

There is ample evidence that tumor angiogenesis is the pathological basis and a necessary condition for the growth and metastasis of solid tumors[15]. VEGF is a strong factor in the angiogenesis of HCC[16], and plays an important role in the development and prognosis of liver cancer. It also has a biological effect by combining its specific VEGF receptor (vascular endothelial growth factor receptor, VEGFR), of which VEGFR-1 and VEGFR-2 are mainly distributed in vascular endothelial cells. Binding of VEGFR-1 and VEGF allows vascular endothelial cell migration, maintains tubular structure and regulates vascular permeability. Binding of VEGFR-2 and VEGF also promotes vascular endothelial cell proliferation and maturation[19]. This study showed that MVD of HCC was positively correlated with the expression of VEGF protein.

Whether angiogenesis of liver cancer after TACE is enhanced is still controversial. Some reports have suggested that the mean MVD of HCC specimens before and after TACE were not statistically significantly

different[20-23]. However, there are other reports which show that the mean MVD was significantly higher in the TACE group than in the non-TACE group[24-26]. The current study showed that the mean MVD of HCC was significantly higher in the TACE groups than in the non-TACE group. The expression of VEGF protein was significantly higher in the TACE groups than in the non-TACE group. After chemoembolization, the expression of VEGF protein increased, and the change in expression and MVD showed a significant positive correlation. It has also been reported that serum VEGF in patients with liver cancers was significantly increased after TACE[27,28]. Tumor tissue ischemia and hypoxia after TACE are important in promoting increased VEGF expression[29]. Therefore, it is necessary to repress angiogenesis in liver cancer after TACE.

The study on the effects of the interval between surgical resection of the tumor and the end of embolization on MVD, revealed that MVD was not significantly different in the group with an interval of less than 30 d or in the group with an interval of more than 90 d, but was significantly increased in the group with an interval of 31-90 d compared with the control group. This showed that after chemoembolization, tumor hypoxia-ischemia results in a series of biochemical changes which cause increased angiogenesis and a gradual increase in MVD, reaching a peak in 1-3 mo. More than three months later, the blood supply to the residual tumor improves, the formation of tumor angiogenesis slows, the residual cancer cells grow, infiltration occurs and damage to generated tumor angiogenesis occurs.

The mean MVD and expression of VEGF protein in liver cancer has a tendency to decrease as the time from TACE therapy increases, although this difference was not significant. It was reported that survival after a number of pre-operative TACE treatments was significantly better than that after a single TACE treatment[30]. Therefore, multiple pre-operative TACEs should be carried out in suitable patients.

In conclusion, the present study demonstrated that angiogenesis of residual HCC cells following treatment with four types of TACE is significantly increased and is positively correlated with the expression of VEGF protein. The effect of TACE on angiogenesis of HCC cells has a close correlation with the number of courses of TACE and the interval of TACE.

COMMENTS

Background

Transcatheter arterial chemoembolization (TACE) has become one of the most popular and effective palliative methods for patients with hepatocellular carcinoma (HCC). Various mixtures of anticancer drugs, lipiodol and gelatin sponge have been used as TACE agents. However, there have been few reports comparing the effects of different TACE regimens on angiogenesis of HCC cells.

Applications

According to the results of this study, it is necessary to repress angiogenesis of liver cancer after TACE.

Terminology

TACE indicates transcatheter arterial chemoembolization; MVD indicates microvessel density.

Peer review

TACE stimulates angiogenesis. It would be very useful from a clinical standpoint to identify which type of TACE stimulates more angiogenesis and give appropriate "prophylactic" anti-angiogenic therapy. The authors have the merit of confirming that TACE is an angiogenesis stimulating procedure and also give new information on this process -- e.g. angiogenesis peaks about 1 mo after TACE and then seems to wane off. The number of observations and the histological specimen (surgical) are adequate.

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CASE REPORT

Pseudolymphoma of the liver associated with primary biliary cirrhosis: A case report and review of literature

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Abstract

We report a case of two pseudolymphomas of the liver in a 63-year-old Japanese woman with primary biliary cirrhosis. One of the lesions was found incidentally during a medical examination, presenting as a 10 mm hypodense nodule that revealed hyperdensity in the early phase and hypodensity in the late phase in computed tomography (CT) after injection of contrast medium. Retrospectively, the 10 mm nodule had first been discovered as a 4 mm nodule during CT 4 years previously. Superparamagnetic iron oxide-enhanced MRI revealed another 4 mm hyperintense nodule in segment 6 in addition to the 10 mm hyperintense nodule in segment 7. CT during arterial portography revealed two hypointense nodules. Findings with other imaging modalities such as ultrasonography, magnetic resonance imaging, and hepatic angiography were consistent with hepatocellular carcinoma. A right posterior segmentectomy was performed, and the lesions were microscopically diagnosed as pseudolymphoma. To the best of our knowledge, only 31 other cases of this disease have ever been reported, with a highly asymmetrical male:female ratio of 1:9.7. Although we could find only one case of transformation of hepatic pseudolymphoma into lymphoma in the liver, the exact nature of development from benign pseudolymphoma to malignant lymphoma is still not fully understood and cases of hepatic lymphoma need to be followed carefully.

Key words: Liver diseases; Pseudolymphoma; Primary biliary cirrhosis

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INTRODUCTION

Pseudolymphoma in the liver is an extremely rare disease. Although the exact etiology remains unknown, it is speculated that the disorder is a reactive immunological response to a chronic infection or inflammation[1]; hepatic pseudolymphoma can develop in patients with autoimmune diseases[2,3], malignancy[4], or hepatitis[5] or who are administered interferon therapy[6]. It has been reported that diagnosis of pseudolymphoma is difficult without histopathological examination, since image findings are quite similar to hepatocellular carcinoma (HCC)[2]. Although pseudolymphoma is generally thought to be benign, the risk of malignant transformation into lymphoma remains controversial.

We report a case of hepatic pseudolymphoma in a female Japanese patient with primary biliary cirrhosis (PBC) and discuss the literature.

CASE REPORT

A 63-year-old female with a history of PBC and resection of the left adrenal gland for primary aldosteronism, was admitted to our hospital for further evaluation of

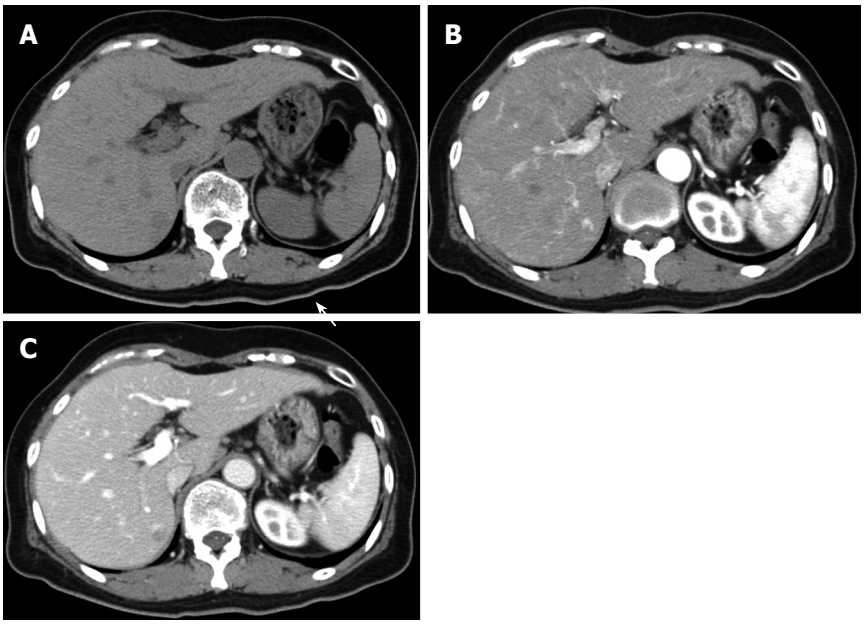

Figure 1 Computed tomography (CT) showing a 10 mm nodule in segment 7. A: A hypodense nodule in plane phase; B: A hyperdense nodule in the early phase after injection of contrast medium; C: A hypodense nodule in the late phase after injection of contrast medium.

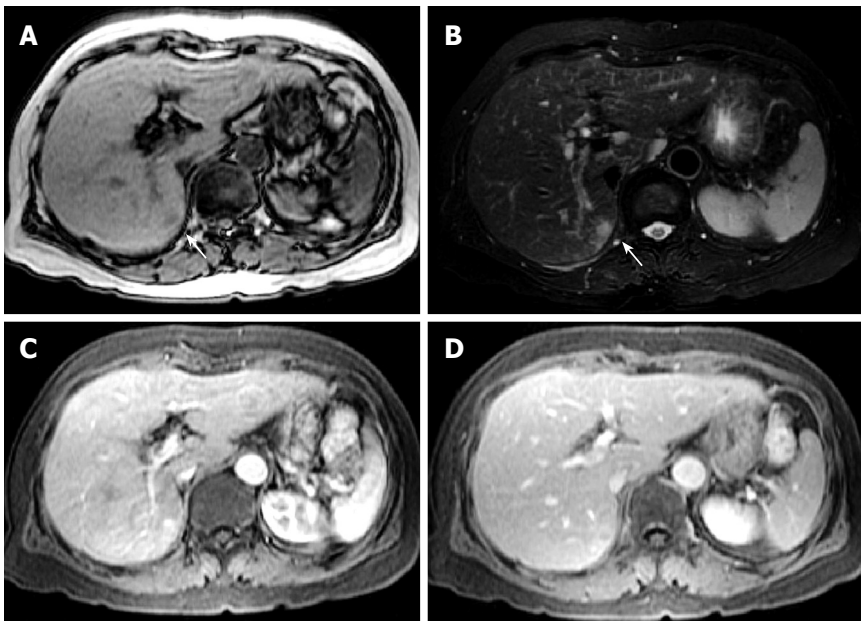

Figure 2 Magnetic resonance imaging (MRI) showing a 10 mm nodule in segment 7. A: A hypointense nodule on T1-weighted images; B: A hyperintense nodule on T2-weighted images; C: A hyperintense nodule in the early phase after injection of contrast medium; D: A hypointense nodule in the late phase after injection of contrast medium.

a hepatic lesion incidentally discovered in abdominal computed tomography (CT). The patient had taken only ursodeoxycholic acid 300 mg/d for PBC for 10 years and had not taken immunosuppressive agents. The patient was asymptomatic on admission and her condition was generally good. She had a surgical scar on her abdomen. We noted no hepatospenomegaly, lymphadenopathy, or peripheral edema. Laboratory tests showed a prothrombin time of 11.8 s (normal, 10.8-13.3), a total bilirubin level of 0.6 mg/dL (normal, 0.3-1.2), and an albumin level of 4.0 g/dL (normal, 4.0-5.0). Her serum alkaline phosphatase level was 350 IU/L (normal, 115-359), γ-glutamyltransferase 48 IU/L (normal, 10-47), aspartate aminotransferase 24 IU/L (normal, 13-33), and alanine aminotransferase 16 IU/L (normal, 6-27). Serology examinations for hepatitis B and C viruses were negative. Rheumatoid factor antibodies, Sjögren syndrome-A antibodies, and Sjögren syndrome-B antibodies were negative. Antinuclear antibodies and antimitochondrial antibodies were positive. Immunoglobulin G, immunoglobulin M, and immunoglobulin A were within normal ranges. Alpha-fetoprotein, protein induced by vitamin K absence, and carcinoembryonic antigen were within normal ranges. A urea breath test was negative. Abdominal ultrasonography showed a hypoechoic lesion, 10 mm in diameter in segment 7 (data not shown). CT demonstrated a 10 mm hypodense nodule that revealed hyperdensity in the early phase and hypodensity in the late phase after injection of contrast medium (Figure 1). Retrospectively, this nodule had previously shown up as a 4 mm nodule in a CT performed for examination of an adrenal tumor 4 years previously (data not shown). Magnetic resonance imaging (MRI) showed a hypointense nodule on T1-weighted images and a hyperintense nodule on T2-weighted images in segment 7. Following injection of contrast MRI, a hyperintense nodule in the arterial phase and a hypointense nodule in the portal phase were revealed (Figure 2). Superparamagnetic iron oxide-

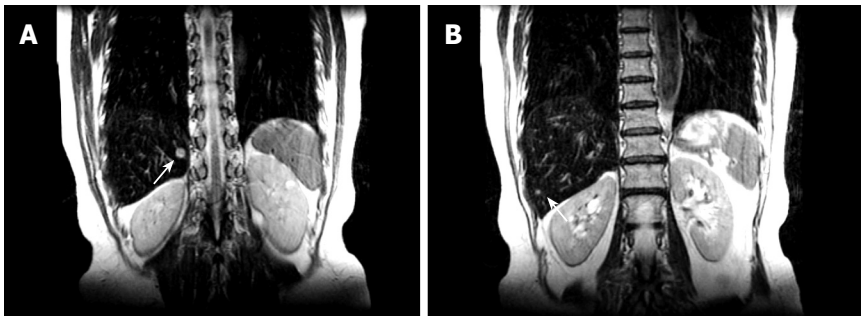

Figure 3 Superparamagnetic iron oxide-enhanced MRI showing hyperintense nodules. A: 10 mm nodule in segment 7; B: 4 mm nodule in segment 6.

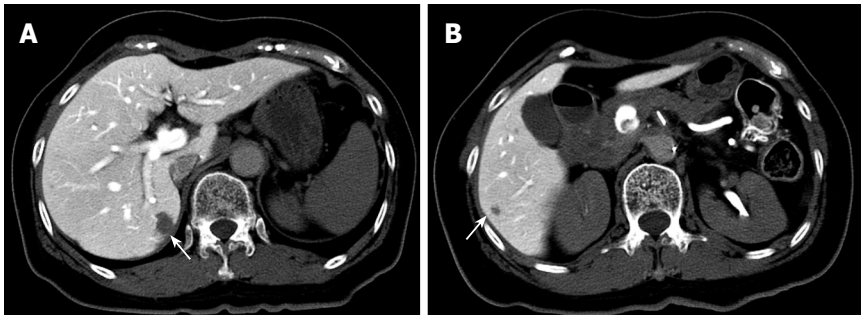

Figure 4 CT during arterial portography showing hypointense nodules. A: 10 mm nodule in segment 7; B: 4 mm nodule in segment 6.

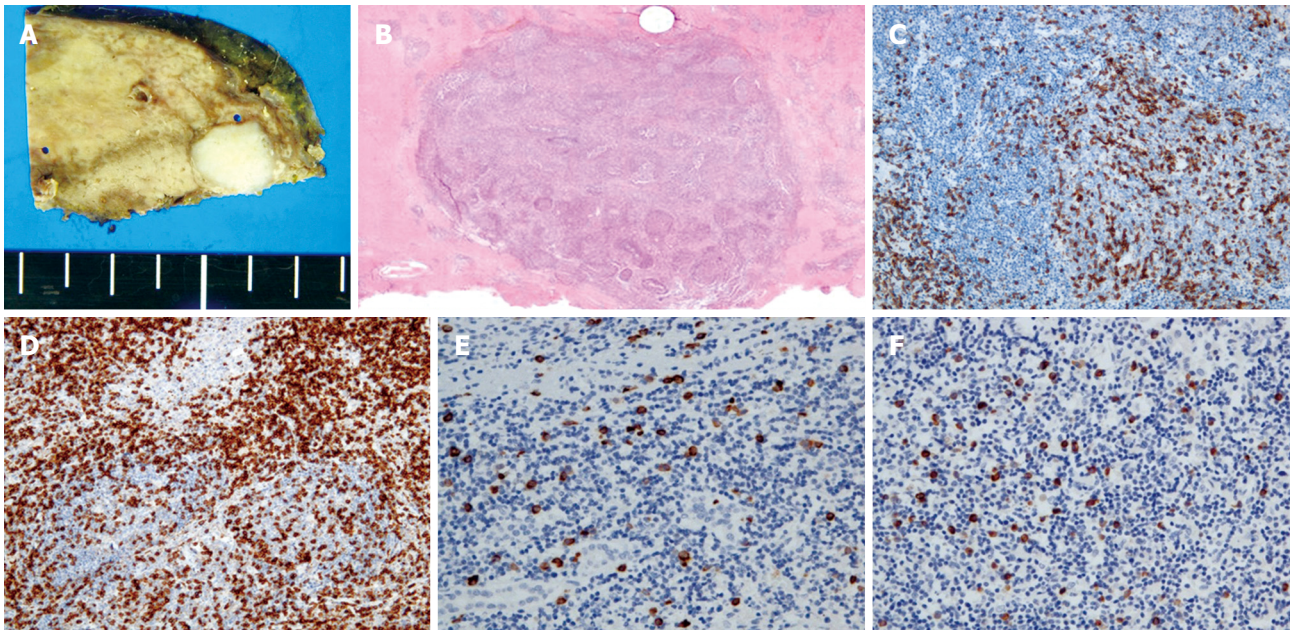

Figure 5 The pathological findings of the lesions. A: Macroscopically, the lesion was white and hard with a clear margin (a lesion in segment 7); B: Microscopically, the lesion consisted of a nodular lymphoid infiltrate with germinal centers (a lesion in segment 7), HE stain (× 40); Lymphocytes in the lesions consisted of CD3-positive T-cells (C) and CD20-positive B-cells (D), Both × 100; E: Stained for κ light chains with *in situ* hybridization (× 200); F: Stained for λ light chains with *in situ* hybridization (× 200).

enhanced MRI revealed another 4 mm hyperintense nodule in segment 6 in addition to the 10 mm hyperintense nodule in segment 7 (Figure 3). Although angiography *via* the common hepatic artery did not demonstrate tumor staining, CT during arterial portography revealed two hypointense nodules (Figure 4). Imaging findings suggested HCC, although no other hypervascular tumor could be excluded. The patient received an explanation of the results, including the possibility of a benign tumor, and expressed a desire for surgical extraction of the nodules. A right posterior segmentectomy was performed. Macroscopically, the lesion in segment 7 was white and hard with clear margins (Figure 5A) and the lesion in segment 6 was not detected. Microscopically, two lesions showed similar histological features. The lesions exhibited a nodular infiltration of mature small lymphoid cells with many lymph follicles. No obvious atypical cells were identified in both lesions. Bile ducts were identified at the periphery of the lesions, although characteristic lymphoepithelial lesions could not be identified. No necrosis or granulomatous inflammation was identified (Figure 5B). Pseudolymphoma and extranodal marginal

Table 1 Reported cases of hepatic pseudolymphoma

No.	Author (reference)	Age	Sex	No.	Size (cm)	Pathological diagnosis	Association
1	Snover *et al*[32]	15	F	1		PL	Combined immunodeficiency, liver fibrosis
2	Grouls *et al*[1]	85	F	2	1.4, 0.8	PL	Gastric cancer
3	Tanabe *et al*[33]	30	F	1	1.5	PL	Acute enteritis
4	Isobe *et al*[34]	59	F	1	0.9	RLH	Diabetes mellitus
5	Ohtsu *et al*[6]	42	F	1	1.5	PL	Chronic hepatitis B, interferon-α therapy
6	Katayanagi *et al*[35]	66	F	2	1.5, 1.0	PL	Diabetes mellitus
7	Tanizawa *et al*[36]	67	F	1	2	RLH	Abnormal liver function
8	Endo *et al*[37]	38	F	1	1.8	PL	Pancytopenia
9	Kim *et al*[5]	72	M	1	1.7	PL	Chronic hepatitis C, gastric cancer
10	Fujinaga *et al*[38]	58	F	1	1.5	PL	Hypertension
11	Nishijima *et al*[39]	58	F	1	1.2	PL	Hypertension, diabetes mellitus
12	Sharifi *et al*[3]	52	F	1	0.4	NLL	Primary biliary cirrhosis
13		56	F	1	1.5	NLL	Primary biliary cirrhosis, CREST syndrome
14		56	M	1	0.7	NLL	Diverticulitis
15	Nagano *et al*[40]	47	F	1	1.7	RLH	Chronic thyroiditis, high titer of ANA
16	Pantanowitz *et al*[41]	69	F	2	1.7, 1.0	RLH	Renal cell carcinoma
17	Okubo *et al*[2]	49	F	1	2	PL	Sjögren's syndrome
18	Mori *et al*[42]	49	F	1	1.8	PL	Chronic hepatitis B
19	Okuhama *et al*[43]	70	M	1	4	PL	
20	Shiozawa *et al*[44]	51	F	1	2	PL	
21	Takahashi *et al*[45]	77	F	1	1.5	RLH	Colon cancer
22		64	F	2	0.9, 0.7	RLH	Colon cancer
23	Maehara *et al*[46]	72	F	2	1.3, 1.0	RLH	
24	Willenbrock *et al*[47]	36	F	1	1.8	NLL	Ovarian cyst, focal nodular hyperplasia of the liver
25	Sato *et al*[4]	75	F	1	1.4	RLH	Gastric cancer, colon cancers, metastatic liver tumor
27	Ota *et al*[20]	63	F	1	1.6	PL	Gastric ulcer, *Helicobacter pylori* infection
28	Machida *et al*[48]	53	F	3	1.5, 1.2, 1.0	RLH	Autoimmune thyroiditis
29	Matsumoto *et al*[49]	67	F	1	1.5	PL	Hypertension
30	Jiménez *et al*[50]	34	F	1	2.3	NLH	Hypothyroidism
31	Park *et al*[51]	46	F	2	1.0, 1.0	RLH	Renal cell carcinoma
32	Present case	63	F	2	1.3, 0,4	PL	Primary biliary cirrhosis, primary aldosteronism

RLH: Reactive lymphoid hyperplasia; PL: Pseudolymphoma; NLL: Nodular lymphoid lesion; NLH: Nodular lymphoid hyperplasia; CREST: Calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia; ANA: Antinuclear antibody.

zone B-cell lymphoma (MALToma) were differential diagnosis. Then, we performed immunohistochemical analysis and *in situ* hybridization. Immunostainings for CD3, CD20, and CD79 revealed regularly distributed T cells and B cells (Figure 5C and D). Plasma cells were not many in number in the lesions, but *in situ* hybridization for immunoglobulin light chains was performed because it is one of the useful tools to discriminate between reactive lymphoid lesions and MALToma. *In situ* hybridization revealed no significant difference between the numbers of cells positive for kappa-chain and lambda-chain (Figure 5E and F). Based on these histological features, the lesions were diagnosed as pseudolymphoma.

In the background liver, liver parenchyma showed bridging fibrosis with lymphoid infiltrate in portal tracts. Granulomas cholangitis and ductopenia were identified. Those histological features were consistent with Stage 2 PBC. The patient had an uneventful postoperative course and has shown no sign of recurrence for 11 mo.

DISCUSSION

In the liver, pseudolymphoma has also been variously termed as reactive lymphoid hyperplasia and nodular lymphoid lesion, and shows histological features of hyperplastic lymphoid follicles with polymorphic and polyclonal cell populations composed of small mature lymphocytes, mature plasma cells, macrophages, stroma fibrosis and often numerous germinal centers. It is usually localized and well demarcated from surrounding tissue[1]. Pseudolymphoma may occur at numerous sites including the stomach[7], lung[8], ocular adnexa[9], hard palate and oral mucosa[10], skin[11], and breast[12].

To the best of our knowledge, 32 cases (including ours) of hepatic pseudolymphoma have been reported in the English and Japanese literature (Table 1). There was a strongly asymmetric male-to-female ratio of 1:9.7 (3 M/29 F). While other sites of pseudolymphoma also tended to show asymmetry, only pseudolymphoma of the breast approached that of the liver in terms of asymmetry magnitude. All seven reported cases of breast pseudolymphoma were female[12], but breast examination is limited almost exclusively to females, which could bias these findings. Reported male-female ratios of pseudolymphoma of the hard palate and oral mucosa[10], lung[8], and skin[11] were 1:2.8, 1:1.2 and 1:1.8, respectively, while ocular adnexal lymphoid hyperplasia affects men and women about equally[9]. Thus the extreme female asymmetry of hepatic pseudolymphoma is a unique characteristic.

Eight of 32 cases of hepatic lymphoma were associated with autoimmune disease and eight with malignant tumor (Table 1). In the lung, cases of pseudolymphoma associated with autoimmune disorders

such as Sjögren's disease were reported[13], which is similar to the liver. In other organs, several factors are thought to be associated with pseudolymphoma, i.e. Epstein-Barr virus (hard palate and oral mucosa[14]), mechanical stimulation (ear[15]), anticonvulsant drugs (skin[16]) and *Helicobacter pylori* (*H pylori*) (stomach[17,18]). Regarding the association of *H pylori* with gastric pseudolymphoma, successful treatment of the pseudolymphoma by eradication of *H pylori* has been reported[19]. Ota *et al*[20] reported a case of hepatic pseudolymphoma in which the diameter decreased after *H pylori* eradication. In the case reported here, the patient had no *H pylori* infection.

Transformation of pseudolymphoma to lymphoma has been discussed. Malignant transformation of pseudolymphomas in the lung[21] and stomach[22] have been reported. These reports, however, either predated or did not include the use of immunofluorescent techniques. It is likely that these cases were in fact the early stage of primary lymphoma misinterpreted as benign[8]. On the other hand, evidence of progression from histologically benign, immunohistochemically polyclonal lymphoid infiltrates to malignant lymphoma in cutaneous pseudolymphoma is well delineated in the literature[23]. A potential association between lymphoma and PBC is suggested on the basis of individual reports in the literature[24-29]. In a retrospective study by Panjala *et al*[30] based on an estimated 2,912 patients evaluated at their institution during a 22-year period, only 13 (an estimated 0.6%) patients were evaluated in referral visits for evidence of lymphoma. Although we could find one case of transformation of hepatic pseudolymphoma into lymphoma[31], we were unable to deduce the long term natural course of hepatic pseudolymphoma since most reported cases of hepatic lymphoma underwent surgical resection.

In conclusion, we have reported a case of hepatic pseudolymphoma associated with PBC. Hepatic pseudolymphoma appears unique in its female preponderance and associated diseases. If hypervascular nodules in the liver of female patients with autoimmune disease are found, the possibility of pseudolymphoma should be considered. Cases of hepatic pseudolymphoma should be followed carefully as the exact nature of this disorder is still not fully understood.

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CASE REPORT

Repetitive response to gemcitabine that led to curative resection in cholangiocarcinoma

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Abstract

This study reports a case of unresectable intrahepatic mass-forming cholangiocarcinoma which showed a dramatic response to gemcitabine that led to curative resection and a long-term survival of more than five years. Six and five cycles of gemcitabine monotherapy were administered separately over a three-year period and a radical excision was performed at 4.5 years after diagnosis. This case indicates the role of gemcitabine as a neoadjuvant chemotherapeutic agent for cholangiocarcinoma and guarantees a randomized controlled prospective study.

Key words: Cholangiocarcinoma; Gemcitabine; Neoadjuvant chemotherapy

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INTRODUCTION

Cholangiocarcinoma is a devastating malignancy with a poor prognosis. Early diagnosis and extensive surgery offers the only chance for cure although other treatment modalities have been applied. Response rates to chemotherapy are relatively low although several kinds of chemotherapeutic agents such as gemcitabine are known to be effective. Furthermore, the role of neoadjuvant chemotherapy for unresectable cholangiocarcinoma has not been established either. Here we report the case of a patient with huge intrahepatic mass-forming cholangiocarcinoma who showed repetitive responses to a gemcitabine single regimen, and eventually underwent curative resection that led to long-term survival of more than five years.

CASE REPORT

A 54-year-old female patient was admitted with mild epigastric discomfort and a liver mass was detected by ultrasonography in January 2004. She had a history of cholecystectomy due to gallstone disease 20 years ago. She denied any recent history of medication or herbal treatments. She had never consumed raw fresh-water fish. An abdominal computed tomography (CT) scan showed a 10 cm sized irregular hypovascular mass occupying the left hepatic lobe and growing inferiorly to compress the gastric antrum and the pancreas (Figure 1A). Invasion of the middle hepatic vein and left portal vein were strongly suggested. Multiple lymph node enlargements were noted at the liver hilum and lesser omentum. Laboratory data showed a white blood cell count of 9560/mm^3 (normal 4800-10 800 mm^3), hemoglobin 15.8 g/dL (normal 13-18 g/dL), platelet count of 203 000 /mm^3, AST 33 IU/L (normal 12-33 IU/L), ALT 20 IU/L (normal 5-35 IU/L), total bilirubin 0.9 mg/dL (normal 0.2-1.2 mg/dL), and albumin 4.6 g/dL (normal 3.5-5.3 g/dL). A tumor marker study revealed AFP 2.3 ng/mL (normal 0-8 ng/mL), CEA 2.2 ng/mL (normal 0-5 ng/mL), and CA19-9 17.6 U/mL (normal 0-36 U/mL). A serologic study showed HBs Ag (-), ANA (-), HCV Ab (+) and HCV RNA PCR (-). Esophagogastroduodenoscopy and colonoscopy were nonspecific except for mild chronic gastritis and a small hyperplasic rectal polyp. An abdominal magnetic resonance imaging (MRI) reported T1 low and T2 slightly high lobulated contour solid mass showing a similar invasion pattern with the CT scan. Biliary dilatation at the upper portion of the mass was seen (image not shown). Percutaneous needle biopsy from the mass reported well differentiated cholangiocarcinoma (image not shown). Histo-

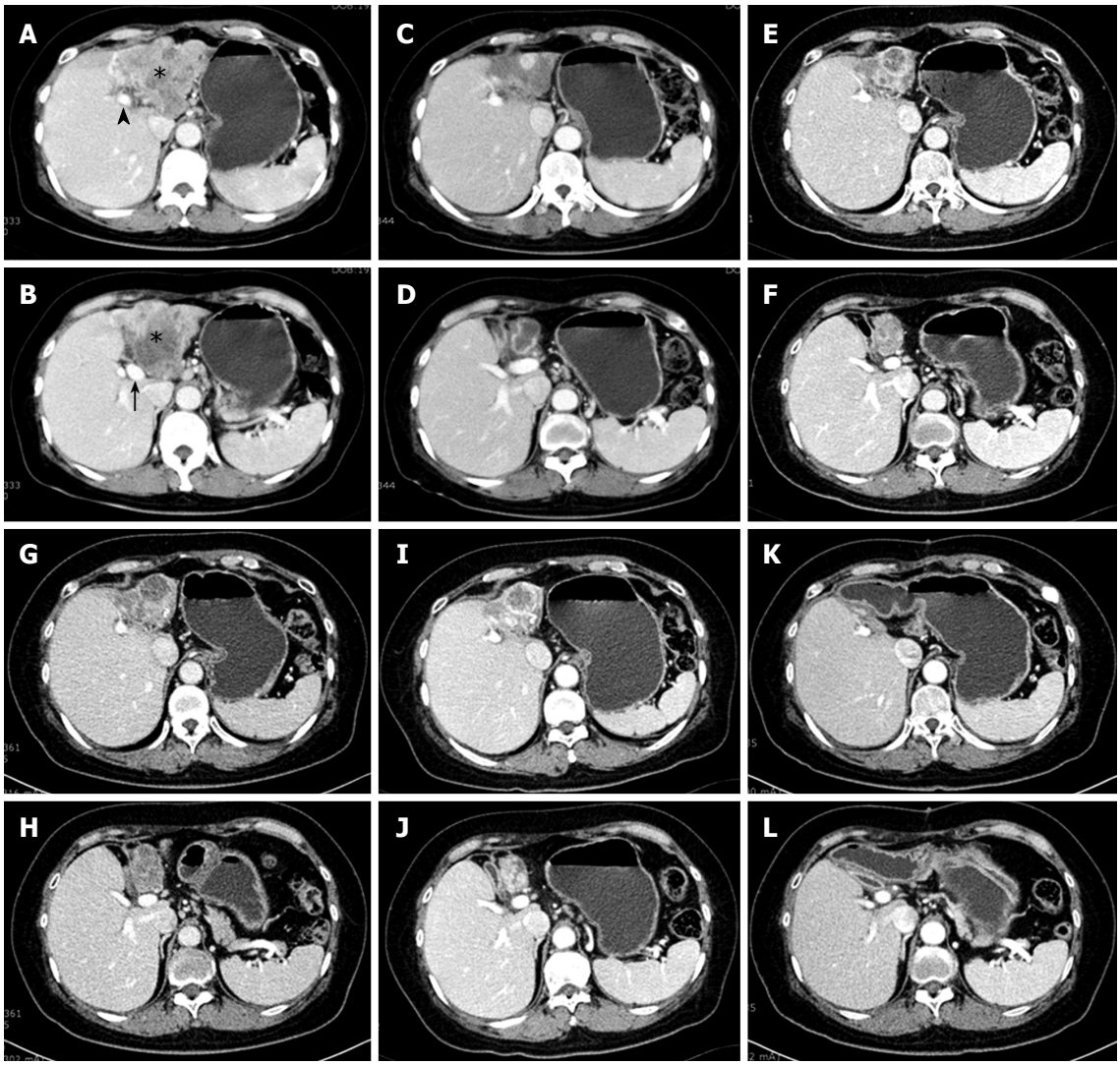

Figure 1 Abdominal CT scan images at two different levels are shown serially. A: At left portal vein level (black arrowhead), a 10 cm sized irregular hypovascular mass (asterisk) occupying left hepatic lobe is shown; B: At lower, main portal vein level (black arrow), the inferiorly grown mass is shown. Invasion of middle hepatic vein and left portal vein were strongly suggested with multiple lymph node enlargements at the liver hilum and lesser omentum; C and D: On CT scan taken after the end of the first 6 cycles (18 times) of gemcitabine chemotherapy, the tumor mass decreased in size dramatically, smaller than half of the previous diameter; E and F: On CT scan on 3 years and 3 mo from diagnosis, mass does not show any significant change except for a little sclerotic change; G and H: On CT scan on 4 years from diagnosis, tumor mass shows re-increment of the size than previous study; I and J: On follow up CT scan after second 5 cycles of gemcitabine chemotherapy, partial response is shown again with slight decrease of the mass size; K and L: On CT scan taken 11 months after R0 resection, there is no any recurrence or new lesion growing.

chemical and immunohistochemical study showed tumor cells were positive for CK-7, and negative for CK-19, and CK-20. An extended surgery was recommended to the patient after explaining the degree of tumor invasion and low possibility of curative resection. However, the patient declined any kind of treatment including surgery and chemoradiotherapy. After 3 mo from diagnosis, the patient was persuaded to undertake chemotherapy. Gemcitabine monotherapy was provided as intravenous 2 h infusions of 1000 mg/m^2 body surface area weekly every three out of 4 wk. A total of six cycles of chemotherapy were treated tolerably.

The CT scan taken on October 2004 (10 mo after diagnosis) showed that the tumor mass dramatically decreased in size and was smaller (half of the diameter) than that of the previous CT (Figure 1B). Surgery was again recommended to the patient. However, the patient declined surgery and further chemotherapy. After ending chemotherapy treatment the tumor mass did not show any significant change on a regular CT scan over 3 years and 3 mo except for a little sclerotic change (Figure 1C).

Unfortunately, an abdominal CT scan in January 2008 showed a slight increase of the mass with a symptomatic complaint of fatigue (Figure 1D). Gemcitabine treatment was restarted under the same protocol as the previous one. The patient barely finished five cycles of treatment due to various symptoms such as weakness or edema. On follow up CT scan in May 2008, a partial response was repeated with a slight decrease in the mass size (Figure 1E). Radical surgery was recommended and the patient agreed to it. A curative resection of atrophied left liver including the mid-hepatic vein and extrahepatic bile duct was performed. A hepatico-enterostomy was followed after the resection. Histological findings

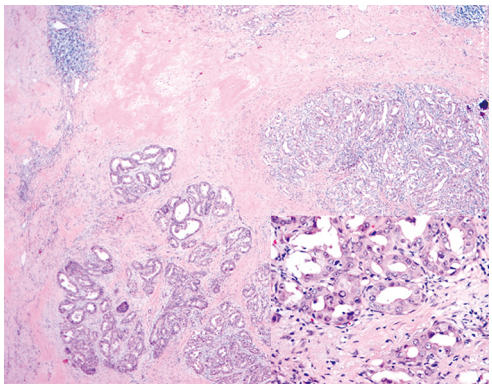

Figure 2 Histological finding of resected specimen is shown. Well differentiated adenocarcinoma is seen with glandular structure and a dense fibrous stroma. Scattered lymphocytes are also present (40 × magnification). In higher magnification, tumor is composed of glands lined by cuboidal mucin-producing epithelium resemble biliary epithelium (Inset, 400 × magnification).

of the resected specimen showed well differentiated cholangiocarcinoma composed of a glandular structure lined by cuboidal mucin-producing epithelium resembling cholangiocytes and a dense fibrous stroma with desmotic reaction (Figure 2). All resection margins were negative for malignancy microscopically. One cycle of adjuvant gemcitabine chemotherapy was administered due to concern about remnant tumor cells. On a CT scan taken 11 mo after R0 resection, there was no recurrence or new growing lesion (Figure 1F). In July 2009, 5 years and 6 mo from diagnosis (1 year from the surgical resection) the patient is living well without any symptoms.

DISCUSSION

The establishment of an optimal chemotherapeutic regimen for cholangiocarcinoma is necessary because most of the patients are diagnosed in an advanced stage and curative resection would not be viable. Gemcitabine is known as one of the most effective agents for cholangiocarcinoma and response rates were reported as 20%-35%[1] for a single regimen[2] or in combination with other agents such as 5-FU (or capecitabine)[3], mitomycin-C[4], or a platinum analog[5]. This case study showed repetitive responses to a gemcitabine single agent regimen given separately over a long-term interval. Tumors were observed to be strongly repressed for more than three years after the first cycle of treatment.

Studies in neoadjuvant settings for unresectable cholangiocarcinoma are very few although there was a remarkable success of a neoadjuvant protocol with the use of radiotherapy and capecitabine prior to liver transplantation[6]. There was no report about gemcitabine based chemotherapy as a neoadjuvant approach combined with surgical tumor excision. In our case, a very large unresectable intrahepatic mass-forming cholangiocarcinoma showed a definite response to gemcitabine alone that eventually led the patient to a curative resection and long-term survival. This result suggests that gemcitabine based chemotherapy should be tried more actively for unresectable cholangiocarcinoma.

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CASE REPORT

Post-gastrectomy acute pancreatitis in a patient with gastric carcinoma and pancreas divisum

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Abstract

Gastrectomy is commonly performed for both benign and malignant lesions. Although the incidence of post-gastrectomy acute pancreatitis (PGAP) is low compared to other well-recognized post-operative complications, it has been reported to be associated with a high mortality rate. In this article, we describe a 70-year-old man with asymptomatic pancreatic divisum who underwent palliative subtotal gastrectomy for an advanced gastric cancer with liver metastasis. His post-operative course was complicated by acute pancreatitis and intra-abdominal sepsis. The patient eventually succumbed to multiple organ failure despite surgical debridement and drainage, together with aggressive antibiotic therapy and nutritional support. For patients with pancreas divisum or dominant duct of Santorini who fail to follow the normal post-operative course after gastrectomy, clinicians should be alert to the possibility of PGAP as one of the potential diagnoses. Early detection and aggressive treatment of PGAP might improve the prognosis.

Key words: Acute Pancreatitis; Gastrectomy; Pancreas divisum; Duct of Santorini; Laparoscopy

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INTRODUCTION

Although gastrectomies for lesions of the stomach and duodenum have been performed safely for more than one hundred years, major complications such as anastomosis leakage, duodenal stump leakage and post-operative bleeding cannot be completely avoided even by experienced surgeons. In the past, most gastrectomies were performed for benign gastric or duodenal ulcer. One of the complications associated with these procedures was post-gastrectomy acute pancreatitis (PGAP). The pathogenesis of this condition is hypothesized to be related to pancreatic parenchymal injury secondary to severe adhesion of peripancreatic tissue, compromised pancreatic micro-circulation, hyperpressure of the duodenum, and edema or spasm of the major papilla[1,2]. The incidence of PGAP has decreased in recent years, coinciding with the dramatic reduction in the need for surgical intervention of complicated peptic ulcer disease in the advent of improved medical and endoscopic management. Gastrectomies nowadays are performed mainly for patients with gastric malignancy. The "pancreatitis-like" presentation is mostly seen in patients with afferent loop obstruction after gastrectomies, mostly following Billroth II reconstruction. The true PGAP might be related to the extended lymph node dissection and resection of adjacent organs (such as splenectomy and distal pancreatectomy) in radical gastrectomies for gastric cancer[3-5]. However, the correlation between pancreatic anomalies and PGAP has not been well documented in the literature. This article, therefore, reports a patient with concurrent advanced gastric cancer and pancreas divisum (PD) who had PGAP and died from multiple organ failure post-operatively.

CASE REPORT

A 70-year-old gentleman with a medical background of hypertension and asthma, presented with a 3 mo history

of poor appetite, 10 kg weight loss, postprandial fullness and nausea. There was, however, no history of tarry stool, hematemesis, fever or abdominal pain. The physical examination did not reveal any palpable abdominal mass, lymphadenopathy, anemia, or jaundice. All laboratory data, including biochemistry examination and hemogram, were within normal ranges except for a low hemoglobin level (10.3 g/dL). Esophagogastroduodenoscopy (EGD) was performed and one ulcerative mass over the posterior wall of antrum was identified (Figure 1). The histopathological examination of the biopsy of the gastric lesion confirmed that it was an adenocarcinoma. Dynamic CT was arranged for pre-operative staging. It revealed several cystic lesions of the liver and possible deformity of the duodenal bulb without definite evidence of metastatic disease (Figure 2). In addition, the gallbladder was contracted with suspicious wall thickening and heterogeneous content. At laparotomy, one unsuspected nodule was seen in segment 3 of the liver. The lesion was confirmed to be a metastatic liver nodule by frozen section. At the time of the operation, severe adhesion between the head of the pancreas and the peripyloric tissue was noted. As a result, a palliative subtotal gastrectomy with Billroth II gastrojejunostomy was performed due to partial outlet obstruction.

Post operatively, the patient had low-grade fever and persistent epigastric pain without jaundice, tarry stools, or active upper gastrointestinal hemorrhage. The laboratory tests on the 3rd postoperative day demonstrated a raised white cell count with left shift (11 500/μL), a platelet count of 176 000/μL and a hemoglobin of 9.5 g/dL. Serum biochemistry revealed a normal liver function and renal function but a raised C-reactive protein (CRP) of 193.27 mg/L. Slightly elevated amylase (185 U/L) and normal lipase (39 U/L) level were also noted. Chest X ray and urinary analysis were obtained and disclosed no pneumonia or urinary tract infection. Although the drainage fluid was clear, there was some yellowish and cloudy discharge from the abdominal wound. We collected the fluid for microbial analysis only and culture, which grew a yeast-like organism. Fever persisted despite the administration of intravenous antibiotics. Abdominal CT arranged on the 8th postoperative day demonstrated irregular pancreatic contours with diffuse enlargement and fluid accumulation in the lesser sac with retroperitoneal extension (Figure 3), suggestive of either an acute pancreatitis or duodenal stump leakage. The laboratory tests on the 10th postoperative day demonstrated a white cell count with marked left shift (8400/μL, 86% in segment form and 10% in band form), a decreased platelet count of 58 000/μL, a raised CRP of 247.95 mg/L, and normal serum level of pancreatic enzymes (amylase level was 79 U/L and lipase level was 26 U/L). In view of continuing clinical deterioration associated with peritonitis and intra-abdominal sepsis from a possible anastomosis leak rather than acute pancreatitis (according to the normal amylase and lipase levels), the patient underwent an exploratory laparotomy instead of percutaneous drainage of the fluid 10 d after the first operation. At laparotomy, colorless turbid fluid accumulation was found at the same regions as reported on the abdominal CT scan. There was no evidence of bile leak, and the duodenal stump and anastomosis were intact. The necrotic tissues around the cavity of the loculated fluid collection had a similar appearance to fat necrosis in acute pancreatitis. Limited debridement was performed and a sump drain tube was placed for post-operative irrigation and drainage.

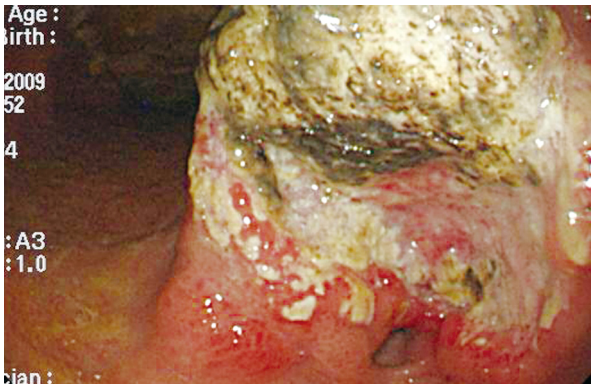

Figure 1 The esophagogastroduodenoscopy (EGD) showed an ulcerated, annular lesion over the gastric antrum.

Bile-stained fluid was noted in the sump drain 2 d after the second operation, and the biochemistry study of the drainage fluid disclosed lower amylase and lipase levels (14 U/L and 9 U/L). Blood culture grew *Bacteroides fragilis* and hemogram showed progressive pancytopenia. A repeat CT scan suggested possible duodenal stump leakage with abscess formation (Figure 4). Despite total parenteral nutrition, hemodialysis for acute renal failure, and sump drain irrigation-drainage with intravenous antibiotics for septicemia and intra-abdominal infection, the patient eventually died of profound septic shock and multiple organ failure on day 30 after the first operation. A retrospective review of the patient's abdominal CT demonstrated a previously undiagnosed, asymptomatic PD (Figure 5).

DISCUSSION

Gastrectomy is a commonly practiced procedure for both benign and malignant lesions of the stomach. Major complications such as postoperative bleeding, anastomotic leak and delayed gastric emptying are well documented[6]. In the past, most gastrectomies were performed for benign gastric or duodenal ulcer. The incidence of PGAP might be as high as 40.8% and the mortality rates in this group of patients ranged from 12.6% to 62.5%[1,2]. Recently, gastrectomies have mostly been performed for gastric malignancies, and the complication and mortality rates are much less because of improved surgical technique and postoperative care. The incidence of PGAP nowadays, although difficult to accurately estimate, has been reported to be less than 5%[7]. The mortality rate, however, can be up to 33.3%-50%[8,9] and is higher than acute pancreatitis of other etiologies[10]. Chen *et al*[3] reported a higher incidence of PGAP in patients having total gastrectomy compared with other types of gastrectomies (7.4% *vs* 0.8%), with a 33.3% mortality rate. Another study reported higher

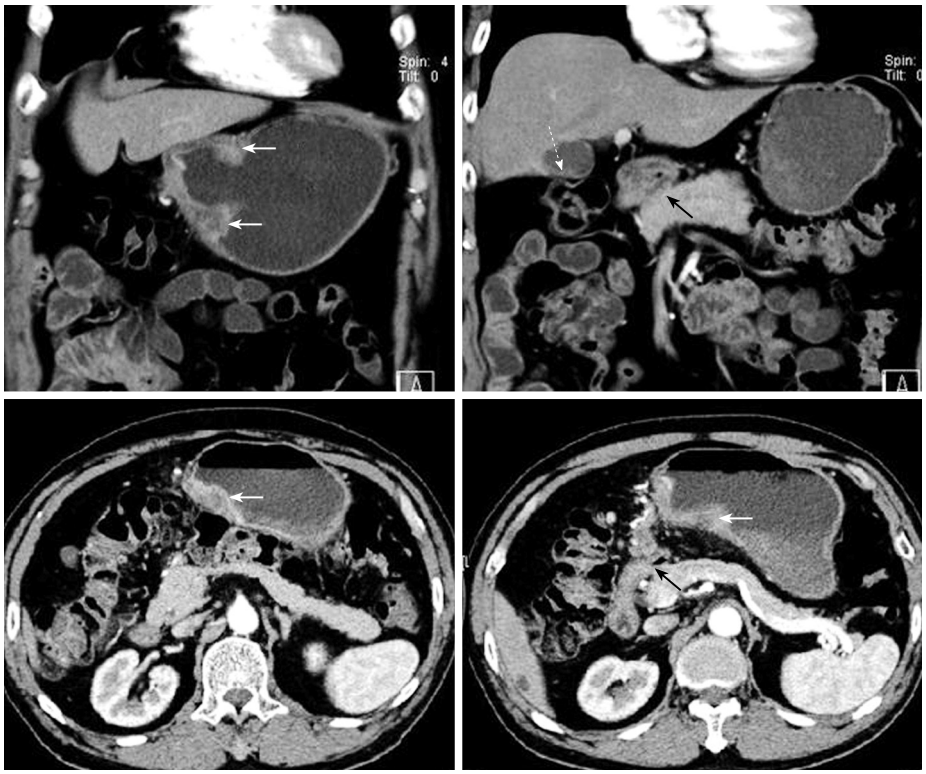

Figure 2 Preoperative abdominal CT images in coronary and transverse sections. The white solid arrows indicate diffuse wall thickening at the gastric antrum. The white dotted arrow indicates a contracted gallbladder with eccentric wall thickening. The black solid arrows point to the apparent deformity of the duodenal bulb with adhesion to the head of the pancreas. There is no evidence of intra-abdominal metastasis in this study.

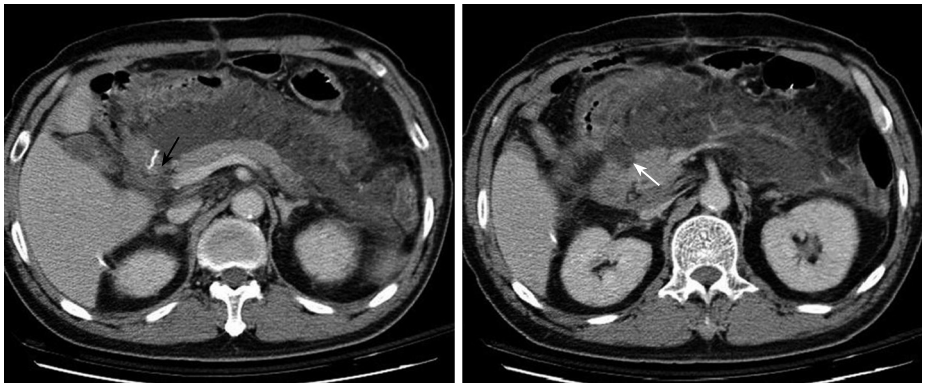

Figure 3 Abdominal CT 8 d after the first operation demonstrates massive fluid accumulation in the peripancreatic area and the lesser sac. The homogenous fluid extends to the retroperitoneal space. The status of the duodenal stump (black arrow) cannot be clearly assessed. The pancreas is well enhanced and enlarged, and the head shows an uneven and infiltrative margin (white arrow).

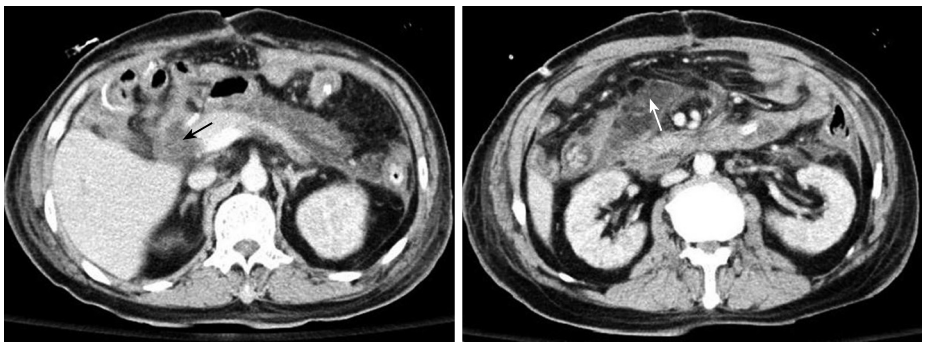

Figure 4 Follow-up abdominal CT after the second operation. Severe fat stranding is seen. An edematous duodenal stump is observed without a clear fat plane surrounding it. This is highly suspicious of a stump leak (black arrow). The loculated fluid with heterogeneous changes indicates the possibility of an abscess formation (white arrow).

PGAP rate in patients undergoing extended lymph node dissection[4]. Despite the various advantages of laparoscopically assisted gastrectomy (LAG), the rates of acute pancreatitis after LAG have also been shown to range from 0.7% to 2.3%[8,9,11]. The poorer prognosis of PGAP might be related to postoperative immunosuppression, increased pancreatitis-related hemorrhage because of extensive soft tissue/lymph node dissection, or anastomotic leak secondary to necrotizing pancreatitis and intra-abdominal sepsis.

PGAP may be a result of several factors. The stomach/duodenum might adhere to the underlying pancreas because of severe peptic ulceration or desmoplastic change from malignancy. Sometimes, the pancreas might actually be the base of the ulcer. Injury to the pancreatic parenchyma by electrocauterization or traction, microcirculation compromise, and direct injury to the pancreatic duct might occur during gastrectomy[7,11]. Other causes

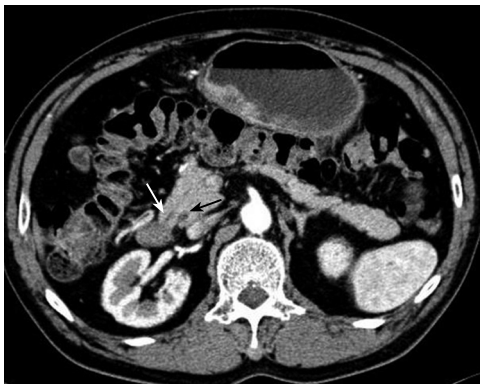

Figure 5 The preoperative CT scan. The dominant dorsal duct (duct of Santorini) drains into the minor papilla (white arrow). The CBD is seen in the section (black arrow) but the ventral duct (duct of Wirsung) can not be traced. These features are consistent with pancreas divisum.

of PGAP such as duodenal hyperpressure[1] and post-operative spasm of the major papilla[2] have been hypothesized, but there is a general lack of evidence in the literature to substantiate these theories. Although some studies suggested insertion of a nasogastric tube or a duodenostomy tube after gastrectomy for decompression and releasing duodenal hyperpressure, Hsu *et al*[12] reported no difference in PGAP in spite of NG tube insertion. On the contrary, the "pancreatitis-like" presentation (such as hyperamylasemia and epigastric pain) caused by afferent loop obstruction can be cured under careful management of operations or decompression procedures[7,13]. The condition should be taken into consideration because the prognosis, timing of intervention and treatment options are far different from PGAP without A-loop obstruction.

The diagnosis of acute pancreatitis is mainly dependent on suggestive clinical features and laboratory studies. Physical examination may be variable and non-specific in postoperative patients because of pain and symptoms related to the operation. Elevated lipase levels 3-fold or more above the normal range appearing within 48 h is the most reliable test[14]. However, normal serum pancreatic enzyme levels cannot exclude acute pancreatitis absolutely[14]. Abdominal CT scan is particularly helpful in making a definitive diagnosis and excluding other differential diagnoses such as anastomotic leak, intra-abdominal abscess and hemorrhage[15]. However, the anatomical distortion post-operatively often makes the interpretation of CT images difficult[16]. This is exemplified by the present case report whereby a definitive diagnosis of PGAP was delayed because of equivocal pancreatic enzymes and non-specific CT findings of intra-abdominal fluid collection.

PD is a common congenital anomaly of the pancreas, with an incidence rate of 4% to 10%[17-19]. Anatomically, the dorsal duct (duct of Santorini) becomes the dominant duct draining the majority of the pancreatic juice through the minor papilla. The ventral duct (duct of Wirsung) does not fuse with the dorsal duct and only drains a small portion of the pancreatic head through the major papilla. The diagnosis of PD is made with endoscopic retrograde pancreatography (ERP) or magnetic resonance pancreatography (MRP). However, with the advent of multi-detector CT scans, high sensitivity and specificity for diagnosis of PD can be achieved by CT images in some particular conditions[20-22]. The CT features consistent with PD include the presence of the dominant dorsal duct sign (the dorsal duct being larger than the ventral duct, or a missing ventral duct) and the miss-communication between the two pancreatic ducts[20]. Both of these features were observed on the patient's CT images retrospectively (Figure 5). About 5% to 45.5% of patients with PD present with pancreatitis or chronic abdominal pain[23,24]. The pathogenesis of this is attributable to the "relative stenosis" of the minor papilla as it drains the majority of the pancreatic juice through a small opening[24], resulting in an increase in the intra-ductal pressure of the dorsal duct system (20-28 mmHg *vs* 8-14 mmHg in normal pressure of major papilla)[25]. In addition, the unusual anatomical arrangement of the sphincter of the minor papilla could also contribute to the development of intra-ductal hypertension leading to pancreatitis[26]. Kamisawa *et al*[27] reported the high incidence of pancreatitis or pancreatic-type abdominal pain in patients with dominant duct of Santorini but without pancreas divisum.

According to these findings, it is reasonable to suppose that patients with PD might be more sensitive to minor injury of the pancreas and disturbance of its microcirculation. Once developed, it might progress precipitously to a more severe form and patients are slower to gain full recovery. Prophylactic administration of octreotide in the post-operative setting has been suggested in an attempt to prevent the development of PGAP[7]. The roles of preoperatively prophylactic cannulation of minor papilla, stent placement, and sphincterotomy have not been reported yet. The potential benefits of these invasive procedures in patients with PD or dominant duct of Santorini undergoing gastrectomy warrant further investigation.

In conclusion, PGAP is a less frequent post-gastrectomy complication in the current era. However, diagnosis and intervention should be made as early as possible because of the relatively high mortality rate associated with PGAP. Theoretically, patients with pancreas divisum or dominant duct of Santorini might connect to PGAP but the absolute relationship should be explored. Although serum pancreatic enzymes play an important role in the diagnosis of acute pancreatitis, imaging study such as an abdominal CT scan is particularly helpful in patients at high risk for PGAP. The roles and benefits of prophylactic octreotide, preoperative cannulation/stenting of the minor papilla, and sphincterotomy of minor papilla have not been well documented and would therefore warrant further investigation.

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CASE REPORT

Hemorrhagic hepatic cysts mimicking biliary cystadenoma

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Abstract

The hemorrhagic simple hepatic cyst is extremely rare and can sometimes be confused with biliary cystadenoma or cystadenocarcinoma. Here we present two cases of huge hemorrhagic simple hepatic cysts. Case 1 was a 43-year-old man with a cystic lesion measuring 13 cm × 12 cm in the right hepatic lobe. Ultrasound and computed tomography showed several mural nodules on the irregularly thickened wall and high-density straps inside the cyst. Case 2 was a 60-year-old woman with a huge cyst measuring 15 cm × 14 cm in the central liver. Ultrasound and magnetic resonance imaging showed the cystic wall was unevenly thickened and there were some flame-like prominences on the wall. The iconographic representations of the two cases mimicked biliary cystadenoma. Cystectomy and left hepatectomy were performed for the two patients, respectively. Both patients recovered quickly after their operations and showed no recurrence.

Key words: Simple hepatic cyst; Intracystic hemorrhage; Biliary cystadenoma; Diagnosis; Treatment

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INTRODUCTION

Intrahepatic cysts are generally classified as congenital cyst, traumatic cyst, infectious cyst, parasitic cyst, or neoplastic cyst. Congenital hepatic cysts include simple hepatic cysts and adult polycystic liver disease. A few simple hepatic cysts can reach large sizes and occasional complications, such as rupture, infection, hemorrhage, obstructive jaundice, or portal hypertension may occur.

Intracystic hemorrhage is an extremely rare complication of simple hepatic cysts and few cases have been reported worldwide. The hemorrhage usually occurs in solitary huge hepatic cysts in older patients. Massive bleeding into the cysts can significantly enlarge the cysts in a short time, which might partly contribute to the huge size of the cysts. The clinical manifestations of the hemorrhagic hepatic cysts are usually lack of specificity and the iconographic representations often mimic biliary cystadenoma or cystadenocarcinoma. Therefore, it is sometimes very difficult to make a precise diagnosis and select the appropriate treatment for this disease in clinical practice.

Here we present two cases of huge hemorrhagic simple hepatic cysts mimicking biliary cystadenoma, and discuss the diagnosis and the treatment of the disease.

CASE REPORT

Case 1 was a 43-year-old man admitted to hospital complaining of right upper quadrant abdominal pain for seven days. No positive clinical signs were found upon physical examination. The patient's serum carbohydrate antigen 19-9 (CA 19-9) concentration was four times higher than normal. Abdominal ultrasound examination showed a liquid anechoic area measuring 13 cm × 12 cm in right hepatic lobe. The cyst had an irregularly thickened wall with several mamelons on it. There were flocculation echoes inside the cyst. A computed tomography (CT) scan showed a low density cystic lesion with high density straps inside. The thickened cyst wall and mural nodules were not enhanced on contrast CT (Figure 1).

Case 2 was a 60-year-old woman admitted to hospital with a huge cystic liver mass found by regular ultrasound examination without any symptoms. The mass could be partly palpated below the right costal margin. The patient's serum concentration of CA 19-9 was normal. Abdominal ultrasound examination showed

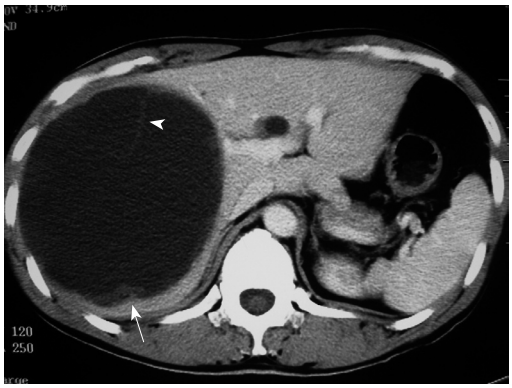

Figure 1 CT scan showing a huge low density cystic lesion in the right liver with high density straps (arrowhead) inside and mural nodules (arrow) on the cyst wall.

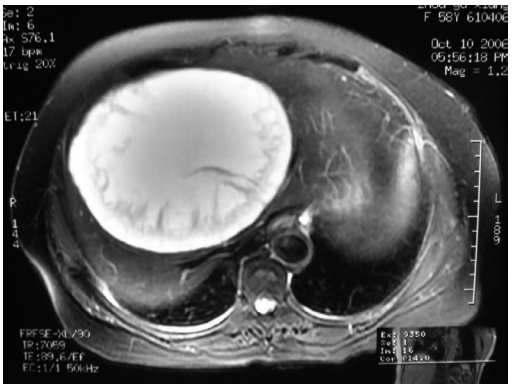

Figure 2 MRI (T2 weighted sequence) showing a huge cyst in the central liver with liquid content and flame-like prominences inside.

a huge echoless cyst measuring 15 cm × 14 cm in the central liver. The margin of the cyst was clear but its wall was unevenly thickened. There were some flame-like prominences on the wall. MRI showed the cyst content was liquid; the flame-like prominences were seen but not enhanced with contrast (Figure 2).

It was difficult to make a diagnosis in either case preoperatively. Biliary cystadenoma was highly suspected. Operations were performed for both cases. Case 1 received a cystectomy and case 2 received a left hepatectomy. The pathological diagnosis was hepatic simple cysts accompanied by intracystic hemorrhage. The mural nodules and prominences on the cyst wall were blood clots. Both patients recovered quickly after their operations and have been alive for six years and two years, respectively.

DISCUSSION

The clinical manifestation of this disease is lack of specificity. Generally the patients might have acute or chronic upper abdomen pain, but some patients have no symptoms[1-4]. Extra attention should be paid to patients whose symptoms are relative to the severe complications caused by the intracystic hemorrhage. For example, Iguchi *et al*[5] reported a 76-year-old man with severe edema of both lower extremities and a CT scan revealed an enlarged hemorrhagic hepatic cyst compressing inferior vena cava (IVC) in which massive thromboses formed. Buyse *et al*[6] reported a patient with simple hepatic cyst developed acute dyspnoea. It was found that the intracystic bleeding led to the enlargement of the cyst and compression of the IVC, followed by pulmonary embolism and dyspnoea. The clinical signs of hemorrhagic simple hepatic cysts depend on the size, location, and complications of the cysts, in which abdominal mass is more common.

The iconographic representations of hemorrhagic hepatic cyst sometimes mimic biliary cystadenoma or cystadenocarcinoma. The common manifestation on ultrasound includes hepatic cystic mass, hyperechoic intracystic straps, and irregularly thickened cyst wall with flame-like prominences or convex palliates. The mural nodules can sometimes be seen to be mildly enhanced on contrast enhanced CT and MRI[2], which increases the difficulty in differential diagnosis with cystic neoplasms. In fact, as proved by surgery and histological examination, the mural nodules are usually blood clots or organized hematomas[1,2]. In our cases, although the mural nodules and prominences on the cyst wall were not enhanced on contrast CT and MRI, the suspicion of biliary cystadenoma could not be precluded.

The distinction between hemorrhagic simple hepatic cysts and biliary cystic neoplasms can prove difficult to determine by clinical and iconographic features alone. For differential diagnosis, percutaneous transhepatic aspiration might be helpful if serosanguineous fluid is obtained[4]. However, the puncture should be forbidden if malignancy is highly suspected. Naganuma *et al*[7] reported that contrast-enhanced ultrasound clearly showed microbubbles oozing from the cyst wall into the cyst cavity in a case of hepatic cyst with intracystic bleeding, suggesting contrast-enhanced sonography might be a useful diagnostic tool for the disease. Akiyama *et al*[8] also reported that Levovist ultrasonography imaging could play an important role in the correct diagnosis of a simple hemorrhagic cyst, by demonstrating the avascularity of the visualized intracystic structures. Horsmans *et al*[9] found in a four-patient small group that both serum and cystic fluid CA 19-9 levels were elevated in the two patients with cystadenoma or cystadenocarcinoma, but remained normal in the other two patients with hemorrhagic simple cyst. This suggested that the determination of serum and cyst fluid CA 19-9 levels might be of help in distinguishing between hemorrhagic simple cyst and cystadenoma or cystadenocarcinoma. However, other studies revealed that the CA 19-9 levels in neither serum nor cyst fluid had a relationship to the benign or malignant property of hepatic cystic lesions[10-12]. In the present two cases, the serum CA 19-9 level was high in one case but normal in the other case, which seems to agree with the latter standpoint. Except for biliary cystadenoma and cystadenocarcinoma, other hepatic lesions such as hemangioma[13], abscess, and parasitic cyst should also be differentially diagnosed.

Treatment for the hemorrhagic simple hepatic cysts should be active because intracystic bleeding can enlarge

the cysts significantly and cause severe complications, such as rupture, infection, and compression of the IVC leading to venous return obstruction or thrombosis. For poorly conditioned patients, transcatheter arterial embolization, transhepatic cyst drainage, intracystic ethanol injection or noninvasive methods are indicated. These methods can obtain therapeutic effect in some patients, though the bleeding may recur afterwards[3-5,14,15]. Surgery should be performed for most well conditioned patients, especially those whose diagnosis does not preclude malignancy. The operations can include partial hepatectomy, cystectomy, and fenestration. Partial hepatectomy and cystectomy can remove the whole cyst and reach curative effect[1-4,8,9,11,13,16]. Occasionally, a fenestration operation is performed to simplify the procedure, which can also obtain a good therapeutic effect[6].

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Meetings

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Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
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http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
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Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
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Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

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Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
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World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
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July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
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Methods in Clinical Cancer Research

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19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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